The Effects of Phosphatidylserine Expression on Older Red Cell Units in Adults with Sickle Cell Disease

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The Effects of Phosphatidylserine Expression

on Older Red Cell Units in Adults with Sickle Cell Disease

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Study Sponsor: NHLBI

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And Emory University

A randomized trial to determine if there is a mechanistic
or clinically important difference between the PS-high vs. PS-low units
on patient immune systems & clinical outcomes

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Quick List of Abbreviations:

RBC – Red blood cell
SCD – Sickle cell disease
PS – Phosphatidylserine
PE – Phosphatidylethanolamine
NTBI – non transferrin bound iron
MFI – mean fluorescence intensity

Concept Synopsis and Study Schema

The desired impact of red blood cell (RBC) transfusion in patients with sickle cell disease (SCD) is to reduce the burden of patient sickled red cells with the goal of reducing stroke risk and reducing the burden of frequent vaso-occlusive pain events that is otherwise refractory to other treatment modalities. However, controversy exists over transfusions with blood banked for short vs. long storage intervals. Pre-clinical animal studies and observational human models suggest that increased phosphatidylserine exposure (PS), seen often from red cells units with longer storage intervals, may lead to downstream immune effects that have detrimental consequences, such as increased clinical infections. No randomized prospective human study has attempted to correlate age of transfused RBCs with PS-exposure, and then evaluate how this exposure modulates patient immune systems and subsequent clinical outcomes.

Primary Hypothesis:

There is a clinically significant difference between the effect of PS-high RBCs vs. PS-low RBCs on patient macrophages.

Study Schema:

Trial design: Adults and adolescents (post puberty) with SCD who are treated with outpatient red cell transfusions will be randomized in blocks of 5 (S. Piantadosi block randomization software, 2010) to receive ≥30 day or ≤10 day old units for 3 months (3 consecutive transfusion events). **The study statistician** will provide the blood bank with the schema who will then be responsible for providing the units for these subjects. Participants and all other investigators will be blinded to treatment assignment.

Two baseline venous blood samples will be obtained to be used as a baseline measurement of the patients underlying inflammatory status. The first will be drawn about 1- week prior to the first randomized transfusion, and the second sample just prior to the first randomized transfusion. Subjects will be asked to hold their iron chelation 72 hours before the study transfusion. Pre-transfusion, sterile samples will be extracted from red cell units. Patient venous blood samples will be also obtained 2 hours post transfusion, 24 hours post-transfusion, and 2 weeks post-transfusion (**Table 1**). Participants will complete standardized diaries daily to document symptoms of infection, SCD pain, medications and emergency department (ED) or hospital use. Diaries will be collected and participants will be assessed with each transfusion encounter, up to 3 transfusion events, and 4 weeks after the last transfusion. To ensure compliance, coordinators will

contact subjects regularly to remind them to complete diaries. All data will be captured with standardized case report forms and entered into an electronic database.

Subject Recruitment and Anticipated Numbers: We intend to recruit and enroll 40 subjects. Participants will be recruited from the Adult SCD Clinic at UNC and Emory/Grady Health System. We expect that UNC will recruit between 5-10 subjects over the next 2 years (15 subjects have already been enrolled at Medical College of Wisconsin), and Emory will recruit between 10-20 subjects to reach the study goal of 40 randomized subjects.

Subject Inclusion/exclusion criteria:

***Inclusion criteria*:**
1) Ages 16 to 60 years old
2) Subject must have sickle cell anemia confirmed by hemoglobin analysis
3) Must be receiving red cell transfusion therapy
4) Must be outpatient at the time of study transfusions

***Exclusion criteria*:**
1) History of severe reactions to transfusion therapy
2) Receive red cell transfusions that are crossmatch incompatible
3) Current participation in another therapeutic trial for sickle cell disease
4) Current pregnancy
5) History of HIV infection
6) Having an uncontrolled inter-current illness, or psychiatric illness/social situations that would limit compliance with study requirements as determined by the principal investigator.

Study procedures:

Red cell unit preparation: Red cell units for transfusion will be crossmatch-compatible, sickle-negative, and phenotype-matched for D, C, E, and K red cell antigens per current hospital protocols. Red cell unit age will be documented by the blood bank at the time of randomization. Study transfusions will take place *only* in the outpatient infusion clinic.

Pain and infection diary: The diary is adapted from a model widely used in SCD studies. Daily, participants will rate pain on a numeric ordinal pain rating scale (0-10), indicate whether the pain was consistent with a “crisis,” indicate whether they utilized a healthcare facility, and record the amount of opioids used. Infection symptoms will also be documented daily along with the type, duration, and dose of antibiotics, if applicable.

Physical exam: A basic, focused, physical exam will be performed on each subject by the PI or trained delegate prior or during each transfusion and prior to the completion of the study. The physical exam will assess a general review of systems, a review of the pre-transfusion vital signs, and a basic evaluation of patient general status, heart, lung,

and neurological organ systems. We will also document if the patient has an indwelling catheter.

Blood measurements:

Unit samples: Red cell characteristics: 2 ml blood from each red cell unit will be obtained from 6 sterile unit links pre-transfusion. RBC PE and PS surface exposure, and plasma microparticles will be quantified by flow cytometry using the LSR2-green, and all flow analyses will be recorded using FACSDiVa software. The plasma fraction from each unit sample will be diverted to EPR (Bruker Elexys X-band system, Billerica, MA, USA), a Bio-Plex cytokine assay kit (Bio-Rad Laboratories), and a fluorescent immunoassay (DxFerr kit) to measure concentrations of free heme/hemoglobin, cytokines (IL-1, IL-6, IL-8, MCP-1), and NTBI, respectively. A small sample will also be frozen for future metabolomic analysis.

Patient Samples: 10-30 ml per lab draw of whole blood will be obtained for each blood draw as defined in **Table 1**, and will be stored per tube storage requirements. . Blood will be obtained by port or peripheral access as available. Patient white cells will be isolated with established Ficoll separation techniques.. White cells will be tested by flow cytometry as above for key markers of activation (macrophages: CD64, CD62L, CD80, CD86, Tim1, neutrophils: The plasma fraction from each sample will be diverted to EPR (Bruker Elexys X-band system, Billerica, MA, USA), a Bio-Plex cytokine assay kit (Bio-Rad Laboratories), and a fluorescent immunoassay (DxFerr kit) to measure concentrations of free heme/hemoglobin, cytokines (IL-1, IL-6, IL-8, MCP-1), and NTBI, respectively. Clinical measurements obtained from patients will also include: CBC (4ml EDTA), reticulocyte count (4ml EDTA), HbS% (3ml EDTA), haptoglobin (1ml red top), chemistry labs (ferritin (0.5ml red top), iron saturation (1ml red top), lactate dehydrogenase (1ml red top), high sensitivity CRP (HS-CRP, 1ml red top)), urinalysis (12 ml) and blood culture (8-10ml/bottle). These clinical labs will be measured at intervals specifically defined in **Table 1**. Individual human samples of plasma and RBC will also be frozen and sent to Angelo D'Alessandro at the University of Colorado Denver to measure lipid metabolites and metabolomic profiles pre vs. post transfusion as an additional exploratory goal of this trial. The laboratory of Dr. Steven Spitalnik and his representatives will be receiving 30-200 individual samples of plasma and RBCs for this purpose. All samples will be de-identified.

