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Transfusion Related Necrotizing Enterocolitis in Very Low Birth Weight Infants

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Transfusion Related Necrotizing Enterocolitis in Very Low Birth Weight Infants

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B.S., Emory University, 2001

M.D., Medical College of Georgia, 2005

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ABSTRACT

Transfusion Related Necrotizing Enterocolitis in Very Low Birth Weight Infants
By Ravi Mangal Patel

Background: Necrotizing enterocolitis (NEC) is a leading cause of morbidity and mortality in very low birth weight (VLBW) infants. The risks of NEC associated with red blood cell (RBC) transfusion and severe anemia have not been prospectively characterized.

Methods: We performed a secondary analysis of a prospective, multicenter observational cohort study from Jan 2010 to Sept 2013 at 3 hospitals in Atlanta, Georgia to prospectively investigate the association between the exposure to RBC transfusion and severe anemia (hemoglobin ≤ 8g/dL) and the subsequent development of NEC in VLBW infants. We tested the primary hypothesis that the risk of NEC is greater in VLBW infants exposed to RBC transfusion compared to non-transfused VLBW infants. As a secondary aim, we evaluated if severe anemia was an independent risk factor for NEC. Multivariate Cox regression analyses were performed to evaluate the association between time-dependent covariates (RBC transfusion and severe anemia) and NEC (defined as Bell's Stage 2 or greater), after adjustment for birthweight and center.

Results: A total of 539 VLBW infants were evaluated and 40 (7.4%) infants developed NEC. Fifty-four percent (291/ 539) of enrolled VLBW infants received at least 1 RBC transfusion. After adjustment for birthweight, center, and severe anemia, the risk of NEC was higher for infants exposed to RBC transfusion in a given week, compared to non-transfused infants (hazard ratio (HR) 2.37; 95% confidence interval (CI) 1.06-5.30; P=0.036). Similarly, the risk of NEC was higher for infants with severe anemia in a given week compared to infants without severe anemia (HR 5.55; 95% CI 2.44-12.64; P<0.0001).

Conclusions: In this prospective study, RBC transfusion and severe anemia were both associated with an increased risk of NEC in VLBW infants. Further study is needed to determine if severe anemia is an important effect modifier of the risk of RBC transfusion.

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INTRODUCTION

Necrotizing enterocolitis (NEC) is a leading cause of neonatal morbidity and mortality with a poorly understood pathogenesis and no safe and effective prevention strategy (1, 2). NEC-related mortality increased by 35% from 2000 to 2011 (3), highlighting the urgent need for prevention strategies. The increase in NEC-related deaths has coincided with a newly documented association between the exposure to red blood cell (RBC) transfusion and the subsequent development of NEC (4). Transfusion-related NEC (TR-NEC) has emerged as a new clinical entity as many centers have shifted to conservative transfusion practices that tolerate increased neonatal anemia. However, the effect of severe anemia independent of RBC exposure on the development of NEC is unknown.

Improving our understanding of TR-NEC is critical because half of very low birth weight (VLBW, birthweight ≤1500 grams) infants receive 1 or more transfusions during hospitalization (5). Prior studies characterizing TR-NEC have been limited by lack of data regarding RBC irradiation and storage, small sample size, case-control design, lack of evaluation of time-varying exposures and limited control for baseline illness severity and other confounders. These limitations have underscored the need for prospective study (4), in which each RBC exposure, including processing characteristics, and NEC can be systematically and consistently evaluated.

Here we present the first prospective study of TR-NEC. Our primary objective was to test the hypothesis that NEC is increased in VLBW infants receiving RBC transfusion compared to non-transfused VLBW infants. Further, we determined if exposure to severe neonatal anemia, defined as a hemoglobin level ≤ 8g/dL, is an independent risk factor for NEC. In addition, we tested the hypothesis that an increased duration of RBC storage age after gamma irradiation is associated with a greater risk of NEC among RBC transfused VLBW infants.

