POINT-OF-CARE RBC WASHING TO PREVENT TRANSFUSION-RELATED PULMONARY COMPLICATIONS

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PRIMARY INVESTIGATOR

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Contents

ABBREVIATIONS

ALI	acute lung injury
APACHE	acute physiology and chronic health evaluation
ARDS	acute respiratory distress syndrome
BNP	brain natriuretic peptide
BRM	biological response modifiers
CABG	coronary artery bypass graft
CATS	continuous autotransfusion system
CCL5	chemokine ligand 5
CFH	cell free hemoglobin
CHF	congestive heart failure
Co-I	Co-Investigator
CTMS	Clinical Trials Management System
DUMC	Duke University Medical Center
EDTA	ethylenediamine tetraacetic acid
EHR	electronic health record
FDA	Food and Drug Administration
FiO2	fraction of inspired oxygen
Hb	hemoglobin
Hct	hematocrit
ICU	intensive care unit
IgA	Immunoglobulin A
IL-6	interleukin 6
IL-8	interleukin 8
IQR	interquartile range
ITT	intention to treat
LC	liquid chromatography
LR-RBCs	leukocyte reduced red blood cells

LVBT	large-volume blood transfusion
LVEF	left ventricular ejection fraction
MAP	mean arterial pressure
MAR	missing at random
MC	Mayo Clinic
MCAR	missing completely at random
MS	mass spectrometry
NO	nitric oxide
OR	operating room
PAI-1	plasminogen activator inhibitor-1
PaO2	partial pressure of oxygen in arterial blood
PCWP	pulmonary capillary wedge pressure
PEEP	positive end expiratory pressure
PI	Principal Investigator
RAGE	receptor for advanced glycation end-products
RANTES	regulated on activation, normal T expressed and secreted
RAP	right atrial pressure
RBC	red blood cell
RBC-MPs	red blood cell microparticles
sCD40L	soluble CD40 ligand
SD	standard deviation
SOFA	sequential organ failure assessment
SpO2	peripheral capillary oxygen saturation
SVR	systemic vascular resistance
TACO	Transfusion-associated circulatory overload
TRALI	Transfusion-related acute lung injury
USCIITG	United States Critical Illness and Injury Trials Group
VFD	ventilator free days

Table of Events

Event	Study Day 1	Study Day 2	Study Day 3	Study Day 4	Study Day 5	Study Day 6-28
Baseline Research Blood Draw	X					
Research Blood Draw #2	X*					
Research Blood Draw #3 (6 hours)	X*					
Research Blood Draw #4		X				
Research Blood Draw #5					X	
Daily Assessment for ARDS/TRALI / TACO	X	X	X	X	X	
Monitoring of blood products	X	X	X	X	X	X
Oxygen titration monitoring**	X	X	X	X	X	X
Adverse Event Monitoring	X	X	X	X	X	X

*May actually occur on the next study day if the patient is a late surgical case

**Oxygen titration only occurs on extubated patients

A. Specific Aims

With the **long-term goal** of reducing pulmonary morbidity following perioperative transfusion, **the objective of this proposal** is to test the feasibility, safety, efficacy, and clinical impact of point-of-care washing of allogeneic Leukocyte-Reduced (LR) Red Blood Cells (RBCs). Specifically, we will evaluate the impact of this intervention on intermediate mechanistic and physiologically-relevant markers of transfusion-related acute lung injury (TRALI) and transfusion-associated circulatory overload (TACO). To this end, we propose a multicenter, randomized, phase I/II clinical trial in adult cardiac surgery patients receiving LR-RBCs on the day of surgery.

TRALI and TACO are common and important causes of morbidity following cardiac surgery and are the leading causes of transfusion-related death in the US.[1] Although successful TRALI prevention interventions exist for non-RBC components (e.g. plasma/platelets), **the lack of effective prevention interventions for RBC-associated TRALI and TACO represent critical knowledge gaps**. In this context, the mechanisms underlying these RBC-associated complications remain poorly defined. Increasingly, soluble biological response modifiers (BRMs) within the RBC storage solution are suggested to play an important role.[2-6] **Point-of-care RBC washing may remove these BRMs, thereby mitigating their influence on postoperative transfusion-related complications.**[7-10] Such measures bear significant clinical importance in cardiac surgery which consumes over one million RBC units each year in the US alone.[11] While the pro-inflammatory stimulus of cardiopulmonary bypass and postoperative reduction in left ventricular compliance may partly contribute to respiratory dysfunction, infusion of BRMs residing within stored RBC supernatant may explain why RBC transfusion also represents a major risk factor for postoperative pulmonary complications in this surgical population. Indeed, numerous BRMs in the RBC product [e.g. neutral lipids,[6,12] soluble CD40 ligand (sCD40L),[3] chemokine (C-C motif) ligand (CCL5),[13,14] RBC-derived microparticles (RBC-MPs),[15-17] cell-free hemoglobin (CFH) [18-20]] have been associated with acute transfusion reactions. Accordingly, safe, efficacious, and logistically feasible interventions to reduce RBC-associated respiratory dysfunction are needed.

Washing blood products prior to transfusion has been shown to reduce inflammatory markers in pediatric cardiac surgery patients[21] and improve survival in patients with acute leukemia[22] However, widespread adoption of this practice has been limited by ongoing equipoise regarding its clinical impact as well as the logistical limitations of washing RBCs in the blood bank prior to transfusion.[8] Our preliminary data demonstrate that: **A** we can predict which patients will receive RBC transfusions, thereby facilitating the targeted enrollment of a high-risk cohort, and **B** we can effectively remove BRMs from allogeneic LR-RBC units with a practical, point-of-care, Autotransfusion device. Therefore, to demonstrate the safety, efficacy, feasibility and clinical impact of this approach, we propose the following specific aims and testable hypotheses.

AIM 1: *To determine the feasibility, safety, and efficacy of point-of-care RBC washing using the Continuous Autotransfusion System (CATS) in adult cardiac surgery patients receiving allogeneic LR-RBC transfusion*.

Aim 1a - We hypothesize that point-of-care washing of allogeneic LR-RBCs will be feasible in the time-sensitive environment of cardiac surgery with minimal or no protocol violations.
Aim 1b - We hypothesize that when compared to standard-issue allogeneic LR-RBCs, washed allogeneic LR-RBCs will be associated with a comparable rise in hemoglobin concentration and will not be associated with evidence of increased hemolysis (CFH, haptoglobin) or acute kidney injury in the recipient.
Aim 1c - We hypothesize that RBC washing will reduce TRALI (neutral lipids, sCD40L, CCL5) and TACO (RBC-MPs, CFH) related BRMs in the RBC unit by greater than 80%.

AIM 2: *To demonstrate the extent to which point-of-care washing of allogeneic LR-RBCs impacts the recipient response to RBC transfusion when compared to standard-issue allogeneic LR-RBCs.*

Aim 2a - We hypothesize that RBC washing will reduce post-transfusion concentrations of validated lung injury-associated biomarkers (IL-6, IL-8, vWF, ICAM-1, RAGE, SP-D, PAI-1) in the transfusion recipient.

Aim 2b - We hypothesize that RBC washing will mitigate adverse physiologic consequences of RBC transfusion that have been associated with TACO (elevated mean arterial pressure, systemic vascular resistance, and pulmonary capillary wedge pressure).

Aim 2c - We hypothesize that lower levels of putative BRMs (neutral lipids, sCD40L, CCL5, RBC-MPs, CFH) in transfused RBC components (and in the RBC recipient) will be associated reduced levels of lung injury biomarkers and an attenuated cardiopulmonary response to RBC transfusion.

AIM 3: *To evaluate the impact of point-of-care washing of allogeneic LR-RBCs on recipient clinical outcomes.*

Aim 3a: We hypothesize that compared to standard-issue RBCs, point-of-care washed RBCs are associated with improved clinical outcomes (shorter duration of mechanical ventilation, oxygen supplementation, and ICU stay; improved oxygenation; and improved organ function).

Our large and accessible at-risk population, established clinical trial infrastructure, and multidisciplinary experience/expertise in translational, patient-centered transfusion research will assure this proposal's success.