1. BACKGROUND AND SIGNIFICANCE

Sickle cell disease (SCD) is an autosomal recessive disorder that affects 1 of every 400 African-American newborns in the United States.[42,43] Abnormal hemoglobin, due to a mutation in the sixth codon of the β-globin gene, is the basis for SCD.[44] Under hypoxic conditions, the abnormal hemoglobin polymerizes, causing red blood cells to become sickle-shaped and unable to transverse the microvasculature.[44] Occlusion of small vessels results in tissue ischemia, infarcts and organ damage.[44,45] Commonly affected organs include the brain, lungs and bone, which result in stroke, acute chest syndrome and pain, respectively.[45] Red cell transfusion is the most critical therapy to treat and prevent complications of SCD.[1]

Adults with SCD depend on red cell transfusion to prevent stroke and critical illness. Red cell transfusion corrects anemia, decreases sickle hemoglobin (HbS) percent, suppresses HbS synthesis, and reduces red cell hemolysis.[1,44,45] These beneficial effects are used to successfully manage multiple complications from SCD, including severe anemia, stroke, acute chest syndrome and multi-organ failure.[1,44-55] When administered on a chronic basis, typically monthly, transfusion decreases rate of cerebral infarcts by up to 45%[51] and vaso-occlusive events by up to 50%.[53-57] Other than transfusion, hydroxyurea is the only medication available for SCD.[1,58] Although hydroxyurea decreases rate of vaso-occlusion and prolongs life, the drug is not indicated for stroke prevention and is ineffective in 50% of patients.[59-64] Since red cell transfusion remains the cornerstone of therapy for this population, provision of the safest units possible is critical.

Changes occur in the physiology of red cell units with prolonged storage that may trigger a cascade of deleterious events in the transfusion recipient: macrophage activation, inflammation and release of toxic iron species (Figure 1). Red cells preserved with Adsol solution can be stored up to 42 days,[65] a recommendation based on integrity, not red cell efficacy or safety. Significant oxidative and metabolic changes occur with storage: hemolysis with release of cell-free hemoglobin, heme, and non-transferrin bound iron (NTBI), as well as alterations to the lipid composition of the red cell membrane.[6-15] Surface exposure of phosphatidylserine (PS), a phospholipid normally found on the inner leaflet of the red cell membrane but which becomes externally exposed with storage, may be the most important of these changes.[23-27] Macrophages recognize PS as an “eat me” signal via specific receptors (Tim1, Tim4, and Stabilin-2) and rapidly phagocytize these red cells.[66-71] In pre-clinical studies, phagocytosis of old red cells results in activation and secretion of pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, CXCL-1 and monocyte chemoattractant protein-1 (MCP-1).[19,28] Phagocytosis of a bolus of old red cells also overwhelm the macrophage’s metabolic capacity, leading them to release cell-free hemoglobin, heme and NTBI with downstream effects.[21,22,29-31] NTBI has been associated with fatal proliferation of ferrophilic bacteria and the generation of toxic reactive oxygen species.[19-22,37-39] Of note, these effects on macrophages are only seen after the transfusion of older intact red cells, not from the products of lysed red cells, which suggests that phagocytosis of red cells is required to generate this pathophysiology.[29]

Although some patients may be able to tolerate the aberrant physiology of older red cells, others may be at risk of poor outcomes.

Adults with SCD may be uniquely susceptible to the deranged physiology of older units because they are prone to infections, inflamed and poorly equipped to handle excess red cells with high PS. Vaso-occlusion-induced tissue damage activates macrophages and leukocytes, which in turn leads to a chronic pro-inflammatory state.[72-76] Reperfusion injury further exacerbates inflammation with the generation of reactive oxygen species as well as additional endothelial and leukocyte activation.[77,78] Activated macrophages, leukocytes and endothelium then promote more vaso-occlusion in what becomes a vicious cycle of ischemia, injury and inflammation.[78] Intravascular hemolysis with release of cell-free hemoglobin and heme also contributes to this pathophysiology as cell-free hemoglobin is a sink for nitric oxide, a potent vasodilator and platelet antagonist,[32-34,78] and heme activates endothelial cells via TLR4 receptors.[35,36] Prior to penicillin prophylaxis, infection was the primary cause of death in patients with SCD due to splenic dysfunction.[79-81] The cumulative result of SCD's pathology is an adult who is prone to infections, chronically inflamed and susceptible to the pathology of PS-high, older red cells, and the consequent increased levels of cell-free hemoglobin, heme and NTBI in circulation. In fact, on sickle erythrocytes (not transfused cells) PS exposure is well-known to cause adhesion to the endothelium and thrombin generation, both of which worsen the pathology of vaso-occlusion.[82,83] Since older red cell units with increased PS exposure can promote a similar physiology as SCD, they may exacerbate the illness of these vulnerable patients.

No prospective study has evaluated the effects of an older unit with high PS exposure in an adult with SCD. There has only been one recently published prospective study in patients with SCD to examine the effects of age of blood.[84] This was a randomized trial, conducted in Africa, of young children with either SCD or malaria transfused with > 25 day-old units versus < 10 day-old units to treat severe anemia (mean Hgb 3.7g/dl) with evidence of lactic acidosis. Although the trial found no difference between the two arms in the primary outcome measure, blood lactate levels, the results do not address the question of the current proposal: do PS-high, older units trigger a pathobiology in chronically transfused adults with SCD that leads to infection? Differences in unit physiology, patient inflammation, infection rates, and pain were not examined in the prior study, which is why the current study needs to be conducted. Other than that one prospective study, the only data about the effects of older units in SCD comes from three observational cohort studies, all of which showed an adverse outcome (increased red cell alloimmunization and decreased vascular dilatation) associated with older units.[85-87]

The effects of the older units have been equally inconsistent in other patient populations, mostly trauma and surgery. While multiple observational studies have associated receipt of old units with poor outcomes, including infection and death,[88-90] recent randomized prospective studies did not show a difference in mortality.[91,92] Limitations of these other previous studies were a small difference in age between older and younger units, as well as the fact that no evaluation was performed of the *unit's*

physiology. Not all older units show the same degree of pathology.[66] Most pertinent to our study, however, is that the patients were not adults with SCD. Organ dysfunction, inflammation, hemolysis, along with a dependence on blood to prevent critical illnesses, creates a situation in adults with SCD that is different from other patient populations, including children with SCD, and makes studies in other populations difficult to apply. To address a critical gap in knowledge, **we propose for the first time, a prospective, randomized study to elucidate the immunological and clinical effects of an older unit with high PS exposure on adults with SCD.**

Preliminary Studies

In Milwaukee, 1/3 of units transfused to adults with SCD are >30 days old, but nation-wide some restrict the transfusion of older units. Four hundred adults receive care in the Adult Sickle Cell Clinic at UNC in Chapel Hill, 47 of who receive chronic outpatient transfusions for chronic pain or stroke prophylaxis. Transfusions are administered to adults regardless of storage age. In a 3-year retrospective review of transfusions administered to adults with SCD, 896 units were transfused over 407 simple transfusion encounters. Median storage age was 23 days (range: 2-42 days); 32% of units provided were ≥ 30 days-old **(Karafin et al., 2015)**.[41] To evaluate the opinions and policies of other hospital blood bank directors about the use of older red cell units for patients with SCD, we conducted a nation-wide survey (n=90). While only 23% of respondents had a storage age restriction policy for patients with SCD, 31% thought that older units were not as effective as younger units, and 65% believed that evidence-based policies were needed **(Karafin et al., 2015)**.[40] These data from our institution and across the US demonstrate equipoise among blood bank directors about the potential harm of older units for patients with SCD. At UNC, we have determined that the average unit age transfused is 18 days, with a standard deviation of 10 days (range: 5 days- 39 days).

