Study Protocol

Kinetics of Red Blood Cell Clearance in Chronically Transfused Children with Sickle Cell Disease

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Title: Kinetics of Red Blood Cell Clearance in Chronically Transfused Children with Sickle Cell Disease

Short Title: Biotin-RBC Transfusion in SCD

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SUMMARY

Sickle cell disease (SCD) carries significant morbidity as a result of red blood cell (RBC) sickling and hemolysis. Stroke is one of the most devastating sequelae of SCD. Chronic transfusion therapy (CTT) reduces stroke risk by (1) supplying normal, non-sickle RBC to circulation, thereby reducing the percentage of endogenous sickle RBC in circulation, and (2) maintaining a higher hemoglobin (Hb), thereby suppressing erythropoiesis of new sickle RBC. While the efficacy of CTT in stroke prophylaxis is well-established, nearly 45% of children continue to have silent or overt strokes despite CTT. The failure of CTT to prevent stroke events may be related to inadequate reduction of circulating sickle RBC and erythropoiesis. The amount of circulating sickle-RBC is related to the survival kinetics of both transfused RBC and endogenous sickle RBC. In a large, longitudinal analysis of CTT in SCD, we found wide variation in the survival of donor RBC following transfusion, with faster clearance associated with patient immune features (historical RBC alloimmunization and spleen presence) and with donor RBC glucose-6-phosphate-dehydrogenase (G6PD) deficiency. To better understand the roles of patient and donor factors in the survival and clearance of transfused RBC, we propose a mechanistic, clinical trial during chronic transfusion episodes in patients with SCD, in which a small aliquot of each transfused unit is labeled with biotin conjugated to RBC surface proteins, to safely identify and measure the *in vivo* survival of donor RBC. Aim 1 will examine the relationships of the recipient's immune system (past alloimmunization, splenic volume, and markers of reticuloendothelial system function) on the post-transfusion survival of biotin-labeled donor RBC. Aim 2 will examine the relationships of donor RBC G6PD levels and donor RBC metabolomics with the *in vivo* survival and changes in donor RBC senescence markers. Completion of these aims will increase our understanding of mechanisms for the variability in RBC survival during CTT, identifying donor and recipient risk factors for decreased RBC survival. Ultimately this knowledge will inform the management of CTT to improve the prevention of strokes in SCD. Aim 3 will compare the *in vivo* survival and clearance rate of phenotype-matched RBCs prepared with an investigational pathogen-reduction system (INTERCEPT Blood System) vs. the *in vivo* survival and clearance rate of conventional, phenotype-matched RBCs (not treated with INTERCEPT). Biotin labeling of donor RBC will be used to measure RBC survival.

1. BACKGROUND

Sickle cell disease (SCD), an inherited hemoglobinopathy carries significant morbidity and mortality related to the pathophysiology of red blood cell (RBC) sickling, vaso-occlusion, and chronic hemolysis which promotes vasoconstriction and vascular endothelial dysfunction.[1,2] Stroke, one of the most devastating consequences of SCD, is a prevalent complication in young children and adults. In the absence of appropriate screening and intervention, up to 10% of children will have overt stroke, while up to 40% may have silent cerebral infarcts (SCI).[3,4] For the majority of SCD patients, chronic transfusion therapy (CTT) is the only available treatment to reduce the risk of stroke and neurocognitive insult.[5,6] Following a first stroke in children with SCD, secondary stroke can be reduced from >66% to ≤13% with a CTT regimen that targets a sustained HbS level below 30%.[7-10] CTT has also reduced the incidence of primary stroke when used for at-risk children identified by abnormal cerebral arterial velocities on transcranial Doppler screening.[11-13] In those with prior SCI, CTT has been shown to reduce the incidence of new stroke and SCI.[6] Despite these protective benefits of CTT, up to 45% of children may have progressive stroke or SCI even when adherent to a CTT regimen. CTT remains an essential but imperfect therapy for many individuals with SCD. It is critical to understand the reasons why CTT often fails to achieve the desired hematologic goals of increased Hb, low HbS, and reduced reticulocytes, in order to improve the safety and efficacy of this much-needed SCD therapy.

CTT is effective in reducing the acute and chronic complications of SCD in a two-fold manner. First, repetitive transfusions occurring approximately once a month dilute endogenous HbS-containing RBC (HbS RBC) with transfused HbA-containing RBC (HbA RBC). Second, by effectively reversing the anemia that occurs with SCD, CTT suppresses erythropoiesis and thus reduces the production of new HbS-RBC and reticulocytes which mediate sickling, vascular adhesion, and cerebrovascular damage. A simple CTT cycle typically has an interval of 3-5 weeks. The initial rise in Hb with transfusion suppresses endogenous erythropoiesis, leading to decline in HbS over the next several days. Transfused HbA-RBC survive longer than HbS-RBC, further reducing the percent of HbS. As transfused RBC age and are cleared, total Hb declines, causing erythropoietic production of sickle-RBC to increase prior to the next transfusion (Figure 1; solid lines). Clinical CTT protocols provide guidelines for transfusion volume and frequency based primarily on pre-transfusion total Hb, with the typical goal of reducing HbS to HbS ≤30% of total Hb. While this formulaic approach to CTT achieves the desired hematologic and clinical outcomes of stroke prevention in the majority of patients, the lack of a personalized approach to CTT fails in many patients. In cases of reduced survival of HbA-RBC following transfusion, patients may not achieve the desired degree of HbS suppression (Figure 1; dashed lines). Hematologic response to CTT is a function of transfusion survival, endogenous RBC survival, and other factors driving erythropoietin (EPO) production,[14,15] but individual RBC kinetics and erythropoietic drive are not specifically evaluated when providing CTT to patients. While the target of HbS<30%, widely used in the initial studies of CTT, has become commonly accepted as a standard goal of clinical care and in numerous randomized trials of CTT;[5,6,12,16-18] there are no comparative studies of HbS levels that have identified the optimal minimal level of HbS for

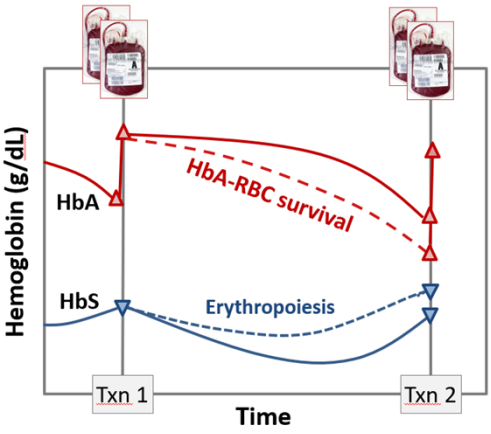


Figure 1. Survival of transfused HbA-RBC and secondary changes in erythropoiesis during a CTT cycle. The dashed line depicts increased transfusion clearance allowing increased erythropoiesis (HbS).

stroke prevention nor any other proven laboratory markers with which to evaluate the clinical efficacy of CTT.[19-21]

Our two-center prospective study of pediatric SCD patients followed for 1 year while receiving CTT for stroke prevention – the Sickle Cell Chronic Transfusion Cohort (SCTT Cohort) – has provided key preliminary data on the causes of variability in hematologic outcomes in CTT. We found that in 24% of patients, the median pre-transfusion HbS was higher than the standard threshold of <30%, despite good patient adherence to the CTT regimen.[22] The failure to reach HbS targets by following generalized CTT protocols underscores the need for a more personalized Precision Medicine approach to transfusion therapy, by identification and modification of patient or donor factors that affect RBC clearance and erythropoiesis in SCD.

In the SCTT Cohort, the rate of decline in HbA between transfusion events (ΔHbA, an estimate of transfused RBC clearance), was strongly associated with increased HbS, increased reticulocyte count, and lower total Hb at the end of the transfusion cycle, demonstrating the relevance of transfusion clearance to the suppression of erythropoiesis and thus to CTT hematologic and clinical outcomes.[22] We found ΔHbA had broad inter-patient and intra-patient variability (Figure 2). Greater declines in HbA were associated with past RBC alloimmunization and splenomegaly, while less rapid clearance of HbA was found in those with past splenectomy. Additionally, broad intra-patient variability in HbA decline with different transfusion episodes suggests the influence of blood donor and unit-specific characteristics on transfusion survival. Glucose 6-phosphate dehydrogenase (G6PD) deficiency, found in 9% of the donor units, was associated with more rapid HbA decline and increased erythropoiesis during CTT.[23] G6PD is the key enzyme in the metabolic pathway protect RBC from reactive oxygen species and organic peroxides that contribute to RBC damage and hemolysis.[24]

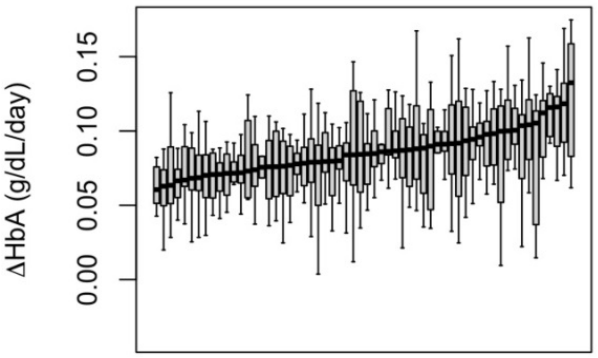


Figure 2. Variability in ΔHbA during CTT. Each box and whisker plot represents 1 patient with multiple CTT episodes. The dark line is median ΔHbA; whiskers are 95% range.

Variability in donor RBC metabolites including G6PD may affect storage hemolysis and *in vivo* post-transfusion recovery (PTR) at 24 hours. RBC metabolomics, the comprehensive profiling of metabolite levels in RBC enzymatic pathways, allows for characterization and identification of metabolic variations that may be associated with poor *in vivo* PTR.[25] The NHLBI Recipient Epidemiology Donor Evaluation Study III (REDS-III) characterized the effects of donor genomic, metabolomics, and epidemiologic factors on storage and oxidative-induced hemolysis in >13,000 blood donors of different backgrounds.[26,27] Similarly, our group has quantified metabolites in donor RBC units stored for 42 days, identifying biochemical profiles that change significantly during storage, thus acting as either markers or mediators of decreased pre-transfusion RBC viability. Significant changes were seen in intermediary metabolites of the glycolysis and glutathione antioxidant pathways, increased RBC membrane peroxidation, and changes in arachidonic acid and linoleic acid metabolism.[25,28] These RBC metabolomics may comprise a biochemical signature to predict cellular aging and decline in RBC viability during blood storage.