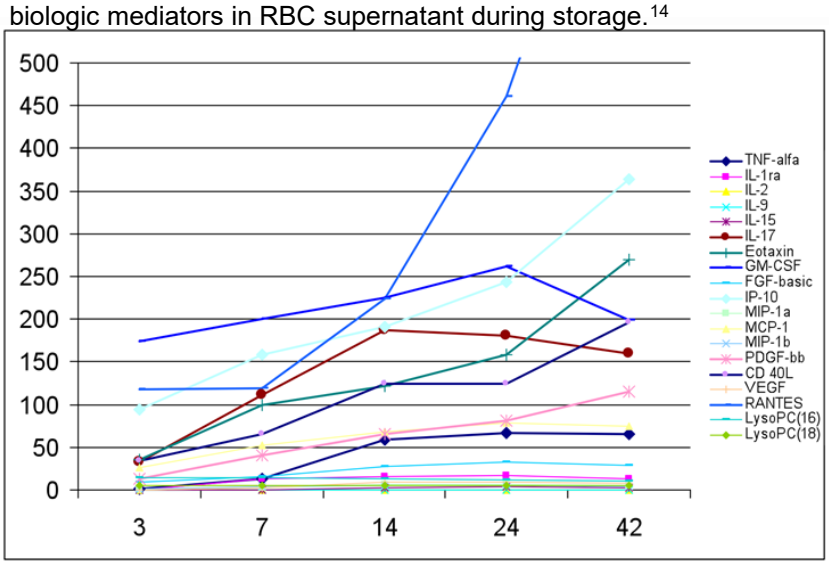
B. RESEARCH STRATEGY

B1. SIGNIFICANCE

Problem statement: Transfusion-related pulmonary complications are the leading cause of serious transfusion-related adverse events. Transfusion-Related Acute Lung Injury (TRALI) remains the leading cause of transfusion-related death in the US and although seemingly less appreciated, Transfusion-Associated Circulatory Overload (TACO) has been the second leading cause of transfusion-related death over the past 5 years.[1] In addition to their associated mortality, both syndromes result in substantial resource utilization and associated health care cost. The majority of patients who develop TRALI will require intensive care unit (ICU) admission and ventilator support, which typically lasts from 3 to 10 days.[23,24] Clinical evidence also notes that up to 21% of TACO cases are life-threatening and associated with increased lengths of ICU and hospital stay.[25-27] Although specific preventative strategies have dramatically reduced the incidence of plasma-associated TRALI (e.g. male-only plasma donation), no prevention strategies exist for RBC-associated TRALI or TACO. Indeed, **the lack of safe and feasible strategies that can mitigate risk of RBC-associated TRALI and TACO represent critical knowledge gaps in transfusion medicine.**

TRALI mechanisms: Although TRALI and TACO share a similar clinical phenotype (pulmonary edema and hypoxemic respiratory insufficiency), each is the result of distinct pathophysiologic processes.[2,4,5,26,28] TRALI is believed the result of a two-hit process that results in leukocyte priming, sequestration, and activation followed by endothelial injury and inflammatory lung edema. The first insult typically relates to recipient factors (e.g. surgery, trauma, or infection) and the second "hit" from the infusion of factors residing in the blood component. For high-plasma volume components (e.g. plasma, apheresis platelets), this is most often the result of anti-leukocyte antibodies within the donor product reacting with recipient cognate antigens. In contrast, multiple lines of evidence suggest alternate mechanisms are at play with RBC-associated TRALI.[2-4,6] Here, the second insult is generally believed the infusion of soluble biologic response modifiers (BRMs) residing in the RBC supernatant. Evidence supporting this theory include: 1) identification of specific BRMs

Figure 1. Temporal changes in the concentration of soluble biologic mediators in RBC supernatant during storage.[14]



which can generate an acute inflammatory response [e.g. bioactive lipids[6,12], soluble CD40 ligand[3], and CCL5 (also known as RANTES)[13,29,30] and RBC-MPs[17,31,32]; 2) association of prolonged storage duration with adverse respiratory outcomes (BRMs accumulate during the storage process, Figure 1)[12,14,15,33,34]; and 3) the potential for mitigating transfusion reactions by washing RBCs prior to transfusion.[21,35-38] Notably, RBC washing has been associated with abrogation of adverse immunologic effects resulting from transfusion in trauma patients, and improved survival in transfusion recipients with acute leukemia.[22,35] Although promising, washing stored RBCs has been largely discounted due to concerns related to cost/feasibility.[38]

TACO mechanisms: The primary mechanism underlying TACO has historically been believed fluid overload.[25,26] However, several lines of recent evidence suggest the potential for additional and potentially synergistic pathophysiologic processes. First, the supposition that excessive fluid volume is the primary mechanism underlying TACO has not been consistently supported by clinical data. We have recently shown the median (IQR) transfusion volume in patients who develop TACO is only 3 (2-7) units.[39] Indeed, a large proportion of reported TACO cases present after a single blood unit exposure. [40,41] Moreover, Roubinian et al. recently compared fluid and transfusion volumes in patients with TACO and TRALI. 42 These investigators reported no statistically significant differences in hourly fluid balance or the number of blood component units transfused in the 24-hour interval preceding the TACO or TRALI episode.

Second, TACO is characteristically associated with a substantial elevation in the systemic blood pressure.[16,29,43] This hypertensive response exceeds what would be expected from the volume challenge alone and suggests the existence of vasoconstricting substances in the transfused product. To this point, Donadee et al. have recently shown significant differences in the blood pressure response to RBC transfusion as a function of storage duration.[18] These responses were strongly correlated with time-dependent increases in nitric oxide (NO) consumption as well as with increased concentrations of RBC-derived microparticles (RBC-MPs) and cell free hemoglobin (CFH), both of which are known to scavenge NO. As a potent microvascular vasodilating substance, NO has substantial influence on endothelial function and systemic vascular resistance (SVR). Indeed, the role of NO scavenging, RBC-MPs, and CFH in endothelial dysfunction and transfusion-related complications has been increasingly described. [16,17,19,20,31,44,453] In regards to TACO, a sudden increase in the SVR has the clear potential to compromise left ventricular function (particularly in transfusion recipients with altered baseline cardiac function), resulting in elevated left atrial pressures and ultimately hydrostatic pulmonary edema (the hallmark of TACO). Notably, recent evidence also suggests that the delivery of therapies specifically targeting NO-mediated pathways (e.g., inhaled [NO44,46] and haptoglobin[47]) can mitigate these adverse responses. In further support of a potential role for RBC-MPs, CFH, and NO scavenging as a mechanism for adverse cardiopulmonary response to allogeneic RBC transfusion, it should also be noted that the primary adverse effect identified with hemoglobin (Hb)-based RBC substitutes is profound hypertension associated with nitric oxide scavenging.[48-51]

As a third line of evidence suggesting alternative TACO mechanisms, our own preliminary data failed to correlate central venous pressure (a measure of intravascular volume status) with post-transfusion hypoxemia in closely monitored, mechanically ventilated patients, receiving single unit RBC transfusion. In contrast, an acute increase in SVR was associated with worsening gas exchange after RBC transfusion (Figure 2).

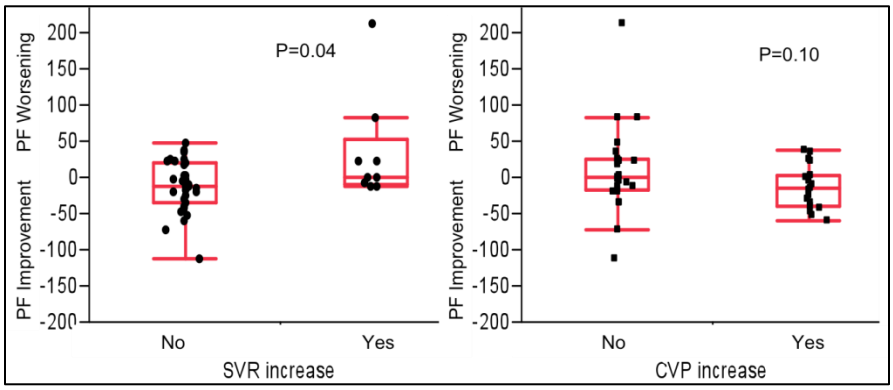


Figure 2. Relative contribution of post-transfusion increase in central venous pressure (CVP) and systemic vascular resistance (SVR) in worsening gas exchange (change in PaO_2/FIO_2 after transfusion) in a prospective study of 48 mechanically ventilated patients receiving an elective transfusion of one unit of stored RBCs. Post-transfusion increase in SVR was associated with worsening gas exchange (left) while CVP increase had no effect on oxygenation (right)

Finally, Blumberg et al. recently evaluated temporal changes in transfusion outcomes and identified a reduced rate of cardiopulmonary complications (including TACO) with implementation of universal leukoreduction.[52] More importantly, **the investigation noted a complete absence of reported TRALI and/or TACO cases associated with washed allogeneic RBCs (n = 28,120 RBC units).** Again, these findings suggest alternative TACO mechanisms and further imply that these mechanisms may be modifiable with RBC washing.

Transfusion-related complications following cardiac surgery: Cardiac surgery is a high-risk endeavor under the best of circumstances. In addition to this baseline risk, multiple studies have associated RBC transfusion with increased risk of adverse events in this population.[53-60] Numerous studies also suggest that respiratory complications are chief among the risks related to transfusion in this setting.[53,59-61] In light of this, as well as the fact that patients who undergo cardiac surgery receive a substantial proportion of the greater than 15,000,000 units of allogeneic RBCs transfused in the US each year[11], the significance of improving transfusion safety and mitigating transfusion-related respiratory complications in this population is clear.