Transfusion of older units to adults with SCD stimulates expansion and activation of macrophages. Adults with SCD may be susceptible to the effects of an older unit's physiology because they are prone to infection, chronically inflamed, and poorly equipped to handle excess iron. In a cohort of 40 steady-state adults with SCD, we specifically measured markers of inflammation and iron excess. High sensitivity C-reactive protein, a marker of global inflammation, was found to be markedly elevated (median 5.6 mg/L, range 0.4-60 mg/L; reference range <1.0 mg/L), as was ferritin, a marker of iron stores (median 2,969 ng/ml, range 20-12,300 ng/ml; reference range 13-400 ng/ml) **(Karafin et al., 2015)**.[93]

To characterize whether older units would exacerbate the adult's pro-inflammatory physiology, we next examined activation of macrophages, neutrophils, B and T cells in the peripheral blood of 8 adults with SCD before and after transfusion and 8 adults with SCD who do not receive transfusion (ages 19-35). All transfused units were greater than 14 days old (range, 14-36 days). In support of our hypothesis, we identified that absolute macrophage and neutrophil numbers were higher post-transfusion compared to pre transfusion in 5/8 (63%) and 6/8 (75%) subjects, respectively. There was also evidence of macrophage activation post-transfusion (**Figure 2**). Among transfused subjects,

macrophages increased in size in 7/8 (88%) subjects, and showed greater expression of CD64+ (12% increase) and CD62L (34% increase) as measured by mean fluorescence intensity (MFI) 24 hrs post-transfusion as compared to pre-transfusion values.

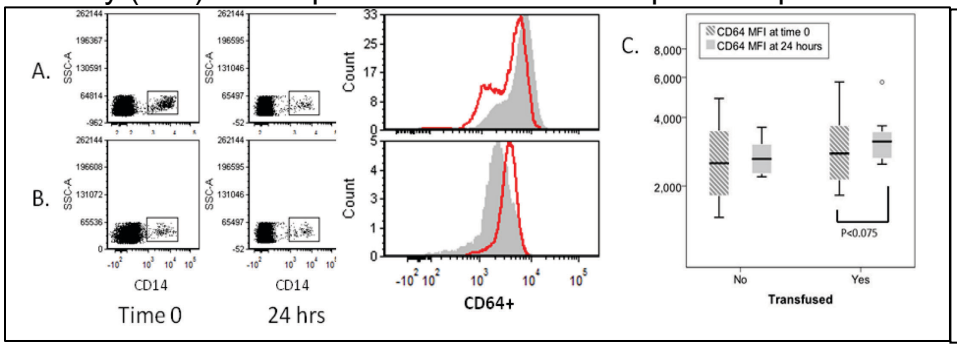

Figure 2: CD64 macrophage activation at time 0 and 24 hours in adults with SCD not transfused **(A)**, transfused **(B)** and a comparison between not transfused and transfused **(C).** Macrophages were identified by CD14 expression (rectangles on scatter plots in (A) and (B)), and further analyzed for activation by CD64 expression (histograms gray=time 0, red=24 hours). There is a trend suggesting transfused subjects have a greater increase in CD64+ macrophages 24 hrs post transfusion.

Transfusion of older red cell units to adults with SCD increases rate of infection. To explore whether the pro-inflammatory state stimulated by older units was associated with infection risk, as has been shown in pre-clinical models, we examined 29 adults with SCD who were treated with a chronic transfusion program. A random effects model, which accounts for a subject's contribution to repeat encounters, was used to define the association between infection and storage age. This model revealed that a mean unit age > 25 days old was associated with a significant increase in ED or hospital admissions for infection (p=0.03). After receipt of older units, adults developed pneumonia (n=6), urinary tract/kidney infections (n=2), gynecologic infections (n=2), an ear infection (n=1), a wound infection (n=1), and sepsis (n=1). Eight (62%) of these infections contributed to a subsequent inpatient admission **(Karafin et al., *submitted for publication)***.

Our preliminary data demonstrates the need for a prospective, randomized trial to characterize the effects of an older unit's physiology on adults with SCD. Because little data exist, blood bank directors across the country have varied policies about the provision of older units to adults with SCD. At our institution, adults are frequently transfused with older units, which we have shown activate macrophages and, ultimately, promote infection.

Summary: Finding ways to maximize the safety and efficacy of red cell transfusion therapy for patients with SCD is desperately needed. Our group has data suggesting that older units of stored red cells contribute to immune activation and the increased risk of infection. If found to be effective, restricting transfusions to PS-low, younger units of blood in this population has the potential to improve patient outcomes and significantly alter the field of transfusion medicine.

2. OBJECTIVES

2.1: Primary Aims

Note: This randomized clinical study should be considered a feasibility study to determine the ability to design and run a protocol of this nature. Each Aim was specifically selected

to maximize the possibility that novel information could be obtained from this trial. However, power calculations and associated precision estimates should be considered preliminary, as the data derived from this study will inform the calculations necessary to design a larger, more definitive study.

Aim 1:
To characterize the physiologic differences between ≥30 vs ≤10 day-old units. As part of the randomized study, we will measure PS exposure on donor red cells, as well as PE, microparticles, plasma free hemoglobin, heme, and NTBI. We hypothesize that ≥30 day-old units will have significantly more ***biochemically old units*** (see section 10 for the definition) than <10 day-old units. With a total sample size of 40 patients (20 in each group), we will have at least 80% power (α=0.05) to detect a difference of at least 47% between the two randomized groups for biochemically old red cell units (close to a 100% difference is expected).

Aim 2:
To quantify whether physiologic differences between ≥30 day-old units versus ≤10 day-old units promote immune cell activation in transfused adults with SCD. We will perform flow cytometry to measure the activation of monocytes and neutrophils, as well as measure cell-free hemoglobin, heme and NTBI on blood samples before, 2, and 24 hours after transfusion. We hypothesize that those administered ≥30 day-old (biochemically old) units will have a greater increase in the number of CD62L + activated monocytes at 2 hours post-transfusion compared to those administered <10 day-old units. With a sample size of 40 patients, we will have 80% power (α=0.05) to detect a change in MFI ratio of 1.35.