BACKGROUND

Necrotizing enterocolitis is the most common gastrointestinal emergency in neonates and approximately 1 in 10 premature infants less than 29 weeks gestation will develop the disease (6). Mortality attributable to NEC is high, with case-fatality rates of 20% to 30% for affected VLBW infants (7). Infants who survive the disease have long-term complications, including short bowel syndrome, poor growth, and neurodevelopmental impairment (8). NEC results in a considerable financial burden, with the cost of care for affected infants in the United States estimated to be between $500 million to $1 billion per year (9).

NEC can frequently progress from early clinical symptoms to extensive intestinal necrosis within hours, limiting the efficacy of therapeutic intervention. As such, approaches to prevent NEC have become a focus of research efforts (10), although a limited understanding of the etiology of NEC has hampered these efforts. Therefore, investigations aimed at understanding NEC pathophysiology are crucial to the development of targeted prevention strategies. Researchers have identified several factors contributing to the development of NEC: prematurity, enteral feeding, intestinal bacterial colonization, immature gut barrier, pro-inflammatory propensity of the immature gut and impaired intestinal blood flow (1, 2, 9-13). The multifactorial nature of the disease suggests that preventative efforts would be more successful if they targeted multiple components, including the emerging risk factor of RBC transfusion and its potential involvement in the pathogenesis of NEC. Investigating the role of transfusion is particularly relevant given the finding that approximately 25% to 38% of NEC cases in retrospective studies have been reported to occur within 48 hours of RBC transfusion (14-16).

Transfusion Practices in Very Low Birth Weight Infants.

VLBW infants frequently require RBC transfusion to treat anemia related to prematurity. However, optimal transfusion practices in neonates are limited by inconclusive published studies. Two previous randomized trials, the Premature Infants in Need of Transfusion (P.I.N.T) trial (17)

and the smaller Iowa trial (18), investigated the effect of comparative transfusion practices on neonatal morbidity. The primary focus of these trials involved comparing the effect of different hemoglobin transfusion thresholds on neonatal morbidity. However, neither trial evaluated NEC as the primary outcome. The single-center Iowa trial showed a decrease in the number of transfusions in infants randomized to the low (conservative) threshold arm but the small sample size limited assessment of safety. In comparison, the multicenter P.I.N.T. trial randomized 451 infants to similar high and low hemoglobin thresholds for transfusion. There was no difference in the primary composite outcome (Bronchopulmonary dysplasia (BPD), death, retinopathy of prematurity (ROP), or brain injury) between infants in the two treatment arms. After the P.I.N.T. trial showed a reduction in the number of transfusions in infants randomized to the conservative hemoglobin threshold arm with no difference in the primary composite outcome, many centers have shifted towards a conservative transfusion strategy to minimize blood exposure in VLBW infants. As conservative transfusion practices, with increased tolerance of neonatal anemia, have become more common following the publication of the P.I.N.T. trial in 2006, the association between RBC transfusion and NEC has been increasingly reported. Since 2006, twelve published reports have described an association between RBC transfusion and NEC (14-16, 19-27) whereas only one study prior to 2006 reported the association (28). However, the lack of randomized trials evaluating transfusion thresholds with NEC as a primary outcome measure limit the assessment of the effect of conservative transfusion practices on the risk of NEC.

Red Blood Cell Transfusion Associated Necrotizing Enterocolitis.

A meta-analysis of several observational studies evaluating the association between RBC transfusion and NEC have shown an increased risk of NEC in infants exposed to RBC transfusion, compared to unexposed infants (Odds ratio 3.91; 95% CI 2.97-5.14) (4). However, a causal link has not been identified and the studies included in the meta-analysis have been limited by small numbers of patients and retrospectively identified end-points. Importantly, these studies

have identified several candidate neonatal risk factors that may predispose VLBW infants to the development of NEC. These factors include severity of anemia and a developmental window at which NEC occurs. However, none of these studies have accounted for the repeated exposures of anemia and RBC transfusion that an infant receives during hospitalization. In addition, no study has prospectively investigated the specific characteristics of the exposure of RBC transfusion, including measures of the duration of storage of blood after gamma irradiation. Therefore, knowledge gaps remain regarding the contribution of blood banking practices, such as irradiation storage time prior to transfusion on development of NEC following RBC transfusion.

