TRANSFUSE
STandaRd Issue TrANsfusion versuS Fresher red blood cell Use in intenSive carE– a randomised controlled trial.

A multi-centre randomised double blinded phase III trial of the effect of standard issue red blood cell blood units on mortality compared to freshest available red blood cell units

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ABBREVIATIONS

2,3-DPG	2,3-diphosphoglycerate
ANZ	Australia and New Zealand
ANZIC RC	Australia & New Zealand Intensive Care Research Centre
ANZICS CTG	Australian & New Zealand Intensive Care Society Clinical Trials Group
APACHE III	Acute Physiology and Chronic Health Evaluation
ARBCS	Australian Red Blood Cross Service
ATP	Adenosine triphosphate
CIDMU	Clinical Informatics and Data Management Unit
CPMP	Committee for proprietary medicinal products
CRF	Case report form
DEPM	Department of Epidemiology & Preventive Medicine
DSMC	Data & Safety Monitoring Committee
FNHTR	Febrile Non-Haemolytic Transfusion Reaction
GCP	Good clinical practice
ICH	International conference on harmonisation
ICU	Intensive care unit
LOS	Length of stay
MV	Mechanical ventilation
NHMRC	National Health and Medical Research Council
POD	Persistent Organ Dysfunction
RBC	Red Blood Cells
RC	Research Coordinator
RCT	Randomised Controlled Trial
RRT	Renal Replacement Therapy
SOFA	Sequential Organ Failure Assessment
TRALI	Transfusion Related Acute Lung Injury

SYNOPSIS

Background	Red blood cell (RBC) transfusion is a very common and potentially life-saving treatment in intensive care units (ICUs). However, RBC transfusion has also been associated with an increased risk of morbidity and/or mortality in critically ill, surgical and trauma patients. Although this association is multifactorial, attention has increasingly focused on the possible adverse impact of transfusing RBC which have been stored for a prolonged time.
Aim	The primary aim of the trial is to determine if transfusion of the freshest available RBC in critically ill patients compared to standard care decreases patient mortality.
Design	Multi-centre, randomised, double blinded, phase III trial
Patient population	Intensive care patients who require a RBC transfusion
Sample size	5000
Methods	Eligible patients who require a blood transfusion will be randomised by the ICU team using a web based system. The study number generated at randomisation will be conveyed to the transfusion service by telephone and on the blood request slip. The hospital transfusion service will allocate the appropriate blood to the patient as designated by a randomisation schedule. Allocation will be either standard practice (the oldest compatible available blood, front of fridge) or freshest compatible available (back of fridge). The patient will continue to receive the randomised treatment arm for any additional blood transfusion episodes during their hospital stay. A telephone follow-up will be conducted at 90 and 180days.
Outcomes	**Primary Endpoint:** • 90 day mortality **Secondary Endpoints:** • 28 day mortality • Persistent Organ dysfunction combined with death at day 28 • Days alive and free of mechanical ventilation (post randomisation) at day 28 • Days alive and free of RRT post randomisation at day 28 • Blood stream infection rate in ICU, post randomisation • Length of stay in ICU and in hospital post randomisation • Proportion of patients who suffer at least one febrile non-haemolytic transfusion reaction in ICU • Quality of life (EQ5D) at 180 days

STUDY ADMINISTRATION STRUCTURE

Coordinating Centre & Data Management Centre

Responsibilities

- overall management of the study including assistance with HREC applications
- management of study budget and liaison with funding bodies
- protocol and case report form (CRF) design and production
- database design and management
- protocol training of research coordinators and TRANSFUSE study team
- preparation and arrangement of investigator payments
- study set-up
- randomisation
- coordination of data entry and feedback of data enquiries
- monitoring and close-out site visits
- organisation of investigator meetings
- serious adverse event notification
- data analysis and collaboration on publications

Executive committee

Responsibilities

- Day to Day study management
- Liaise with Management Committee and sites

Members

Ms	Bridget	Ady	TRANSFUSE Project Manager	ANZIC-RC, Monash University
Dr	Cecile	Aubron	Adjunct Senior Research Fellow	ANZIC-RC, Monash University
Prof	Jamie	Cooper	Co-Director	ANZIC-RC, Monash University
Dr	Craig	French	ICU Director	Western Hospital, Intensive Care Unit
Dr	Dashiell	Gantner	Senior Research Fellow	ANZIC-RC, Monash University
Dr	Maija	Kaukonen	Senior Research Fellow	ANZIC-RC, Monash University
Mrs	Lynne	Murray	Research Manager	ANZIC-RC, Monash University
Dr	Alistair	Nichol	Assoc/Professor	ANZIC-RC, Monash University
Dr	Ville	Pettila	ICU Director	Helsinki University Meilahti Hospital (Finland)
Dr	Zoe	McQuilten	Clinical Research Fellow	Transfusion Outcome Research Collaborative, Monash University

Management Committee

Responsibilities

Overseeing all aspects of the study management including

- liaison with coordinating centre staff
- liaison with steering committee
- liaison with ANZICS CTG
- overseeing funding applications
- overseeing disbursement & administration of funds
- ensuring fiscal responsibilities are maintained
- development and approval of final protocol & study materials
- development and approval of data collection tools and methods
- general study management issues

Members

Ms	Bridget	Ady	TRANSFUSE Project Manager	ANZIC-RC, Monash University
Dr	Cecile	Aubron	Adjunct Senior Research Fellow	ANZIC-RC, Monash University
Assoc/Prof	Michael	Bailey	Senior Biostatistician	ANZIC-RC, Monash University
Prof	Rinaldo	Bellomo	Co-Director	ANZIC-RC, Monash University
Mr	Andrew	Webb	Senior Scientist	The Alfred, Blood Bank
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Dr	Maija	Kaukonen	Senior Research Fellow	ANZIC-RC, Monash University
Dr	Colin	McArthur	Clinical Director	Auckland City Hospital NZ (Dept Critical Care Medicine)
Dr	Phillip	Mondy	Transfusion Medicine Specialist	Australian Red Cross Blood Service
Mrs	Lynne	Murray	Research Manager	ANZIC-RC, Monash University
Dr	Alistair	Nichol	Assoc/Professor	ANZIC-RC, Monash University
Dr	Neil	Orford	ICU Director	The Geelong Hospital, Barwon Health
Dr	Ville	Pettila	ICU Director	Helsinki University Meilahti Hospital (Finland)
Dr	Louise	Phillips	Head, Transfusion Research Unit	DEPM, Monash University
Dr	Zoe	McQuilten	Clinical Research Fellow	Transfusion Outcomes Research Collaborative, Monash University
Dr	Jeff	Presneill	Deputy Director Campus Wide Adult ICU	Mater Health Services
Prof	Michael	Reade	Defence Professor of Military Medicine & Surgery	Royal Brisbane & Women's Hospital
Ms	Shirley	Vallance	Research Manager	The Alfred, Intensive Care Research

Steering Committee

Responsibilities

- Oversight and advisory role
- Data analysis, collaboration and approval of study publications

Members

- Management committee (as above)
- Associate site investigators (4 from each site – suggested 1 Intensive Care Physician, 1 Transfusion Medicine Specialist, 1 Research Coordinator, 1 Blood Bank Scientist)

Contact Details

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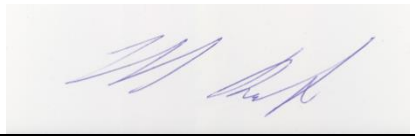
MANAGEMENT COMMITTEE AUTHORISATION PAGE

We the management committee have read the attached protocol and authorize it as the official protocol for the study entitled "A multi-centre randomised double blinded phase III trial of the effect of standard issue red blood cell blood units on mortality compared to freshest available red blood cell units"

Management Committee Bridget Ady		**Date**	
Management Committee Cecile Aubron		**Date**	
Management Committee Michael Bailey		**Date**	
Management Committee Rinaldo Bellomo		**Date**	
Management Committee Andrew Webb		**Date**	
Chief Investigator Jamie Cooper		**Date**	
Management Committee Craig French		**Date**	
Management Committee Dashiell Gantner		**Date**	
Management Committee David Irving		**Date**	
Management Committee Maija Kaukonen		**Date**	

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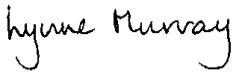
Management Committee		**Date**	
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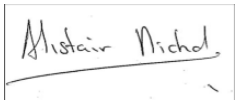
Management Committee		**Date**	
Alistair Nichol			
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Neil Orford			
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Ville Pettila			
Management Committee		**Date**	
Louise Phillips			
Management Committee		**Date**	
Zoe McQuilten			
Management Committee		**Date**	
Jeff Presneil			
Management Committee		**Date**	
Shirley Vallance			

LAY DESCRIPTION

In Australia, blood for transfusion has a “use by” date of 42 days after collection. The actual age of blood given to patients depends on what is available at the time and the rate of usage. During the last decade, it has been reported that blood transfusion in patients admitted to intensive care was associated with an independent increase in mortality. Some research suggests that transfusion of fresher blood might help patients in the intensive care unit to reach a better recovery. This project will test whether patients who receive ‘fresher’ blood do better than patients who receive ‘standard issue’ blood.