Aim 3:
To explore whether the physiology of ≥30 day-old units versus ≤10 day-old units is associated with adverse clinical outcomes in transfused adults with SCD. We will perform a systematic evaluation of hospital admissions, pain scores, medications, and clinical symptoms prior to each transfusion and at the end of the study period (**Table 1**). *We hypothesize that reports of infection will be greater in subjects randomized to receive* ≥30 day-old *older units.*

2.2 Secondary Aims

Aim 1:
To explore whether: ≥30 day-old units will have significantly greater mean 1) phosphatidylethanolamine expression, 2) cell free hemoglobin concentration, 3) cell free heme expression, and/or 4) NTBI expression in comparison to ≤10 day-old units.

Aim 2:
To explore whether:

1) Those *administered ≥30 day-old units will have a greater increase in the number of activated monocytes or neutrophils as defined by flow cytometry markers of activation at 2 or 24 hours post-transfusion compared to those administered ≤10 day units.*

2) Those *administered ≥30 day-old day units will have a greater increase in the concentration of NTBI, cell free hemoglobin, and free heme, compared to those administered ≤10 day-old units at 2 or 24 hours post transfusion.*
3) Recipient RBC PS, PE, and microparticle expression will increase more between 2 and 24 hours after receiving ≥30 day-old units than after receiving ≤10 day units.

Aim 3:
To explore whether those *administered ≥30 day-old units will have a more rapid increase in the concentration of HbS% and a more rapid decrease in hemoglobin over time compared to those administered ≤10 day-old units.*

Aim 4:
To explore whether those administered *≥30 day-old units will have a significantly different RBC metabolomic profile 2 and 24 hours post transfusion than those provided ≤10 day-old units.*

Aim 5:
We will address aspects of study feasibility, such as subject drop-out, rates of missing data, and the confidence intervals/precision of our recorded variables, including PS, PE, microparticles, CD62L, and the other recorded clinical variables.

3. STUDY POPULATION

3.1 Enrollment Inclusion Criteria

1) Ages 16 to 60 years old
2) Subject must have sickle cell anemia confirmed by hemoglobin analysis
3) Must be receiving chronic red cell transfusion therapy
4) Must be outpatient at the time of transfusion

3.2 Enrollment Exclusion Criteria

1) History of severe reactions to transfusion therapy
2) Receiving red cell units that are crossmatch incompatible
3) Current participation in another therapeutic trial for SCD
4) Current pregnancy (due to the rapidly changing blood volumes that would alter the number of units needed for successful randomization, and due to the increased likelihood of hospitalization during the study protocol)
5) History of HIV infection

6) Having an uncontrolled inter-current illness, or psychiatric illness/social situations that would limit compliance with study requirements.

4. TRIAL ENROLLMENT

4.1 Screening/Recruitment

Patients ≥ 16 years of age undergoing outpatient red cell transfusion therapy will be identified through either the adult outpatient sickle cell clinic at UNC, the Infusion Clinic at UNC and at Emory/Grady Health System. Emory/Grady Health System will only enroll subjects who are ≥ 18 years of age. Basic features of patient medical and surgical histories (i.e. age, gender, reason for receiving chronic transfusion therapy) will be recorded.

Potentially eligible patients, as described in the previous paragraph, will be approached for study consent prior to their transfusion. Individual center scheduling practices will influence how this contact is arranged. Subjects who consent to the study will be assigned a study ID number and have their eligibility status evaluated. If the subject is eligible for the study based on the inclusion/exclusion criteria (section 3.1 and 3.2), they will be enrolled in the study and will be randomized. The availability of suitable products (see sections 4.2 and 5.1) will determined when the study will start for that individual.

If a subject is enrolled and receives a red cell transfusion prior to randomization, that subject will still be randomized, and will start the study at their next outpatient red cell transfusion appointment, pending the availability of the appropriately aged units.

4.2 Stratification and Randomization

Subjects will be randomized to receive RBCs stored either ≤ 10 days at time of transfusion or ≥ 30 days at the time of transfusion. Storage arm assignment applies to up to 3 consecutive outpatient transfusions, beginning at the time of randomization and continuing through the end of the 3rd randomized outpatient transfusion. Randomization assignment can occur at any time between the date of consent till 2-3 days prior to the first study transfusion. Only the transfusion service will have access to the randomization assignment as designated by the study biostatistician.

Due to the pilot nature of this study, randomization will not be stratified by gender, age, ethnicity, or hemoglobinopathy type.

For the first study transfusion, after the number of units requested for crossmatch is known, but no earlier than 2-3 calendar days before transfusion, a study staff member will contact the institution’s transfusion service to determine whether the transfusion service has enough suitable units stored ≤ 10 days and ≥ 30 days to meet the crossmatch request for this subject.

If there is not sufficient inventory to meet the randomization criteria, the subject's study participation will be postponed, and unit availability will be assessed again prior to their next outpatient transfusion.

Once the subject has received their first study transfusion, the remaining 2 outpatient study transfusion events will occur regardless of whether the transfusion service can meet the transfusion needs of the study.

4.3 Masking of Treatment Allocation

At enrollment, the subject's eligibility status and ABO/Rh type will be entered into the data management system. If eligible, the subject will be randomized at the time of consent, but no later than 2-3 calendar days before the first outpatient study transfusion. Unit age availability will be assessed no earlier than 2-3 calendar days before the first outpatient study transfusion, and the study will commence if unit availability is satisfactory for that subject. The study start will be delayed until the next outpatient transfusion if blood unit availability is deemed insufficient for that first study transfusion. Only blood bank staff with the appropriate security level in the data management system will be able to access the treatment arm assignment. Access to case report forms containing information about the age of the RBC products sent for each subject will also be restricted at the site to the appropriate blood bank staff. Clinical staff overseeing the subject's participation in the trial, collecting data, and reporting the data into the data management system will not have access to the treatment arm assignment or information about the age of the RBC products transfused.

No alteration will be made to the labels on the RBC units. The expiration date, collection date, and any processing dates (e.g. irradiation dates) will not be obscured. Medical infusion clinic personnel physically providing the RBC transfusions will verify product and patient identity according to hospital-specific procedures. These personnel will be instructed to not divulge the patients' randomization assignments. Other infusion clinic staff (other than those actually infusing RBCs), will be instructed not to seek to identify the age of the products the patients are receiving. The subjects themselves will not be informed of their randomization assignment and will also be instructed not to seek to identify the age of the products they are receiving. However, as the key components of this study are objective, inadvertent unblinding of the randomization assignment will not compromise the validity of the study.

5. INTERVENTIONS

5.1 Preparation

In Section 5 of this protocol, the term "study RBC units" includes all RBC units provided to a randomized subject, beginning at the time of the first study transfusion and continuing through the end of study. Transfusions will always be ordered based on patient need, following all institutional policies and practices. The decision to order (or not order) an RBC transfusion is not dictated in any way by the study protocol

5.1.1 Preparation criteria applying to both treatment arms

The following criteria apply to all study RBC units in both treatment arms. Any study RBC unit which does not meet all these criteria will be considered a protocol violation.

1. Institutional processing standards will be maintained for all units.
2. All study RBC units should be pre-storage leukoreduced.
3. All study RBC units should be stored in either AS1, AS3, or AS5.
4. No study RBC unit should be washed before release from the transfusion service.
5. No study RBC unit should be volume-reduced.
6. No study RBC unit should include a frozen product.
7. No study RBC unit should be deglycerolized.
8. No study RBC unit should be irradiated.

