Red blood cells treated with the amustaline (S-303) pathogen reduction system: a transfusion study in cardiac surgery

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BACKGROUND: Nucleic acid–targeted pathogen inactivation technology using amustaline (S-303) and glutathione (GSH) was developed to reduce the risk of transfusion-transmitted infectious disease and transfusion-associated graft-versus-host disease with red blood cell (RBC) transfusion.

STUDY DESIGN AND METHODS: A randomized, double-blind, controlled study was performed to assess the in vitro characteristics of amustaline-treated RBCs (test) compared with conventional (control) RBCs and to evaluate safety and efficacy of transfusion during and after cardiac surgery. The primary device efficacy endpoint was the postproduction hemoglobin (Hb) content of RBCs. Exploratory clinical outcomes included renal and hepatic failure, the 6-minute walk test (a surrogate for cardiopulmonary function), adverse events (AEs), and the immune response to amustaline-treated RBCs.

RESULTS: A total of 774 RBC units were produced. Mean treatment difference in Hb content was −2.27 g/unit (95% confidence interval, −2.61 to −1.92 g/unit), within the prespecified equivalence margins (±5 g/unit) to declare noninferiority. Amustaline-treated RBCs met European guidelines for Hb content, hematocrit, and hemolysis. Fifty-one (25 test and 26 control) patients received study RBCs. There were no significant differences in RBC usage or other clinical outcomes. Observed AEs were within the spectrum expected for patients of similar age undergoing cardiovascular surgery requiring RBCs transfusion. No patients exhibited an immune response specific to amustaline-treated RBCs.

CONCLUSION: Amustaline-treated RBCs demonstrated equivalence to control RBCs for Hb content, have appropriate characteristics for transfusion, and were well tolerated when transfused in support of acute anemia. Renal impairment was characterized as a potential efficacy endpoint for pivotal studies of RBC transfusion in cardiac surgery.
Improvements in donor selection, infectious disease testing, and donor deferral policies have effectively reduced but not eliminated the risk of transfusion-transmitted infectious disease (TTID). The risk persists due to undetected low titers of recognized agents during the infectious window period and the emergence of novel pathogens such as *Babesia*, dengue, chikungunya, and Zika viruses (ZIKVs),\(^1\)\(^-\)\(^4\) which may persist in cellular components where they are undetectable by conventional nucleic acid testing.\(^5\) Emerging pathogens are not predictable and may be present in the blood donor population for a period of time before discovery and there are limits to the level of sensitivity achievable with testing, especially early after initial infection.\(^5\)\(^,\)\(^7\) US Food and Drug Administration (FDA)-approved tests are not available for many agents known to contaminate blood (e.g., *Babesia microti*)\(^4\) and, finally, bacterial contamination remains a concern for red blood cells (RBCs).\(^8\)\(^,\)\(^9\)

The risk of TTID is compounded by the cumulative exposure that many patients experience with chronic transfusion support.\(^6\) In addition, patients at risk for transfusion-associated graft-versus-host disease (GVHD) may not be recognized and consequently fail to receive irradiated blood products when these are selectively prescribed.\(^10\)

An alternative proactive approach to reduce the risk of TTID and transfusion-associated GVHD is pathogen reduction treatment of blood components targeted to nucleic acids. Currently, pathogen reduction treatment platelet (PLT) and plasma products prepared with aminosalen and ultraviolet A light technology (INTERCEPT Blood System, Cerus Corporation) are licensed for use in the United States and in many other countries.\(^11\)\(^,\)\(^12\)

A pathogen reduction technology using a reactive small molecule (amustaline, S-303) and glutathione (GSH) is in clinical development (Fig. 1).\(^13\) Amustaline irreversibly forms adducts on, and crosslinks DNA and RNA, preventing replication of infectious pathogens and white blood cells (WBCs). RBCs treated with a first-generation process were evaluated for safety and efficacy in Phase III clinical trials\(^14\)\(^,\)\(^15\) and the system exhibited effective in vitro inactivation of multiple blood-borne pathogens (≥4 log) and residual WBCs.\(^13\)\(^,\)\(^16\)\(^,\)\(^17\) Low-titer antibodies to amustaline-treated RBCs were identified in two patients participating in a chronic transfusion clinical trial\(^15\) and were found to be specific for the acridine moiety of amustaline. Although the antibodies did not cause clinical hemolysis nor support in vitro phagocytosis of sensitized RBCs, the amustaline treatment process was modified to reduce reactivity with the RBC cell surface to lower the potential for immune responses.\(^13\) In a Phase II clinical trial in healthy volunteers, second-generation amustaline-treated RBCs met FDA criteria for posttransfusion recovery and were shown to be metabolically and physiologically sufficient for transfusion following 35 days of storage.\(^18\)\(^,\)\(^19\) Inactivation of pathogens and WBCs is conserved with the higher GSH concentration, resulting in broad and efficient inactivation with the modified process. The primary objective of this study (named “STARS”) was to assess the characteristics of S-303–treated RBCs in a Phase III device study to meet European regulatory requirements for Conformité Européenne (CE) mark certification and to evaluate safety and efficacy in support of acute anemia in patients undergoing cardiac surgery using exploratory endpoints. A secondary objective was hypothesis generation for endpoints informative of tissue oxygenation to be used in a subsequent powered Phase III clinical efficacy study.