Point-of-care washing of allogeneic RBCs for the prevention of RBC-associated respiratory dysfunction:

Standard issue, 120-micrometer transfusion set filters permit the transfusion of cellular debris, microparticles and soluble mediators present in the supernatant of an RBC unit.[62] RBC washing can remove soluble contaminants in the RBC supernatant including chemokines, biologically active lipids, cellular debris/microaggregates and possibly microparticles.[7-10] **Importantly, however, washing RBCs in the blood bank may not always be the best solution**. A typical device used in blood banks, the Cobe 2991 Cell Processor (Terumo BCT, Denver, CO), is an example of a device designed to remove all traces of plasma, essential for IgA-deficient RBC recipients.63 Importantly, such aggressive washing may damage RBCs. Indeed, this is illustrated in Figure 3 [adapted from O'Leary et al.[63]] where progressive hemolysis of the resuspended, washed RBCs occurred in the post-wash storage period. Notably, post-wash hemolysis appears to be attenuated when using cell-salvage devices. To this point, potassium release is a marker of hemolysis and has been proposed as a washed product quality marker.[64] Recently, O'Leary et al. demonstrated reduced hemolysis when comparing the Continuous AutoTransfusion System (CATS; Fresenius Kabi, Bad Homberg, Germany) to the Cobe 2991 Cell Processor.[63]

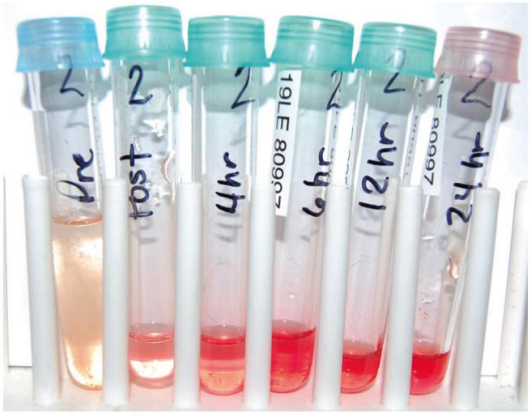


Figure 3. Progressive hemolysis following standard AABB-approved RBC washing procedures (Cobe 2991).

Perhaps even more limiting is the time required for standard blood bank RBC washing procedures (typically greater than 1 hour).[8] This time requirement precludes the feasible implementation of "on-demand" RBC washing in time-sensitive environments such as the operating room and ICU. Moreover, RBC washed units have a 24-hour outdate.[8] Therefore, pre-washing RBCs prior to a known transfusion need risks wastage of this scarce and expensive resource. In contrast, point-of-care bedside RBC washing with cell-salvage devices may provide a practical, and possibly preferable option. Indeed, use of cell salvage devices to reduce allogeneic RBC transfusion during cardiac surgery is recommended by published guidelines[65] and is currently standard of care in most cardiac surgery practices. If washing of allogeneic RBCs using such a device proves effective in mitigating RBC-associated respiratory dysfunction, all RBCs (cell-salvaged and allogeneic) could quite feasibly be washed with such a bedside device.

Of note, cell-salvage devices are also not identical. CATS is based on continuous cell separation with constant application of a low centrifugal force (330-680g)[6658] during separation or "skimming" of the red cells, whereas the more typical Latham bowl-based cell separation devices employ an interrupted/discontinuous, higher centrifugal force (1220-2000g).[66,67] The CATS design is associated with less hemolysis[63] and has proved superior to Latham bowl based cell-salvage devices in the removal of oil emulsions[7], RBC microaggregates[10] and separation of plasma from RBCs.[9] Indeed, Gruber et al. have recently shown elimination rates exceeding

90% (a limit used for quality control in cell salvage) when washing allogeneic RBCs with the CATS device.[8]

Summary on Significance: In light of: 1) the impact of transfusion-related pulmonary complications on patient-important outcomes; 2) the lack of effective preventative strategies for RBC-associated TRALI and TACO; 3) the biologic plausibility for the role of soluble BRMs in RBC-associated respiratory dysfunction; and 4) the ability to remove these BRMs with point-of care RBC washing, we believe this proposal is highly significant. Furthermore, this proposal specifically addresses multiple highlighted topics in this funding opportunity announcement including: 1) Non-infectious complications of blood transfusion including transfusion-related acute lung injury (TRALI); 2) Immunomodulatory, inflammatory and vasoregulatory properties of transfused blood components; and 3) Evaluation of the impact of introducing a new blood product preparation or transfusion strategies on transfusion-associated adverse events and recipients' clinical outcomes.

B2. INNOVATION

At present, there are no effective strategies that can mitigate the occurrence and/or impact of RBC-associated TRALI or TACO. This proposal seeks to evaluate the novel repurposing of a cell-salvage device (CATS) in an effort to provide an innovative strategy to prevent allogeneic RBC-associated pulmonary complications. We will also evaluate novel mechanisms underlying TRALI and TACO. **While specifically focused on BRMs and their association with respiratory dysfunction, our findings will more broadly differentiate the role of soluble BRMs (removed with washing) versus the role of the red blood cell itself (not removed with RBC washing).** We believe this application is highly innovative for the following reasons.

Testing the feasibility and efficacy of point-of-care allogeneic RBC washing: Although washing of stored RBCs has shown promise in isolated settings (e.g. pediatric cardiac surgery[21] and acute leukemia[22]), concerns related to feasibility have prevented larger scale testing of this novel approach to improving transfusion safety. Major limitations with current RBC washing protocols include cost and the time requirements (typically one to several hours).[8] In time-sensitive environments such as cardiac surgery or the ICU, this time delay is not feasible. To address this limitation, this proposal takes an FDA-approved autotransfusion device (CATS) and proposes to repurpose it for point-of-care washing of allogeneic RBCs; an approach that is clearly innovative. If both feasibility and efficacy are established, this would present a novel, feasible and cost effective approach to improving transfusion safety. Importantly, additional costly equipment would not be required as autotransfusion is standard of care for many surgical populations.

There are no current strategies which mitigate the risk of RBC-associated TRALI or TACO: Despite their impact on patient-important outcomes, no effective prevention strategies exist for RBC-associated TRALI or TACO. Development of new strategies that can mitigate the onset and/or severity of these respiratory complications would be novel and highly significant. We proposed to test a promising intervention, point-of-care washing of allogeneic RBCs, in a high-risk population undergoing cardiac surgery. There is clear biologic plausibility for cause-effect relationships between the infusion of soluble BRMs and development of these life-threatening transfusion-related respiratory complications.[3,4,6,13,29,30] Moreover, recent evidence as well as our preliminary data (see below), suggest the washing procedures proposed in this application are effective at removing RBC supernatant (and the BRMs contained within).[7-10] Therefore, we believe our proposed intervention provides an innovative, feasible approach to preventing RBC-associated respiratory complications.

Evaluating new mechanisms for RBC-associated TRALI: Though our understanding of the mechanisms underlying TRALI has improved in recent years, these advances have been primarily limited to antibody/antigen interactions.[2,68] Importantly, these specific episodes typically result from high-plasma containing blood components (plasma, apheresis platelet concentrates, whole blood). In contrast, our current understanding of the processes underlying non-antibody mediated TRALI (the primary mechanism believed responsible following RBC transfusion) remain quite limited. Indeed, the great preponderance of data in this regard has been generated by Dr. Christopher Silliman, a consultant on this grant application.[4-6,12,38] We believe our innovative approach (evaluating the concentration of potentially putative BRMs in the donor unit, their change with RBC washing, the relationship between their concentration in the transfusion recipients and

intermediate markers of lung injury) will provide essential new hypothesis-generating mechanistic insights into the associations between RBC transfusion and post-transfusion lung injury.

Evaluating new mechanisms for RBC-associated TACO: Despite its clear significance to transfusion recipients and the healthcare team, our understanding of the mechanisms underlying this life-threatening syndrome remain incomplete.

Although volume overload likely plays a key role in many cases of TACO, a growing body of evidence suggests the potential for alternate and potentially synergistic mechanisms that may present important opportunities for TACO mitigation. Increasingly, evidence suggests a role for NO consumption, via RBC-MPs[16,17] and CFH[18-20,46,47,69] in the hemodynamic response to RBC transfusion. However, our understanding of how these processes impact TACO pathogenesis remains incomplete. This is the first significant attempt to improve our understanding of the pathophysiology underlying TACO. The targeted study of RBC-MPs and CFH in the RBC unit (as well as the transfusion recipient), and the evaluation of their association with the recipient's physiologic response to transfusion, is novel and with high scientific merit.

APPROACH

We propose a multicenter, phase I/II clinical trial evaluating the feasibility, safety, efficacy, and clinical impact of point-of-care washing for allogeneic LR- RBC units in patients undergoing cardiac surgery. **Our group has demonstrated experience and expertise in the translational study of transfusion therapies and patient-important transfusion-related respiratory complications.** We also have significant experience with the design and conduct of clinical trials. Pertinent preliminary data are summarized here.