BACKGROUND & RATIONALE

Clinical and Scientific Rationale

Anaemia is common in critically ill patients: up to 90% of patients will be anaemic by day three of their intensive care unit (ICU) stay [1]. The RBC transfusion rate in critically ill patients is reported between 20-40% in ICU [1-3] , with a mean of 2 to 4.8 RBC units transfused per patient [2, 4]. In Australia, a mean of 786,097 RBC units per year were delivered by the Blood Service between 2007 and 2010 [5]. Approximately 18% of these (~140,000) are administered to critically ill patients. While potentially life-saving for individual patients RBC transfusion has also been associated with an increased risk of morbidity and/or mortality in critically ill, surgical and trauma populations [6, 7]. Literature reports have increasingly focused the attention on the possible harmful impact of RBC storage on the outcome of ICU patients [8-11].

Storage Lesion

Storage of RBCs in a preservative medium is associated with metabolic, biochemical and molecular changes to erythrocytes commonly referred to as "storage lesion" [9, 12]. These biochemical and biomechanical changes include: depletion of adenosine triphosphate (ATP) and of 2,3-diphosphoglycerate (2,3-DPG), membrane phospholipid vesiculation, protein oxidation and lipid peroxidation of RBC membrane, and loss of deformability [9, 12, 13]. Bioreactive substances (lipids and cytokines) accumulate during storage [12]. Over time, RBC shape changes with increased osmotic fragility and loss of deformability [12, 14]. Decreased membrane flexibility may compromise microcirculatory flow and leads to increased red cell-endothelial cell interaction, with activation of inflammation. Other consequences of the "storage lesion" include: alteration of oxygen delivery (secondary to a decrease of 2,3-DPG and pH) and cell lysis [9]. In addition, haemolysis during RBC storage increases free iron, which is also bio-reactive [15]. Free haemoglobin also affects the bioactivity of nitric oxide (NO) leading to endothelial dysfunction, intravascular thrombosis, vasoconstriction and leukocyte adhesion [16]. Recently, a study pointed out the higher extravascular haemolysis after old blood transfusion (storage of 40 to 42 days) compared to fresh blood (storage of 3 to 7 days) in healthy volunteer and the possible harmful effect of iron delivery [17]. White blood cells may participate in the storage lesion, but do not fully explain it[12].

The severity of illness of a blood transfusion recipient may further increase their susceptibility to deleterious effects of RBC storage. Critically ill patients frequently have disease states leading to impaired microcirculatory blood flow. In addition, patient neutrophils may first be primed by an event (e.g. sepsis or trauma) and then subsequent exposure to bio-reactive substances in a RBC unit may initiate enhanced activation of adherent leucocytes. This hypothetical "two-hit" model was confirmed during transfusion of aged RBC to lipopolysaccharide-pre-treated rats. Lung injury was more severe when compared with transfusion with fresh RBC in another group of pre-treated animals or with transfusion of aged RBC in healthy rats [18]. These data support the hypothesis that critically ill patients may be particularly sensitive to the adverse effects of the storage lesion (i.e. older transfused RBC) and that the transfusion of fresher blood may decrease mortality.

Clinical significance

Studies in trauma, ICU and patients undergoing cardiac surgery have demonstrated adverse outcomes potentially attributable to storage lesion. In 1993, Marik *et al.* demonstrated an inverse association between change in gastric intramucosal pH and age of the transfused blood and concluded that patients receiving old RBC developed splanchnic ischemia[19]. More recently, a prospective 66 patient study showed that transfusion of blood stored for more than 19 days failed to increase brain oxygenation, whereas the freshest RBC were effective[20]. Six, single centre retrospective observational studies conducted in trauma patients, and one in critically ill patients reported clinical adverse events related to RBC storage. Adverse events included multiple organ failure, increased ICU and hospital length of stay, deep vein thrombosis, nosocomial infections and mortality [21-27]. However, these studies are of insufficient quality to confirm the impact of duration of RBC storage (currently recommended up to 42 days) on RBC adverse effects, or to change current transfusion practice.

In the last two years, 6 large observational studies have shown that older compared to fresher blood increased patient mortality and morbidity [28, 29] [30] [31] [32]. Koch *et al.* reported prolonged mechanical ventilation (9.7% versus 5.6%, p<0.001) and an increased incidence of sepsis (4% versus 2.8%, p=0.01) and mortality in 6,002 cardiothoracic patients. Transfusion of older RBCs was also independently associated with an increased risk-adjusted rate of a composite of serious adverse events (25.9% versus 22.4%, p=0.001)[28]. A Canadian prospective observational study in 4,933 acute care cardiovascular patients reported an adjusted relative risk for death of 1.48 (95% CI 1.07 to 2.05) for patients in the highest quartile of maximum age of blood compared with those in the lowest quartile [29]. A retrospective observational study conducted in 1,478 post cardiac surgery patients showed an increase risk of infections when the patients were transfused with blood older than 14 days [32]. Two prospective paediatric ICU studies, in 455 and 296 patients respectively, showed an independent association between duration of blood storage and morbidity [30, 31]. In 296 paediatric ICU patients, the odds ratio for development of new or progressive multiple organ dysfunction syndrome in patients receiving older blood (stored for more than 14 days) was 1.87 (95% CI 1.04 to 3.27, p=0.03) after correction for confounding variables. Patients receiving older blood (≥14 days) had a longer ICU length of stay (9.9±8.3 versus 14.0±10.4 days, p<0.0001)[31]. Similar results were reported by Gauvin *et al.* Furthermore, these authors found that a RBC storage time of more than 21 days compared to less than 21 days was associated with higher mortality (9.2% versus 3.8%, p<0.025)[30]. In 2008 we conducted a prospective observational study to assess any association between age of transfused RBC and outcome in 757 ANZ ICU patients. After adjustment for confounding factors including APACHE III score, number of transfusions, pre-ICU transfusions, fresh frozen plasma and platelet transfusions, leucodepletion status, pre-transfusion haemoglobin concentration, clustering of study sites, and cardiac surgery, the administration of “fresher” RBC was independently associated with decreased mortality in a heterogeneous group of critically ill patients: odds ratio for hospital mortality of the older three quartiles compared to the freshest quartile 2.01% (95% CI, 1.07 to 3.77) [3].

However some investigators have not demonstrated any association between RBC storage lesion and clinical outcomes [33-39]. Vlaar et al. recently reported that storage of RBC did not influence TRALI occurrence [40]. In contrast to other studies of cardiac surgical patients a recent large retrospective study did not demonstrate any association between storage lesion and mortality [36].

Most of the clinical studies in this area are observational: they are mostly retrospective, small, and subject to bias. Only two small RCTs have been performed: they were very small and under-powered to answer the question [41, 42]. Accordingly, systematic reviews [8-10, 43] and a meta-analysis[44] have been inconclusive.

The impact of storage lesion on clinical outcomes remains uncertain and no definitive conclusions may be made. The available literature supports further research to determine the effect of storage lesion on clinical outcome in critically ill patients.