**MATERIALS AND METHODS**

**Trial design and study population**

This was a randomized, controlled, double-blind, parallel-group, multicenter study performed at the German Red Cross Blood Donor Service (GRCBDS; DRK-Blutspendedienst Baden Württemberg–Hessen, Germany), the Johann Wolfgang Goethe University Hospital in Frankfurt, and the Kerckhoff-Klinik in Bad Nauheim. The trial was approved by the ethics committees of all participating institutions. Study patients, treating physicians, patient caregivers, clinical study personnel, clinical investigators, and sponsor personnel were blinded to...
treatment assignment. Blood center personnel involved in processing, testing, and distribution of RBCs were not blinded.

**Study RBCs**

Blood donors were screened and qualified according to the German “Guidelines for the Collection of Blood and Blood Components and for the Use of Blood Products.”20,21 Leukoreduced RBCs in the additive solution SAG-M were derived from buffy coat–depleted whole blood collections with a volume of 220 to 340 mL. Control RBCs were transferred to an identical storage bag for the purpose of blinding and test RBCs were treated with the amustaline process as previously described (Fig. 1).13 Test RBCs were mixed with a processing solution containing GSH, followed by amustaline (20 mmol/L GSH-0.2 mmol/L amustaline, based on 280-mL RBC input). GSH reduces nonspecific reactions of amustaline with membrane proteins. Pathogen inactivation is complete within 3 hours, and unreacted amustaline (half-life 20 min) decomposes to undetectable levels plus nonreactive by-products after 18 to 24 hours of room temperature storage. RBCs were centrifuged, and the treatment solution was expressed and replaced with SAG-M before storage up to 35 days. Test and control RBCs were sampled postproduction (PP), stored at 2 to 6°C, and either transfused or if unused at end of storage (EOS) assessed for quality variables on Days 35 to 38.

The release criteria for study RBCs were at least 40 g hemoglobin (Hb), normal visual inspection, and a volume of 190 to 330 mL. Test units were released into clinical inventory on Day 2 and control units on Day 1.

**Quality control (QC) data on study RBCs**

Study RBCs were evaluated PP and after 35 to 38 days for untransfused components. Variables assessed included Hb content, hematocrit (Hct), hemolysis, normalized adenosine triphosphate (ATP), plasma-free Hb, mean corpuscular volume (MCV), mean corpuscular Hb concentration (MCHC), pH, extracellular potassium, glucose, lactate, residual plasma protein, and sterility. Test RBCs were analyzed for the primary degradation products of amustaline: acridine (S-300), 9-glutathiol acridine, 9-(amino glutathione)-acridine [9A-GSH], 9-(amino glutathione disulfide)-acridine [9A-GSSG]), and GSH (GSH/glutathione disulfide [GSSG]). Degradation products were measured using high-performance liquid chromatography.

**Clinical study population**

The clinical study population included adults (≥18 years old) scheduled to undergo elective coronary artery bypass graft (CABG) only (first procedure), valve repair or replacement only (first procedure), or a combination of first-time CABG and valve repair or replacement. Only patients with a high likelihood of receiving a RBC transfusion, as determined by a transfusion risk understanding scoring tool (TRUST) score of at least 3, were enrolled.22 Patients and their treating physicians were blinded as to the randomization.

To reduce the number of RBCs discarded and to prevent extensive use of D– RBCs, only group O D+ and group A D+ patients were enrolled. Patients requiring gamma irradiation of blood products were excluded. Further exclusion criteria included active autoimmune hemolytic anemia or a positive direct antiglobulin test (DAT), pregnancy, renal failure (creatinine level, >1.8 mg/dL) within 7 days before surgery, existing cross-reactive antibodies to amustaline-treated RBCs identified by gel card assay with paired amustaline-treated and untreated RBC cell panels, or an emergency or salvage surgical procedure. Written informed consent was obtained from all study subjects before screening.

**Transfusion strategies**

Blood transfusions were ordered according to the national German and local institutional guidelines for therapy with blood components and plasma derivatives.21 Study RBCs were provided during a 7-day treatment study period (day of surgery plus 6 postoperative days) as ordered by the treating physician. After the treatment period, all patients received conventional RBCs. Patients with existing alloantibodies were supported with compatible RBCs. Depending on the availability of study RBCs, patients with blood group A were treated with study RBC blood group O as needed. All study RBCs were crossmatched with fresh (≤3 days old) patient plasma using gel card technology incorporating an anti-human immunoglobulin step (BioRad). Depending on the patient characteristics and the planned procedure, 3 to 6 study RBCs units were crossmatched before the planned day of surgery. Unused RBCs with records of appropriate storage were reincorporated into the study RBCs repository at GRCBDS. In instances of severe bleeding, study RBCs were delivered to University Hospital in Frankfurt within less than 10 minutes and to Kerckhoff-Klinik in Bad Nauheim in less than 1 hour.