5.1.2 Preparation of RBC units for subjects randomized to receive units stored ≤ 10 days

All study RBC units released from the blood bank for subjects randomized to receive units stored ≤10 days should have been stored ≤10 days at the time of transfusion.

If there are not enough suitable units stored ≤10 days at the time a study RBC transfusion is needed, the blood bank should release suitable units with the minimum available storage time. The transfusion itself should not be cancelled or postponed because there are not enough suitable units stored ≤ 10 days.

Study units stored longer than 10 days will be considered protocol deviations for subjects in this treatment arm if used for an outpatient transfusion and after the first successful randomization.

Subjects randomized to this treatment arm are likely to receive units with shorter storage times than the units they would receive if not in the study, but this is not guaranteed to be true for any particular subject.

5.1.3 Preparation of RBC units for subjects randomized to receive units stored ≥ 30 days

All study RBC units released from the blood bank for subjects randomized to receive units stored ≥ 30 days should have been stored ≥ 30 days at the time of transfusion.

If there are not enough suitable units stored ≥ 30 days at the time a study RBC transfusion is needed, the blood bank should release suitable units with the maximum available storage time. The transfusion should not be cancelled or postponed because there are not enough suitable units stored ≥ 30 days.
Units stored less than 30 days will be considered protocol deviations for subjects in this treatment arm.

5.2 Administration

RBC units will be administered according to local institutional policy and safety standards as ordered by the medical team for patient care needs.

5.3 Concomitant Treatments

Standard medical care according to the local institutional standard will be provided to the subject.

6. SCHEDULE OF MEASUREMENTS

6.1 Red Cell Unit Sample Collection:

2 ml blood from each donor unit will be obtained from 6 sterile unit links/segments on the day of the 3 randomized outpatient transfusion events. PE and PS exposure, and microparticles, will be measured by flow cytometry using the LSR2-green, and all flow analyses will be recorded using FACSDiVa software. The plasma fraction from each red cell unit will be diverted to EPR (Bruker Elexys X-band system, Billerica, MA, USA), a Bio-Plex cytokine assay kit (Bio-Rad Laboratories), and a fluorescent immunoassay (DxFerr kit) to quantify concentrations of free heme/hemoglobin, cytokines (IL-1, IL-6, IL-8, MCP-1), and NTBI, respectively.

6.2 Patient Sample Collection (Table 1):

A. One - ***four weeks prior to the first transfusion:***
- 15ml whole blood for flow cytometry and EPR measurements

B. ***Immediately prior to each red cell transfusion:***
- 15ml whole blood for flow cytometry and EPR measurements
- 15ml to McLendon Labs
- blood culture to McLendon Labs (8-10ml blood/culture bottle)
- urine to McLendon Labs (12 ml urine)

C. ***Two hours after each red cell transfusion (1 ½- 3 hours)***
-15ml whole blood for flow cytometry and EPR measurements
-15ml to McLendon Labs

D. **1 day *after each red cell transfusion (about 21-27 hours after)***
-15ml whole blood for flow cytometry and EPR measurements
-15ml to McLendon Labs

E. ***2 weeks after each red cell transfusion (+/- 2 days)***
- 10ml McLendon Labs

Patient Sample Processing: Blood will be obtained by port or peripheral access as available. Patient white cells will be isolated with established Ficoll separation techniques.. White cells will be tested by flow cytometry as above for key markers of activation (macrophages: CD62L, CD64, CD80, CD86, Tim1, neutrophils: The plasma fraction from each sample will be diverted to EPR (Bruker Elexys X-band system, Billerica, MA, USA), a Bio-Plex cytokine assay kit (Bio-Rad Laboratories), and a fluorescent immunoassay (DxFerr kit) to measure concentrations of free heme/hemoglobin, cytokines (IL-1, IL-6, IL-8, MCP-1), and NTBI, respectively. Clinical measurements obtained from patients will also include: CBC (4ml EDTA), reticulocyte count (4ml EDTA), HbS% (3ml EDTA), haptoglobin (1ml red top), chemistry labs (ferritin (0.5ml red top), iron saturation (1ml red top), lactate dehydrogenase (1ml red top)), urinalysis (12 ml) and blood culture (8-10ml/bottle). These clinical labs will be measured at intervals specifically defined in **Table 1**. Individual human samples of plasma and RBC will also be sent to Angelo D'Alessandro at the University of Colorado Denver to measure lipid metabolites and metabolomic profiles pre vs. post transfusion as an additional exploratory goal of this trial. The laboratory of Dr. Steven Spitalnik and his representatives will be receiving 30-200 individual samples of plasma and RBCs for this purpose. All samples will be de-identified.

6.3 Patient Clinical Outcomes:

A daily pain diary will be used and subjects will rate their pain on a ordinal numeric pain rating scale (0-10), indicate whether the pain was consistent with a "crisis," indicate whether they utilized a healthcare facility, and record the amount of opioids used. Infection symptoms will also be documented daily along with the type, duration, and dose of any antibiotics taken if applicable. Subject ED and hospitalization events, and etiologies (*see definitions in 6.7*), will be recorded at regular intervals by the study coordinator during the study period using the available electronic medical record. Of note, diary completeness will be assessed at each face-to-face visit.

6.4 Patient Physical Exam:

Prior to each randomized transfusion, the PI or appropriately trained designee will perform a basic assessment of the patient. This exam will include a basic interval history (including review of any ED or hospital visits), a focused review of systems, and a focused physical exam.

Table 1: Schedule of Measurements

Study Measure	***Daily***	***1-4 weeks pre-transfusion***	***Pre-every transfusion***	***2hr post every transfusion***	***24 hr post every transfusion***	***2 weeks post every transfusion***	***End of Study***
Physical exam AE assessment Med assessment			***X***				***X***
CBC w/ differential*			***X***		***X***	***X***	
HbS%			***X***		***X***	***X***	
Blood culture			***X***				
Urinalysis w/ reflex culture			***X***				
Haptoglobin			***X***	***X***	***X***		
Plasma Hemoglobin			***X***	***X***	***X***		
Basic Metabolic panel+			***X***				
Ferritin & Iron Panel#			***X***	***X***	***X***		
Lactate dehydrogenase			***X***	***X***	***X***		
HS-CRP			***X***	***X***	***X***		
RBC Unit blood donor sample			***X***				
Flow cytometry sample		***X***	***X***	***X***	***X***		
Study Diary	***X***		***X***			***X***	***X***

*WBC, RBC, Hgb. Hct. Plt ct
+Creatinine, Bicarb, Chloride, BUN, Potassium (K), Sodium (Na), Glucose, Calcium
#Iron, Transferrin, TIBC, % Saturation

Table 2: Lab Sample Type and Storage Conditions

Blood Samples	**Sample required**	**Tube type**	**Storage**	**Processed By**
Red cell units	6 unit segments	NA	Room temp	PI or designee
Flow	8ml	Green top	Room temp (cold after 30 minutes)	PI or designee
Flow/EPR Labs	10 ml	Cytochex	Room temp	PI or designee
Hematology Labs	10 ml	EDTA	Room temp	McLendon Labs
Chemistry Labs	5 ml	Red top	Room temp	McLendon Labs
Microbiology Blood Culture	10 ml	Culture vial	Room temp	McLendon Labs
Microbiology Urine Culture	12ml	Sterile cup	Room temp	McLendon Labs