Our study design does not use an arbitrary cut-off age for defining the youngest RBC, as others have proposed[45] [41],[46], but instead uses the “freshest available” RBC versus standard care. This design best reflects the clinical practice change which would be practical to implement if fresher blood was beneficial in ICU patients. Importantly if we find no difference between the 2 treatment arms, such practice change would not be necessary.

The pragmatic design of the 5,000 patient TRANSFUSE-RCT expands and complements two current international studies. ABLE (n=2500), a Canadian multicentre RCT will evaluate transfusion of RBC units stored for less than eight days compared to standard care in critically ill patients [45]. This design is logistically demanding and cannot translate to current practice where freshest available blood varies in age. With 2500 patients ABLE is likely to be underpowered unless a much larger than anticipated treatment effect is observed. RECESS is a United States multicentre RCT comparing cardiac surgery patients transfused with leucodepleted RBC stored for 10 days or less, versus 21 days or more. The very low mortality in cardiac surgery patients requires a surrogate outcome (change in multiple organ dysfunction score); this reduces the clinical significance of any study outcome [46].

Only a large multicentre RCT can address whether the age of RBC transfusion affects mortality. Accordingly the TRANSFUSE study will have important health policy implications regardless of the outcome, in Australia and internationally.

OBJECTIVES

Aim

This study aims to determine whether, compared to standard care, transfusion of the freshest available allogeneic RBC decreases mortality of patients admitted to ICU.

Hypothesis

In critically ill patients who require a RBC transfusion, compared to standard practice, the administration of the freshest available compatible RBC reduces 90-day patient mortality.

STUDY OUTCOME MEASURES

Primary outcome

90 day mortality

Secondary outcomes

1. 28 day mortality
2. Persistent Organ Dysfunction combined with death at 28 [47]
3. Days alive and free of mechanical ventilation at day 28 post randomisation
4. Day alive and free of renal replacement therapy at day 28 post randomisation
5. Blood stream infection in ICU (post randomisation)
6. Length of stay in ICU and in hospital post randomisation
7. Proportion of patients who suffer at least one febrile non-haemolytic transfusion reaction in ICU
8. EQ-5D score at day 180 post randomisation [48]

OVERALL STUDY DESIGN

Study design

TRANSFUSE-RCT is a multi-centre, randomised, double blind, controlled trial, testing the effect of the freshest available RBC compared to standard practice, on mortality in critically ill patients who require RBC transfusion.

Study treatments

- Freshest available blood group: These patients will receive the freshest available group-specific compatible RBC unit in the transfusion service.
- Standard care group: These patients will receive standard practice, which is the oldest available group-specific compatible RBC unit in the transfusion service.

Study population

ICU patients or emergency department patients accepted for ICU admission in participating hospitals who meet all inclusion criteria and have no exclusion criteria. The study will enrol 5000 patients, in approximately 50 centres in Australia and New Zealand (ANZ).

Our observational study indicated that the recruitment of 40 patients per week is feasible and conservative [3]. At such a rate study recruitment would be completed in approximately 2.5 years. Allowing for study set up (12 months), 90 day outcome assessments, monitoring and analysis, a study time period of 4 years is anticipated.

Inclusion criteria

ICU patients* with an anticipated ICU stay of at least 24 hours, in whom the decision has been made by medical staff to transfuse at least one RBC unit.

*A patient can be included into the study in the Emergency Department if the decision to admit the patient into the ICU has been taken.

Exclusion criteria

- Age younger than 18
- Previous RBC transfusion during the current hospital admission (including transfusion in another hospital for transferred patients)
- Diagnosis of transplantation or hematologic malignancy
- Pregnancy
- Cardiac surgery during the present hospital admission
- Expected to die imminently (<24hrs)
- The treating physician believes it is not in the best interest of the patient to be randomised in this trial.
- Known objection to the administration of human blood products
- Participation in a competing study (see below)

Co-enrolment

- Co-enrolment into any RBC transfusion studies is not permitted
- TRANSFUSE patients cannot be co-enrolled into the POLAR, ARISE, TRISS, and IRONMAN studies.
- Co-enrolment in the ADRENAL, EPO-TBI, PHARLAP, and SPICE studies is permitted by the TRANSFUSE management committee
- Co-enrolment in single site studies is permitted (excluding blood transfusion studies)

STUDY PROCEDURES

Eligible patients

Patients will be screened in the ICU and emergency department for their potential eligibility by the ICU research coordinator (RC) and clinicians. The ICU RC will place a sticker on the patient's chart to assist the ICU team with identifying eligible patients when a blood transfusion is ordered.

Enrolment

The inclusion and exclusion criteria will be checked when a clinical decision is made to transfuse. Transfusion indication, timing and number of the RBC units will be determined by the treating clinicians. However, we recommend following the current ANZ transfusion guidelines for ICU patients [49].

Randomisation

Randomisation will be performed by the ICU research staff using a Web-based computer system.

The patient will be allocated a unique study identification number at randomisation to identify their study involvement for the first and each subsequent RBC transfusion during their hospital stay. This number will be recorded on a sticker placed in the patient's chart and also on the RBC request form sent to the hospital transfusion service. The treatment allocation will be determined using variable block randomisation in a 1:1 ratio, stratified by centre.

The ICU staff (the physician or the nursing staff caring for the patient, or the research coordinator) will telephone the transfusion service to: 1) order the RBC units, 2) inform them of the patient's study inclusion and unique study identification number. The hospital transfusion service will be given the randomisation schedule for their site and the allocated study number will be identified on the schedule and the appropriate group (freshest or standard care) will be allocated to the patient.

For the freshest blood group, the transfusion service scientist will take the required number of compatible RBC units from the area of the fridge containing the freshest blood. For the standard care group, the transfusion service scientist will select the RBC units in accordance with the current practice and will take the required number of compatible RBC units from the area of the fridge containing the oldest blood.

The blood scientist will flag the patient's allocation in the transfusion service computer system to ensure they remain in the same study group throughout their primary hospital admission. In addition to this measure, while the patient is in ICU clinical staff will remind the transfusion service of the patient's study enrolment when ordering RBC. For any subsequent transfusion episodes in the operating theatre or on the ward, the blood scientists will be reminded by the computer flag.

At the time of the patient's primary hospital discharge the ICU RC will notify the blood bank to remove the computer flag. Patients who are readmitted to hospital and receive further transfusion will receive standard issue RBC.

Blinding

Study group allocation will be concealed from the treating medical and nursing staff and the research coordinator.

Only transfusion service staff will have access to patient allocation. The transfusion service staff will treat the randomisation schedule confidentially and will not reveal the study group to the research or clinical staff. In keeping with normal hospital protocol and transfusion practices, the RBC unit details including collection and expiry dates will be checked; however in ICU this will be performed by 2 nurses not involved in the direct care of the patient. One of the 2 non-bedside nurses will put the RBC unit in a bag with opaque panels to conceal the collection and expiry dates before commencing the transfusion. For those patients receiving transfusion in the operating theatre or ward following ICU discharge, RBC checking will be performed according to usual practice by treating staff. For the purposes of later data analysis, RBC collection and expiry dates will be obtained directly and sequentially from the ARBCS. Due to considerable variation in stock levels in the transfusion service, knowledge of these dates will not identify the study group.

Our pilot study showed that clinical staff remained blind to treatment group, confirming the feasibility of the blinding procedures [50].

ETHICS

Guiding principles

This study is to be performed in accordance with the ethical principles of the Declaration of Helsinki (June 1964 and amended 1975, 1983, 1989, 1996, 2000 and Note of Clarification 2002, 2004, 2008)[51], ICH GCP Notes for Guidance on Good Clinical Practice (CPMP/ICH/135/95) annotated with Therapeutic Goods Administration comments[52] and NHMRC National Statement on Ethical Conduct in Research Involving Humans (March 2007)[53]

Ethical issues of the study

Patients will not have capacity to consent for themselves.

Ethics Committee approval

In Australia, this protocol will be submitted to a Human Research and Ethics Committee constituted according to the NHMRC National Statement on Ethical Conduct in Research Involving Humans (March 2007)[53] for each institution. In New Zealand, this protocol will be submitted to the appropriate Health and Disability Ethics Committee, accredited by the Health Research Council and constituted in accordance with the Operational Standard for Ethics Committees March 2002. Approval of the protocol and related documents will be obtained prior to the start of the study at each site.