Candidate biological mechanisms of TR-NEC.

Two potential mechanisms with biologic plausibility may explain the causal pathway in which RBC transfusion leads to the development of NEC. These two factors are decreased intestinal oxygen delivery due to severe anemia and the "storage lesion" of RBCs related to decreases in nitric oxide as RBCs age. These candidate mechanisms are discussed in further detail below.

Severity of anemia. Intestinal injury and predisposition to NEC may be due to impaired intestinal perfusion during periods of compensated and uncompensated anemia. Physiologic studies support this rationale. Alkalay and colleagues, using echocardiographic measurements, demonstrated that infants who appeared clinically "stable" with significant anemia (hematocrit levels $\leq$ 21%) and some infants with milder anemia (hematocrit levels of 22% to 26%) were in a high cardiac output state with restricted gut perfusion despite having no clinical symptoms (29). Krimmell and colleagues evaluated mesenteric blood flow in anemic infants who required RBC transfusion. The authors found that premature infants demonstrated decreased mesenteric blood flow responses to feeding after RBC transfusion, potentially predisposing these infants to intestinal hypo-perfusion and the development of NEC (30). There is conflicting data about the association between anemia and NEC. These studies are limited by inadequate control of RBC exposure and the lack of longitudinal assessment of anemia during hospitalization. In a recent

study, Singh and colleagues demonstrated that each percentage point decrease in the lowest measured hematocrit was associated with an increased risk of NEC in VLBW infants (odds ratio 1.10 per 1% drop in nadir hematocrit, 95% CI 1.02 to 1.18) (23). In contrast, the P.I.N.T. trial showed no significant difference in NEC between liberally and conservatively transfused groups with differing levels of tolerated anemia (17). No study to date has used prospectively identified longitudinal measures of hemoglobin to investigate the association between severe anemia and NEC.

Storage Lesion. During RBC storage, blood cells undergo progressive changes and deterioration that reduce function and viability and may cause detrimental clinical effects (31-34). The risks of prolonged RBC storage were highlighted in a study by Koch and colleagues in which adults undergoing cardiac surgery who received blood that was stored for more than 14 days were at significantly increased risk of postoperative complications and mortality (35). This may be partly mediated through the decreased availability of nitric oxide (NO) in stored RBCs as concentrations of NO have been shown to decrease with prolonged RBC storage (36). In addition, NO is a primary vasodilator for the newborn intestine and alterations in the levels of NO may promote vasoconstriction resulting in intestinal ischemia and the possible development of NEC (37). Importantly, the RBC storage lesion is worsened by RBC irradiation (38, 39), which is a widespread practice to reduce the risk of graft-versus-host disease in transfused premature infants (40, 41). Although a recent randomized trial in Canada showed no difference in a composite outcome of neonatal morbidity between preterm infants receiving fresh compared to old blood (42), the trial did not study the timing of RBC irradiation. In addition, the external validity of the study was limited as the Canadian study did not reflect US transfusion practices as we highlighted in a response letter (43). No studies have evaluated the association between an increased duration of RBC storage between irradiation and transfusion and the subsequent development of NEC.

METHODS

Research goal and hypothesis

Our research goal was to test the hypothesis that risk of NEC is greater in VLBW infants exposed to RBC transfusion compared to non-transfused VLBW infants. As secondary aims, we evaluated if severe anemia, defined as a hemoglobin concentration ≤ 8 g/dL, is an independent risk factor for NEC and if prolonged RBC storage after irradiation is associated with the development of NEC among transfused VLBW infants.