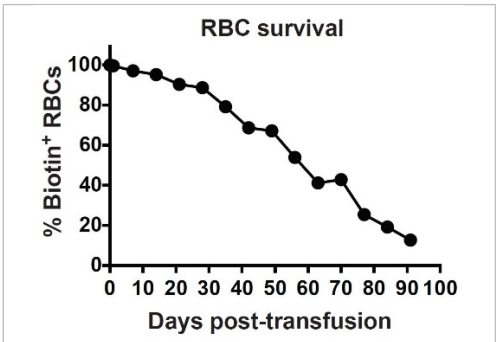


Figure 3. Biotinylated autologous BC survival and marker analysis from a representative volunteer.

RBC Survival *in vivo* can be determined by biotin labeling of transfused RBC**.** The primary limitation of the SCTT Cohort was the use of estimated ΔHbA as an imprecise surrogate to reflect survival of the most recently transfused units. Labeling RBC prior to transfusion provides a method to precisely measure survival of a single transfused RBC population over time. Our investigative group currently uses this technique to study RBC survival following autologous transfusion to healthy volunteers (Figure 3). Donor RBC are treated with sulfosuccinimidyl biotin (sulfo-NHS-biotin) to covalently bond the carboxyl group on biotin to lysine residues on RBC surface membrane proteins.[29] Biotin-labeled RBC (BioRBC) can be safely transfused, and their circulatory survival accurately determined by flow cytometry using sequential blood samples over several weeks' time.[30] This method allows simultaneously tracking of multiple RBC populations (e.g. multiple donor units), using multiple different densities of biotin for labeling.[31,32]

The feasibility, safety, and efficacy of this technique has been demonstrated in studies of allogeneic RBC transfusion survival in preterm and low birth weight infants.[33] Autologous transfusion of BioRBC in individuals with SCD has been reported previously in a series of studies examining the survival of various RBC populations. In 1998, Franco et al. first reported this technique in 7 adults with SCD (as well as healthy controls), demonstrating that the technique provided similar survival data in comparison to the previous gold-standard of ^{51}Cr labeling.[34] In this and in subsequent studies, RBC were collected from SCD patients in their baseline state of health, washed and incubated with NHS-biotin, then reinfused into the patient on the same day. The lifespan of the cells was determined at 24 hours and long term through serial blood sampling and flow cytometric identification of BioRBC.[34] Through this technique, the survival of different populations of sickle RBC (dense sickle cells, reticulocytes, F cells, and cells with externalized phosphatidylserine) have been elucidated.[35-37] No adverse events related to maintenance of sterility, accelerated hemolysis, or immunogenicity of BioRBC were reported in these studies.

Immunogenicity of BioRBC:

Experience in healthy adult donors

The development of antibodies to BioRBC (anti-BioRBC antibodies) has been observed in approximately 15% of healthy adult subjects following autologous BioRBC infusions. The likelihood of antibody development appears to be dose related (both density of biotin on the RBC surface and total volume of BioRBC transfused). In fact, anti-BioRBC antibodies have only been described in subjects who received BioRBC at densities >18 µg/mL (with BioRBC enrichment of total RBC ranging 1.7% - 6.0% at day 1 post-transfusion).[30,38,39]

Methods and results of screening for BioRBC antibodies have been reported only for healthy adult subjects. BioRBC antibody screens are performed by the conventional RBC antibody screen technique of IgG gel card, in which reagent RBC are biotinylated at a high density (e.g. 250 µg/mL) and combined with patient plasma to screen for reactivity.[39] Increasing the density of reagent RBC labeling leads to increased sensitivity but decreased specificity for detecting true BioRBC antibodies. Screening for reactivity to BioRBC among 161 individuals

never exposed to BioRBC transfusion demonstrated no reactivity to reagent cells biotinylated at 18-54 µg/mL, however 1.2% had reactivity to reagent BioRBC at 162 µg/mL; 4.3% had reactivity at 486 µg/mL, and 13% had reactivity at 1458 µg/mL, implying both a loss of specificity for detection of BioRBC antibodies and the occurrence of naturally occurring BioRBC antibodies.[39] Naturally occurring antibodies against biotinylated proteins had previously been identified in 10% of unexposed individuals, typically with weak avidity.[40]

Anti-BioRBC antibody emergence has not been detected until approximately 3 – 5 months from initial exposure in healthy subjects, and reactivity had disappeared (antibody evanescence) between 6 – 12 months after emergence.[39,41] Importantly, BioRBC survival, as reflected by both 24-hour recovery and half-life were not different among subjects with or without BioRBC antibody development, and no clinical hemolysis was observed in any subject.[41]

In 2022, Mock et al reported their summary experience to-date of BioRBC transfusion and antibody detection in 42 healthy adults, of whom 6 (14%) developed BioRBC antibodies after exposure.[38] Among patients who did develop antibodies, all were detected in a time frame ranging 84 – 154 days from initial exposure to BioRBC, **thus providing a framework/reference for future investigators to appropriately screen for this potential outcome, if re-exposure is to be considered.**

Three of these BioRBC immunized individuals (who had initial antibody detected between 84 – 154 days post exposure) underwent re-exposure to autologous BioRBC at intervals ranging from 4.8 – 22.1 years from first exposure. Initial BioRBC exposure was significantly higher than the re-exposure, for both densities (up to 162 µg/mL for initial exposure) and BioRBC enrichment (% of total RBC at day 1). Following re-exposure, BioRBC antibodies were detected within 5-7 days in all 3 subjects, indicating an anamnestic response. A series of re-exposures to different doses of autologous BioRBC were performed in these 3 immunized patients to assess hypotheses that BioRBC antibodies lead to accelerated RBC clearance in a dose/density-dependent fashion:

- One subject (#1) had an initial exposure to 4 different densities of autologous BioRBC (6, 18, 54, and 162 µg/mL) then underwent re-exposure to these same densities of BioRBC 5 years later. Clearance of all densities of BioRBC was accelerated on re-exposure, in a density-dependent rate (54 µg/mL > 18 µg/mL > 6 µg/mL; data excluded for BioRBC >54 µg/mL due to potential for non-immune clearance).
- Subject #1 subsequently had 2 additional exposures. Exposure to BioRBC at 2 µg/mL stimulated another anamnestic antibody response yet did not accelerate BioRBC clearance, as compared to the rate of clearance of the initial exposure. This subject had a third re-exposure to BioRBC at 2 and 6 µg/mL while BioRBC antibodies were still present; post-expsoure the antibody titer remained constant and RBC survival was shortened for the 6 µg/mL but not the 2 µg/mL BioRBC.
- Subject #2 had a similar initial exposure (6, 18, 54, and 162 µg/mL), but only 1 lower dose re-exposure of BioRBC at 2 and 6 µg/mL. This patient had a strong anamnestic antibody response (titer 1:4096) with accelerated clearance of both 2 and 6 µg/mL BioRBC.
- Subject #3 had a lower dose initial exposure (20 µg/mL only) and a 22 year interval to re-exposure to BioRBC at 2 and 6 µg/mL. Post-exposure, antibodies detected after re-exposure were only detected in undiluted plasma, and the survival of BioRBC was only slightly shortened as compared to initial RBC survival studies.

The experience of these 3 subjects demonstrates that anti-BioRBC alloimmunization can result in accelerated clearance with subsequent re-exposures to BioRBC particularly at higher labeling densities, which would impact the validity of RBC survival studies. From a safety perspective, appearance of anti-BioRBC antibodies in these subjects was not associated with any clinical adverse events or laboratory changes in hemoglobin, RBC indices, reticulocyte counts, or white blood cells.

Additionally, Mock et al. report 3 healthy adult subjects who did not form anti-BioRBC antibodies after initial infusion of biotinylated RBC (at densities of 25-30 µg/ml) also did not form anti-BioRBC antibodies after a re-exposure to BioRBC. From these studies of BioRBC exposures, Mock et al published recommendations for BioRBC survival studies, aimed at minimizing initial BioRBC alloimmunization and reducing the risks of accelerated BioRBC clearance for re-exposure studies. These recommendations are summarized in Table 1:

Table 1. Recommendations for BioRBC red cell survival studies, Mock et al.[38]

For initial BioRBC studies:
• Prior to study: Screen subject's plasma for naturally occurring BioRBC antibodies using IgG gel card assay with reagent BioRBC ~256 µg/mL
• **Use the lowest practical BioRBC density, such that density x volume ≤ ~180 µg**. As examples:
  ○ Single density study: ≤18 µg/mL, ≤10 mL volume
  ○ Two density studies: ≤7 mL of BioRBC-18 + ≤7 mL of BioRBC-6
  ○ Three density studies: ≤7 mL of BioRBC-18 + ≤7 mL of BioRBC-6 + ≤7 mL of BioRBC-2- µg/mL.
• Monitor for plasma BioRBC antibodies between 8 – 24 weeks post exposure

For BioRBC re-exposure studies: • Do not re-expose to BioRBC if antibodies have been previously detected. • Prior to study: screen plasma for BioRBC antibodies and do not proceed if detected. • Dose BioRBC as per recommendations for initial studies • Monitor for plasma BioRBC antibodies at frequency recommended after initial studies

BioRBC Antibody screening in SCD subjects

Screening for BioRBC antibodies following BioRBC red cell survival studies were not reported in any of the earlier studies of autologous BioRBC in SCD by Franco et al.[34-37] In our experience to-date **on this research study** of biotinylation of allogeneic RBC infusions in chronically transfused SCD patients, we have screened patient plasma against reagent BioRBC at 250 µg/mL (BioRBC-250) using IgG gel card methods, as described by Schmidt et al, with repeat screens against BioRBC at 50 µg/mL (BioRBC-50) for plasma samples that reacted to BioRBC-250.

Of 15 enrolled subjects, all had negative screens for BioRBC antibodies prior to exposure.

- 10 subjects have completed 6 months of screening following BioRBC transfusion
- 5 subjects remain on study (<6 months from BioRBC transfusion).
- 3 subjects have had weak (1+) reactivity (graded 1-4) to BioRBC-250 and BioRBC-50 during the follow up period, indicating a weak antibody to biotinylated RBC. In Subject 3 this reactivity was present only at weeks 1-3, then was not detectable. In Subject 4,

BioRBC-specific reactivity appeared at week 12-13 (initially masked by development of a warm autoantibody at week 7, but confirmed). In subject 9, the reactivity was present only at weeks 2-4, then was not detectable.