It is the principal investigator's responsibility to ensure that all conditions for approval of the study are met and that amendments to the protocol or serious adverse events are also reported to the HREC (or equivalent) as required by that committee.

Confidentiality of patient data

Patient's will be randomised via a secure website and will be allocated a unique study number. The Research Coordinator will compile an enrolment log including the patient's name, date of birth, hospital identification number, unique study number and date and time of randomization. Subsequent data will be identified by the unique study number only. The enrolment log and study data will be kept separately. Follow up details of the patient and their family will be collected including name, address and contact telephone numbers to allow the site research coordinator to conduct the telephone interview at 90 days. Follow up contact details will not be sent off site. All data collected in the follow up assessments will be identified by the unique study number only. Study data will be entered into a secure website managed by the Clinical Informatics and Data Management Unit (CIDMU, Monash University). No identifying data will be entered into the website. All contact details and study data will be kept in a locked office at both the study site and in a locked office at Monash.

Consent

The NHMRC National Statement on the Ethical Conduct of Research in Humans (March 2007)[53] acknowledges in Chapter 4.4 that research involving patients who are heavily dependent on medical care, such as the patients in this study, is necessary to assess and improve the efficacy and safety of interventions used in their treatment. Critically ill patients requiring blood transfusion are unlikely to be able to provide informed consent. This project involves emergency care research; recruitment has to be achieved rapidly as the treatment (RBC transfusion) is urgent. The Statement also acknowledges (4.4.6) that where the research involves emergency treatment a waiver for consent may be provided. Accordingly a waiver for consent is sought. The Statement also outlines the conditions when such a waiver may be granted (2.3.6).The reasons we believe such a waiver may be granted to this project include: it carries no more than low risk as defined by the National Statement (2.1.6 and 2.1.7); the potential benefits to society, irrespective of the result of the study, are significant; it is impractical to obtain consent given the emergency nature of the treatment; the study involves no investigational drug, both arms of treatment are within current practice, and there is no known reason for thinking the participants would not have consented if asked. As soon as practicable following

recruitment the participant and/or their legally authorised representative will be informed of the participant's inclusion in the research and of the option to withdraw without any reduction in quality of care. If they choose to withdraw the patient, permission will be asked to use the data collected up to that time (4.4.13).

Any interaction between research staff, participants and their person responsible will take into consideration the stress or emotional factors associated with critical illness and ensure that the dependency of potential participants and their relatives on medical personnel providing treatment does not compromise the freedom of the decision to continue participation (4.4.11).

DATA MANAGEMENT

Data collection methods

All data will be collected by trained staff at each study site using a paper source document developed by the coordinating centre. Data will then be entered into a web database designed by the CIDMU. Data queries will be automatically generated via the electronic data collection database.

Randomised patients will be followed up to death or 180 days post-randomisation. Data collection will be restricted primarily to those variables necessary to define clinical patient characteristics including: baseline demographics, primary diagnoses, physiological parameters, diagnostic interventions, therapeutic interventions and documentation of deaths and other adverse events.

Patients and/or their legal surrogate will be asked to provide three possible points of contact (home and close family contact details) to the research staff prior to discharge. For patients discharged alive from ICU, follow-up will be restricted to information concerning duration of hospital stay and vital status at hospital discharge, 90 days and 180 days post randomisation. In addition, patients (or a proxy – generally a close family member) that are alive at 180 days after randomization will be interviewed by the site RC using a simple standardized structured telephone questionnaire to measure quality of life EQ5D [48].

Data variables collected

- Demographic data:
    - Identity (initials, study number, date of birth, gender, postcode)
    - Blood group: A, B, O, AB, Rhesus status (positive or negative)
    - Alloantibodies (if present)
    - Co-morbidities: heart disease including angina, myocardial infraction and previous CABG, immuno-compromised
    - Ethnicity (for patients enrolled in New Zealand only)
- Hospital data
    - Hospital admission and discharge dates
    - Hospital discharge destination
- ICU data
    - ICU admission and discharge dates
    - Acute Physiology and Chronic Health Evaluation III [APACHE III] score and risk of death at ICU admission
    - Acute Physiology and Chronic Health Evaluation III [APACHE III] diagnosis code at admission
    - Current "unstable or active" heart disease –(cardiac arrest, arrhythmia, cardiogenic shock, angina, acute myocardial infarction)

  - Sequential Organ Failure Assessment [SOFA] score at randomisation and daily during ICU admission
  - Haemoglobin at ICU admission
  - Requirement of invasive mechanical ventilation at randomisation
  - Duration of mechanical ventilation
  - Requirement of renal replacement therapy at randomisation
  - Duration of renal replacement therapy
  - Requirement of catecholamines at randomisation
  - Duration of catecholamine administration
  - Blood stream infection (date and pathogen)
- Transfusion information
  - Haemoglobin pre transfusion episode
  - Date and time of each RBC unit transfused
  - RBC Blood group (A,B, O, AB) / rhesus status (positive or negative)
  - Donation number (=pack number)
  - Fresh frozen plasma (number of units post randomisation)
  - Platelets (number of units post randomisation)
  - Blinding status (ie: units checked by non-beside nurse)
  - Adverse events including febrile non haemolytic reaction
- Vital status at
  - ICU discharge
  - Hospital discharge
  - 28 days post randomisation
  - 90 days post randomisation
  - 180 days post randomisation
- Quality of life
  - 180 day EQ5D score [48]

Data management

Data entry and data management will be coordinated by the Project Manager and the ANZIC-RC, including programming and data management support.

Data quality & monitoring

Several procedures to ensure data quality and protocol standardisation will help to minimise bias and to optimise data quality. These include:

- Start-up meeting for all research coordinators and investigators will be held prior to study commencement to ensure consistency in procedures;
- A detailed data dictionary will define the data to be collected on the case report form;
- CIDMU & the Project Manager will perform timely validation of data, queries and corrections.

The study will be monitored by quality control reviews of data queries and protocol violations. On site monitoring of data quality will be performed on 10% of the data. Additional onsite monitoring will be performed on a case by case basis if quality control issues are flagged by electronic review of data.

STATISTICAL CONSIDERATIONS

All statistical analyses will be conducted under the supervision of Michael Bailey at Monash University Department of Epidemiology and Preventive Medicine.

Sample size calculation

We estimated the baseline mortality using the prospective ANZ observational study data [3]. Over 5 weeks in 2008, 757 patients were recruited. The exclusion criteria of TRANSFUSE leaves 45% recruitable patients. These patients had a hospital mortality of 25% and we conservatively estimate 90-day mortality to be 28%. Our sample size calculation is based on a 15% relative decrease in 90-day mortality, or an absolute decrease of 4.2% from 28% to 23.8%. This effect size would be clinically highly relevant (Number needed to treat to save one life [NNT]=24). This relative decrease is much less than observed in the Koch study (30% RRR) [28], and in our observational study where we found a mortality of 21.3% in the older three quartile versus 13.2% in the freshest quartile (38% RRR) [3]. With a type I error of 0.05 and a type II error of 0.1 (power 90%), the required patient number is 2332 per group. According to our previous studies [54, 55], loss to follow up should not exceed 5%, giving an accurate number of 4898 patients which has been rounded up to 5000 patients.

Statistical and analytical plan

This study will be analysed on an intention-to-treat basis. Analysis will primarily be conducted using SAS version 9.2. (SAS Institute Inc., Cary, NC USA). Baseline comparisons will be performed using chi-square tests for equal proportion, student t-tests for continuously normally distributed variables and Wilcoxon rank sum tests otherwise. The primary outcome, 90 day all-cause mortality will be analysed using a Chi-square test statistic, with results reported as frequencies and percentages per arm. Adjustment for strata (centre) will be made using logistic regression for binomial outcomes and Cox Proportional Hazards regression for time to event data with results reported as Odds Ratios (95% CI) or Hazard ratios (95% CI) respectively. In the unlikely event that baseline imbalances are found to exist between treatment groups, sensitivity analysis will be performed with imbalanced variables included as covariates. A two-sided p-value of 0.05 will be considered to be statistically significant. Additionally a per protocol analysis will be undertaken to account for patients with alloantibodies for whom freshest blood is unavailable. A detailed statistical analysis plan will be published prior to analysis being performed.