Study design and characteristics of the study population

We performed a secondary analysis of a prospective, multicenter observational birth-cohort study investigating the transfusion-transmission of cytomegalovirus in preterm infants (TT-CMV study, registered at clinicaltrials.gov under identifier NCT00907686). The study design and methods of the TT-CMV study have been previously published (44). Parents or guardians provided a signed informed consent before enrollment of their VLBW infant in the TT-CMV study. All enrolled VLBW infants were followed from birth to 90 days, hospital discharge or death.

We included VLBW infants born at three level III neonatal intensive care units (NICUs) in Atlanta, Georgia. Two of these NICUs were academically-affiliated (Grady Memorial Hospital and Emory University Hospital Midtown) and part of a single regional perinatal center. The other NICU was a private, non-academically affiliated hospital (Northside Hospital). Our inclusion criteria were: 1) birth weight ≤ 1500 grams and 2) postnatal age ≤ 5 days. Our exclusion criteria included: 1) Infant not expected to survive beyond 7 days of life based on the assessment of the treating neonatologist; 2) severe congenital abnormality; 3) transfusion prior to enrollment; or 4) maternal refusal to participate. This study was approved by the Institutional Review Board and/or Research Oversight Committees at all participating centers.

Measurements and sources of data, including definition of outcome, predictors, and covariates

All patient, laboratory and transfusion variables were collected as part of standardized data collection for the TT-CMV study. DataFax (Clinical DataFax Systems, Ontario, Canada) was used for data collection in case report forms (CRFs) by the TT-CMV study research nurses. Data from CRFs were entered into a SQL database managed by the TT-CMV data coordinating center. De-identified data was used for the analysis of the study aims, which was performed using SAS 9.2 (SAS Institute, Cary, NC).

The primary outcome of interest was NEC, defined as Bell's Stage II or greater according to the established Bell's criteria (45). The primary outcome was ascertained by active surveillance and systematic outcome assessment for all enrolled VLBW infants. The staging of all cases of NEC were adjudicated by an independent, board-certified neonatologist (Dr. Sarah Keene, MD) by review of clinical notes and abdominal radiographs to minimize ascertainment bias. Dr. Keene was not directly involved with aims of the study. The primary exposure (predictor of interest) was RBC transfusion. All RBC transfusion exposures were recorded by the TT-CMV study research nurses in CRFs. For patients who developed NEC, only transfusion exposures that occurred before the onset of NEC were analyzed. In addition, the age of blood transfused and timing of irradiation were systematically recorded for each RBC transfusion exposure. The secondary exposure of interest was severe anemia, defined as a hemoglobin concentration ≤ 8 g/dL. The use of the cut-point of 8 g/dL for severe anemia was determined taking into consideration the lower thresholds of anemia tolerated in two randomized controlled trials comparing transfusion practices in preterm infants (17, 18). Additional measured covariates are detailed below:

A) *Baseline characteristics.* Maternal and neonatal demographic and baseline information was collected by TT-CMV research nurses. Baseline illness severity was determined using the score for neonatal acute physiology (SNAP), a validated measure of neonatal illness severity (46).

B) *Laboratory testing.* Hemoglobin and hematocrit values were recorded at weekly intervals up to 90 days of age or discharge by the TT-CMV study research nurses.

C) *Additional Clinical Outcomes.* Standardized assessments for the following neonatal comorbidities were performed: intraventricular hemorrhage (IVH) grade II or higher, defined according to the scoring system by Papile et al. (47), patent ductus arteriosus (PDA) and infection, defined as the presence of a positive blood culture.

Missing data issues

Missing data was minimized through the use of regular quality assurance and quality control reports by the TT-CMV data coordinating center to ensure that all key variables were collected for enrolled participants. If any variables were missing in the prospectively collected CRFs, retrospective chart review was performed. There were no instances of missing data for any of the variables included in the multivariable Cox model, including the exposures (RBC transfusion, severe anemia) and outcome (NEC) of interest.