- In all of these subjects, there was no increase in hemolysis, decline in hemoglobin, or accelerated clearance of BioRBC, and there was no reported clinical adverse symptom or event.

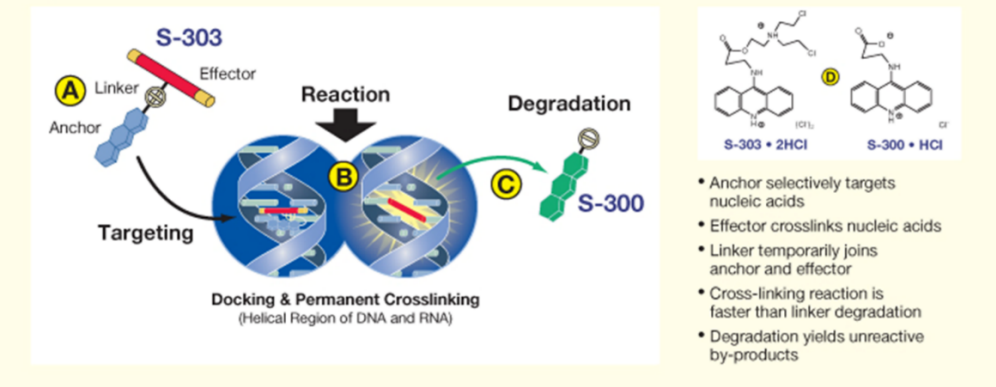
The conclusions from both the published literature, as well as our experience on this trial, shows that the incidence of antibodies to BioRBC in individuals with SCD after 1 exposure to biotinylated RBC transfusion is similar or slightly higher than that reported in healthy adult recipients of autologous BioRBC (14%). In these cases, antibodies showed relatively weak *in vitro* reactivity and demonstrated no adverse clinical outcome or laboratory evidence of increased hemolysis or BioRBC accelerated clearance. Since the publication of Mock et al. recommendations, all subjects have received cumulative volumes of BioRBC transfusion less than or equal to those recommended by Mock et al., with no BRBC antibodies detected in subsequent subjects.

Optional Investigation Arm (Aim 3): INTERCEPT Blood System for Red Blood Cells

The safety of RBCs and other labile blood products prescribed for patients undergoing CTT has historically been assured through a combination of donor selection practices, antiseptic collection and safe storage techniques, and a limited number of laboratory screening assays. However, serious transfusion-transmitted infections (TTI) caused by known and emerging viruses, bacteria and protozoa;[42,43] and fatal transfusion complications such as transfusion-associated graft-versus-host disease (TA-GVHD) still pose a threat to transfusion recipients.[44]

To proactively address the risk of TTIs and TA-GVHD in patients transfused with RBCs, the INTERCEPT Blood System for RBCs (Cerus Corporation, Concord, CA), is an investigational device designed to inactivate contaminating pathogens, including donor leukocytes (IDE #13803). The INTERCEPT Blood System for RBCs uses a nucleic acid-targeting compound called amustaline (S-303) that intercalates into and upon activation, covalently bind to nucleic acids of viruses, bacteria, parasites and leukocytes that may contaminate RBCs. These covalent bonds form irreversible adducts and crosslinks, thus preventing replication and TTI and reducing the risk of TA-GVHD. (**Figure 4**) Glutathione is used with amustaline to quench side reactions between the acridine moiety in amustaline and RBC surface membranes.

Figure 4: Amustaline (S-303) Mechanism of Action

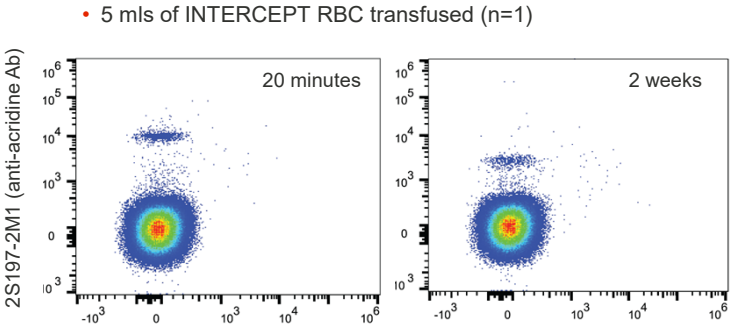


Additional details regarding the physical, chemical and pharmaceutical properties of the amustaline formulation, the investigational INTERCEPT RBC device, and the indications for use of INTERCEPT RBCs are available in the Investigator's Brochure (INB 00012).

Recovery and survival of INTERCEPT RBCs has been studied in healthy volunteers transfused with 35-day-old biotin-labeled INTERCEPT RBCs.[45] 24-hour recovery was comparable for biotin-labeled conventional RBC, INTERCEPT RBCs, and $^{51}Cr/^{99m}Tc$ radiolabeled INTERCEPT RBCs. Overall lifespan was improved in both categories of biotin-labeled RBCs; however, lifespan and half-life in ^{51}Cr-labeled INTERCEPT RBCs were ~12% and ~17% lower than biotin-labeled and INTERCEPT RBCs, respectively. This finding suggests that ^{51}Cr radiolabeling may underestimate RBC survival. These results were also aligned with similar recovery and survival data derived from a larger randomized recovery and survival study with $^{51}Cr/^{99m}Tc$ radiolabeled INTERCEPT RBCs.[46]

RBC clearance kinetics may also be assessed by flow cytometry for surface concentration of acridine adducts on the INTERCEPT-treated RBCs. An exploratory (n=1) study demonstrated that INTERCEPT RBCs persist for at least 90 days and the surface concentration of acridine diminishes rapidly on transfusion. (**Figure 5)** These changes can be quantified by phycoerythrin (PE) labelling of surface acridine captured by flow cytometry.

Figure 5: Circulating INTERCEPT RBC Detected Post-Transfusion by Flow Cytometry for RBC Surface Acridine.



Intensity of surface acridine is reduced in vivo by ~40% within 24 hours of transfusion, and by 72% by 1 week.

Immunogenicity of INTERCEPT RBCs: In early studies of the pathogen inactivation process for RBCs there was evidence of treatment-emergent anti-INTERCEPT RBC antibody formation in two patients participating in a chronic RBC transfusion study.[47] In addition, positive crossmatch reactions between INTERCEPT RBCs and serum from two Control patients (who had never received INTERCEPT RBCs) prompted further evaluation. A larger study demonstrated that a small proportion of individuals (0-1.6%) with no prior amustaline

exposure had positive crossmatch reactions with INTERCEPT RBCs ("naturally occurring" antibodies). Initial characterization of these antibodies determined the IgG subtypes to be non-IgG_1, non-IgG_3 which are of lower clinical significance. The INTERCEPT Blood System for RBCs has been reformulated to reduce the level of RBC cell surface labelling. Animal models confirm lower levels of immunogenicity.

Three clinical studies have been completed with the current INTERCEPT Blood System for RBCs and two Phase III studies are underway. Additional detail related to clinical data produced by these studies is provided in the Investigators Brochure. Several treatment emergent antibodies to INTERCEPT RBCs have been detected, all without evidence of clinical significance. The studies remain blinded after FDA and DSMB review. It is not known whether the subjects received Test or Control RBCs.

2. SPECIFIC AIMS

Aim 1: In patients with SCD or or transfusion-dependent thalassemia (TDT) receiving CTT, to assess the relationships of recipient RBC alloimmunization and reticuloendothelial system (RES) activity to the *in vivo* survival of donor RBC.

Hypothesis: In patients with past alloimmunization responses and increased RES activity, donor RBC will show decreased survival in vivo and an increase in senescence markers.

Aim 2: In the same population, to assess the relationships of donor G6PD status and global RBC metabolomics to the *in vivo* survival and senescence changes on donor RBC.

Hypothesis: Donor G6PD deficiency and specific RBC metabolomic patterns during storage will be associated with earlier appearance of senescence changes on donor RBC and shorter post-transfusion in vivo survival.

OPTIONAL (Aim 3) SECONDARY AIMS: The following aims are specific to an additional optional investigational treatment arm using pathogen reduced (INTERCEPT) RBCs. Subjects will follow the structure of Aim 1 in which they receive phenotype-matched allogenic RBCs treated with the INTERCEPT Blood System for RBCs. The specific aims described below are related only to subjects who choose to participate in this optional arm.

1. **Compare *in vivo* survival and clearance rate of pathogen-reduced, phenotype-matched RBCs with *in vivo* survival and clearance rate of untreated (conventional not pathogen-reduced), phenotype-matched RBCs as assessed by biotin labeling.**

2. **Compare *in vivo* RBC clearance kinetics assessed by surface acridine flow cytometry and by biotin labeling in individuals transfused pathogen reduced RBCs in order to validate the surface acridine flow cytometry assay for tracking pathogen reduced RBCs.**

The relationships of RBC survival and clearance to recipient characteristics (including immunologic measurements and alloimmunization history) and to donor characteristics (including donor RBC metabolomics) may be examined in both the primary and the optional arms through completion of these specific aims.

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This study will help to establish better benchmarks for hematologic response to CTT, allow for early identification of suboptimal response to CTT in patients with SCD or TDT, and identify donor metabolomic profiles associated with decreased transfusion survival, allowing for more specific identification of optimal donor and unit selection for patients with SCD. These findings will directly inform future transfusion medicine trials in SCD to bring a Precision Medicine approach to the bedside, allowing for consideration of individual patient immune and hematologic responses and donor characteristics to provide the safest, most effective delivery of CTT to improve stroke prevention outcomes in children with SCD or TDT.

3. METHODS

3.1 Rationale: There is large inter-patient and intra-patient variability in the survival of phenotype-matched, transfused red blood cells in patients with SCD and TDT. How recipient immunologic characteristics (alloimmunization and spleen function) and donor unit characteristics (metabolomics of cellular energy and antioxidant pathways and storage duration) affect the *in vivo* survival of transfused donor RBC and the subsequent outcomes of CTT in SCD or TDT is unknown.

Rationale for Optional Arm (Aim 3): Use of biotin-labeled INTERCEPT RBCs will allow for a direct comparison and description of RBC survival and lifespan in pathogen reduced and non-pathogen reduced (conventional) phenotype-matched RBCs transfused in the same patient. Residual acridine present on the surface of INTERCEPT RBCs offers a novel approach to measure RBC *in vivo* survival using a surface acridine flow cytometry assay. Use of INTERCEPT RBCs will generate new data regarding the feasibility and effectiveness of acridine tracking as an alternative RBC survival measurement approach compared to biotinylation in INTERCEPT RBCs.