Subgroup analyses

A priori subgroup analyses will include:

- Group O versus non group O
- Illness severity according to APACHE III and SOFA scores
- Blood < 8 days old

Interim analysis

One planned interim safety analysis will be performed by the independent data and safety monitoring committee (DSMC) at 50% patient enrolment. It will examine safety and efficacy with stopping rules to be determined by the DSMC at their first meeting and to be published in the statistical analysis plan. Additionally an independent statistician will assess treatment separation by reviewing the average age of blood in the freshest and standard issue groups.

SAFETY

Data Safety Management Committee

An independent Data and Safety and Monitoring Committee (DSMC), comprising experts in clinical trials, biostatistics, transfusion medicine and intensive care medicine will be established before patient enrolment to review all trial protocols. The DSMC will conduct one planned interim safety analysis at half (2500) of the specified overall (5000) patient recruitment target, and will assess the differential proportion of all-cause mortality between the two treatment groups (a safety assessment of the trial primary outcome) as well as the differential cumulative "serious adverse events" reports. The DSMC may, in its absolute discretion, request assessment of any other trial data at any time.

Adverse events

Defined adverse events will be collected. Any undefined adverse events will be collected as free text bearing in mind that it is recognised that the patient population with critical illness and requiring RBC transfusions will experience a number of common aberrations in laboratory values, signs and symptoms due to the severity of the underlying disease and the impact of standard therapies. These will not necessarily constitute adverse events unless they are considered to be causally related to the study intervention or are otherwise considered to be of concern in the investigator's judgement.

Serious adverse events

The baseline mortality of intensive care patients enrolled in trials will be high due to the critical illness that has necessitated their ICU admission. They will frequently develop life-threatening organ failure(s) unrelated to study interventions and despite optimal management. Therefore, consistent with the advice of Cook et al[56], events that are part of the natural history of the primary disease process or expected complications of critical illness will not be reported as serious adverse events in this trial. Additionally, events already defined and reported as study outcomes (total mortality, febrile non-haemolytic transfusion reactions, bacteraemia, need for mechanical ventilation or renal replacement therapy) will not be labelled and reported separately as adverse events or SAEs unless they are considered to be causally related to the study intervention or are otherwise of concern in the investigator's judgement.

Reporting

SAEs will be recorded on separate case report forms. SAEs which occur from the time of commencement of study treatment to 90 days post randomisation will be reported to the coordinating centre (ANZIC-RC) by faxing the provided SAE form. SAEs should be reported to the ANZIC-RC within 24 hours of study staff becoming aware of the event. Minimum information to report will include:

- Patient initials and study number
- Nature of the event
- Commencement and cessation of the event
- An investigator's opinion of the relationship between study involvement and the event (unrelated, possibly, probably or definitely related).
- Whether treatment was required for the event and what treatment was administered.

SAEs must be reported on the fax form. Follow up reporting may be required. The SAE reports will then be forwarded to all sites for reporting to their HRECs in accordance with local requirements.

Attention:
Bridget Ady
Transfuse Project Manager
Fax number for SAE Reporting:+ 61 3 9903 0035
Telephone Numbers:+61 3 9903 0071

FUNDING

The TRANSFUSE RCT is funded by a project grant from the National Health and Medical Research Council (NHMRC#1020694) and by the Australian Red Cross Blood Service. The ANZIC-RC will supply infrastructure and administrative support.

PUBLICATION

The study will be conducted in the name of the TRANSFUSE investigators, the ANZIC RC and the ANZICS CTG. The central project coordination and data management will be provided by the ANZIC RC at Monash University, Melbourne. The principal publication from the study will be in the name of the TRANSFUSE Investigators with full credit assigned to all collaborating investigators, research coordinators and institutions. Where an individuals' name is required for publication it will be that of the writing committee, with the chair of the writing committee listed first and subsequent authors listed alphabetically.

Funding bodies will be acknowledged in the publication.

RESEARCH TIMELINES

Time frame indicator	Milestone
February 2012	Appoint Project Manager
March 2012	Protocol & HREC approval (Monash University)
April –Aug 2012	CRF & database development
April – Sept 2012	Staff training & ethical approval at collaborating sites
Oct 2012	Commence recruitment
Jan 2015	Complete 50% recruitment
Mar 2015	Interim analysis
Aug 2016	Complete 100% recruitment
Nov 2016	Complete 90 day follow up
Jan 2017	Query resolution & database lock of primary endpoint data
Feb 2017	Complete 180 day follow up
Mar 2017	Query resolution and database lock of secondary endpoint data
April 2017	Primary manuscript submitted

APPENDIX 1 – SCHEDULE OF EVENTS

Assessments / Procedures		ICU admission	Pre-1st RBC transfusion	Daily ICU Admission	ICU Discharge	Hospital Discharge	Follow-up – 28 days post-randomisation	Follow-up – 90 days post-randomisation	Follow-up- 180 days post randomisation
Screening log		X							
Inclusion & Exclusion criteria			X						
Demographic data			X						
Primary diagnosis		X							
Comorbidities			X						
Physiological parameters		X	X						
APACHE III		X							
SOFA			X	X					
Patient's blood group (ABO, Rh)			X						
Randomisation			X						
RBC Transfusion (each unit)	Haemoglobin pre-transfusion		X	X					
	Time & date of transfusion			X		X			
	RBC blood group (ABO, Rh)			X		X			
	Pack number			X		X			
	Collection and expiry dates			X		X			
	Blinding status (units checked by non-bedside nurse)			X					

Assessments / Procedures	ICU admission	Pre-1st RBC transfusion	Daily ICU Admission	ICU Discharge	Hospital Discharge	Follow-up – 28 days post-randomisation	Follow-up – 90 days post-randomisation	Follow-up- 180 days post randomisation
Adverse Events			X					
Serious Adverse Events			X					
Number of units (ml) FFP post-randomisation			X					
Number of units platelets post-randomisation			X					
Febrile non-haemolytic transfusion reaction			X					
Requirement for catecholamine		X	X					
Requirement for Invasive Ventilation		X	X					
Requirement for Renal Replacement Therapy (RRT)		X	X	X				
Blood stream infection (date(s), pathogen(s))			X					
Vital status & cause of death				X	X	X	X	X
EQ5D								X

APPENDIX 2 – EURO QUALITY OF LIFE (EQ 5D)

By placing a cross in one box in each group below please indicate which statement best describes your own health state today

- Mobility

☐ I have no problems in walking around
☐ I have some problems in walking around
☐ I am confined to bed

- Personal care

☐ I have no problems with personal care
☐ I have some problems with personal care
☐ I am unable to wash or dress myself

- Usual activities(eg: work, study, housework, leisure activities)

☐ I have no problems with performing my usual activities
☐ I have some problems with performing my usual activities
☐ I am unable to perform my usual activities

- Pain/discomfort

☐ I have no pain or discomfort
☐ I have some pain or discomfort
☐ I have extreme pain or discomfort

- Anxiety/depression

☐ I am not anxious or depressed
☐ I am moderately anxious or depressed
☐ I am extremely anxious or depressed

APPENDIX 3 – THE IRON TRANSFUSE SUB STUDY (THE IT STUDY)

Investigators (in alphabetical order)

Dr Cecile Aubron, Dr Melinda Dean, Dr Robert Flower, Dr Chris Hogan, Dr Zoe McQuilten, Dr Sant-rayn Pasricha, Dr Rosemary Sparrow and A/Prof Erica Wood on behalf of the TRANSFUSE investigators.

Aims:

To investigate changes in iron metabolism, bacterial growth in plasma and inflammatory profile after red blood cell (RBC) transfusion, and the role played by the duration of RBC storage in these changes, in critically ill patients.