Adequacy of the study population to ensure feasibility of the protocol: Cardiac surgery remains a leading consumer of RBC component therapies.[11] At Mayo Clinic in Rochester, MN, there were 2,344 unique patients who underwent cardiac surgery in 2011. A total of 1,059 of these (45%) were transfused on the day of their surgical procedure with 310 patients receiving 4 or more units. In the cohort of 10,653 patients undergoing CABG or CABG/valve surgery at Duke University Medical Center between 2000 and 2010, 28% of patients received between 4 and 20 units of allogeneic RBC. Last year, Duke University Medical Center performed 1,343 cardiac operations in total of which ~350 were isolated Aortic or Mitral Valve repair/replacement. The remaining 1,000 cases would be of equivalent or greater risk of transfusion than the CABG and CABG/valve cohort. Therefore, at least 250 patients per year will be eligible for screening for this proposal. Of note, approximately 75% of allogeneic transfusions in this population are administered on the day of the surgical procedure (based on our preliminary data from 2011).

A prediction model for RBC requirements in patients undergoing cardiac surgery[70]: Utilizing a 10-year study population of isolated CABG surgery patients at Duke University Medical Center (n = 5,887), our team (Co-PI: Welsby) developed a prediction model for estimating perioperative RBC requirements in this setting.[70] The model performed well with derivation and validation data set c-indices of 0.79 and 0.78, respectively. Applying this model in more complex cardiac surgery populations (combined CABG/valve, aortic repair/replacement, etc.) suits our purposes well, as it will likely underestimate RBC needs for these more complex procedures. Entering the predictive variables (see Table 1) into an on-line calculator tool, will enable screeners to enroll patient expected to receive 4 or more units of allogeneic RBCs into the proposed clinical trial.

Table 1. Factors predictive of RBC transfusion in CABG surgery.

Variable	Maximum likelihood estimate	p value
Hct	−0.0310	0.0006
Age	0.00670	0.6090
Female sex	0.7934	<0.0001
Number of grafts	0.4292	<0.0001
LVEF	−0.0794	<0.0001
Weight	−0.0132	<0.0001
Serum creatinine	0.2967	<0.0001
Diabetes	2.0958	<0.0001
Stroke	0.3250	0.0042
Hct < 40%	0.7965	<0.0001
Weight < 70 kg	0.5010	<0.0001
Hct*diabetes	−0.0472	0.0003
Age*LVEF	0.00101	<0.0001

* Denotes an interaction term.

Clinical trial experience in transfusion medicine and lung injury prevention[71,72]: Our team (Co-PI: Kor; Co-I: Gajic) has experience in the design and conduct of multicenter clinical trials evaluating the impact of storage duration on respiratory and immunologic outcomes in populations similar to those targeted in this

proposal. More specifically, we recently completed a two-center (Mayo Clinic and the University of California-San Francisco) randomized, double-blind clinical trial which compared fresh (< 5 days storage) to standard-issue single-unit RBC transfusion in adult, intubated and mechanically ventilated patients.[71] Novel automated hemoglobin screening algorithms were employed to identify, enroll, and randomize fifty patients to receive fresh RBCs an additional 50 to standard-issue RBCs. The primary outcome evaluated was the change in partial pressure of arterial oxygen to fraction of inspired oxygen concentration ratio ($\Delta PaO_2/FIO_2$) with secondary outcomes evaluating changes in immunologic and coagulation status. Although no significant differences were noted in the endpoints evaluated (Table 2), the limited sample size, restriction to single unit RBC transfusion, and short duration of follow-up (median of 1.9 hours) were notable limitations with this investigation.

Table 2: Pertinent outcomes in a randomized controlled trial evaluating single-unit fresh versus standard issue RBC transfusion.

Outcomes*†	Fresh RBC Cohort ($n = 49$)	Standard Issue RBCs ($n = 50$)	Difference of the Means for Standard Issue and Fresh RBCs (95% CI)	*P* Value
Pulmonary				
$\Delta Pa_{O_2}/F_{I_{O_2}}$, mm Hg	2.5 (49.3)	−9.0 (69.8)	−11.5 (−35.3, 12.3)	0.22
$\Delta Vd/V_T$, %	−1.8 (3.7)	0.3 (5.4)	2.1 (0.2, 4.0)	0.10
ΔStatic compliance, ml/cm H_2O (n = 52)	−0.5 (11.0)	1.3 (10.9)	1.8 (−4.2, 7.8)	0.34
ΔDynamic compliance, ml/cm H_2O (n = 66)	−0.4 (7.8)	0.7 (7.8)	1.1 (−2.7, 4.9)	0.16
Immunologic				
ΔTNF-α, pg/ml	0.7 (5.8)	0.1 (1.1)	−0.6 (−2.3, 1.1)	>0.99
ΔIL-8, pg/ml	5.3 (153.7)	−23.2 (100.3)	−28.5 (−80.6, 23.6)	0.50
ΔCRP, mg/dl	0.4 (1.1)	0.7 (2.2)	0.3 (−0.4, 1.0)	0.88
Coagulation				
ΔFibrinogen, mg/dl	0.9 (71.4)	−2.2 (42.1)	−3.1 (−27.0, 20.8)	0.36
ΔATC	0.1 (8.7)	−1.2 (8.1)	−1.3 (−4.7, 2.1)	0.59

Using the multicenter, interdisciplinary infrastructure of the US Critical Illness and Injury Trials Group, our investigative team (Co-PIs: Kor, Welsby; Co-Is: Gajic, Carter) has also helped to design and conduct the first multicenter clinical trial evaluating the safety and efficacy of a promising ALI prevention agent (ClinicalTrials.gov ID: NCT01504867).[72] Specifically, this trial is evaluating the safety and efficacy of aspirin as an ALI prevention agent in patients at high risk for ALI. Sixteen geographically diverse institutions throughout the US are enrolling patients into this trial and enrollment is currently exceeding plan.

Experience with the study of transfusion-associated circulatory overload [39] : Our team (Co-PI: Kor; Co-I: Gajic) has significant experience with the study of TACO epidemiology and case recognition. In a retrospective observational cohort study, we evaluated risk factors for TACO among 901 patients who were transfused in the medical ICU setting.[39] Fifty-one (6%) of these patients developed TACO and 16 were associated with RBC transfusion. Several baseline predictors (e.g. left ventricular dysfunction; plasma ordered for the reversal of anticoagulant therapy) and transfusion factors (Table 3) were identified as TACO risk predictors. RBC storage duration was longer in the TACO cohort, but this did not meet significance in the underpowered subgroup analyses.

Table 3: Transfusion characteristics of TACO cases and matched transfused controls.

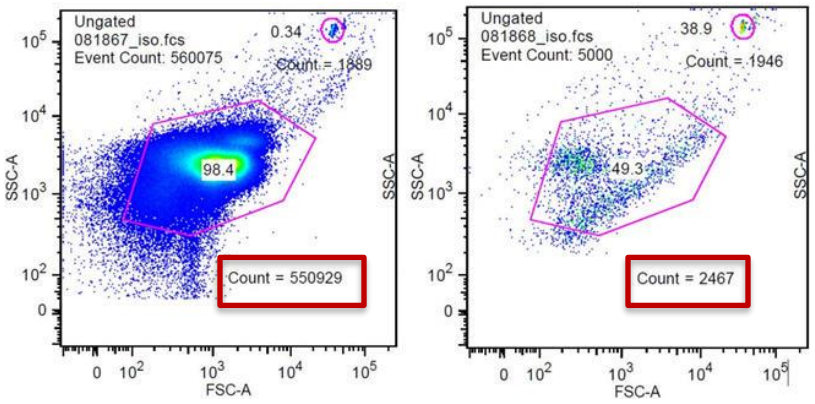
Variables	Matched pairs	Cases	Controls	OR (95% CI)	p value
Lowest Hb*†	51	8 (7-9)	8 (7-9)	0.78 (0.58-1.03)	0.081
Number of units (total)†	51	3 (2-7)	2 (2-3)	1.45 (1.12-1.88)	0.005
RBCs†	51	2 (1-4)	2 (1-2)	1.30 (0.99-1.70)	0.06
FFP†	51	0 (0-4)	0 (0-0)	1.39 (1.07-1.80)	0.005
PLTs‡					
Storage days for RBCs†	34	22 (17-30)	19 (16-29)	1.02 (0.97-1.08)	0.472
Total plasma (L)†	51	0.41 (0.07-1.02)	0.07 (0.07-0.15)	4.88 (1.55-15.36)	0.007
Transfusion rate (mL/hr)†	51	225 (135-350)	168 (100-205)	1.88 (1.06-3.33)	0.031
Fluid balance (mL)†	51	1445 (830-3520)	830 (350-1700)	1.38 (1.12-1.71)	0.003

Recently, our team (Co-PI: Kor; Co-I: Gajic) also developed innovative electronic health record (EHR) surveillance algorithms which can be used to facilitate the detection of transfusion-related pulmonary complications.[54] Using a study population acquired from a NIH-sponsored prospecrtive cohort investigation,[55], we developed optimal TRALI and TACO case detection algorithms using data contained within the EHR.