**Randomization**

The randomization assignments were generated by an interactive Web response system and provided to the blood center by a contract research organization (Nextrials). Patients were randomized based on a stratified scheme that included the clinical site and elective cardiac procedure (simple valve or CABG vs. complex procedure). Randomized patients who did not require any RBC transfusions were replaced and excluded from analysis. Randomized and transfused patients comprised the modified-intention-to-treat (MITT) population.
Endpoints
As this study was focused on supporting CE mark certification for the investigational device, the primary and secondary endpoints were selected to demonstrate quality of amustaline-treated RBCs compared to conventional RBCs. PP RBC Hb content was selected as the primary endpoint because it is reasonably expected to be proportional to the posttransfusion Hb increment in nonbleeding patients, although not necessarily so in actively bleeding surgical patients in which it is not feasible to measure Hb increments. The viability of amustaline-treated RBCs characterized by posttransfusion recovery has been established by prior radiolabeling studies.18,19 The secondary endpoints included proportion of RBCs that met European Directorate for the Quality of Medicines (EDQM) guidelines for Hb content, Hct, and hemolysis at the EOS; the proportion of RBCs that had adenosine triphosphate (ATP) levels of more than 2 μmol/L; and the proportion of RBCs that had plasma-free Hb of less than 0.8% of the total Hb mass.

The clinical safety and efficacy of amustaline-treated RBCs was assessed by measuring RBC usage and by evaluating adverse events (AEs) and exploratory clinical outcomes reflective of tissue oxygenation. The exploratory outcome of interest was renal insufficiency defined as serum creatinine level of more than 2 mg/dL with at least a 50% increase from preoperative baseline and/or a new requirement for renal replacement therapy (i.e., dialysis). The study also evaluated hepatic insufficiency defined as total bilirubin that is more than two times the upper limit of normal and at least a 50% increase from preoperative baseline and/or a new requirement for renal replacement therapy (i.e., dialysis). The study also evaluated hepatic insufficiency defined as total bilirubin that is more than two times the upper limit of normal and at least a 50% increase from the preoperative baseline and global cardiovascular function as assessed by the 6-minute walk test (6MWT).23 Clinical data were collected from the day of surgery through Day 13 postsurgery or death or discharge from hospital whichever occurred first. Baseline, follow-up (Days 28-40), and end-of-study (Day 90) blood samples were screened at the GRCBDS for RBC alloantibodies using standard indirect antiglobulin tests (IATs) as well as DAT gel card assays and for amustaline antibodies with a screening panel that consisted of 3 group O RBC units with or without amustaline treatment. To evaluate amustaline-specific antibodies in the presence of antibodies to intrinsic RBC alloantigens, an additional panel of up to six alloantigen-characterized, corresponding amustaline-treated and untreated RBCs were available for testing.

Data safety monitoring board
A data safety monitoring board (DSMB) reviewed grouped, blinded study data after half of the patients had been transfused. No data safety monitoring board safety concerns were raised.

Sample size calculation
A minimum of 400 RBC units (200 for each treatment arm) were required to demonstrate equivalence between the mean Hb content of test and control components with 97.8% power at the 5% significance level, based on an equivalence margin of ±5 g/RBCs for the mean treatment difference. For the exploratory clinical assessments, a minimum of 50 cardiovascular surgery patients that received at least one study transfusion were evaluated to assess the clinical and safety endpoints. The study was not powered to detect differences in the exploratory endpoints, but was used for hypothesis generation to identify potential endpoints for a subsequent Phase III study powered for clinical efficacy.

Statistical analysis
The primary efficacy analysis assessed the mean treatment difference (test – control) in PP Hb content from all RBCs using an equivalence design. A fixed-effects analysis of covariance (ANCOVA) model was used while controlling for potential confounders including the sex, donor blood group (group O vs. non-O), and RBC volume and Hct. Equivalence was declared at the 5% significance level if the two-sided 95% confidence interval (CI) for the mean treatment difference (test – control) was within the equivalence margins of ±5 g/unit.

The exploratory clinical endpoints were analyzed using the MITT group. The proportion of patients experiencing renal insufficiency or hepatic insufficiency was compared using a Cochran-Mantel-Haenszel (CMH) test (controlling for the site and surgical procedure performed). The 6MWT was compared using analysis of variance (ANOVA; controlling for the site and surgical procedure). The physical mobility assessment performed before the 6MWT was measured using the Walking Impairment Questionnaire. Walking Impairment Questionnaire total scores were calculated. Summaries for the frequency of all serious, related, and severe (grade ≥3) TEAEs were reported by treatment group, system organ class, and preferred term (PT). All other quantitative safety data were summarized descriptively. A p value of less than 0.05 was considered significant. Statistical analysis used computer software (Statistical Analysis System, SAS Institute, Inc.).