6.5 RBC Transfusions

The following data will be collected for each RBC unit ordered for the subject while on study:

- Unit ID number
- Source of unit (apheresis or whole blood collection)
- Blood group of unit (ABO and Rh)
- Leukoreduction status
- Irradiation status
- Volume reduction/washed unit status
- Storage medium (AS1, AS3, or AS5)
- Antigens matched (D, Cc, Ee, Kk,or other)
- Collection or expiration date (specifically recorded by the blood bank to maintain study coordinator blinding)
- Start time and end time of transfusion

6.6 Unblinding

Investigators will remain blind to treatment assignment until the entire study is complete. Only the DSMB, blood bank staff, and the designated study statistician at Medical College of Wisconsin should be aware of the treatment that is assigned to each subject. Unintentional unblinding of study participants will not be a reason for study discontinuation. Adverse events or urgent clinical care may lead to study termination as documented in section 6.7.

6.7 Ending of Study Participation

1) The subject completes the 3 transfusions
2) The subject decides to withdraw from the study
3) The subject moves away, dies, or is lost to follow-up
4) The patient no longer needs outpatient red cell transfusions as determined by the patient's provider
5) Subjects may be removed from the study, or treatment stopped by the Investigator for any of the following reasons:

    - Occurrence of CTCAE Criteria of grade 3 or higher that are deemed attributable to the transfusion per the opinion of the PI
    - PI decides to withdraw the subject due to noncompliance
    - Withdrawal by the PI or subject because of treatment side effects or complications

6.8 Key Definitions

1) Vaso-occlusive pain crisis: an episode of acute pain with no cause other than a vaso-occlusive event lasting at least 24 hours that requires the administration of oral or parenteral opioids in a medical facility.

2) Acute Chest Syndrome: an acute illness requiring medical facility attention characterized by fever and/or respiratory symptoms, accompanied by a new pulmonary infiltrate on a chest X-ray.

3) Priapism: an acute illness requiring medical facility attention characterized by *an* erect penis does that does not return to its flaccid state, despite the absence of both physical and psychological stimulation, within four hours.

4) Acute infection: an acute illness requiring medical facility intervention (i.e prescription of antibiotics or hospital admission) characterized by any subjective symptom.

5) *Chronic transfusion*: receiving red cell units on a 3-8 week schedule as part of routine, non-acute care).

7. SPECIMEN COLLECTION PROCEDURES

Blood samples will be obtained per institutional protocol and assayed in local laboratory facilities as defined in **Section 6**.

8. ADVERSE EVENT CRITERIA AND REPORTING

This study will be using the descriptive terminology developed by the National Cancer Institute for use in reporting adverse events: Common Toxicology Criteria for Adverse Events (CTCAE) version 4.0, dated May 28, 2009. The CTCAE includes a grading (severity) scale for each adverse event term. Grades were developed using the following guidelines:

Grade 1: Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.

Grade 2: Moderate; minimal, local or noninvasive intervention indicated

Grade 3: Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL.

Grade 4: Life-threatening consequences; urgent intervention indicated.

Grade 5: Death related to AE.

In general adverse events will be reported as diseases or syndromes whenever possible, instead of reporting individual component symptoms, signs, laboratory abnormalities or sequelae.

8.1 Definitions

Adverse Event (AE) –For this study, it shall be defined as any untoward or unfavorable medical occurrence in a human subject requiring medical intervention (grade 2 or above) and outside of standard treatment for their underlying illness (i.e. sickle cell disease), including any symptom, or disease state, temporally associated with the subject's participation in the research, whether or not considered related to the subject's participation in the research

Serious Adverse Event (SAE) – Any adverse event temporally associated with the subject's participation in research that meets any of the following criteria:

- results in death;
- is life-threatening (places the subject at immediate risk of death from the event as it occurs);
- requires inpatient hospitalization or prolongation of existing hospitalization (not including usual sickle cell disease pain crises);
- results in a persistent or significant disability/incapacity;
- any other adverse event that, based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition

Unexpected Adverse Event – Any adverse event occurring in one or more subjects in a research protocol, the nature, severity, or frequency of which is not consistent with either:

- the known or foreseeable risk of adverse events associated with the procedures involved in the research that are described in (a) the protocol–related documents, such as the IRB-approved research protocol or the current IRB-approved informed consent document, and (b) other relevant sources of information, such as product labeling and package inserts; or
- the expected natural progression of any underlying disease, disorder, or condition of the subject(s) experiencing the adverse event and the subject's predisposing risk factor profile for the adverse event.

Unanticipated problem involving risks to subjects or others (UP): Any incident, experience, or outcome that meets all of the following criteria:

- unexpected (in terms of nature, severity, or frequency) given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the subject population being studied;
- related or possibly related to a subject's participation in the research; and

- suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) related to the research than was previously known or recognized.

Attribution – the determination of whether an adverse event is related to a transfusion procedure.

Attribution categories:
1. Not Related - Event clearly related to other factors (e.g., clinical state, other therapies; concomitant drugs)

2. Possibly Related - Sequence of event is compatible with study drug, device, or procedure, but could have been produced by other factors

3. Probably Related - Sequence of event is compatible with study drug, device, or procedure and cannot be explained by other factors without much doubt

4. Definitely Related - Sequence of event is compatible with study drug, device, or procedure and beyond doubt cannot be explained by other factors

8.2. Types of Adverse Events to be Collected

Specific information about the following events will be collected for this study on the adverse event checklist case report form:

1. Serious adverse events, regardless of attribution to red cell transfusion

2. Specific adverse events, regardless of attribution to red cell transfusion
   - Myocardial infarction
   - Pulmonary embolism
   - Stroke
   - Renal failure or acute injury
   - Infection/sepsis
   - Ventricular tachycardia
   - Ventricular fibrillation
   - Transfusion-associated congestive heart failure/transfusion-associated circulatory overload (TACO)
   - Transfusion-related acute lung injury (TRALI)
   - Anaphylaxis
   - Graft vs. Host Disease (GVHD)
   - Grade 2 (medical intervention needed) or higher events of the following types:
     - Allergic reaction
     - Sinus bradycardia
     - Sinus tachycardia
     - Hypertension
     - Hypotension
     - Dyspnea
     - Hypoxia

- Wheezing
- Fever
- Hemolysis
- Hyperbilirubinemia
- Hemoglobinuria.

3. Unexpected adverse events (all grades), attributed as possibly, probably or definitely related to red cell transfusion

4. Unanticipated problems

8.3. Timeline for Reporting of Adverse Events

Study staff must fill out an Adverse Event Checklist form within 7 days of each randomized transfusion. The Adverse Event Checklist will indicate whether or not one or more of the events listed in Section 8.2 occurred during the transfusion. The PI will be also be notified of the identified event within 24 hours of learning of the event by phone or email.