These algorithms accurately identified TRALI cases with a sensitivity and specificity of 83.9% (95% confidence interval [CI], 74.4%-90.4%) and 89.7% (95% CI, 80.3%-95.2%), respectively. For TACO, the sensitivity and specificity were 86.5% (95% CI, 73.6%- 94.0%) and 92.3% (95% CI, 83.4%-96.8%), respectively. Notably, of true-positive cases identified using the screening algorithms (TRALI/transfused ALI, n = 78; TACO, n = 45), only 11 (14.1%) and five (11.1%) were reported to the blood bank by clinical service, respectively.

The Continuous AutoTransfusion System (CATS) can eliminate potentially dangerous BRMs from stored allogeneic RBCs: In addition to previous work demonstrating effective eliminiation of protien[8], CFH[8], and RBC microaggregates[10], our preliminary data suggest effective elimination of additional BRMs as well. Specifically, 4 LR-RBC units were washed with the CATS device using a 4:1 dilution process as previously described.[8] RBC-MPs, sCD40L, and CCL5 concentrations were assessed before and after the washing procedures. The reduction in microparticle concentration exceeded 80 % in all cases evaluated with a median (IQR) percent change of 92.2% (82.2% – 98.7%). Similar reductions were noted in CCL5 for all 4 units evaluated and in sCD40L for 2 of the 4 units. The remaining two units had low levels of sCD40L in the pre-wash sample. A representative pre-, post-wash sample evaluating RBC-derived microparticle counts is presented in figure 4.

Figure 4. RBC microparticle counts before (left) and after (right) washing with the CATS.



Biomarkers as intermediate outcomes for clinical lung injury (Grant #:K23HL112855-01): Our team has substantial experience in the study and use of biomarkers as intermediate surrogate outcomes for clinical lung injury. Our current K23 award (PI: Kor) specifically aims to evaluate the use of biomarkers to better understand the mechanisms underlying postoperative acute respiratory distress syndrome (ARDS). As prelminary data for the present proposal, we evaluated the biomarker profiles of 12 patients who developed ARDS following cardiovascular surgery with 12 matched controls who did not. As can be seen in Figure 4, differing biomarker profiles were noted in those who developed postoperative ARDS when compared to those who did not. In light of this preliminary data, we have refined our biomarker panel in this A1 resubmission to include IL-6, IL-8, PAI-1, and RAGE as the intermediate outcomes for Aims 2a/c of this proposal.

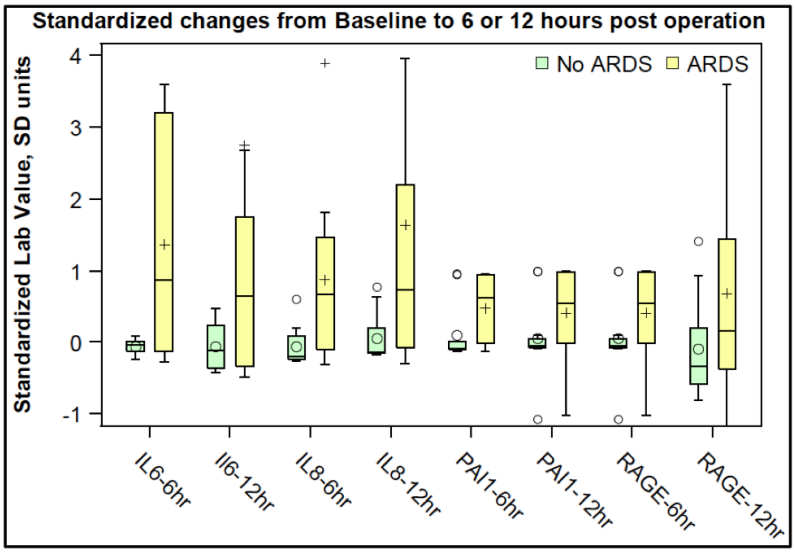


Figure 4. Lung injury biomarkers in patients who do and do not develop ARDS.

PHASE I/II CLINICAL TRIAL EVALUATING POINT-OF-CARE RBC WASHING TO PREVENT RBC-ASSOCIATED RESPIRATORY COMPLICATIONS FOLLOWING CARDIAC SURGERY

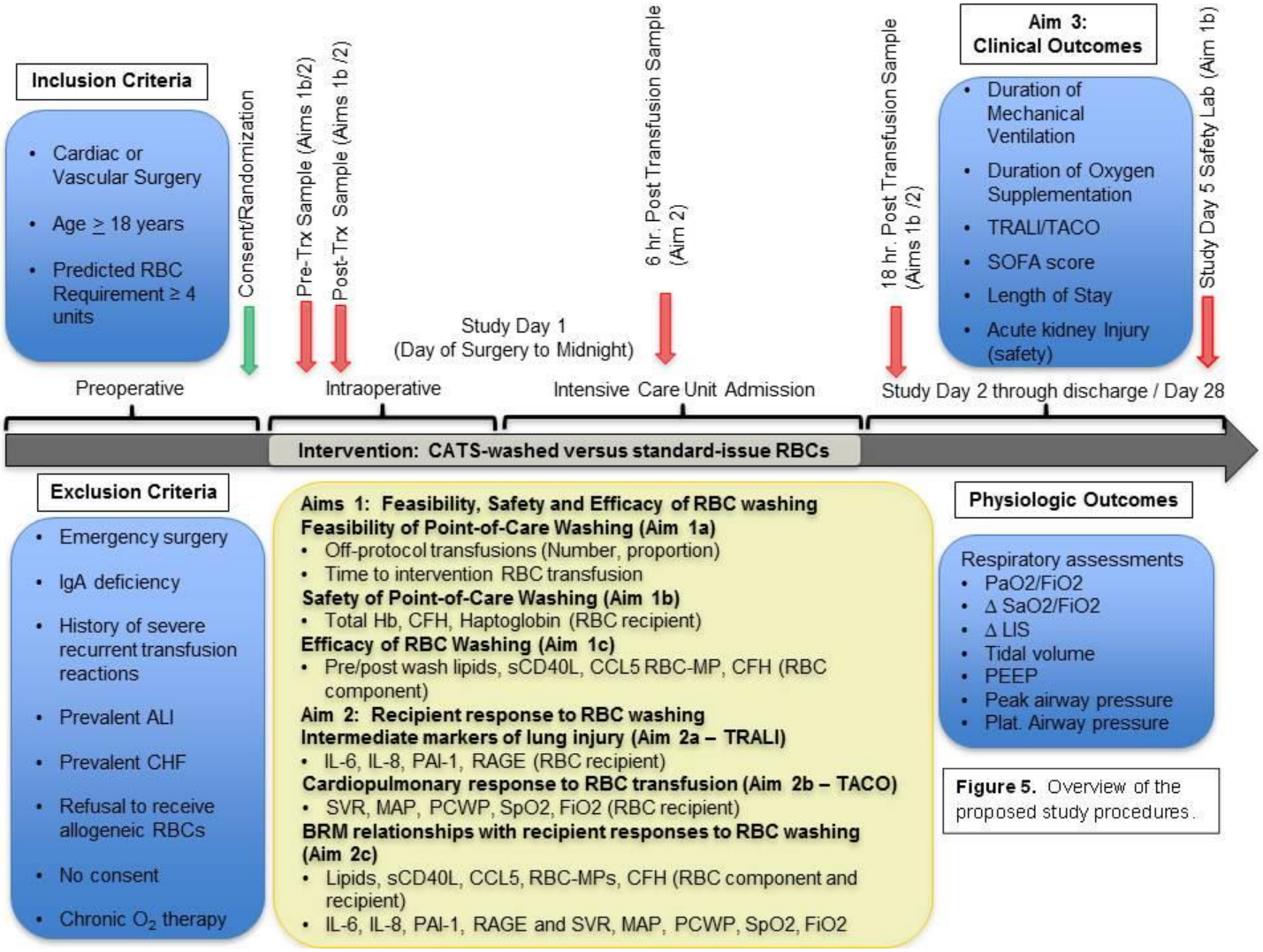


Figure 5. Overview of the proposed study procedures.

Study population: Study procedures are summarized in Figure 5. We will enroll a total 154 patients receiving at least one RBC transfusion on the operative day. We anticipate this will require randomizing up to **190 patients** in this trial (95 per group). Adult patients (age ≥18) who are planned to undergo cardiac surgery at one of the two participating institutions and who have a predicted RBC transfusion requirement of ≥ 4 units will be screened for the exclusion criteria outlined in Table 4. Predicted RBC transfusion requirements will be determined utilizing a recently published large-volume blood transfusion (LVBT) prediction model (>4 units of RBC).[73] A cut off of 4 predicted units of RBC administration was chosen because: 1) this transfusion volume has been associated with increased risk of pulmonary complications following cardiac surgery[58]; 2) we have previously identified this transfusion volume as a common "dose" in patients who experience TRALI and/or TACO[28, 39,42]; and 3) this threshold would still identify a sizable cardiac surgery population, ensuring study feasibility.