RESULTS
RBC production and in vitro assessment
A total of 774 study RBC units were manufactured, 659 were released into clinical inventory, and 148 were transfused (Fig. 2). Amustaline-treated RBCs demonstrated equivalence for Hb content compared to untreated RBCs based on the PP Hb content (Table 1), meeting the primary efficacy endpoint. Secondary endpoints were also
met: the proportion of test RBCs satisfying the EDQM guidelines for Hb content (EOS) and Hct (PP and EOS) were not different compared to control RBCs. The proportion of test RBCs satisfying the EDQM guidelines for EOS hemolysis was higher than control (p < 0.001). Normalized ATP was higher for test RBCs and plasma-free Hb was lower for test RBCs than conventional RBCs (p < 0.001).

QC data on study RBCs

All variables, except PP Hct, were significantly different between test and control RBCs for at least one time point (Table S1, available as supporting information in the online version of this paper). MCHC (PP and EOS) and glucose (EOS) were higher in test compared to control (p < 0.05; Table S1), while MCV (PP and EOS), pH (EOS), potassium (EOS), lactate (EOS), and residual plasma protein (EOS) were lower (p < 0.05). These differences were not considered to be of physiologic or clinical significance. EOS residual plasma protein was 3.4 ± 1.4-fold lower in test compared to control (Table 1; p < 0.05). All test and control RBCs were culture negative (no growth of bacteria) at EOS.

Test RBCs had a mean PP S-300 (the primary degradation product) level of 18.4 ± 3.4 μmol/L (range, 9.6-35.5 μmol/L). PP extracellular mean GSH levels were 5.3 ± 0.8 mmol/L in test RBCs. GSSG PP was below the limit of quantitation, 0.75 mmol/L, for both treatment groups. Despite a decrease in GSH over storage, the level of GSSG remained less than 0.75 mmol/L (the limit of quantitation) in test RBCs at EOS. The mean level of 9-glutathiyldiacidine of test RBCs at EOS was 3.2 ± 1.1 μmol/L (range, <1-6.5 μmol/L) and the mean level of 9A-GSH at EOS was 4.1 ± 1.1 μmol/L (range, 1-7.5 μmol/L). 9A-GSSG was below the limit of quantitation (1.0 μmol/L) in all PP and EOS test components. The levels of these reaction by-products were consistent with the high safety margins established in preclinical studies.24

Clinical endpoints

Eighty-seven patients were randomly assigned and 51 received study RBCs, comprising the MITT population (Fig. 3). None of the patients screened for enrollment had existing cross-reactive antibodies to S-303 RBCs. The 51 transfused patients were equally distributed between the test and control groups in terms of demographics and surgical characteristics; however, test patients had significantly higher serum creatinine levels at baseline (mean serum creatinine, 1.12 mg/dL vs. 0.95 mg/dL, p = 0.033; Table 2). The proportion of MITT patients with abnormal serum creatinine levels at baseline was significantly higher for test (14 of 25 [56.0%] patients) compared to control patients (four of 26 [15.4%] patients; p < 0.01).

At transfusion, the mean storage age of the study RBCs before transfusion was 18.9 days (± 8.4), with no difference between treatment groups (p = 0.253). There were no differences between treatment groups in the mean number or in the mean volume of study RBCs transfused (Table 3). Three (12.0%) test and four (15.4%) control patients received off-protocol RBCs due to excessive urgent demand for RBCs transfusions. Although not statistically different, there were numerical differences...
between treatment arms in the exposure to other blood components (Table 3) that were explained by an outlier patient in the test arm who received 21 Hct components (6.83 L) and 5.29 L of plasma for treatment of an aortic dissection with intraoperative bleeding resulting in death due to uncontrolled hemorrhage after surgery.

### Clinical safety and efficacy

The overall incidence of renal insufficiency was 15.7% and there was no significant difference between the treatment groups (p = 0.41; Table 4). None of the renal insufficiency events occurred in relationship to an acute decrease in Hb levels or administration of study RBCs and a correlation of the effect of transfusion to renal organ perfusion could not be established. Renal insufficiency resolved in five of the eight patients (test, four; control, one). The incidence of hepatic insufficiency was low (2%), with no detectable difference between treatment groups (p = 0.37). Thirty-seven of 51 patients (73%) were able to perform the 6MWT at the time of first ambulation. There were no differences between treatment groups in the mean distance walked in meters between Day 0 and 6 or on Day 13 or discharge (Table 4).