8.4. Review of Events for Subjects Enrolled

The Data Safety Monitoring Board (DSMB) is an independent board appointed by the PI. The principal role of the DSMB is to regularly monitor the data from the clinical trial, review and assess the performance of its operations, and make recommendations, as appropriate, to the PI and IRB.

The PI will be notified within 24 hours of learning of an event when a serious adverse event possibly, probably or definitely related to red cell transfusion or an event resulting in death (regardless of attribution) is reported. The PI will review the event as soon as the materials are available. The PI may request additional information regarding the event and may request the subject's treatment arm assignment. Following the review, the PI will sign a log summarizing the event.

The DSMB chairperson will receive information on all serious adverse events possibly, probably, or definitely related to red cell transfusion and all events resulting in death (regardless of attribution). The DSMB chair has expertise in benign hematology and transfusion medicine, will review the serious adverse event materials, and determine if the information is complete.

The DSMB members will meet annually via teleconference. All adverse events will be reviewed during the call, and the group will vote if additional DSMB review is required, and make recommendations about the study.

8.5. Reporting Events to Local Institutional Review Boards

The investigator will notify the local Institutional Review Board (IRB) of all adverse events in accordance with institutional policy.

8.6. Annual DSMB Reports

The DSMB will meet once a year, either in-person or via teleconference. The PI will provide the DSMB prior to the meeting with:

- Accrual totals entered into Velos
- Adverse event log reports for each study subject entered into the electronic data system
- Serious adverse event reports for each study subject entered into the electronic data collection system
- Study compliance issues if applicable
- The DSMB will also be provided each subject's study arm assignment by the primary biostatistician, Dr. Pippa Simpson.

The DSMB will define:

- All-cause mortality, with p-value to compare.
- Number, type, and severity of serious adverse events, overall and by treatment arm. No formal stopping rule is set for this comparison.
- Number of subjects in each treatment arm with at least one clinically documented infection leading to an ED or hospital admission with p-value. No formal stopping rule is set for this comparison.
- Unexpected adverse events and unanticipated problems overall and by treatment arm.
- Evaluate statistically significant difference in serious infection or pain admissions (**Section 10**) between the two arms.
- Recommend stopping guidelines as needed

9. SAFETY MONITORING AND STOPPING GUIDELINES

Interim monitoring plans and stopping guidelines will be defined by the DSMB. This section describes proposed plans. The study is expected to take three years to complete. Stopping guidelines are proposed for two outcomes, serious infection and pain crisis requiring ED or hospital admission. For these two outcomes, yearly looks at the data will take place. Therefore, three looks at the data (including the final look) are anticipated.

In designing the study, a serious infection difference of 20% between the 2 arms would be of clinical interest. As we do not expect to have adequate power in this study of 30 subjects to detect this difference, with an alpha of 0.05 with 15 in each arm, we will be able to detect an infection rate difference of 47-56% between the two arms. Any statistically significant difference in serious infection or pain admissions between the two arms will be considered as evidence for stopping the clinical trial.

10. STATISTICAL CONSIDERATIONS

10.1. Sample Size and Power

Participants will be recruited from UNC and Emory for this proposal. There are about 50 eligible patients at UNC and about 100 chronically transfused subjects at Emory. We anticipate no difficulties reaching our 40 patient study target. We anticipate that we will enroll at least 40 total individuals aged 16-60 years who are managed with chronic transfusions at UNC or Emory and will meet the study inclusion and exclusion criteria. This study will not attempt to stratify the study population for age or gender.

10.2. Analysis Plan

The analysis will be an intent to treat (ITT) with all those randomized being included in the study. For all aims, the distribution of, and relationship between, variables will be explored with summaries, plots, and tree analysis. All statistical estimates of population parameters will be tabulated along with corresponding confidence intervals (CIs). All hypothesis tests yielding large p-value (e.g., $p > \alpha$) will be reported as being inconclusive. Dr. Pippa Simpson, the lead biostatistician, or her designee, will perform these analyses. Where necessary for parametric assumptions, appropriate transformations will be employed with justification for their use; otherwise, non-parametric tests will be used. P-values will be reported without dichotomization wherever applicable. Data obtained from transfusion events where units of the appropriate age could not be obtained (study deviation) will be excluded from a subanalysis of the dataset, and the same distribution of, and relationship between, variables will be explored with similar summaries, plots, and tree analysis as in the ITT analysis. All subjects, regardless of age and gender, will be analyzed together as one cohort. Statistical software used in this study will be Cytel, StatXact and LogXact, SAS version 9.4, and Salford systems CART (Classification and Regression Trees). To adjust for multiple comparisons, we will use the false discovery rate approach which is less conservative than the Bonferroni approach. *Missing data:* Logistic regression will be used to investigate the possible causes for missing data and to investigate if data are missing at random (MAR). Reasons for missing data will be documented in the study database. We do not anticipate any missing data or drop-outs, but we will monitor these events. Assuming data are MAR, multiple imputations will be used for items, and for repeated measures, a random effects models will be used; the structure for the variance covariance matrix will be explored under the constraints of sample size. A sensitivity analysis will also be performed to evaluate the robustness/fragility of the study's main results to reasonable perturbations of the statistical methods and assumptions used. Results of the sensitivity analyses will only be used to guide trust in the main results.

10.2.1 Primary Outcome Analysis

Key variables of interest:
PS – Measured as a percent of RBC positive
PE – Measured as a percent of RBC positive

Microparticles – Measured by number positive per microliter
CD62L – Measured as percent of monocytes positive
Plasma free hemoglobin – Measured as mg/dL
Non-transferrin bound iron – Measured as mg/dL
Rate of clinical infections – Measured as number per days on study

Aim 1:
The primary outcome measure is the proportion of biochemically old red cell units. Previous studies and our own work have suggested that PS, PE, and microparticles increase in a sigmoidal fashion over time.[7,10] For this study, the cut-off for a biologically old unit would be the point at which the sigmoidal curve enters its log phase, which for PE, PS, and microparticles will be the mean value at day 21, or 8%, 3%, and 3/µl respectively based on these preliminary studies. This cutoff is biologically meaningful and is supported temporally by the metabolic changes that occur with red cell storage.[119] Consequently, biochemically old units will be defined as having either a surface PE, surface PS, or a microparticle concentrations ≥ the *a priori*-defined cutoff. We will compare the transfusions provided to the two groups (the proportion of biochemically old units when stored ≥30 days compared to when stored ≤10 days) using a Fisher exact test at an alpha of 0.05. *Power*: With a total sample size of 40 patients (20 in each group), we will have at least 80% power (α=0.05) to detect a difference of at least 47% between the two randomized groups for biochemically old red cell units (close to a 100% difference is expected).

Exploratory: We will calculate the mean, minimum, and maximum levels of PS, PE, and microparticles for each study group, and we will compare the secondary outcome measures of plasma free hemoglobin, heme, and NTBI and also PS, PE, and microparticle concentrations using a two sided two sample t-test at an alpha of 0.05.We will also plan for auxiliary analyses that characterize the relationship between the PS level and the age (days) of the RBC units. This analysis will focus on estimation of the mean PS, PE, and microparticle level as a function of age (days), visualization of the data values, estimation of correlation coefficients, and evaluation of how well age (days) predicts PS, PE, and microparticle levels.