Study procedures:

Screening: Study coordinators will screen all adult (age ≥ 18) patients who are scheduled to undergo coronary artery bypass graft surgery with or without valve repair/replacement, complex cardiac valve surgery, pericardial resection, and/or ascending aortic surgery in one of the two participating institutions. Eligible patients with a predicted transfusion requirement of 4 or more units will be approached before their elective surgical procedure by a study coordinator for consent. After confirming the patient's inclusion and exclusion criteria, the study coordinator at the respective institution will obtain informed consent and assign a study ID number to the study participant. Screening logs will be maintained at each site to allow generation of a CONSORT diagram.

Table 4. Exclusion criteria.

Exclusion Criteria	Justification
• Emergency surgery	Inability to randomize/perform study procedures
• IgA deficiency	Not ethical to randomize to standard issue RBCs
• History of severe recurrent transfusion reaction	Not ethical to randomize to standard issue RBCs
• Refusal to receive allogeneic RBCs	Inability to administer intervention of interest
• Refusal to provide informed consent	Not ethical to enroll into trial
• Prevalent Acute lung injury prior to randomization	Inability to adequately assess outcome
• Prevalent hydrostatic pulmonary edema prior to randomization	Inability to adequately assess outcome
• Expected hospital stay < 48 hours	Incomplete study procedures and outcome data
• Not anticipated to survive > 48 hours	Incomplete study procedures and outcome data
• Previously enrolled in this trial	Violation of the independence assumption
• No intention to measure cardiac output parameters during surgical procedure	Inability to assess key physiologic parameters outlined in the study protocol
• Use of home oxygen therapy	Inability to assess oxygen use outcome
• Complex RBC antibody profiles	Washing not feasible due to testing delays
• Need for the use of irradiated RBCs	Intervention contraindicated
• Intention to place a cardiac mechanical assist device	Inability to assess key physiologic parameters outlined in the study protocol

Randomization: Randomization will occur at the time of consent and analyses will be conducted using a modified intention-to-treat principle (See statistical considerations below). Randomization to RBC washing or control group (standard-issue RBC) will be conducted by the study's electronic data management system's Balance (Medidata) algorithm. This software uses dynamic minimization stratified by clinical center. The software will return a confirmation of the randomization indicating the study participant's treatment allocation status. A note will be placed into the EHR identifying the patient as a study participant and the transfusion medicine service will have unblinded, electronic access to the treatment assignment. Randomization and screening will halt after the 154th study participant has undergone surgery and received at least one RBC transfusion on the operative day. Patients randomized but awaiting surgery will continue study participation.

Study intervention: The intervention in this investigation will be implemented for all allogeneic RBCs administered on the day of sugery (intraoperative and postoperatively on Study Day 1). The decision to administer an allogeneic RBC transfusion will be left to the responsible clinical service and will not be prespecified in the study protocol. We have elected to leave this decision to the clinical service for the following reasons: 1) the target population frequently experiences major acute blood loss; in these circumstances, typical measures assessing the need for RBC transfusoin (e.g. Hb) are often associated with unacceptable time delays and frequently do not reflect true red blood cell mass nor need for RBC transfusion; 2) this design facilitates a more meaningul understanding of the feasibility of RBC washing in clinical practice. Upon receiving an order for allogeneic RBC transfusion, the transfusion medicine service will prepare the RBC product according to the allocated treatment assignment (intervention versus control). Of note, all allogeneic RBC units at the two participating institutions undergo pre-storage leukocyte reduction.

-Intervention cohort: For patients randomized to receive washed RBCs, all allogeneic RBCs **administered** on the day of surgery will be washed with the CATS device prior to transfusion. To account for the time

required to complete the ordering, delivery, washing, and administration procedures, we will restrict RBC washing to those RBC units ordered prior to 23:30 on the day of surgery. Any RBC unit ordered after 23:30 on the day of surgery will no longer be considered an "intervention unit" and will therefore be standard issue (non-washed) as it anticipated that the time required to perform the activities mentioned above (ordering, delivery, washing, and administration) would otherwise result in the RBC component's actual administration time to occur on the day after surgery. When the need for transfusion is identified by the primary team, the RBC product will be prepared. As previously described[8] and confirmed in our preliminary data, pre-dilution of stored, allogeneic RBCs results in the most effective elimination of supernatant. Therefore, an approximate 4:1 dilution will be added to the reservoir of the CATS by gravity drainage. The "High Quality" wash mode option will be selected for processing; this takes approximately 7-10 minutes to complete.[74] Washed RBCs will then be drained from the reinfusion reservoir into sterile transfer bags (Fenwal Inc, Lake Zurich, ILL) for transfusion.

-Control cohort: For patients randomized to the control group arm, all RBCs administered on the day of surgery will be standard-issue RBCs.

Off-protocol transfusions: In the setting of cardiac surgery, it is occasionally necessary to provide allogeneic RBCs in an emergent fashion (e.g. acute, life-threatening bleeding). In this particular circumstance, time-delays relating to study-related activities may prove unsafe. To address this potential scenario, our study protocol will allow the administration of emergency "off-protocol" allogeneic RBCs. "Off-protocol" RBC transfusions will be administered as per standard institutional practice. These RBC transfusion episodes will be specifically noted as "off-protocol" and will be summarized and analyzed to assist in assessing the feasibility of point-of-care RBC washing in patients underoing cardiac surgery (see statistical description below). In addition, autotransfusion ("cell-saver") is frequently used in this patient population. Cell-salvage will be implemented at the discretion, and under the direction, of the clinical team. If cell-salvage is employed, the device used for this procedure will be distinict and separate from the intervention CATS device.

Blinding: In light of the time-sensitive, point-of-care nature of the intervention being proposed, the patient, clinical team, and members of the study team involved in the RBC washing procedure will not be blinded to the patient's treatment allocation status. Blinding will be ensured for Drs. Norris and Silliman who will be running the proposed biomarker analyses.

Other aspects of care: ARDSnet trials have demonstrated the benefit of standardizing clinical processes in ICU trials.[75,76] In this study, clinical care that may affect development of respiratory dysfunction and associated outcomes will be standardized to the extent possible. To this end, our protocol will specify optimal ventilator strategies for both the OR and ICU environments (tidal volume $\leq$ 8 mL/kg predicted body weight; peak inspiratory pressures < 35 cm H_20, PEEP ≥ 5 cm while ventilated in the ICU). Similarly, although we will not pre-specify RBC transfusion thresholds in this protocol, we will advise restrictive transfusion practices in the postoperative period (hemoglobin target > 8 g/dL in the absence of acute bleeding and/or ischemia). This transfusion threshold was chosen as it is the current standard of care at the two participating institutions. Standardization of best practices in at-risk patients will decrease the heterogeneity of the risk modifiers that may otherwise confound our associations of interest (RBC washing and intermediate markers of respiratory outcomes). Additionally, each center has adopted protocols on daily spontaneous awakening and spontaneous breathing trials to facilitate standardized weaning from ventilators (see attached appendix for specific protocols). Non-intubated patients will undergo standard titration of oxygen twice daily (at 0700 and 1900, +/- 2 hours). Patients saturating 92% or greater on room air will not receive supplemental oxygen, unless specifically requested by the primary service. If the primary care service requests oxygen supplementation for a patient saturating greater than or equal to 92% on room air, the reason for the deviation from oxygen weaning will be documented. Patients will continue to undergo evaluation for oxygen titration until liberation from oxygen therapy for 24 hours, hospital day 28, or hospital discharge, whichever comes first.

Outcome Measures: To help ensure the integrity and quality of the laboratory analyses, all assays will be performed by our collaborators, Drs. Norris and Silliman. The safety outcome analyses (aim 1b) will be an exception as these will be run in "real-time" at each enrolling institution. All clinical outcome variables will be assessed by trained study coordinators using standardized definitions and operating procedures. To the

extent possible, these study coordinators will also be blinded to the study participant's treatment allocation status.