Most patients in both groups (84.3%) experienced an AE (Table 5). There were no statistical differences in the overall incidence of AEs, or in possibly related AEs, although there was a trend toward more severe AEs (p = 0.07) in the test group. No trend in serious AEs was apparent when examined by system organ class. The three most frequent AEs were pleural effusion (eight test patients [32.0%], 10 control patients [38.5%]), atrial fibrillation (six test patients [24.0%], seven control patients [26.9%]), and delirium (four test patients [16.0%], five control patients [19.2%]); these were similarly distributed between treatment groups. There were no trends or safety signals identified within specific system organ classes. Three serious AEs (SAEs) were considered possibly related to the transfusion of study RBCs by the clinical investigators in one test patient (acute myocardial infarction on the day of the surgery) and two control patients (mantle cell lymphoma on Day 63 and cerebrovascular accident.

### Table 1. In vitro efficacy evaluations

| In vitro parameter | Test | Control | p value (95% CI)* |
|--------------------|------|---------|------------------|
| Hb content, PP (g/component) | Number | 389 | 365 | <0.001† |
| Mean (± SD) | 53.6 (±5.6) | 56.3 (6.0) | (-2.61 to -1.92) |
| Satisfy EDQM criteria, n (%) | 387 (99.5) | 365 (100) | |
| Hb content, end-of-storage (g/component) | Number | 301 | 261 | <0.001 |
| Mean (± SD) | 53.1 (±5.7) | 55.8 (±5.9) | (-2.76 to -1.92) |
| Satisfy EDQM criteria, n (%) | 297 (98.7) | 259 (99.2) | |
| Hct, PP (%) | Number | 389 | 367 | 0.209 |
| Mean (± SD) | 57.4 (±2.0) | 57.3 (±2.9) | (-0.10 to 0.45) |
| Satisfy EDQM criteria, n (%) | 389 (100) | 362 (98.6) | |
| Hct, end-of-storage | Number | 301 | 261 | 0.149 |
| Mean % (± SD) | 60.4 (±3.2) | 60.9 (±3.5) | (-0.81 to 0.12) |
| Satisfy EDQM criteria, n (%) | 299 (99.3) | 259 (99.2) | |
| Hemolysis, end-of-storage | Number | 301 | 261 | <0.001 |
| Mean (± SD) | 0.28 (0.12) | 0.35 (0.16) | (-0.09 to -0.04) |
| Satisfy EDQM criteria, n (%) | 301 (100) | 256 (98.1) | |
| Normalized ATP, end-of-storage (μmol/g) | Number | 294 | 262 | <0.001 |
| Mean (± SD) | 2.8 (±0.9) | 2.4 (±0.7) | (0.33 to 0.59) |
| >2 μmol/g, n (%) | 249 (84.7) | 185 (70.6) | |
| Plasma-free Hb, end-of-storage (g/dL) | Number | 263 | 225 | <0.001 |
| Mean (± SD) | 1.42 (±0.64) | 1.79 (±0.88) | (-0.49 to -0.23) |
| Satisfy EDQM criteria (%) | 263 (100) | 224 (99.6) | |
| Residual plasma protein, end-of-storage (mg/dL) | Number | 301 | 298 | <0.001 |
| Mean (± SD) | 68.0 (±25.6) | 228.8 (±34.7) | (-165.7 to -155.9) |

* p values and 95% CIs for the mean treatment difference (test – control) are based on the following: 1) analysis of covariance model controlling for the treatment, sex, blood type, input Hct, and input volume for all assays other than residual plasma protein and 2) t test with unequal variances for the residual plasma protein assay.

† Equivalence was declared at the 5% significance level if the two-sided 95% CI for the mean treatment difference (test – control) was within the equivalence (noninferiority) margins of ±5 g/unit.
on Day 16). Although a relationship to study RBCs of the myocardial infarction was not ruled out, assessment of relatedness was complicated by proximity to the surgery, the subject’s medical history, the use of catecholamines, and the fact that the patient continued receiving transfusion support with study RBCs without additional cardiac SAEs.

No transfusion reactions or hemolytic events were reported. Five patients died during this study with the following causes of death: three test patients (ventricular fibrillation on Day 26 postsurgery, hemorrhage during surgery, and multiorgan failure on Day 2 postsurgery) and two control patients (septic shock on Day 53 postsurgery and multiorgan failure on Day 4 postsurgery). The deaths were not considered related to the administration of study RBCs. All of these events were related to the patients’ primary disease, comorbid conditions, and/or postsurgical complications unrelated to RBC transfusion exposure.

Two test patients with a negative DAT at baseline converted to positive on Days 28 to 40 and Day 90 (end of study). One of them also converted a negative IAT at baseline to a positive IAT on Days 28 to 40 and returned to a negative IAT at the end of Study Day 90. This patient developed an antibody against the Jk(b) antigen, most likely a reactivation detected by IAT gel-card assay. No patients exhibited an immune response specific to amustaline-treated RBCs during the acute transfusion period or at any of the follow-up visits and no patients reported any symptoms suggestive of immune hemolysis (data not shown).