Background:

Age of blood, iron metabolism and the TRANSFUSE trial

One third of patients hospitalised in intensive care unit (ICU) will be administered an average of 3 to 4 RBC units during their ICU stay [1, 2]. While RBC transfusion is considered lifesaving, their administration is independently and significantly associated with an increase in mortality and morbidity in this population [7]. Mechanisms for this potential harmful effect are not understood, however experimental studies suggest that blood storage duration may play a key role through RBC storage lesions, including accumulation of bioactive substances over the storage period.

One of the main consequences of the RBC storage lesion is the increase in extra vascular haemolysis in both the RBC unit and recipient, which results in a large amount of iron delivered to the recipient. Studies in animals, healthy volunteers and non-critically ill patients have described that transfusion of older RBC, compared to fresher RBC, leads to an increase in extra vascular haemolysis, an increase in non-transferrin bound iron (NTBI) and to a pro-inflammatory cytokine storm within the first hours after transfusion [57, 58]. NTBI is also involved in oxidative damage [59] [60], expression of endothelial adhesion molecules [61] and bacterial growth [62] [60]. For instance, it was reported, in 33 preterm infants, that transfusion of older RBC units was associated with a significantly higher concentration of malondialdehyde (MDA) 4 hours after transfusion compared to fresh RBC [59], suggesting elevated oxidative stress. However, these studies are restricted to animals or a non ICU-adult population and are limited in their sample size and design. Underlying disease appears to be a determinant in the occurrence of post transfusion adverse events [63].

A large prospective study on pathophysiological consequences of RBC transfusion and RBC storage duration in critically ill patients is required to: i) better understand how old RBC may promote inflammation, bacterial growth, oxidative damage and leucocyte adhesion, and ii) to define the patients most at risk of these adverse effects.

The TRANSFUSE (sTandaRd Issue TrANsfusion versuS Fresher red blood cell Use in intenSive carE)-trial (ACTRN12612000453886) enrols heterogeneous critically ill patients who did not receive blood before intensive care unit (ICU) admission and aims to determine whether freshest available allogeneic RBC compared to standard practice is associated with a decrease in mortality. This randomised controlled trial (RCT), conducted in Australia, New-Zealand and Europe, is the largest ongoing RCT on age of blood in critically ill patients at this time.

The TRANSFUSE-trial provides a unique opportunity to study how the RBC storage lesion may be harmful in critically ill patients. This study will provide valuable data on in vivo iron metabolism after RBC transfusion and other pathophysiological consequences of RBC storage lesions. These include: i) changes in bacterial growth in plasma before and after transfusion and between patients who received old and fresh RBC post-transfusion; ii) inflammatory profile post-RBC transfusion in patients receiving fresh vs. old RBC, and iii) plasma induced changes to leukocyte-endothelial interaction and the difference in leucocyte adhesion in patients receiving fresh vs. old RBC.

Significance

The project aims to address important and unresolved questions regarding potential mechanisms for any adverse effect from the storage age of RBC on patient outcomes. These data will provide detailed measurements of the potential pathways (iron, inflammation, propensity to infection) by which RBCs may produce adverse outcomes in patients. Importantly, by utilising the randomised design of the TRANSFUSE trial, this study will be able to investigate the effect of age of RBCs on these potential pathways. Identification of this mechanism could help future researchers develop specific approaches to preventing these adverse effects. The findings of this project will contribute to the development of strategies to address these effects and inform clinical practice, both in the intensive care and other clinical settings. They will also form the basis for a future potential NHMRC project grant to fund further studies into the mechanisms of adverse effects of transfusion. The collection of patient samples while the TRANSFUSE-trial is recruiting will be an invaluable resource for further research on the mechanisms of adverse effects from RBC transfusion in critically ill patients.

Objectives

Hypothesis

Transfusion of older RBC compared with freshest available RBC leads to greater extravascular haemolysis and subsequent increase in NTBI resulting in an increase in bacterial growth and inflammation.

Primary Outcome

Indices of increased erythrocyte damage (extravascular haemolysis, iron indices including NTBI and labile plasma iron [LPI]) at 4-hour and 24-hours post the first RBC.

Secondary Outcomes

Bacterial growth in plasma at 4-hour and 24-hours post the first RBC.

Inflammatory cytokines at 4-hour and 24-hours post the first RBC transfusion

Leucocyte adhesion at 4-hour and 24-hours post the first RBC transfusion

Study Design

A sub study of patients enrolled in the TRANSFUSE randomised control trial.

Recruiting Site

The Alfred Hospital Melbourne, the Austin Hospital Melbourne and Monash Medical Centre Melbourne

Inclusion criteria

- Patients randomised in the TRANSFUSE trial and who are going to receive their first RBC transfusion in the current admission in ICU

Exclusion Criteria

- Current therapy with iron supplementation or iron chelation.

Ethics and consent

Appropriate Human Research and Ethics Committee (HREC) approval and Research Governance, including the use of opt-out consent has been obtained for the TRANSFUSE trial at each participating site. Application for an amendment will be made to the HREC to approve the inclusion of this study. Prospective consent will be obtained from the patient or the person responsible in the first instance, and if this is not possible telephone consent will be sought. If no person responsible is contactable after reasonable attempts, patients will be enrolled under section 42T.

Researchers will ensure that the requirements for section 42T are met before proceeding –

- Patient will not regain capacity within a reasonable time
- The person responsible cannot be ascertained or contacted despite making reasonable efforts
- The medical research procedure is not contrary to the person's best interests
- The medical research procedure is not against the wishes of the person
- The HREC has agreed that the procedure may proceed for a person where that person or their person responsible is unable to consent
- The purpose of the research is to test the effectiveness of the proposed therapy
- The procedure poses no greater risk than the risk inherent in the person's condition and alternative treatment
- The researcher must tell the patient or their person responsible of the medical research procedure once the patient has regained capacity or the person responsible has been ascertained or located and that they are advised that they may withdraw from the treatment.

A copy of the section 42T form will be submitted to the HREC and also to the Office of the Public Advocate.

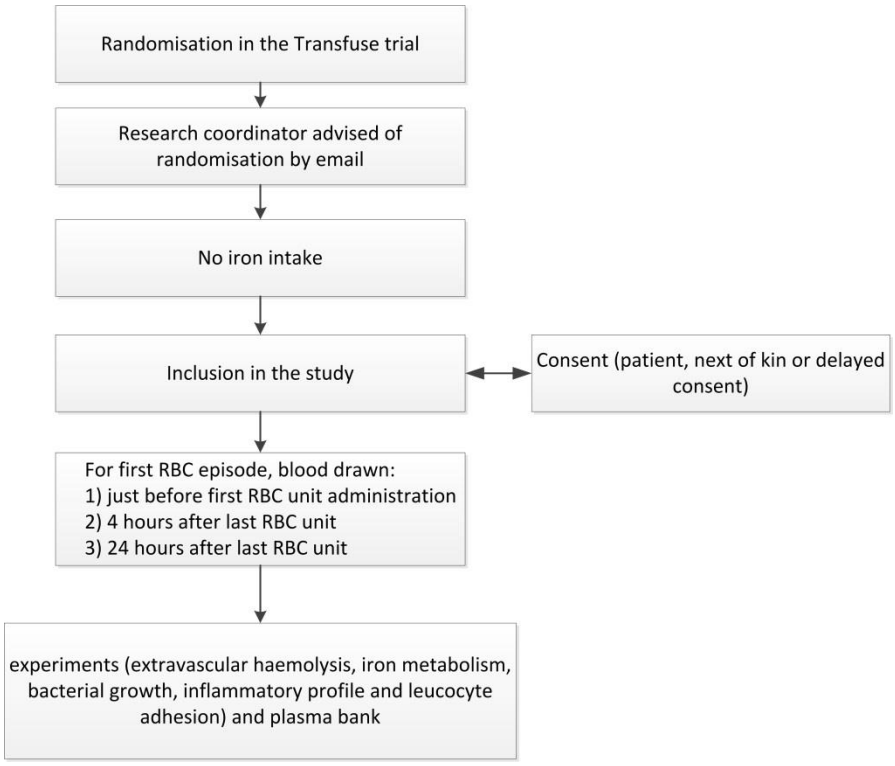
Patients and the person responsible (if applicable) will be given the plain language and information form including details about the study design, methodology and significance.