3.2 Study Design: This is a single-arm, mechanistic clinical trial to measure predictors of senescence and the *in vivo* survival of transfused RBC in individuals with SCD or TDT receiving CTT. Labeling of the clinically-indicated RBC transfusion with biotin is the primary "exposure" that will be used to measure the primary outcome of *in vivo* BioRBC survival. Survival measurements include half-life (T_{50}) and long-term life span (linearly extrapolated as mean potential lifespan, MPL). Both T50 and MPL survival are defined only for BioRBC that remain in circulation for at least 1-day post transfusion (24-hour post transfusion recovery, PTR_{24}). A secondary (exploratory) outcome is changes in surface immunoglobulin, complement, sialic acid glycosylation, CD47, and phosphatidylserine (PS) exposure on transfused bio-RBC (markers of RBC senescence).

Predictor variables of interest are: (1) Patient alloimmunization, defined by historical detection of ≥1 RBC alloantibodies; (2) Patient RES function as measured quantitatively by RBC Howell Jolly Body (HJB) count; (3) spleen dimensions by abdominal ultrasound and by review of annual abdominal MRIs (routinely done for liver iron quantification) will be assessed. Spleen measurements will be conducted in all patients, even those with past surgical splenectomy due

to the possibility of accessory spleen regrowth; (4) Donor RBC G6PD activity level and metabolomic profile.

Optional Arm (Aim 3): The same basic study design described above will apply to the optional investigation with pathogen reduced INTERCEPT RBCs with the addition of comparative analyses to describe differences between pathogen reduced and conventional RBCs transfused simultaneously with RBCs labeled with different surface biotin concentrations. Patients who participate in the optional pathogen reduced RBC arm will also be assessed against the following additional endpoints:

1. The same recovery and survival variables described in the primary endpoint assessed via surface acridine flow cytometry compared to biotin labeling in individuals transfused pathogen reduced, phenotype-matched RBCs.
2. Development of antibodies with specificity to INTERCEPT RBCs.

3.3 Study Population: Individuals with SCD or TDT receiving CTT who meet the following selection criteria:

Inclusion criteria:

1) Age 2 – 65 years

2) Hemoglobinopathy:

a) Any sickle cell disease genotype, or

b) Transfusion-dependent thalassemia (TDT)

(3) Receiving CTT for ≥3 months prior to enrollment.

(4) for subjects with past BioRBC transfusion exposure, BioRBC antibody screens must have been conducted through at least 6 months post exposure, with negative results.

Exclusion criteria:

(1) Anticipated cessation of CTT in the next ≤2 months;

2) Ongoing consumption of biotin or raw egg dietary supplements

3) Antibody specific of INTERCEPT RBCs at baseline (for subjects consenting to the optional arm)

4) BioRBC-specific antibodies ever detected in the past, or detected on post-enrollment screening prior to first infusion of Bio-RBC.

Subjects will not be excluded based on any sickle cell therapies, including (but not limited to) the use of hydroxyurea, chronic transfusion therapy, hematopoietic stem cell transplantation, or gene therapy.

We will enroll non-English-speaking participants. We anticipate subjects will speak either Spanish or Swahili but may speak other languages. The study team is English speaking. An IRB approved short form or a fully translated and IRB approved consent/assent will be used. The consent process will take place with a qualified interpreter present.

Subject Screening and Recruitment: Patients who receive CTT are pre-identified by existing

clinical lists at the study locations. During the recruitment phase, scheduled patients will be routinely screened to determine eligibility and approached for consent by the P.I. Recruitment targets will average 1 patient per month. An equal number of alloimmunized and non-alloimmunized patients will be enrolled. To ensure feasibility, we will target enrollment of 1 or more patients per month for a minimum of 40 patients over approximately 36-40 months. Target enrollment will be approximately 25-30 pediatric (<18 years old) and 10-15 adult (≥18 years old) patients. The optional arm of the study (Aim 3) will enroll at least 6 patients.

3.4 Study Procedures and Measurements/Data collection: Patient and/or parental consent to participate in research will be obtained prior to the planned transfusion. For a single transfusion episode, a small fraction of each RBC unit (20 mL) will be labeled with biotin. Selection of RBC units for transfusion as part of this study will follow the rigorous, institutional standard of care requirements for blood products provided to SCD and TDT patients: leukoreduced, HbS negative, universal minor antigen matching for C/c, E/e, K, with extended matching for Fy^a, Jk^b, and past alloantibodies (for alloimmunized patients). Institutional protocol for CTT will be followed to determine transfusion volume and interval.

3.4.1 Clinical data collection: Patients' lifetime transfusion and alloimmunization histories will be recorded, including seeking full transfusion histories from any outside hospital facility. Donor units transfused during the study (including both biotin-labeled and non-labeled units transfused later in the study period) will be identified by unique unit number; unit donation date, additive solution, volume, blood type and minor antigen typing will be recorded for each unit. Laboratory testing that will be performed through the clinical laboratory is indicated in Table 1. Case report forms will be managed using REDCap (Research Electronic Data Capture), a secure, web-based application. Quality control will be applied to each phase of data handling to ensure that all data are reliable and have been processed correctly, including generation of regular reports to track routine data collection and maintain data completeness.

Table 1. Study Procedures and Tests

	Transfusion *	PRE-TXN Studies	Clinical Labs			Research Labs			
								OPTIONAL ARM (Aim 3)	
			CBC, Retic, HbA,S †	EPO	LDH, DAT	BioRBC	Anti-BioRBC antibody	RBC Surface Acridine	INTERCEPT RBC Antibody
Day -1 (range -3 to 0)		US, RBC PIT, Urine HCG	X	X	X		X***		X***
Day 0	SOC **Biotin labeled**		X (post)			X (post)		X (post)	
Day 1			X		X	X		X	
1 week			X	X	X	X	X	X	X
2 weeks			X	X	X	X	X	X	X
3 weeks	SOC (unlabeled)		X	X	X	X			
4 weeks			X	X	X	X	X	X	X
5 weeks			X	X		X			
6 weeks			X	X		X	X	X	X
7 weeks	SOC (unlabeled)		X	X		X			
8 weeks			X	X		X	X	X	X
9 weeks			X	X		X			
10 weeks			X	X		X	X	X	X
11 weeks			X	X		X			
12 weeks	SOC (unlabeled)		X	X		X	X	X	X
13 weeks **			X			X			
14 weeks **			X			X		X	X
15 weeks **			X			X			
16 weeks **			X			X		X	X
16-18 wk (4 months)	SOC (unlabeled)						X		
20-22 wk (5 months)	SOC (unlabeled)						X		
24-26 wk (6 months)	SOC (unlabeled)						X	X	X

*Prior to each monthly transfusion event (including optional INTERCEPT RBCs), a blood draw, including an Antibody screen, CBC, reticulocyte count, and Hb electrophoresis (HbA, HbS levels) will be performed a clinical standard of care (SOC), not a study procedure. The exact frequency of unlabeled transfusions (after BioRBC transfusion) is a clinical care decision, not a study procedure. US = ultrasound.
**Laboratory testing at weeks 13-16 (CBC, retic, Hb electrophoresis, BioRBC) are optional. *Optional studies are marked in color on the above table.*
***Afer a subject consents we will use discarded samples to test anti-BioRBC and INTERCEPT RBC Antibodies. These tests will be repeated at Day -3 to Day 0.
† HbA and HbS (Hb electrophoresis): only for subjects with sickle cell disease

Laboratory testing

	Volume	Tube type
CBC, reticulocyte, Hb electrophoresis (HbA,S)	2-3 mL	Lavender/EDTA
EPO (serum erythropoietin)	1 mL	Serum or plasma separator
LDH (lactate dehydrogenase)	1 mL	Green/lithium heparin
DAT (Direct Antiglobulin Test)	3 mL (minimum 1 mL)	Lavender/EDTA
Anti-BioRBC antibody	3 mL	Lavender/EDTA
BioRBC (enumeration, senescence studies)	1 mL	Lavender/EDTA

Additionally, a urine pregnancy test (urine HCG) will be performed at enrollment in females of childbearing age (post-menarche).

3.4.2 Biotinylation and transfusion of RBC: Investigational New Drug (IND) approval for the biotinylation and transfusion of autologous RBC in healthy volunteers is held by investigator Dr. Marianne Yee (IND #16716, effective 12/18/15); Study #3 under this IND, titled "Kinetics of Red Blood Cell Clearance in Chronically Transfused Children with Sickle Cell Disease," for biotinylation of allogeneic RBC transfusions in patients with SCD was approved on 01/20/2020; modification for TDT and BioRBC re-exposure approved 4/2023) .
All studies will follow current good manufacturing processes (cGMP) with standard operating procedures currently established within the Emory CTCT (Dr. Roback's laboratory) for labeling samples of RBC units with biotin at different densities that can be easily differentiated by flow cytometry (2-18 μg/mL of packed RBCs), while ensuring no introduction of bacterial contamination to the unit. Prior to transfusion, an aliquot will be sterilely withdrawn from each unit, washed and labeled with sulfo-NHS-biotin for 30 minutes, washed to stop the labeling reaction, then resuspended in plasma to a hematocrit (Hct) of ~60% (acceptable Hct range: 35 – 75%). (Currently, RBCs are collected in AS-1 system of blood storage (Fenwal Laboratories) or AS-5 (Terumo BCT). INTERCEPT RBCs are suspended in SAG-M.) For transfusions of >1 unit (or for the untreated Control and INTERCEPT-treated Test RBCs), each unit will be labeled with a different density of biotin (up to 3 units). The biotin-labeled RBC (BioRBC) will be transfused along with the remainder of the RBC unit (unlabeled volume). Standard blood bank and CTT protocols and minor antigen matching for SCD patients will be followed. Exact transfusion volume will be determined based on pre-transfusion Hb, HbS, and body weight, per clinical protocol.

For individuals consenting to the optional arm (Aim 3), study aliquots for biotin labeling for an INTERCEPT RBC unit will be drawn from INTERCEPT-treated and untreated pedi-packs that accompany the main INTERCEPT RBC unit as shown in Figure 6.