**DISCUSSION**

The results of this Phase III device trial demonstrate that amustaline-treated RBCs retained Hb content equivalent to that observed in conventional RBCs and met EDQM guidelines for Hb content, Hct, and plasma-free Hb when produced for clinical use. When transfused to cardiac surgery patients, no treatment differences were observed in usage of RBCs to support acute anemia or in clinical outcomes indicative of insufficient tissue oxygenation, such as renal or hepatic failure, although this study was not powered to differentiate these exploratory clinical endpoints. Renal insufficiency was more correlated to surgical complications during complex cardiac surgery, with random distribution between the test and control groups. The overall incidence (15.7%) was consistent with the incidence for renal failure reported in comparable patient

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**Fig. 3. STARS study CONSORT flow diagram.**

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populations and in a prior study utilizing a first-generation version of the amustaline RBC treatment process.\(^{15,25-27}\)

To explore the value of renal injury as a potential endpoint informative for tissue oxygenation in a subsequent powered clinical efficacy study, post hoc reanalyses of the serum creatinine levels and the proportions of patients with abnormal serum creatinine levels at baseline were conducted. Acute kidney injury (AKI) is recognized as a sensitive prognostic indicator in critically ill, including cardiac surgery patients.\(^{26,28-31}\) Using the KDIGO definition of AKI\(^{32}\) we observed an overall rate of 31.4% (test 11 (44%) vs. control five (19.2%); \(p = 0.11\)), suggesting that this might be an appropriate primary endpoint on which to power a pivotal Phase III clinical trial. A baseline difference in serum creatinine levels (Table 2) and in the proportion of patients with abnormal baseline serum creatinine concentrations, a known risk factor for AKI, was noted; 14 of 25 test patients had serum creatinine concentrations above normal reference ranges at baseline versus four of 26 control patients (\(p = 0.003; \text{data not shown}\)). Baseline renal status must be considered as a covariate when using AKI as a clinical endpoint in this patient population.

We included the 6MWT as an exploratory endpoint.\(^{33,34}\) Thirty-seven of 51 (72.6%) enrolled patients were assessed on Days 0 to 6 and 32 of 51 (62.7%) patients were available for assessment on the day of discharge or Day 13 postsurgery, whichever occurred first. Failure to assess 6MWT was due to patient withdrawal from study (five patients), death (five patients), patient discharge to local/regional facilities before they could ambulate, or patient inability to perform the test (Fig. 3). The variable clinical condition of patients in the immediate postoperative period and the variable date of discharge and/or death after surgery contributed to missing data in approximately one-third of the participants. For all patients evaluated there was no difference between groups, but the standard deviation (SD) was broad (50%-60% of the mean value), suggesting that the 6MWT had limited power for detecting differences between test and control subjects at these time points. Taken together, we conclude that the 6MWT is not an informative indicator of RBC function for future studies of pathogen-inactivated RBCs immediately

| TABLE 2. Baseline demographics and surgical characteristics: MITT population* |
|---------------------------------|------------------|------------------|------------------|-------------------|
| Characteristics                 | Test (n = 25)    | Control (n = 26) | Overall (n = 51) | p value (95% CI)† |
| Age (years)                     | 73.9 (±7.7)      | 74.3 (±6.5)      | 0.861 (–4.3 to 3.6) |
| Female                          | 11 (44.0)        | 16 (61.5)        | 0.192            |
| Body mass index (kg/m\(^2\))    | 27.8 (±5.8)      | 26.4 (±4.2)      | 0.317 (–1.4 to 4.3) |
| Baseline Hb (g/dL)              | 12.7 (±0.8)      | 12.4 (±1.2)      | 0.217 (–0.2 to 0.9) |
| Blood group                     |                  |                  |                  |
| A                               | 12 (48.0)        | 14 (53.8)        | 0.585            |
| O                               | 13 (52.0)        | 12 (46.2)        |                  |
| Rh factor positive              | 25 (100)         | 26 (100)         |                  |
| TRUST score                     | 3.3 (±1.0)       | 3.3 (±1.1)       | 0.835 (–0.5, 0.6) |
| Baseline serum creatinine       | 1.12 (0.27)      | 0.96 (0.22)      | 0.033 (0.01, to 0.29) |
| Baseline total bilirubin        | 0.40 (0.14)      | 2.20 (8.93)      | 0.421 (–4.93 to 2.09) |
| CABG procedure                  |                  |                  |                  |
| Vessels bypassed                | 2.7 (±1.0)       | 2.6 (±1.0)       | 0.909 (–0.7 to 0.6) |
| Grafts placed                   | 2.5 (±0.6)       | 2.3 (±0.8)       | 0.621 (–0.3 to 0.5) |
| Valves repaired or replaced     |                  |                  |                  |
| Aortic                          | 9                | 8                |                  |
| Mitral                          | 5                | 7                |                  |
| Tricuspid                       | 3                | 1                |                  |
| Use of bypass pump              | 22 (88.0)        | 23 (88.5)        | 0.912            |
| Use of aortic cross-clamp       | 22 (88.0)        | 23 (88.5)        | 0.912            |
| Use of cell saver               | 13 (52.0)        | 15 (57.7)        | 0.781            |
| Surgical complications leading to additional blood usage | 1 (4.0) | 2 (7.7) | 0.631 |
| Estimated volume of surgical blood loss (L) |                  |                  |                  |
| Mean (±SD)                      | 1.57 (±2.13)     | 1.32 (±0.93)     | 1.45 (±1.63)     | 0.625 (–8.38 to 11.83) |
| Median                          | 0.80             | 1.00             | 0.85             |
| Min to max                      | 0.3 to 9.999     | 0.1 to 3.5       | 0.1 to 9.999     |