Specific Aim 1: The **primary feasibility outcome in aim 1a** will be the number and proportion of "off-protocol" allogeneic RBC transfusions administered during the study intervention period (day of surgery). A secondary feasibility outcome will be the time required for the RBC washing procedures (defined as the time from determination of RBC need by the clinical team to time of delivery of the RBC unit to the clinical team). This time will be computed for all patients and all transfusions during the study intervention period (in both the intervention and control cohorts). The **primary safety outcome in (aim 1b) include** the change in the RBC recipient's Hb concentration from pre- to post-transfusion as well as the concentration of CFH and haptoglobin following RBC transfusion. To assess the primary safety outcomes, samples for total Hb, CFH, and haptoglobin will be obtained prior to transfusion, as soon as possible after the first transfusion, but no later than 30 minutes following the first intervention/control RBC transfusion, as well as 6 hours (+/- 30 minutes) and 18 hours (+ / - 30 minutes) from the end of first study RBC transfusion for all study participants. We will also assess for clinical evidence of acute kidney injury throughout the hospitalization. Safety labs will also be drawn anytime on study day #5 if the patient is still in the hospital. The **primary efficacy outcome in aim 1c** will be the change in the concentration of neutral lipids [arachidonic acid, 5-hydroxyeicosatetranoic acid (HETE), 12-HETE, 15-HETE], sCD40L, CCL5/RANTES, RBC-MPs, and CFH in the RBC component from the pre- to the post-wash phase. These data will allow us to calculate the CATS-related elimination rates for these BRMs, which have previously been associated with transfusion-related respiratory dysfunction and hemodynamic alterations. To balance assay costs while ensuring the scientific success of aim 1c, pre- and post-wash samples will be obtained/analyzed for the first 75 washed RBC units. If efficacy is not clearly established, we will continue to obtain both pre- and post-wash samples for 75 additional units.

Specific Aim 2: The outcomes measures for aim 2a will be the change in concentration of multiple, validated lung injury biomarkers representing the primary pathways leading to development of lung injury (Table 5). Patients will have blood drawn as soon as possible after the decision to transfuse, as soon as possible after transfusion, but not later than 30 minutes after the end of first study RBC transfusion, 6 hours (+ / - 30 minutes) from the end of first study RBC transfusion, and 18 hours (+ / - 30 minutes) from the end of first study RBC transfusion.

Table 5. Lung injury biomarkers and exploratory potentially pathogenic biologic response modifiers for specific aim 2.

Validated lung injury biomarkers (Aim 2a)	Primary process evaluated	Supporting Reference
Interleukin-6	Inflammation	77-79
Interleukin-8		77-81
Von Willebrand Factor	Endothelial activation/injury	77,80-8570,73-76
Intercellular Adhesion Molecule-1		77,80,86-89
Receptor of Advanced glycation end-products	Epithelial Injury	80,93
Surfactant Protein-D		77,80,90,91
Plasma activator inhibitor-1	Dysregulated coagulation	79,86,94-96
Exploratory biomarkers (Aim 2c)	**Primary process evaluated**	**Supporting Reference**
Neutral lipids	Lung inflammation	6,12
sCD40L		3
CCL5/RANTES		13,29,30
RBC-derived microparticles	NO scavenging	16-18
Free Hemoglobin		18-20
N-terminal Brain Natriuretic Peptide	Ventricular stretch/volume-overload	97-99

In addition to these biomarker analyses, we will proceed with detailed assessment of the cardiopulmonary responses to study RBC transfusions administered in the operating room (**Aim 2b**). Specific variables to be assessed and recorded are listed in Table 6. Systemic vascular resistance will be calculated at each time point using the following equation: SVR (dyns/cm^5) = [(MAP – RAP)/CO] x 80. Each of these variables will be assessed and recorded immediately prior to the intervention RBC transfusion and again immediately after the RBC transfusion. Standard operating procedures for these cardiopulmonary assessments will be defined prior to study onset according to previously established recommendations.[100] These secondary outcomes will allow a more detailed assessment of the cardiopulmonary response to RBC transfusion and will be expected to provide important insight into the pathophysiology of TACO. Of note, pulmonary artery catheter placement is standard of care for this patient population at both of the enrolling institutions.

Table 6. Physiologic parameters to be measured during the study intervention period.

Respiratory Variables	**Hemodynamic Variables**
PaO_2, SpO_2, FiO_2	Mean arterial pressure
Tidal Volume	Heart Rate
Peak inspiratory pressure	Cardiac output
Plateau airway pressure	Central venous/right atrial pressure
Positive end-expiratory pressure	Pulmonary artery wedge pressure

The exploratory putative BRMs in Table 5 have been selected to evaluate specific potential mechanistic pathways for TRALI and TACO (**aim 2c**). N-terminal BNP has been included as a marker of volume overload/ventricular strain. To better elucidate the relationship between the "dose" of these soluble BRMs in the RBC components, their subsequent concentration in the recipient, and their ultimate relationship to recipient's cardiopulmonary response to transfusion, levels of these potential putative agents will be determined in the RBC components prior to transfusion (in both the washed and standard issue cohorts) as well as in the transfusion recipient. As we are targeting surgical patients who are expected to receive > 4 units of allogeneic RBCs, we will obtain samples from the RBC component for all RBC units administered up to and including the fourth unit for each study participant. This will be a post-wash sample in those randomized to the RBC washing arm. Of note, we recognize the potential for incomplete capture of relevant information in those who receive larger volumes of RBC transfusion. However, we believe the four-unit cutoff to be the best compromise between study feasibility and scientific validity. In the recipient, samples for the putative BRM assessments will be obtained at the same time points outlined above for the lung injury biomarker samples.

Specific Aim 3: To facilitate the design and conduct of future clinical trials, we will also pursue a number of exploratory clinical outcomes. **The clinical outcome measure of interest for aim 3** is the duration of postoperative mechanical ventilation. Study coordinators will collect data daily until endotracheal extubation, death or hospital discharge regarding the need for mechanical ventilation. The primary clinical outcome will then be determined by calculating the duration of mechanical ventilation in hours for each study participant, determined by subtracting the time of ICU admission from the time of endotracheal extubation. If the study participant is extubated prior to ICU admission, the duration of mechanical ventilation will be assigned as 0 hours. Recognizing the potential for early death (intraoperative or early postoperative) biasing our primary clinical outcome, the number of ventilator free days (VFD) at postoperative day 28 will also be determined. Those who die prior to day 28 will be determined to have had 0 VFD. Those who are discharged from the hospital alive prior to day 28 will be assumed to have had no additional days of mechanical ventilation following hospital discharge. Additional secondary outcomes for Aim 3 will include evaluations of hypoxemia (SpO_2, PaO_2/FiO_2), duration of oxygen supplementation, sequential organ failure assessment scores, and durations of ICU and hospital stay. The clinical diagnoses of TRALI, possible TRALI, and/or TACO will be measured through study day 5, or hospital discharge, whichever comes first.

Specimen Handling and Laboratory Assays: **Aim 1b:** 5 ml of blood will be drawn from each study participant at baseline, following the first RBC unit administered, 6 hours after the end of the first RBC transfusion, 18 hours after the end of first RBC transfusion, and on hospital day 5 if the patient remains hospitalized (see *Specific Aim 1* and *Specific Aim 2* for more details on the timing of blood draws). These safety laboratory

assessments (total Hb, CFH, haptoglobin) will be analyzed locally at the enrolling sites using standard clinical assays. **Aim 1c:** A 6-ml sample will be taken pre- and post-wash from the already anticoagulated intervention RBC units. **Aim 2:** 10 mls of blood will be drawn from each study participant at baseline, following the first RBC unit administered, at 6 hours after the end of first study RBC transfusion, and 18 hours after the end of first study RBC transfusion. All samples will be centrifuged, alloquoted and frozen. Samples will be batch shipped to Mayo Clinic Rochester and analyzed in Dr. Norris' laboratory at Blood Systems Research Institute (BSRI), and a separate aliquot provided for Dr. Silliman's laboratory (neutral lipids only).

Biomarkers: Thawed platelet poor plasma will be diluted with assay buffers and measured (a**ims 1c/2c**: sCD40L, CCL5/RANTES; **aims 2a/2c**: IL-6, IL-8, PAI-1; Plasma will be used to perform ELISA-based measurements of RAGE (a**im 2a/2c**). NT-proBNP (a**im 2c**) will be measured.RBC-derived microparticles **(aims 1c/2c):** Thawed platelet-poor plasma will be spun, then labeled in preparation for flow cytometric measurement Vesicles will be lysed with NP-40 detergent and samples re-run to confirm results and allow setting of gates. Free hemoglobin **(aims 1c/2c): will be measured.** Neutral lipids **(aims 1c/2c):** Following the addition of ice-cold methanol, proteins will be precipitated, and non-polar lipids will be extracted/analyzed using high-pressure liquid chromatography (LC) interfaced into the electrospray source of a triple quadrupole mass spectrometer (MS) (liquid chromatography coupled to electrospray ionization mass spectrometry [LC/MS/MS]). Lipid concentrations will be estimated using ratios to an internal standard ($^{2}H^{8}$-5-HETE), as previously described.[101,103]

Predictor variables other than the intervention: Pertinent baseline demographics and clinical characteristics such as age, sex, race, and comorbidities will be recorded. Additional predictor variables will include: vital signs and laboratory values that are obtained during the course of routine care, APACHE IV score, administration of statins, ace-inhibitors, insulin, amiodarone, or steroids; blood product administration for the up to day 28 or hospital discharge, whichever comes first, daily fluid status, and vasopressor requirements.