All of the interventions described for **Aims 1 and 2** will apply to the optional arm (Aim 3) with the following alterations:

a) For subjects opting in to receiving a pathogen reduced INTERCEPT RBC unit, recovery and survival of biotinylated allogeneic INTERCEPT RBCs will be compared with biotinylated allogeneic control RBCs (not pathogen reduced) from the same donor. Overall recovery and survival results from subjects transfused with INTERCEPT RBCs in the optional arm will be compared to recovery and survival results from subjects transfused with conventional RBCs in the main study. See figure 5 for RBC production and transfusion procedures for Aims 3 and 4.

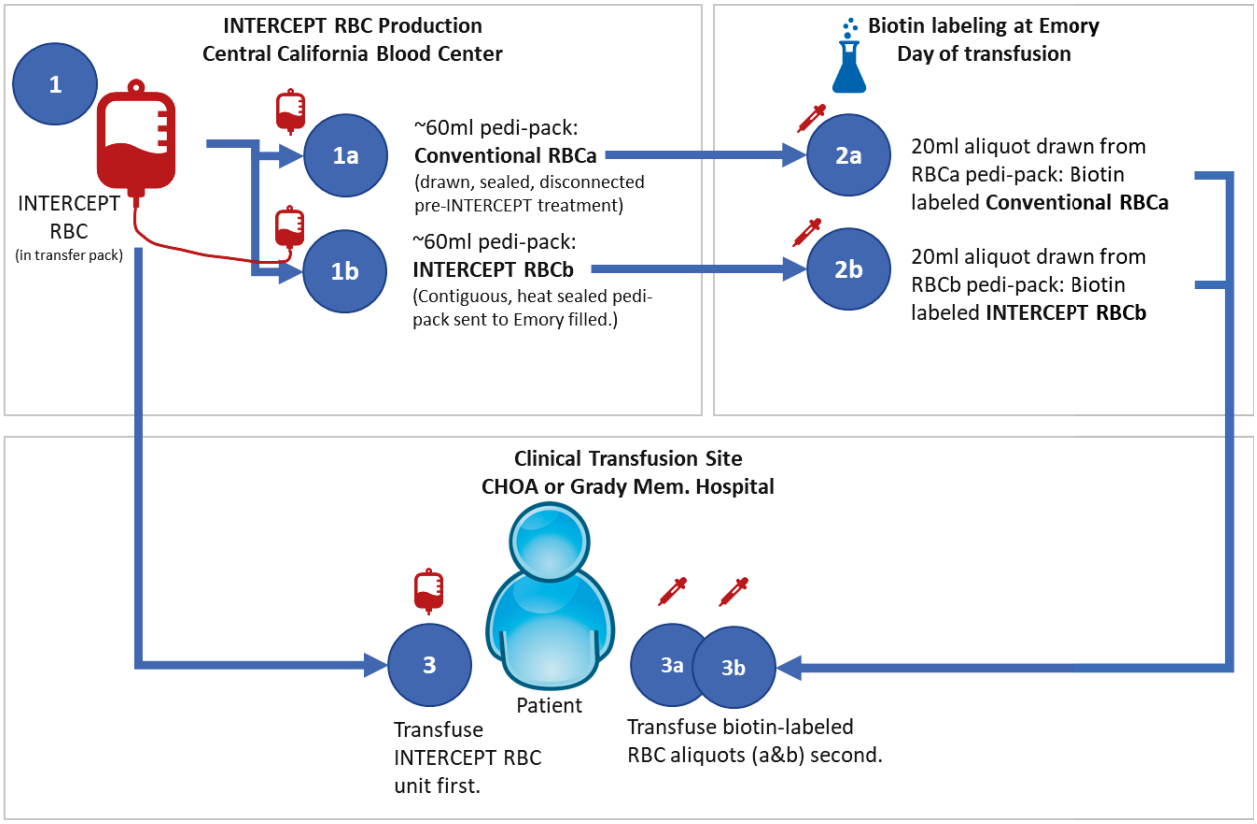
b) Two aliquots will be drawn from and accompany each pathogen reduced INTERCEPT RBC unit. The pathogen reduced INTERCEPT RBC unit will be provided by a blood center that has been qualified to manufacture INTERCEPT RBCs (Cerus IDE 13803). One aliquot will contain non-pathogen reduced RBCs drawn from the study RBC unit prior to the INTERCEPT pathogen reduction process. The second aliquot will contain pathogen reduced RBCs from the same study RBC unit drawn after the INTERCEPT treatment process. Both aliquots will be biotinylated using different surface biotin levels and tracked in vivo independently at the same time points as described in Aim 1.

c) Biotinylated aliquots drawn from the pathogen reduced INTERCEPT RBC unit will be referred to as “INTERCEPT BioRBCs.” The non-pathogen reduced biotinylated RBCs in the second aliquot will be referred to as “BioRBCs.”

d) Only one unit of INTERCEPT RBCs will be transfused with the biotinylated RBC aliquots as part of the optional investigation. If a subject enrolled in the optional investigation requires > 1 RBC units for their transfusion, conventional RBCs meeting the same production criteria (leukoreduced, HbS-negative, phenotype-matched) will be used as described in Aim 1. The INTERCEPT RBCs can be tracked in vivo as non-biotinylated RBCs with surface acridine labeling.

e) Subjects enrolled in the optional pathogen reduced RBC investigation will participate in all of the blood draws and assays described in Table 1. Additional tubes of peripheral venous blood will be drawn for: evaluating treatment-emergent antibodies specific to INTERCEPT RBCs and acridine surface label monitoring. Assays associated with these additional blood draws are described in Table 1 and sections II.5.c and II.5.e below.

Figure 6: Study RBC production and transfusion procedures



3.4.3 BioRBC enumeration: After transfusion completion and sufficient *in vivo* distribution (≥10 minutes), a blood sample will be drawn to determine RBC volume for each of the multiple biotin densities. At ~24 hours, another sample will be drawn to determine 24-hour post-transfusion recovery (PTR_{24}) of BioRBC. To determine long-term BioRBC survival, sequential blood samples will be obtained weekly until week 12 post-transfusion. Complete blood counts (CBC), reticulocyte counts, and hemoglobin electrophoresis (hemoglobin A, S) will be obtained at the same intervals as BioRBC enumeration. Blood samples will be labeled with avidin-FITC, and BioRBC will be quantified by flow cytometry. Mathematical modeling (regression analysis) of absolute BioRBC count from days 1 – 70 will be used to calculate BioRBC half-life (T_{50}, or 50% recovery), and mean potential lifespan (MPL, or 100% of BioRBC disappeared from circulation). During this time, patients will continue to receive regular monthly transfusions as part of CTT (not biotinylated).

3.4.3.b. Optional study extension: Patients may consent to continue weekly blood draws from weeks 13 through week 16 to measure: CBC, reticulocyte count, hemoglobin electrophoresis and BioRBC enumeration. This may be of utility in accurately determining RBC lifespan if the MPL of BRBC extends beyond the initial 12 week follow-up period.

3.4.4 BioRBC Senescence marker testing: BioRBC will be isolated from patient samples, stained and incubated with the following: Strepavidin (to detect biotinylated RBC), anti-human IgG and IgM, anti-human C3 (total complement deposition) and iC3b (cleaved, active

complement), anti-CD47 (total and oxidized), Sambucus nigra lectin (SNA) and Maakia amurensis lectin II (MALII) which bind sialic acid on terminal galactose of RBC), Ricinus Communis agglutinin (RCA, binds exposed galactose on RBC), and annexin V (binds exposed PS on RBC). Binding of the corresponding immunoglobulin or lectin on BioRBC will be assessed by flow cytometry.

3.4.5 Acridine surface label monitoring (optional arm - Aim 3): Acridine levels on INTERCEPT RBCs will be assessed at baseline, on Day 1 and on weeks 1, 2, 4, 6, 8,10, 12, 14 (optional), 16, (optional) and 24-26. Frozen or refrigerated RBC samples will be shipped to Cerus for this assessment using a flow cytometry assay. The assay to detect acridine levels on INTERCEPT RBCs is an indirect staining assay using a murine monoclonal antibody specific to the acridine moiety found on INTERCEPT RBCs, a secondary antibody conjugated to phycoerythrin (PE), and QuantiBRITE PE beads (QBPE). Fresh or de-glycerolized previously frozen RBCs may be used in the staining process

3.4.6 Anti-BioRBC Antibody testing: Plasma samples will be tested for anti-BioRBC antibodies at baseline prior to BioRBC transfusion and at weeks 1, 2, 4, 6, 8, 10, 12, then monthly until 6 months after BioRBC transfusion (Table 1).[41] Published data and our own unpublished data (presented above) suggest that anti-BioRBC antibodies do not pose a safety risk in SCD, result in biotin deficiency, nor interfere with data interpretation; however the occurrence remains an event of special interest for safety monitoring and would preclude a future re-exposure to Bio-RBC on this or other clinical studies.[40,41] Consequently, any patient with SCD who has formed anti-BioRBC antibodies following initial exposure will not be re-exposed to Bio-RBCs. We are confident that our screening protocol for anti-BioRBC antibodies up to 6 months from initial exposure will detect any anti-BioRBC antibodies given data by Mock et al. which demonstrated that initial anti-BioRBC antibodies were detected between 84 – 154 days (2-5 months) post exposure. **Therefore, it is exceedingly unlikely that a primary immune response to Bio-RBC would go undetected after a first exposure or prior to any potential, future re-exposure.**

Following the technique published by Schmidt et al., IgG gel card assays (Search-Cyte TCS, Grifols, USA) will be used to screen plasma samples with a 3-cell panel of reagent Red Blood Cells (Grifols) that are unlabeled (conventional) and biotin labeled BioRBC from a group O+ convenience donor, following manufacturers instructions. Agglutination will be quantified from 0 (no agglutination) to 4+ based on the manufacturer's gradation-specific definitions of reactivity.[39] If subjects' plasma demonstrates reactivity to biotinylated reagent RBC at an initial high density of 250 µg/ml (BioRBC-250) that is not present in the unbiotinylated reagent RBC, then the plasma samples will be screened against Search-Cyte TCS reagent RBC labeled at a lower densities of 50 µg/mL (BioRBC-50).