Research plan and Methods

One hundred and fifty patients will be included in this sub-study. Wherever possible eligible patients will be enrolled consecutively.

After randomisation in the TRANSFUSE-trial, a sample of 18 ml (31/2 teaspoons) of blood will be taken before the first RBC unit, at 4 and 24 hours after the transfusion episode.

Figure. Study procedures



Patient and transfusion data: will be routinely collected as part of the data collected for the TRANSFUSE-trial. Dates and times when samples are taken and results of the experiments performed in the study sites will be recorded in a separate case report form (CRF).

Blood samples: Blood samples (2 x5 ml in lithium heparin tube, 1 x K2E 3ml EDTA (lilac top) tube and 1 x 5 ml in gel lithium heparin) will be sent immediately to the pathology lab of each hospital where plasma samples will be aliquoted and stored at - 80°C before transfer to the Australian Red Cross Blood Service Melbourne Processing Centre (MPC), where they will be stored until recruitment is complete and experiments are performed. For further details refer to the section below "blood sample schedule."

Experiments:

- Bilirubin, free haemoglobin, haptoglobin, ferritin, serum iron, transferrin and transferrin saturation will be performed in each pathology laboratory at the 3 participating centres.
- The other experiments will be performed at a later date once all patients have been enrolled and samples collected. These experiments are outlined below:

i) Non transferrin bound iron (NTBI) and labile plama iron (LPI, a fraction of NTBI that is redox active) will be measured by FeROS eLPI and LPI assays (Aferrix, Israel), and serum hepcidin will be measured using a commercially available ELISA (DRG, Germany).
ii) Bacterial growth will be performed in plasma before and after transfusion without and with desferroxiamine as described elsewhere [64].
iii) Inflammatory biomarkers: sCD106 (VCAM-1), sCD62L, sCD62P, ICAM-1, MCP-1, MIP-1a, MIP-1β, IL-1α, IP-10, IL-6, IL-8, IL-10, IL-12, IL-4, IL-13, IL-17, IL-21, IFN-γ and IFN-α, will be assessed in plasma samples via multiplex Cytometric Bead Array (BD Biosciences).
iv) Leucocyte adhesion

Blood samples schedule

Samples will be taken within 1 hour prior to transfusion (T0), 4 hours after transfusion initiation (T4) and 24 hours after transfusion initiation. For example, if the first RBC transfusion starts at 10 am, the samples T0 will be taken between 9 am and 9.59 am, at 2 pm (samples T4) and at 10 am the following day (samples T24).

In pathology, samples will be processed by the pathology laboratory staff. Supernatant will be removed and 0.5ml of plasma will be aliquoted and stored in cryovials at -80 degrees Celsius. At least five tubes per patient for each collection time-point will be stored. They will include four cryovials of plasma with heparin and one cryovial of plasma with EDTA. The storage tubes will be pre-labelled with the study number, the date, time and type of collection tube used (i.e. EDTA or lithium heparin). Patient identifiers will not be used on the cryovials.

Each week, a research assistant (Shauna French, PhD student) from the Blood Service will collect the samples from the 3 sites and transfer them with ice to the MPC. The patient samples will be kept at -80 degrees Celsius until the study recruitment has been completed, when the samples will then be transferred for analysis.

		Samples/volume collected	Processing and storage	Tests
T0 Sample	Before transfusion of the first RBC unit	1x5ml lithium heparin tube	Processed in laboratory for immediate analysis as per laboratory protocol	Iron studies: ferritin, transferrin, transferrin saturation, serum iron Haptoglobin
		1 x5ml gel lithium heparin tube	Processed in laboratory for immediate analysis as per laboratory protocol	Free haemoglobin
		1 x5ml lithium heparin	^Centrifuge and aliquot for -80 degrees Celsius storage into: Plasma in heparin, (5	Non-transferrin bound iron Labile plasma iron Hepcidin

			tubes of 0.5ml)	Bacterial growth
		1 x3ml EDTA tube	^Centrifuge and aliquot for -80 degrees Celsius storage into: Plasma in EDTA, (2 tubes of 0.5ml)	Inflammatory biomarkers (as listed above)
T4 Sample	4 hours after transfusion initiation*	1x5ml lithium heparin tube	Processed in laboratory for immediate analysis as per laboratory protocol	Iron studies: ferritin, transferrin, transferrin saturation, serum iron Haptoglobin
		1 x5ml gel lithium heparin tube	Processed in laboratory for immediate analysis as per laboratory protocol	Free haemoglobin
		1 x5ml lithium heparin	^Centrifuge and aliquot for -80 degrees Celsius storage into: Plasma in heparin, (5 tubes of 0.5ml)	Non-transferrin bound iron Labile plasma iron Hepcidin Bacterial growth
		1 x3ml EDTA tube	^Centrifuge and aliquot for -80 degrees Celsius storage into: Plasma in EDTA, (2 tubes of 0.5ml)	Inflammatory biomarkers (as listed above)
T24 Sample	24 hours after transfusion initiation*	1x5ml lithium heparin tube	Processed in laboratory for immediate analysis as per laboratory protocol	Iron studies: ferritin, transferrin, transferrin saturation, serum iron Haptoglobin
		1 x5ml gel lithium heparin tube	Processed in laboratory for immediate analysis as per laboratory protocol	Free haemoglobin
		1 x5ml lithium heparin	^Centrifuge and aliquot for -80 degrees Celsius storage into: Plasma in heparin, (5 tubes of 0.5ml)	Non-transferrin bound iron Labile plasma iron Hepcidin Bacterial growth
		1 x3ml EDTA tube	^Centrifuge and aliquot for -80 degrees Celsius storage into: Plasma in EDTA, (2 tubes of 0.5ml)	Inflammatory biomarkers (as listed above)

*****In the event that multiple RBC units are transfused in the transfusion episode, samples will still be taken 4 hours after initiation of the first RBC transfusion

^Centrifugation will be performed at 16 degrees for 10 minutes with a speed of 2898 G

Data analysis and statistics

Sample size: 150 patients. Enrolment of 150 patients will enable detection of a difference in NTBI at 4 hours of 2.2uM with SD of 3.7 (similar to that shown in healthy volunteers in the study by Hod et al., Blood 2011 [62]),

between patients receiving fresh and older RBC units, accounting for a 20% incomplete data collection rate (which is expected due to the complex management requirements of patients enrolled in this study). Recruitment of 150 patients prior to the completion of the TRANSFUSE-trial (anticipated 18-months from commencement of the sub-study) is feasible based on the 3 centre's current combined recruitment rate of approximately 8 patients per month within usual working hours.

Statistical analysis: Data collected for the sub-study will be treated and analysed separately after the TRANFSUE RCT has been completed. Data will be transformed when skewed and analysis performed on log-transformed values. Parameters before and after the transfusion episode will be compared for each patient using paired t-test. Biological parameters post-transfusion, as well as differences between pre- and post-transfusion values, will be compared according to RBC storage duration category, which will be defined according to the RCT (i.e.: freshest available vs. standard issue) and also according to whether patients were transfused with only RBC younger than 14 days vs. patients transfused with RBC older than 14 days. A correlation between patient severity at randomisation and each parameter (including: post-transfusion extravascular haemolysis and NTBI) will also be performed. A sub group analysis will be performed on the most severe patients according to their SOFA score on the day of transfusion.

Safety

As per the TRANSFUSE trial. There are no additional risks posed by the IT TRANSFUSE sub-study. The results of the study will be forwarded to the Data and Safety Monitoring Committee (DSMC).

Funding

This study is collaboration with the Australian Red Cross Blood Service, who has provided funding for the costs for research staff time, sample collection, processing and testing in addition to in kind support provided by the Blood Service.

Publication

The study will be conducted in the name of Monash University on behalf of the TRANSFUSE investigators. Authorship will be under the names of the sub-study investigators on behalf of the TRANSFUSE investigators. Publication of results will be embargoed until publication of the main study results unless specific permission for early publication is given by the TRANSFUSE management committee.