Aim 2:
The primary outcome measure is the change in CD62L (activated) circulating monocyte/macrophage MFI at 2 hours post-transfusion compared to the subject pre-transfusion. We will compare the two groups (≥30 day-old compared to ≤10 day-old unit exposure) using a two sample two-sided t-test of the log at an alpha of 0.05. *Power:* We will have at least 80% power to detect a change in the ratio of 1.35 post-transfusion if the variability is similar to that seen in our preliminary data.

Exploratory: We will use a general(ized) linear model to include biochemically old units transfused (Aim 1 outcome), regardless of unit age, as a covariate in our analysis. Other co-variates in this model will include free heme, cell free hemoglobin, and NTBI. We will similarly compare other obtained activation measures as outcomes: activation markers for monocytes, neutrophils (as listed in section 6) and measured plasma cytokine

concentrations (section 6). We will also plan for auxiliary analyses of the outcome variables in which the explanatory variable is the age (days) of the RBC units --to characterize the degree to which age (days) can accurately predict the clinical and physiologic outcome measures defined. Lastly, as an exploratory aim, we will evaluate the *in vivo* change in recipient red cell PE/PS positivity at 2 and 24 hours post transfusion.

Aim 3:
The primary outcome variable is the rate of post-transfusion infections in the patient. The primary estimand is the treatment effect defined as the difference between the regimen-specific proportions of patients in the target populations that would experience post-transfusion infections. This population parameter will be estimated as a function of the proportions observed in the sample of patients studied. The point estimate will be reported along with the corresponding 95% confidence interval estimate. The presence of an indwelling catheter will be used as a key co-variate in this analysis. *Power:* A difference of 20% will be of clinical interest. We do not expect to have adequate power for this pilot study but at an alpha of 0.05 with 20 subjects in each group we will be able to detect a difference of 47% between the proportions.

Exploratory: We will explore the relationship of blood age and transfusion of biochemically old red cell units on the change in Hb and HbS% over time, daily pain scores (0-10 as an ordinal scale), opioid use and dose, ED and hospitalization rate, infection symptoms, new alloantibody formation, and antibiotic use during the 3-month study period. We will compare groups using a Fisher exact test. We will also plan for auxiliary analyses of the outcome variables in which the explanatory variable is the age (days) of the RBC units --to characterize the degree to which age (days) can accurately predict the clinical and physiologic outcome measures defined.

AIM 4:
We will calculate the mean, median, minimum, and maximum levels of each metabolic compound analyzed by Dr. Alessandro in his laboratory at the University of Colorado. Analysis tools and methods will be determined by his research team, and will focus on changes in the patient and the unit pre and post transfusion.

AIM 5:
Feasibility Analysis: We will assess important variables necessary to develop a future larger clinical trial on this subject. Areas that we will investigate during the course of this trial will include frequency and content of missing data, subject drop out rates, and estimates of outcome measure precision. For this study, we will evaluate the number of males, females, and adolescents that enroll in the study to inform future studies. We do not expect there to be differences in the key outcomes based on these variables, and plan to analyze all 40 subjects together as one cohort, but differences by age and gender will be evaluated and considered.

11. DATA COLLECTION AND VALIDATION

Data will be collected and entered into a web-based data management system. The lead coordinator will routinely validate that all data entry fields are entered. Validations are question-by-question checks that give immediate feedback to help catch data entry errors, form completion errors, and out-of-range values. Reports of outstanding edits, generated upon completion of data entry, will enable continuous cleaning of data.

Confidentiality – Each subject is assigned a unique number to assure confidentiality. Any publication or presentation will refer to subjects by this number and not by name. The medical records department, affiliated with the institution where the subject receives medical care, maintains all original inpatient and outpatient chart documents. Subject research files will be kept in a locked room or locked cabinet.

Data Management – The principle investigator will serve as the trial coordinator for this study. The PI will monitor timely entry of data into the study database. Access to all source documentation maintained by the lead coordinator, including correspondence and source data, will be available for monitoring and audit purposes.

Data Quality - The principal investigator will routinely check for data accuracy and data completeness. When data are found to be missing, the principal investigator or his designee will attempt to document in the electronic record why the values are missing. The principal investigator and his designee will maintain a data dictionary and will evaluate adherence to the codebook at regular intervals.

12. PROTECTION OF HUMAN SUBJECTS

This study will be conducted according to Good Clinical Practices (GCP), the rules and regulations of the Institutional Review Board at the University of North Carolina, and in accordance with state and federal agencies.

13. INVESTIGATOR RESPONSIBILITY

13.1 Institutional Review Board (IRB) / Approval

No patient will be enrolled in the study until the IRB has approved the protocol and the Informed Consent Form. Documentation of approval will be maintained at all times. At study termination, a study summary will be submitted by the PI to the IRB. Copies of all submissions to and correspondence (approvals and disapprovals) from the IRB/EC will be maintained on file.

13.2 Informed Consent

If a patient is potentially eligible for the study as defined, the patient will be approached to obtain written informed consent. The background of the study and the potential benefits and risks will be explained. The patient or patient’s legally authorized

representative must sign the consent form that has been approved by the IRB prior to enrollment. Failure to obtain signed informed consent renders the patient ineligible for the study. Copies of the signed informed consent shall be kept in subject's study files.

13.3 Subject Data Protection

Subjects will be identified in the electronic case report form (eCRF) by a subject identification number. All information and data concerning subjects or their participation in this study will be considered confidential. All data used in analysis and reports will be used without identifiable reference to the subject. At all times throughout the study, confidentiality shall be observed by all parties involved. All data shall be secured against unauthorized access.

14. MONITORING AND QUALITY CONTROL

14.1 Site Training

Only trained personnel can perform study related procedures. All investigators and relevant staff such as study coordinators and blood bank staff members will be trained on the protocol. These individuals will be trained by the PI or lead coordinator. The study team will be responsible for ensuring that the hospital staff directly responsible for patient care (staff nurses and physicians) is adequately trained in the management of these study patients and blood bank procedures.

14.2 Monitoring of the Study

The PI or designee will monitor the investigation to ensure that it is conducted in accordance with the protocol.

15. RECORDS AND REPORTS

15.1 Case Report Forms

Case Report Forms (CRFs) will be used to collect all subject data during the course of the study. The Principal Investigator or predetermined designated individual shall be responsible for completion of the CRFs. All protocol deviations shall be documented and a justification for any missed assessments shall be provided on the protocol deviation log. Completed CRFs will be verified at regular intervals throughout the study.

15.2 Source Documents

Good Clinical Practice Guidelines require that investigators maintain information in the subject's medical records, laboratory reports, clinic charts, etc. that corroborate data recorded on the CRFs. In order to comply with these requirements, the following information should be maintained:

- Medical history/physical condition of the subject before enrollment sufficient to verify protocol entry criteria
- Dated and signed notes for specific results of procedures and exams

15.3 Record Retention

The Investigator will maintain the signed Informed Consent Forms, CRFs, study documentation (listed above) and source documents for at least 10 years per University of North Carolina protocol after study completion or termination. In addition, the Investigator will not discard or destroy any study-specific materials unless otherwise instructed.

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