* Data are reported as mean (±SD) or number (%). The MITT population comprised all randomized patients who received at least one study transfusion.
† For continuous variables, the CIs and p values for the treatment difference (test – control) in least-square means are based on ANOVA (controlling for the treatment and cardiac procedure performed). For categorical variables, the p values are based on a CMH test of general association (controlling for treatment and cardiac procedure performed).
after cardiac surgery. While it has been utilized during cardiac rehabilitation to assess patient recovery over a longer time period, it is unlikely that an assessment performed during rehabilitation weeks after RBC transfusion\(^3\) when few transfused RBCs remain in the circulation could be related to functional RBC differences.

### TABLE 3. Components transfused: MITT population*

| Components transfused                                      | Test (n = 25) | Control (n = 26) | Overall (n = 51) | p value (95% CI)† |
|------------------------------------------------------------|---------------|------------------|------------------|-------------------|
| Number of patients transfused with RBCs                    |               |                  |                  |                   |
| 1 unit, n (%)                                              | 4 (16.0)      | 7 (26.9)         | 11 (21.6)        | 0.870             |
| 2 units, n (%)                                             | 10 (40.0)     | 8 (30.8)         | 18 (35.3)        | (–1.0 to 1.1)     |
| 3 units, n (%)                                             | 4 (16.0)      | 3 (11.5)         | 7 (13.7)         |                   |
| ≥4 units, n (%)                                            | 7 (28.0)      | 8 (30.8)         | 15 (29.4)        |                   |
| Mean (±SD)                                                 | 2.9 (±1.7)    | 2.9 (±2.0)       | 2.9 (±1.8)       |                   |
| Min to max                                                 | 1.0 to 7.0    | 1.0 to 8.0       | 1.0 to 8.0       |                   |
| Volume of study RBCs transfused (L)                        |               |                  |                  |                   |
| Mean (±SD)                                                 | 0.74 (±0.42)  | 0.75 (±0.53)     | 0.74 (±0.48)     | 0.951             |
| Min to max                                                 | 0.25 to 1.75  | 0.25 to 2.40     | 0.25 to 2.40     | (–0.27 to 0.28)   |
| Patients with off-study RBC transfusions                  |               |                  |                  |                   |
| 1 unit                                                     | 0             | 1                | 1                | 0.724             |
| 2 units                                                    | 2             | 0                | 2                |                   |
| ≥3 or more units                                           | 1             | 3                | 4                |                   |
| Number of patients receiving PLT units                     |               |                  |                  |                   |
| 1-4 units                                                  | 5             | 7                | 12               | 0.156             |
| ≥5 units                                                   | 2             | 1                | 3                |                   |
| Estimated total volume of PLTs transfused (L)              |               |                  |                  |                   |
| Number                                                     | 7             | 8                | 15               | 0.118             |
| Mean (±SD)                                                 | 1.89 (±2.20)  | 0.78 (±0.48)     | 1.30 (±1.59)     | (–0.44 to 3.38)   |
| Median                                                     | 1.04          | 0.68             | 0.78             |                   |
| Min to max                                                 | 0.66 to 6.83  | 0.2 to 1.84      | 0.2 to 6.83      |                   |
| Number of patients with plasma components transfused       |               |                  |                  |                   |
| 1-4 units                                                  | 2             | 1                | 3                | NA                |
| ≥5 units                                                   | 1             | 0                | 1                |                   |
| Estimated total volume of plasma transfused (L)            |               |                  |                  |                   |
| Number                                                     | 3             | 1                | 4                | NA                |
| Mean (±SD)                                                 | 2.24 (±2.64)  | 0.32 (NA)        | 1.76 (±2.36)     |                   |
| Median                                                     | 0.84          | 0.32             | 0.72             |                   |
| Min to max                                                 | 0.603 to 5.29 | 0.318 to 0.318   | 0.318 to 5.29    |                   |

* The MITT population comprised all randomized patients who received at least one study transfusion.
† For continuous variables, the CI and p values for the treatment difference (test – control) in LS means are based on ANOVA (controlling for the treatment and cardiac procedure performed). For categorical variables, the p values are based on a CMH test of general association for binary data or row mean scores for ordinal data, controlling for treatment and cardiac procedure performed.