APPENDIX 4 - THE RENALTRANSFUSE SUBSTUDY

Investigators

Liz Moore, Cecile Aubron, Zoe McQuilten, Bridget Ady, Lynne Murray, Maija Kaukonen, Michael Bailey, Rinaldo Bellomo, Jamie Cooper of ANZIC Research Centre, School of Public Health and Preventive Medicine, Monash University

Aim

This study aims to determine whether, compared to standard care, transfusion of the freshest available allogeneic red blood cell (RBC) unit is associated with a decrease in the incidence of acute kidney injury (AKI) in patients admitted to the intensive care unit (ICU) who require RBC transfusion.

Hypothesis

In critically ill patients who require a RBC transfusion, compared to standard practice, the administration of the freshest available compatible RBC reduces the incidence of AKI and the need for ongoing renal replacement therapy after hospital discharge.

Background

Age of blood, storage lesion and acute kidney injury

There is increasing evidence supporting an association between RBC transfusion and the development of AKI and RBC transfusion is reported to be an independent risk factor for AKI in critically ill patients[7, 65].

Moreover, in critically ill patients, the deterioration of RBC over time as a result of the 'storage lesion' could lead to organ dysfunction, including AKI, through iron toxicity and/or microcirculation dysfunction. Indeed, a human study demonstrated that RBC administration is associated with an increase in plasma iron concentration secondary to transfused RBC haemolysis with the observed changes proportional to the age of the RBC units[17].

In vitro and animal studies have shown that circulating free iron is harmful and leads to glomerular damage and alteration in proximal tubule cell homeostasis[66, 67]. These findings taken together strongly suggest that older RBC may have greater toxicity than fresh RBC on the kidney. One of the hypotheses for this adverse effect is the deleterious effect of free iron secondary to haemolysis which is partly dependent on RBC storage duration.

Age of blood and AKI: clinical evidence

The clinical effects of the age of transfused RBC on kidney function have not been well investigated and therefore remain uncertain. Only a few observational studies have been performed reporting conflicting findings[28, 34, 68, 69]. In a large retrospective single centre study published in the New England Journal of Medicine, Koch et al reported that renal failure was more frequent in the 2872 patients receiving only old RBC (storage duration>14 days) compared to patients (n=3130) receiving only young RBC (storage duration less than 14 days)[28]. Similarly, in 1724 trauma patients, Weinberg et al reported an increase in the incidence of renal failure in patients receiving blood older than 2 weeks[69]. In contrast, a study of 670 patients undergoing cardiac surgery found that the age of RBC was not associated with any outcome, including renal failure[34]. The only study to directly investigate the association between age of RBC and development of AKI in a heterogeneous ICU population was observational and did not find an independent association between age of RBC and AKI. However, with 652 transfused patients this study was likely to be underpowered for this

outcome[70]. Indeed, they did find that the incidence of Stage 3 AKI was significantly greater in patients in the RBC age quartiles 2 to 4 (older) compared to quartile 1 (21.4% v. 11.2% respectively, p=0.006). All these studies have methodological limitations, including their design and/or the definition of AKI used, precluding any definitive conclusion. Furthermore, a meta-analysis has also been inconclusive regarding the impact of blood storage on the occurrence of AKI[11]. This question requires investigation with a randomised controlled trial (RCT) to achieve a more definitive result.

The Canadian Age of Blood Evaluation (ABLE) RCT measured a composite outcome of multi organ failure only, without including a staged consensus definition of AKI in its study outcomes[45]. The TRANSFUSE RCT provides a unique, cost-effective opportunity to properly investigate the impact of RBC storage duration on the incidence of AKI and to better understand transfusion-associated AKI.

Significance

This Renal substudy of the TRANSFUSE trial provides a unique opportunity to evaluate the effect of age of RBC on the incidence, severity and duration of AKI in critically ill patients and may also elucidate mechanisms involved in the body's response to RBC transfusion, thereby enhancing the parent study results. Leveraging the infrastructure and study design of the TRANSFUSE trial, this substudy provides a highly cost-efficient opportunity to test a clinically important hypothesis which cannot be answered outside such a large RCT and which would be unlikely to be funded as a separate study.

Methods

Renal function will be assessed in the TRANSFUSE trial using the KDIGO (Kidney Disease, Improving Global Outcomes) classification system[71] and an additional question at 90 day follow-up on the need for ongoing renal replacement therapy. There are no additional procedures required for this substudy.

Inclusion criteria: The same as for the TRANSFUSE trial. All patients recruited to the trial at Australian, New Zealand and other overseas sites that agree to participate in this substudy will be included.

Exclusion criteria: The same as for the TRANSFUSE trial with the following addition: patients with pre-existing end stage renal failure (receiving chronic dialysis) and those already undergoing renal replacement therapy at randomisation will be excluded.

Measurements

Renal function: AKI will be classified using the KDIGO classification system, the latest consensus classification system for AKI which has now been validated and used extensively[71, 72].

Ongoing renal replacement therapy: A question has been added to 90 day follow-up to ascertain whether the patient is receiving ongoing renal replacement therapy.

The only additional data to collect would be baseline creatinine defined as the most recent creatinine pre-randomisation, then the highest daily creatinine thereafter when available during the ICU stay for a maximum of 28 days (the duration of the trial follow-up in ICU). Collection of routine available creatinine values will be performed for this period, with no extra dedicated blood collection required for the substudy. The use of renal replacement therapy while in hospital will also be recorded as part of the TRANSFUSE parent trial.

Outcome measures

Primary outcome:

Cumulative proportion of patients with any degree of AKI

Secondary outcomes:

a) The proportion of patients who develop stage 3 AKI (KDIGO criteria)

b) The proportion of patients requiring ongoing renal replacement therapy at 90 day follow-up

c) The severity of AKI measured as the change in serum creatinine from baseline (continuous variable)

d) The duration of AKI measured as AKI free days (number of days not in stage 1-3 categories) for ICU stay

e) Comparison of the mean creatinine levels across the first 28 days

The fact that we are including several outcomes related to renal function will allow this study to provide a more comprehensive picture of the impact of RBC storage duration on renal function and will increase the possibility of detecting an effect if there is one.

Power calculations and sample size

This substudy will analyse data for 2000 patients. With 1000 patients per group, we will have an 80% power (2 sided p-value of 0.05) to show a difference of 5.3% (19.5% vs 24.8%) of AKI any stage. This is taken from a recent article reporting an incidence of AKI (defined by the RIFLE criteria) of 24.8% in anaemic transfused ICU patients compared to 16.7 % in anaemic non transfused ICU patients ($p<0.05$)[73]. If the harmful effect of blood is mainly due to storage duration[73] a sample size of 550 patients per group would be enough to detect a difference of this magnitude (8.1%, 16.7% v. 24.8%). However, we don't exclude that fresh RBC may also be deleterious to a degree, therefore, we have decided on a more conservative approach with a sample size of 1000 patients per group.

Alternatively, stage 3 AKI (KDIGO criteria) is the most severe stage of AKI and therefore the category in which we are most likely to detect a difference between groups. With 1000 patients per group we will have more than enough power to detect a difference of 10.2% (11.2% vs 21.4%) in stage 3 AKI[70].

Statistical Analysis

Data will be assessed for normality and log-transformed where appropriate. Univariate analysis will be conducted using student t-tests, chi-square test for equal proportion or non-parametric tests where appropriate. Multivariate analysis will be performed adjusting for baseline imbalances and known covariates and confounders. Multiple logistic regression will be used for AKI as a binomial outcome. Linear regression will be used for analysis of continuous outcomes. The difference between groups for daily serum creatinine will be determined using generalised linear modelling fitting main effect for group and time and an interaction between group and time to determine if the two groups behave differently over time. A two-sided p-value of 0.05 will be considered to be statistically significant. All data will be analysed using Intercooled Stata, version 9 and above (Statacorp, College Station, Tx, USA).

Publication

The study will be conducted in the name of Monash University on behalf of the TRANSFUSE investigators. Authorship will be under the names of the sub-study investigators on behalf of the TRANSFUSE investigators. Publication of results will be embargoed until publication of the main study results unless specific permission for early publication is given by the TRANSFUSE management committee.

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