3.4.6.a. Unexpected antibodies other than BioRBC Antibodies: New RBC antibodies (alloantibodies or autoantibodies to RBC antigens, not previously detected on past screens) are a common occurrence in patients with SCD or TDT who receive transfusion, but are an event of interest, as Aim 1 seeks to determine if RBC antibody history influences donor RBC survival, and new antibody formation may be associated

with increased clearance. When an antibody is detected on routine blood bank screening, antibody identification panels are performed by the blood bank and/or an immunohematology reference lab (IRL), in order to identify the antigen to which the antibody is directed. In some instances, the antibody identity is undetermined by testing, or is discrepant with patient's RBC antigen typing (for example, an antibody directed against an antigen which the patient expresses). In these cases, it may be necessary to perform molecular testing or sequencing of the RBC antigen in question, to determine if genetic variants in the antigen have led to indeterminant antibody identity. If an unexpected antibody is found on routine screens during the study period, which cannot be resolved with traditional, clinical IRL testing, or if further phenotyping of the patient's RBC characteristics are needed for data interpretation, the patient blood sample may be sent to the National Molecular Laboratory of the American Red Cross or to the specialty laboratory services of LifeSouth Community Blood Centers for appropriate molecular testing. No additional blood sample would be required from the patient, and the patient would be informed of clinically relevant results. Genetic variation of RBC antigens is not associated with disease status or predisposition, but is important for safety of appropriately matched RBC transfusion.

3.4.7 Anti- INTERCEPT RBC Antibody testing (optional arm - Aim 3): Tests for treatment-emergent antibodies specific to INTERCEPT RBCs will be performed at baseline (after a subject consents), prior to the study transfusion (Day-3 to Day 0), on weeks 1, 2, 4, 6, 8, 10, 12, 14 (optional), 16 (optional) and 24- 26 according to procedures developed by Cerus Corporation and described in IDE 13803. All subjects with treatment emergent antibodies specific for INTERCEPT RBCs will be offered an opportunity by Cerus to participate in supplemental clinical and laboratory investigations to further explore the potential clinical significance of the antibody.

3.4.8 Delayed hemolytic transfusion reaction (DHTR) monitoring: DHTR will be suspected if there is a decline in hemoglobin within 21 days of transfusion to below the pre-transfusion value plus 1 or more of the following criteria: new RBC antibody detected, hemoglobinuria, accelerated increase in HbS % with concomitant fall in HbA, significant change in reticulocyte count (either reticulocytopenia or reticulocytosis) from baseline, significant rise in LDH from baseline, and exclusion of an alternate cause (American Society of Hematology 2019 Guidelines for Sickle Cell Disease: Transfusion Support). Of note, in up to 30-35% of DHTR in patients with SCD, no new, putative alloantibody is identified,[48,49] thus definitive diagnosis of DHTR cannot rest solely on post-transfusion antibody detection assays. A diagnostic normogram for the grading of DHTR suspicion (high, intermediate, or low) was developed by Mekontso Dessap et al. using decline in HbA over time, as shown in Figure 4. This normogram will be used to grade DHTR suspicion for all transfusion episodes in which total Hb drops to below pre-transfusion baseline within 3 weeks.

Monitoring for DHTR will include the following laboratory testing at baseline (pre-transfusion) and then weekly through 4 weeks after transfusion: CBC, reticulocyte count, Hb electrophoresis (HbA and HbS percentages), LDH, DAT. At each study visit through week 4, participants will be monitored for signs or symptoms of transfusion reaction including the following signs and symptoms: fever (temperature ≥38.0°C) or chills, rash or hives, pain in back or generalized body pain, dark urine, increased jaundiced appearance.

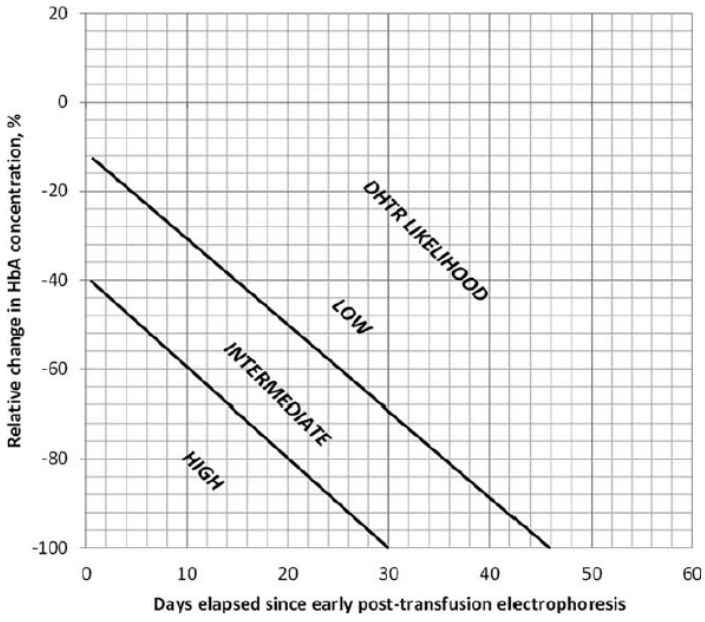


Figure 7. Diagnostic normogram for DHTR, to estimate the likelihood of DHTR following transfusion based on days since transfusion and the relative change in HbA concentration (g/dL) at that time. Relative change in HbA is calculated as: 100 x (HbA at DHTR suspicion – HbA early post-transufsion)/ HbA early post-transfusion. Adapted from Mekontso Dessap et al.[49]

3.4.9 RES and spleen measurements: On the date of BioRBC transfusion, a blood sample will be obtained for RBC pitted cell count, which will be tested through the clinical laboratory. Both RBC pit count and Howell Jolly Bodies are predictors of normal vs. diminished splenic function. As described by Rogers et al. (Blood 2011), "normal spleen function is better predicted by PIT of ≤ 1.2% or HJB ≤ 55/10^6 red blood cells and absent function by PIT ≥ 4.5% or HJB ≥ 665/10^6. [50,51] Patients will also undergo splenic volume estimation by abdominal ultrasound and review of past abdominal MRI imaging for volumetric measurements by the collaborating radiologist.[52]

3.4.10 Collaboration with Blood Center: LifeSouth has tested/recorded the RBC minor antigen genotyping of 50,000 mostly minority donors. Demographic and clinical information on the donors will be obtained from LifeSouth via the unique donor identification number, including age, gender, ethnicity, blood type and RBC minor antigen phenotype or genotype, and donation frequency.

3.4.11 G6PD of donor RBC units: G6PD activity will be measured by quantitative, spectrophotometric assay in batched samples of transfused RBC units. Hemolysate added to a solution with glucose-6-phoshate and NADP will result in the formation of NADPH through G6PD activity. NADPH production is determined by colorimetric detection at a wavelength of 340 nm.[53] G6PD activity is expressed as units of enzyme activity per gram of Hb (U/g Hb); G6PD deficiency is categorized as activity <60% of the normal mean (<3.9 U/g Hb), and severe deficiency as activity <10% of the normal mean (<1.3 U/g Hb).

3.4.12 RBC Metabolomics of donor RBC units: Samples of RBC from transfused units will be split into aliquots for analysis by gas chromatography/mass spectrometry (MS) and liquid chromatography/tandem MS. Specific compounds will be identified by comparison to purified standards, and visualization and interpretation software will be used to confirm the consistency of peak identification between samples.

3.5 Biostatistics: The study is powered and designed to assess the relationship of alloimmunization to the survival of transfused BioRBC. Power analysis is based on RBC survival

studies a sample of 5 SCD patients who had serial measurements of HbA-RBC decline after transfusion (Joiner, unpublished). From this sample, we estimate the mean decline of HbA-RBC to be 0.035 $x10^6$/μL/day (SD 0.004 $x10^6$/μL/day) in non-alloimmunized patients. The expected difference in HbA-RBC decline between alloimmunized and non-alloimmunized subjects is 15%, based ΔHbA estimates in the SCTT Cohort. Assuming a SD of 0.005 $x10^6$/μL/day, which is moderately higher SD than observed in the pilot data, a sample of 17 patients per group (34 patients total) will provide 81% power to detect a ≥15% difference in HbA-RBC decline between alloimmunized and non-alloimmunized patients, at the 2-sided significance level α=0.05 (2-sample equal variance t-test). To account for attrition, the enrollment will be 20% greater than the minimum sample size, to total 40 subjects (20 per group). The univariate associations of the following other secondary and confounding variables on BioRBC survival will be examined: (1) RES function, reflected by HJB count, and (2) splenic volume, measured by ultrasound and MRI. Based on results of the univariate analyses, multivariable linear regression of the outcome variable BioRBC survival will be done.

4. PROTECTION OF HUMAN SUBJECTS

4.1. Risk to Human Subjects

4.1a. Human Subjects Involvement, Characteristics, and Design: The study will enroll children, adolescents, and adults (ages ≥2 years) who have sickle cell disease (SCD) or transfusion-dependent thalassemia (TDT) and who are receiving chronic transfusion therapy (CTT) which is defined as regimen of scheduled blood transfusions (typically occurring once a month, with at least 8 transfusions per year). Targeted enrollment will be 40 patients (20 with RBC alloimmunization, 20 without alloimmunization). The study design is a single arm, mechanistic clinical trial, to measure the *in vivo* survival of donor RBC from 1 chronic transfusion episode over time. Subjects will be recruited from the outpatient sickle cell clinics at Children's Healthcare of Atlanta (Egleston, Hughes Spalding, and Scottish Rite locations), and from the Comprehensive Sickle Cell Center at Grady.

4.1b. Study Procedures: Participants who are presenting for a standard of care RBC transfusion as part of CTT will consent to the labeling of a portion of the RBC transfusion units with biotin conjugated to RBC surface proteins (BioRBC) in order to measure the concentration of transfused RBC in circulation over time. Subjects will undergo research study blood draws prior to transfusion, immediately after and 1 day after transfusion, then weekly for 12 weeks, then monthly (at the time of a clinically indicated blood draw) until 6 months post BioRBC transfusion. The Subjects will also undergo a limited abdominal ultrasound on the day of the BioRBC transfusion, to measure spleen volume.

4.1c Optional Arm (Aim 3): The same basic study design described above will apply to the optional investigation with pathogen reduced INTERCEPT RBCs with the addition of comparative analyses to describe differences between pathogen reduced and conventional RBCs transfused simultaneously with RBCs labeled with different surface biotin concentrations. Patients who participate in the optional pathogen reduced RBC arm will also be assessed against the following additional endpoints:

3. The same recovery and survival variables described in the primary endpoint assessed via surface acridine flow cytometry compared to biotin labeling in individuals transfused pathogen reduced, phenotype-matched RBCs.
4. Development of antibodies with specificity to INTERCEPT RBCs.