Quality Control and Data Management: Mayo Clinic will serve as the DSCC to coordinate data collection, quality control and data analysis. The principal investigators at each study site will be responsible for the quality control of data collection according to standard definitions that are provided in the protocol. In addition, the strategies to achieve a high level of protocol adherence will include 1) refresher education sessions for study coordinators two weeks after the study onset, 2) periodic checks of protocol compliance by the research coordinators at the DSCC and 3) computerized identification of protocol violations in the database.

Mayo Clinic has implemented an enterprise-wide Clinical Trials Management System (CTMS). CTMS is a 21 CFR Part 11 compliant data management infrastructure to support multi-center clinical trials and participant registries. The core of the CTMS project is the Medidata RAVE product. Briefly, Medidata RAVE will serve as the electronic data capture and randomization system for the study. The system has comprehensive audit trails, user authentication, security and/or disaster plans, and standardized training for end users. The system provides real-time, cross-form data integrity checks to maximize data integrity while lessening the need for on-site source document verification (See Facilities and Resources).

Statistical Considerations

(prepared in collaboration with our statistical: Aim 1 is centered on the clinical and technical feasibility of on-demand RBC washing (hypothesis 1a). Feasibility is defined as the administration of protocol RBCs instead of "off-protocol" standard-issue RBCs. Simon's two-stage design will be used to determine if the protocol needs to be modified to prepare RBCs prior to the surgical procedure. The null hypothesis rate for feasibility is $p<=0.75$ versus the alternative that $p>=0.90$, where p is the probability of successfully preparing and administering the intervention RBCs. Stage 1 of the design will examine the first 16 cases randomized to RBC washing. If 12 or fewer transfusions were deemed "feasible", the protocol will be modified to pre-wash RBCs (see limitations). If 13 or more are feasible, an additional 32 cases will be evaluated. Should 39 or fewer (of the 48 studied) not be feasible, the protocol will be modified to pre-wash. If 40 or more cases meet the feasibility definition, the remainder of the study will go as originally planned. (Sample size/ design calculations details: PASS 2008, alpha=0.10, beta=0.10, Optimal design). The safety endpoint for aim 1 is the change in hemoglobin level (hypothesis 1b). The change in hemoglobin after the first transfused unit will be used as the primary safety measure. Wilcoxon rank sum tests will be used to compare change in hemoglobin levels between randomized groups. The change in concentrations of soluble BRMs (e.g.

neutral lipids; hypothesis 1c) will be assessed using paired t-tests. There is > 80% power to detect a 0.4 SD difference pre- to post-washing with 78 washed RBC products. Should there be fewer (due to feasibility), power will decrease. However, the magnitude of differences observed in preliminary data (92% reduction, Figure 4) is many times larger than an effect size of 0.4. Therefore, power for this hypothesis should not be a concern.

Aim 2 examines the changes in biomarkers levels measured over three time points (Baseline, no later than 30 minutes after the end of the first RBC transfusion, 6 hours (+ / - 30 minutes) from the end of first study RBC transfusion, and 18 hours (+ / - 30 minutes) from the end of first study RBC transfusion. Mixed models will be fit to model the linear trajectory of these biomarkers. A model with a random slope and intercept will be considered initially, and the primary parameter of interest will be the treatment group by time interaction. For this analysis, a "modified" intention to treat (ITT) principle will be considered. Only patients randomized to RBC washing who receive at least one unit of the washed cells will be included from RBC washing group in the analysis. Those not receiving RBC washed cells will be in the considered the control group. The reason for this is this aim is focused on mechanistic action and demonstrating biologic plausibility prior to formal evaluation of clinical outcomes (which would be analyzed using traditional ITT considerations). Since the functional form of the changes in biomarkers over time is not known, a discrete (2 D.F. test) representation of time will also be used to gauge the linearity assumption as well as provide a sensitivity analysis to the primary regression model. Standard mixed modeling practices will be utilized (e.g., assessment of residuals, verification of variance components, nested modeling to simply variance components and covariance patterns, etc). This modeling scenario will be conducted for each biomarker of interest. Since prior research have noted that these outcomes are "clustered", the methodology by Shi et al. will be used to adjust for multiple comparisons.[104] We will also compute O'Brien's nonparametric global test statistic to provide an overall measure of treatment effect between the two treatment groups.

The effects of storage duration will also be assessed. The mean age of the transfused blood products will be determined. This value will be used to test for effect modification (interaction with treatment). Specifically, the main effect of RBC age, its interaction action with treatment, time and the treatment-by-time interaction term will be used to determine if the biomarker trajectories are affected by RBC age. It is hypothesized that the washing protocol will attenuate the effects of age resulting in more uniform trajectories over the range of RBC age where as the unwashed RBC will show increases in biomarker values as RBC age increases.

Sample Size Considerations: The sample size for this clinical trial is based on the mechanistic outcomes detailed for Specific Aim 2a. Table 2 in Fremont et al. was used to provide estimates of the range of effect sizes for the biomarkers considered in this study.[80]Using an approximation of the standard deviation from the interquartile range (SD~=IRQ/1.35), the median effect size was found to be 0.4. This magnitude of change would be relevant, and appropriate to power this study on. With equal allocation between the groups, the sample size for this study is estimated to be n=78/group (alpha=0.10, two-sided; see additional statistical considerations below). Actual power is expected to be higher on account of the repeated measures design. To allow for drop out and "non-feasible" cases, we plan to enroll and randomize patients until 154 patients have undergone surgey and had at least one transfusion on the operative day. We anticipate this may require randomizing up to 190 total patients. Any patients randomized and awaiting surgery when the 154th patient is achieved will also be included. The final sample size may be adjusted further based on the feasibility findings observed in the two-stage design.

Aim 3 will utilize standard analytical measures for comparing randomized treatment groups with ITT. For data that may be skewed (e.g., length of ventilation, ICU stay), Wilcoxon rank sum tests will be used to compare groups. Serial measurements (oxygen saturation) will be analyzed using longitudinal summary statistics (e.g., AUC, mean value). It should be noted that the intermediate clinical outcomes are not being powered for in this study. Estimates of precision (confidence intervals) along with range of responses will be used to guide subsequent trial designs (e.g. a larger phase II/III trial with clinical outcomes as the primary outcome of interest).

Additional statistical considerations: Consistent with early phase clinical trials, we have selected a level of significance higher than the traditional 0.05. This will facilitate advancement of the technique should it prove feasible and demonstrates potential efficacy. It is noted that multiple testing may increase the overall family wise error rate, so further research, particularly with clinical events, will be needed to quantify clinical efficacy of the approach. Missing data is expected to be minimal during the course of the study given the close surveillance the surgical and ICU environment provides. Missing specimens may occur in the event of patient discharge, death or administrative issues. Initial analyses will be conducted under the assumption of MCAR/MAR (ignorable missing). Sensitivity analyses using multiple imputation and pattern mixture models will be used to assess the robustness of the model assumptions. The analysis of specific aim 2 proposes to use a modification of the ITT principle. Justification for this is that it is a mechanistic aim with blinded examiners. We are interested in quantifying the effects of washing as clearly (and perhaps ideally) this is an early phase trial. In Aim 3, the clinical outcomes will be assessed using the traditional ITT definition to provide a more realistic measure of the effect size for planning subsequent studies.

Protocol Addendum:

The Aim 1c RBC washing efficacy outcome analyses evaluating the first 75 washed RBC units, comparing the pre-washed soluble biologic response modifier (BRM) concentrations to the post-washed unit concentrations, have been completed in collaboration with Drs. Norris and Silliman. The Aim 1c findings indicate a decrease in the RBC micro-particle and cytokine concentrations, indicating the CATS washing procedure was effective in removing the BRM's that have previously been associated with transfusion-related cardiopulmonary dysfunction. However, the analyses also noted increased concentrations of cell free hemoglobin (CFH).

In an effort to further understand the potential relevance/clinical importance, if any, of the increase in CFH, we will obtain 20 evaluable hematocrit values between the two sites, both before and after the washing procedure. To accomplish this, a small sample of 2 mls. will be drawn from the RBC unit prior to performing the washing procedure and again at the conclusion of the washing procedure prior to administering the unit to the patient. This hematocrit analyses will be conducted locally at each site.

As we have completed the Aim 1c washing efficacy analyses defined a priori, we will no longer be collecting the 6 ml sample from the RBC bag prior to washing. Thus, the total amount of sample drawn from the RBC units will be less than it has been up to this point in the trial. Therefore, we would not anticipate any additional risks to the recipients of these washed allogeneic RBC units.

Our decision to test the hematocrit levels before and after washing in 20 RBC units is the result of our interactions with the lead statistician of this protocol who has estimated this to be a sufficient sample size to better understand the increase in cell free hemoglobin in relation to the total hemoglobin present in the allogeneic RBC units.

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