### TABLE 4. Clinical outcomes: MITT population*

| Clinical outcomes                                      | Test (n = 25) | Control (n = 26) | Total (n = 51) | p value (95% CI)† |
|--------------------------------------------------------|---------------|------------------|----------------|-------------------|
| Renal insufficiency (%)                                | 5 (20.0)      | 3 (11.5)         | 8 (15.7)       | 0.412             |
| Hepatic insufficiency (%)                              | 1 (4.0)       | 0                | 1 (2.0)        | 0.371             |
| 6MWT measured on day of first ambulation (Day 0 to 6) (m) |               |                  |                |                   |
| Number                                                  | 17            | 20               | 37             | 0.742             |
| Mean (±SD)                                              | 44.8 (±46.8)  | 53.1 (±41.8)     | 49.3 (±44.6)   | (–37.0 to 26.6)   |
| Median                                                   | 38.8          | 35.0             | 35.0           |                   |
| Min to max                                              | 1 to 157.5    | 0 to 142         | 0 to 157.5     |                   |
| 6MWT measured at acute follow-up (Day 13 or Discharge) (m) |               |                  |                |                   |
| Number                                                  | 14            | 18               | 32             | 0.626             |
| Mean (±SD)                                              | 95.5 (±69.7)  | 97.7 (±51.1)     | 96.7 (±59.0)   | (–30.8 to 50.3)   |
| Median                                                   | 84.0          | 91.5             | 87.8           |                   |
| Min to max                                              | 10 to 291     | 7.5 to 180       | 7.5 to 291     |                   |

* The MITT population comprised all randomized patients who received at least one study transfusion.
† For continuous variables, the CI and p values for the treatment difference (test – control) in LS means are based on ANOVA (controlling for the treatment and cardiac procedure performed). For categorical variables, the p values are based on a CMH test of general association (controlling for treatment and cardiac procedure performed).
Observed AEs were within the expected spectrum of comorbidity and mortality for patients of similar age undergoing cardiovascular surgery requiring RBC transfusion. Cardiac rhythm disturbances requiring treatment (Grade 3) are common after cardiac surgery. Pleural effusions and delirium are among the most common side effects after CABG and valve replacement surgery with reported incidences of up to 90% and 3% to 73%, respectively. Due to the limited number of patients enrolled, further investigation on possible adverse effects and Hb increment after transfusion of amustaline-treated RBCs is required in nonsurgical patients to evaluate further the safety and efficacy of this novel blood product. The clinical data from this study are consistent with the data from a prior clinical trial utilizing a previous version of the system with amustaline, where amustaline-treated RBCs and conventional RBCs were equivalent with respect to clinical efficacy and safety in supporting the transfusion needs of cardiac surgery patients.

The data suggest that the storage lesion in amustaline-treated RBCs is comparable, if not minimized, relative to that in conventional RBCs. Substantially reduced residual plasma protein levels resulted from the suspension media exchange step incorporated into the amustaline treatment process. This may reduce transfusion reactions to plasma proteins and could improve patient safety, due to lower levels of HLA antibodies implicated in TRALI that are correlated with the volume of transfused plasma. Levels of degradation products of amustaline and GSH were consistent with previous observations (unpublished data) and within the safety margins derived from preclinical testing.

New and emergent pathogens such as the ZIKV underscore the need for a sustainable and proactive approach to reduce the risk of TTIID. Given the proven potential to be transmitted by transfusion and its association with deleterious outcomes such as microcephaly and Guillain-Barre syndrome, the FDA recently issued a final guidance recommending the cessation of blood collections in areas with active transmission of ZIKV, such as Puerto Rico; investigational ZIKV tests; or the use of an approved pathogen reduction device for plasma and for Hct. The guidance specifically states that pathogen reduction systems for RBCs may replace testing, once clinically available, indicating the FDA’s commitment to pathogen reduction as a future risk mitigation intervention for BRC safety.

This trial successfully demonstrated that amustaline-treated RBCs were equivalent to conventional RBCs in terms of Hb content and other in vitro variables correlated with transfusion efficacy. Amustaline-treated RBCs supported patients as effectively as conventional RBCs in the setting of RBC transfusion for acute anemia during cardiac surgery with hemodynamic changes, and the safety profile was comparable given the limited data set. There was no difference in RBC usage between treatment groups, indicative of adequate tissue oxygenation after transfusion of test RBCs.

The study confirmed that AKI may be an informative endpoint and that baseline serum creatinine is an important covariate that must be considered. In conclusion, amustaline-treated RBCs were well tolerated justifying larger Phase III clinical trials to confirm clinical efficacy and safety.

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CONFLICT OF INTEREST

AN, NH, NM, AE, CE, SR, RJB, and LMC are employees of Cerus Corporation. The other authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Supplementary Materials: Laboratory Analytical Methods.

Table S1. Quality parameters postproduction and end of storage study RBCs