4.1d. Potential Risks: While overall the study poses greater than minimal risk, there are no anticipated serious additional risks to receiving transfusion of biotin-labeled packed RBC (BioRBC) units for transfusion, beyond the existing risks of RBC transfusion therapy. Participants in this study are receiving chronic transfusion therapy (CTT) as standard of care for sickle cell disease (SCD) or TDT, thus the transfusion event is not for research purposes but is being observed by the addition of biotin labeling to the transfused units. Based on the growing number of published reports using biotinylated RBCs for measuring RBC volume and RBC survival in humans, the use of biotinylated RBCs is becoming increasingly accepted as a safe and effective method for determining RBC volume and survival.[57,58,68,69] The administration of biotin-labeled RBCs (BioRBC) poses the following potential but manageable safety risks: (1) introduction of infection during biotinylation and transfusion, and (2) formation of antibodies to BioRBC following transfusion. Published studies of biotinylated RBC transfusion have identified antibodies to biotinylated RBC proteins in 12-15% of adult subjects but have not formed antibodies to biotin alone. Individuals who have formed anti-BioRBC antibodies have not had evidence of hemolysis, change in BioRBC survival after the initial Bio-RBC antibody detection, nor any notable adverse signs or symptoms; plasma and urinary biotin levels remain normal for month following the detection of anti-BioRBC antibodies.[54,63,64]

In this study design, subjects receive Bio-RBC transfusion at 1 time point, and cell survival is followed until clearance, with close, frequent monitoring for potential development of anti-Bio-RBC antibodies. Individuals who demonstrate antibody reactivity to BioRBC will not be re-exposed to BioRBC; however individuals who have received Bio-RBC infusion and have had negative screening for anti-BioRBC antibodies through month 6 may be eligible for future exposure to BioRBC at appropriate dose volumes as delineated by Mock et al.(2022).[34]

The dosage of biotin (a water soluble B group vitamin) that will be delivered ranges 25-250 pmol/mL of packed RBC (pRBC), thus in a patient who receives 3 unit of biotin-labeled RBC (≤7 mL aliquots of Bio-RBC per unit labeled with 2, 6, or 18 µg/mL pRBC) will result in the transfusion of an amount of biotin that is within the FDA adequate intake (AI) of biotin for adults and children ≥4 years (30 µg/day). Additionally, there is no known toxicity of biotin in doses even exceeding those that we propose, and no observed risks noted in studies of adults and infants.[54,70-73]

There is minimal risk to study participants for blood draws associated with measuring BioRBC survival after transfusion. Whenever possible, research blood draws will be coordinated to coincide with times in which the patient is undergoing a blood draw for clinical purposes (as noted in Table 1). Each research blood draw will consist of 10 mL blood or less (range 1 – 10 mL). Subjects who participate in the Optional study arm and receive both INTERCEPT and conventional study RBCs will have two ≤5 mL blood samples drawn at each study visit (Table 1). Risks associated with drawing blood from a vein may include momentary discomfort, bruising, bleeding, redness and swelling of the site, feeling of lightheadedness, and rarely infection at the site of the blood draw.

There is the risk of breach of confidentiality during the study. Study participants will be assigned a unique study ID that does not contain any personal identifying information (such as initials, dates, etc). A master list linking study ID number to name, date of birth, and medical record will be kept separate from data records, on a password-protected computer. All blood specimens and case report forms obtained as part of the study will be labeled with the study ID and study visit number only. Due to the need to collect clinical information for study purposes, the following personal identifying information will be recorded: full name, date of birth, medical record number, and address, phone number, and email for purposes of contacting subjects about study visits for study retention.

OPTIONAL ARM: An increase in transfusion reactions due to the INTERCEPT treatment process is not foreseen for patients transfused with INTERCEPT RBCs (both biotin-labeled and unlabeled). A possible known risk linked to INTERCEPT RBC transfusions is the development of antibodies specific to INTERCEPT RBCs.[54] Patients with a confirmed treatment-emergent antibody to INTERCEPT RBCs will be monitored for hemolysis and offered an opportunity to participate in follow-up studies to characterize the clinical significance of the antibody.

4.2. Adequacy of Protection Against Risks

4.2a. Informed Consent and Assent: Participants will be recruited from the Aflac outpatient clinic and infusion centers. Study information may be initially provided by either a trained research coordinator or investigator, with final consent and assent obtained in person by the PI, Dr. Yee. In cases where parent/guardian is unavailable to consent in person the consent process can take place over the phone. The consent would then be sent to the parent/guardian via an encrypted email or regular mail. The consent discussion will explain the purpose, procedures, potential risks and benefits, alternatives to participation, and the right to refuse or withdraw from the study. For children and adolescents under 18 years of age, the consent discussion will occur with both the minor participant and the parent/guardian. Written consent will be obtained from the adult parent or legal guardian. Children ages 6-10 years will provide verbal assent (documented by the PI), and adolescents ages 11-17 years will provide written assent to study participation. For young adult participants ≥18 years old, written consent will be obtained from the participant.

For non English speaking participants we will use either an IRB approved short form or a fully translated consent/assent. We anticipate subjects will speak either Spanish or Swahili but may speak other languages. The study team is English speaking. An IRB approved short form or a fully translated and IRB approved consent/assent will be used. The consent process will take place with a qualified interpreter present.

4.2b. Protections Against Risk:

Risks of breach of confidentiality: All personal facts, identifiers, and data about patients will be kept private and confidential, and the investigators will use these data strictly for research purposes. In order to maintain participant confidentiality, subjects will be assigned and identified by a unique study code number. Only de-identified patient data, per HIPAA guidelines, will be used for data analyses by the PI. Data will be maintained in secure, password-protected excel spreadsheets and/or REDCap databases on a remote server with password-protected PI access, as per Emory Information Security regulations. Access to this data will be controlled by the PI. A record of patient identifiers and corresponding reference codes will be stored in locked file cabinets in a secure office of the P.I. Published results will be presented as anonymous group data. The Emory University Institutional Review Board will oversee the conduct of this study and will have the right to review all records pertaining to this study. We will keep the records private to the extent allowed by law and ensure compliance with HIPAA regulations.

Risks of RBC biotinylation: In order to prevent blood infections related to RBC biotinylation, sterility is maintained in a closed system at all times in the blood bank laboratory and infusion center. All routine procedures for screening for blood products safety, including infectious disease testing and antigen cross-matching will be adhered to, per standard operating

procedures and FDA regulations. To monitor for the occurrence of anti-BioRBC antibodies, plasma samples obtained from the recipients will be tested pre-transfusion, at weeks 1,2,4,6,8,10,12, and then monthly until 6 months post BioRBC transfusion, per FDA requirements.

4.2c. Vulnerable Subjects: Pregnant women, fetuses, and neonates will not be included in the study. Children ≥6 years old will be included in the study. The rationale for the inclusion of children is that children with SCD often require regular blood transfusions to prevent further disease-related damage. Studying the mechanisms of transfused RBC survival and clearance in this population is important to improving transfusion therapy to this unique pediatric population.

4.3. Potential Benefits of the Proposed Research to Research Participants and Others

For individuals with SCD receiving CTT, understanding the kinetics of transfused RBC survival and clearance has potential benefit, as this information may help to improve management of the individuals' transfusion frequency, volumes, as well as choice of donor product characteristics (such as screening for G6PD deficiency or other metabolic characteristics).

4.4. Importance of the Knowledge to be Gained

The purpose of the proposed research is to better understand mechanisms that result in variability in outcomes of CTT in SCD. Transfusion therapy is used commonly in SCD for the long-term prevention of serious disease complications such as stroke; however, a substantial proportion of patients have suboptimal response to therapy, with progressive cerebrovascular findings or recurrent stroke. Additionally, there is variability among different patients in the correction of anemia and suppression of erythropoiesis with CTT, the 2 mechanisms by which CTT prevents SCD complications. This study aims to examine variables that affect the *in vivo* survival of transfused RBC in chronically transfused SCD patients and thus potentially impact the clinical efficacy of CTT. The knowledge gained from this study ultimately may inform efforts to better individualize transfusion therapy practices in SCD. The risk to the study participants of biotinylating the RBC units of 1 chronic transfusion episode is above minimal risk yet is reasonable in relation to the importance of the knowledge expected to be gained.

Data from the optional investigation (Aim 3) will also add to the knowledge base regarding the use of biotinylation with INTERCEPT-treated RBCs and produce new data to describe the use of surface acridine monitoring as an alternative method to biotinylation for future recovery and survival studies with INTERCEPT RBCs. Similar to the risks described for biotinylated RBCs, the risk to study participants exposed to INTERCEPT RBCs is above minimal risk yet is reasonable in relation to the importance of the knowledge expected to be gained.

5. DATA SAFETY AND MONITORING PLAN

Plan for reviewing data for safety: Given past safety data of BioRBC transfusion, we believe this mechanistic clinical trial presents greater than minimal risk but does not pose serious risk to children or adult participants. This study will be conducted in compliance with the Aflac Cancer & Blood Disorders Center Data Safety Monitoring Plan. The Data Safety Monitoring Board (DSMB) consists of 6 members including a chair, a statistician, a pharmacist, and at least one external member. The DSMB meets on a quarterly basis to review current study results, as well

as data available to the DSMB from other related studies. The DSMB will also meet ad hoc if necessary, to review any time sensitive information. For each study reviewed, the DSMB will provide recommendations on whether there needs to be changes in the conduct of the study. The DSMB will provide recommendations to change the study or to continue the study unchanged. The Study Chair assumes responsibility for assuring that the study is carried out in accordance with the DSMB. The DSMB will continue to meet to monitor this study until all subjects are off study. External monitoring of the trial for regulatory and protocol compliance will be the responsibility of the study team. Studies will be monitored on a regular basis according to patient enrollment. Monitoring will be done predominantly via source documentation and case report forms. In addition, summary reports (compiled by the PI Dr. Yee) reviewing study conduct to date, all SAE reports, and accrual data will be shared with the DSMB at the quarterly meeting.

The PI (Dr. Yee) will monitor data and safety, ensuring participants' safety on a weekly basis. Dr. Yee will review all data collection forms at monthly intervals for completeness, accuracy of the data compared to source documents, and compliance with the protocol. Any data inconsistencies will be resolved by study staff in 4 weeks or less. The PI will review this protocol on a continuing basis for subject safety and protocol deviations or study noncompliance. Reportable Events will be communicated to the local institutional IRB as required. The results of the review will also be included in annual progress reports submitted to the local institutional IRB, Georgia CTSA Research Safety Advisory Subcommittee, or any other regulatory agency or sponsor, as required. An ad hoc meeting of the DSMB may be called to review any adverse events of special interest or serious adverse events. All reportable events occurring in subjects who received INTERCEPT-treated RBCs on the optional investigation arm (Aims 3 and 4) will be immediately reported to the study sponsor (Cerus Corporation).

Adverse Event Causality Grading: All adverse events (AEs) will be graded by the PI or designee on a timely basis as to their attribution (unrelated to protocol, or possibly, probably, or definitely related to protocol) and seriousness (see below). Any AE that is reported to either the PI or their designated research associates by a study subject or by medical staff caring for the subject and which meets the AE criteria will be documented as such.

Serious adverse events are defined as: any experience that suggests a significant hazard, such as events which: a) are fatal, b) are life threatening, c) result in persistent or significant disability, d) requires or prolongs inpatient hospitalization, e) results in congenital anomalies/birth defects, or f) in the opinion of the investigators represents other significant hazards or potentially serious harm to research subjects or others.

Unanticipated problems (UPs) are defined as unanticipated events involving risks to participants or others that are *possibly, probably*, or *definitely related* to the research; or anticipated events that occur with a greater frequency, duration, or severity than what is documented in the protocol, informed consent, or investigator's brochure; or other unanticipated information that changes the risk benefit ratio or that indicates participants or others might be at greater risk of harm than was previously known.

Unanticipated Adverse Device Effects (UADE) [Optional Arm (Aim 3)]

Any serious adverse effect on health or safety or any life-threatening problem or death caused by, or associated with, a device (e.g., the INTERCEPT Blood System for RBCs), if that effect, problem, or death was not previously identified in nature, severity, or degree of incidence in the investigational plan or application (including a supplementary plan or application), or any other unanticipated serious problem associated with a device that relates to the rights, safety, or welfare of patients.

All adverse events, unanticipated problems, and UADEs will be reported promptly (within 10 business days from the date the PI first learned about the event) to the Emory University IRB as per reporting guideline policies and procedures. The investigators and staff will track and summarize AE frequency, severity, and relatedness at a frequency appropriate to ensure subject safety. Any SAEs (Grades 3 to 5 based on table below) that are determined to be definitely, probably, or possibly related to the study intervention will also be reported to the study sponsor, FDA, and the Emory IRB promptly (within 24 hours). Serious transfusion reactions associated with subjects enrolled in the Optional arm (Aim 3) must be reported by the Investigator to the INTERCEPT Blood System for RBCs device manufacturer (Cerus Corporation, Concord, CA) immediately (i.e., no more than 24 hours) after the Investigator becomes aware of the event.

Severity of Adverse events will be graded as:

Grade 0 = No adverse event or within normal limits.

Grade 1 = Mild adverse event not requiring treatment.

Grade 2 = Moderate adverse event resolved with treatment.

Grade 3 = Severe adverse event resulted in inability to carry on normal activities and requiring professional medical attention.

Grade 4 = Life threatening or disabling adverse event.

Grade 5 = Fatal adverse event.

Expected adverse events will be detailed in the Consent Form and include the following:

1. Adverse events that might occur during or immediately after BioRBC transfusion:
    - Skin rash or hives
    - Fever, chills
    - Infection
    - Swelling at the needle site

2. Adverse events that might occur days to weeks after BioRBC transfusion:
    - Hemolysis
    - Detection of an antibody against biotin-labeled RBCs.

Adverse events of special interest:

- Detection of anti-BioRBC antibodies
- Detection of a new RBC alloantibody (other than anti-BioRBC antibodies)
- Delayed hemolytic transfusion reaction (DHTR) (intermediate or high suspicion) within 4 weeks of BioRBC transfusion
- Bloodstream infection
- Detection of new INTERCEPT RBC antibody (Optional Arm [Aim 3] only)

Plan for collection of adverse events: Subjects will be monitored closely during the blood transfusion procedure by staff trained to detect adverse events with transufsion. Laboratory monitoring for events of special interest will occur for 4 weeks (for hemolytic transfusion reactions) and up to 6 months (detection of anti-BioRBC antibodies and new INTERCEPT RBC antibody). If such events occur, they will be entered into the case report forms (CRFs).

Plan of Action in Response to Serious Adverse Event or Adverse Event of Special Interest:

1. **Delayed Hemolytic Transfusion Reaction:** DHTR is not an uncommon event following transfusion in SCD, with estimates in adult patients as high as 4-7% of transfusion events of phenotype-matched RBC.[48,49,55] Thus this event may occur during the study period as a consequence of the participant's underlying disease (SCD and alloimmunization) rather than due to study procedures. Nonethless, careful monitoring for DHTR will occur for 4 weeks after each BioRBC transfusion. DHTR will be suspected based on the definition provided by the American Society of Hematology, then suspicion for DHTR graded as high, intermediate, or low as defined by Mekontso et al.[49] (Methods section 3.4.6).
    - If there is intermediate or high suspicion for DHTR following BioRBC transfusion, then enrollment of new participants will be temporarily suspended, pending full evaluation of the event (including testing for potential new alloantibodies) and review by the DSMB.

2. **Anti-BioRBC Antibodies:** Development of anti-BioRBC antibodies is anticipated to occur in some patients; these are not anticipated to result in hemolysis or other clinical consequence or harm, based on experience in healthy adult subjects (without SCD).[30,56] Given the special considerations of SCD (including inherent hemolysis and increased risk of alloimmunization), study participants will be monitored for the development of anti-BioRBC antibodies for up to 6 months post-BioRBC transfusion, with attention to their association with increased hemolysis or suspected delayed hemolytic transfusion reaction (DHTR). The frequency of anti-BioRBC antibody formation in healthy volunteers has been reported to be 12-15%, with no antibodies detected in individuals receiving RBC with biotin label density ≤18μg/mL (the highest concentration to be used in this study).[39,41]
    - If anti-BioRBC antibodies are detected and there is high suspicion for DHTR or excessive hemolysis in the subject, then study enrollment will be suspended, even if the DHTR episode has resolved. The DSMB will review the available data, including any other RBC antibodies associated with the DHTR, in order to determine duration of study suspension.

3. **New RBC Alloantibody:** Development of new RBC alloantibodies is not an uncommon event in individuals with SCD following transfusion. If a new RBC alloantibody (not specific to biotinylated RBC) is detected within 4 weeks of BioRBC transfusion, this will be reviewed by the DSMB.

4. **Bloodstream Infection:** If a subject develops fever during or following transfusion, then clinical care guidelines would direct the individual to present for immediate medical attention for blood cultures and empiric antibiotics. If a blood culture is positive within 2 days after BioRBC transfusion, then study enrollment will be temporarily suspended, pending investigation and review by the DSMB.

5. **INTERCEPT RBC Antibody:** Treatment-emergent antibodies with specificity to INTERCEPT RBCs are a rare but expected event. Based on current data the rate of clinically significant antibodies to INTERCEPT RBCs will be less than 0.1% of transfused patients. Patients will be monitored for treatment-emergent antibodies to INTERCEPT RBCs for six months following the study RBC transfusion. All patients with treatment-emergent antibodies to INTERCEPT RBCs will be offered an opportunity to participate in supplemental clinical and laboratory investigations to further explore the potential clinical significance of the antibody. The supplemental investigation will be conducted under a separate protocol by the manufacturer of the INTERCEPT Blood System and may include agglutination inhibition studies to determine antibody specificitiy, immunoglobulin isotype and IgG subclass (IgM, IgG1, 2, 3, or 4) studies, antibody titer and persistence studies, thermal spectrum and phase of reaction studies, complement-mediated in vitro hemolysis studies, investigation of concurrent antibodies to intrinsic RBC allo-antigens, Monocyte Monolayer Assay for clinical significance, natural vs. treatment-emergent differentiation studies. The occurrence of any (1 patient) hemolytic transfusion reaction with S-303 specific antibodies or hyperhemolysis syndrome in subjects exposed to INTERCEPT RBC may result in an enrollment pause or study stop for the Optional arm while the reaction is fully investigated and reviewed with the DSMB, which will determine if enrollment in the Optional arm may resume.

   If anti-S-303 specific antibody is detected in any participant who has high suspicion for a DHTR (see criteria above), then all study enrollment will be paused (enrollment pause) and all study transfusions in patients already enrolled in the optional arm will be stopped (clinical stop), until a full investigation and review by the DSMB determine if enrollment in the Optional arm may resume. Details of the enrollment pause and clinical stop are detailed below:
   - Enrollment pause: All recruitment activities for eligible patients will be held. New patients will not be enrolled in the optional arm of the study while investigations are ongoing. In addition to cases involving a treatment-emergent hemolytic transfusion reactions described above, an enrollment pause may be requested by Cerus, the DSMB or the FDA in order to allow time to investigate other potential safety signals. Patients who have already received study RBCs will complete all study follow-up visits. The optional study arm may continue enrollment of new patients following consultation with the DSMB. The DSMB and FDA will be notified of enrollment pauses and decisions to continue enrollment.
   - Clinical stop: All study transfusions in patients already enrolled in the optional arm will be stopped. Enrolled study participants will continue to receive clinically necessary non-study (non-INTERCEPT) RBC transfusions. All study-related monitoring tasks for participants who have already received study RBCs will continue per protocol.

Assuring Data Accuracy and Protocol Compliance

An IRB-approved written informed consent will be obtained from each subject at entry into the study; elements of informed consent will include: (a) having the subject and/or their legally authorized representative review the study consent form; (b) having the investigator(s) or study staff meet with the subject and/or guardian/proxy to review the consent, confirm understanding, and answer any questions; and (c) having the consent signed once the investigator(s) or study staff are convinced that the protocol is understood and that there is agreement to participate. A copy of the signed consent form will be provided to subject and/or their legally authorized

representative. Documentation of the informed consent process will be made in the research and/or medical records as appropriate.

Plans for transmission of temporary or permanent suspension actions: Any actions that mandate temporary or permanent suspension of study will be transmitted to the Georgia CTSA SAS, the Emory University IRB, study sponsor, and to the FDA.

Plans for protecting subject confidentiality: All information and materials that are obtained for research purposes only will be kept in strict confidence. Confidentiality will be assured by the use of subject codes rather than personal identifiers. The study database will be secured, and information will only be entered using subject identifier codes rather than personal identifiers. Electronic communication will involve only coded, unidentifiable information.

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