Sample-size and power considerations and calculations

The projected enrollment of the primary TT-CMV study was 700 VLBW infants. Based on an interim analysis in August of 2011 of infants completing the TT-CMV study, 13 (7.5%) of 173 enrolled VLBW infants had been diagnosed with NEC. Assuming proportional hazards with a significance level of 0.05, a statistical power of 80% and a NEC incidence of 7%, a sample size of 535 enrolled VLBW infants was calculated to achieve an 80% statistical power to detect a hazard ratio for NEC of approximately 2.5 for transfused VLBW infants relative to non-transfused VLBW infants.

Analytic plan

The cumulative incidence of NEC was estimated using the Kaplan-Meier method and compared between RBC transfusion exposed and unexposed infants. Univariable and multivariable Cox regression models were utilized to determine the hazard ratio of NEC with fixed and time-varying exposures. The following fixed patient covariates were compared between NEC and non-NEC patients: gestational age, birth weight, SNAP score at birth, 5 minute Apgar score, and breast feeding status. In addition, the following time-dependent patient and transfusion variables were compared between NEC and non-NEC patients: RBC transfusion, severe anemia, time in days from irradiation to RBC transfusion (IST), age of the blood at transfusion (storage age), and number of transfusions within a given week.

Multivariable models included adjustment for known and potential confounders. To protect against model overfitting, the number of parameters were limited in the multivariable model and parameter selection was driven by available knowledge and biological plausibility of potential confounders, taking into consideration the hypothesis of interest **(Figure 1)**. Correlation and co-linearity between parameters in the Cox regression model were evaluated using correlation plots, box plots and the Spearman correlation coefficient. Additional analysis was performed among patients exposed to RBC transfusion to evaluate for the association between the two specific donor RBC characteristics (IST and age of transfused RBC) and NEC. We categorized IST and storage age of donor RBCs using pre-specified cut-points of 4 days and 7 days, respectively. Categorization was favored over specification of these parameters as linear covariates because the effects of aging and storage of blood on RBC function and viability are non-linear. We also performed *post-hoc* sensitivity analysis to our cut-points and the effect of center by using an IST cut-point of 1 day and comparing full- and reduced models with and without center.

We were unable to evaluate for statistical interaction between exposure to RBC transfusion and severe anemia on the risk of NEC as these two variables were time-varying exposures with sparse episodes of co-occurrence. As a pre-planned alternative, logistic regression models were utilized to evaluate statistical interaction with fixed covariates, where patients were deemed to have severe anemia and RBC transfusion if they had one or more of these exposures during hospitalization. Due to the competing outcomes of NEC and non-NEC death, a composite outcome of NEC or death was also fit to the logistic regression model in addition to the outcome of NEC. The presence of statistical interaction was determined using a Wald chi-square test for the interaction term. For the logistic regression model, fit was evaluated using the Hosmer and Lemeshow goodness-of-fit test, collinearity between parameters was evaluated using condition indices and variance decomposition proportions. Finally, the c-statistic (a measure of area under the curve) was used to determine how well included covariates predicted NEC. As with our approach to the Cox regression analysis, we limited the number of parameters in the logistic regression model to protect against model overfitting. We defined statistical significance as a P-value < 0.05.

RESULTS

From January 2010 to September 2013, we screened a total of 1455 infants, of whom 541 VLBW infants met study criteria and were enrolled **(Figure 2)**. Ninety-nine percent (539/541) of enrolled infants completed the study. Baseline characteristics of infants demonstrated a lower mean birth weight and gestational age for infants who received RBC transfusion, compared to non-transfused infants (birthweight [mean ± standard deviation] 860 ± 237g vs. 1188 ± 196g; gestational age 26.4 ± 2.1 wk vs. 29.5 ± 2.2 wk; **Table 1**). There were no clinically significant differences in sex or race between transfused and non-transfused infants. However, RBC transfused infants had a higher baseline illness severity compared to non-transfused infants (mean SNAP score 12.5 ± 4.5 vs. 8.4 ± 5.3).

A total of 40/539 (7.4%) infants developed NEC Bell's stage II or greater. Infants with NEC, compared to those without NEC, had a lower birthweight and gestational age but no difference in gender or baseline illness severity **(Table 2)**. The median postnatal and postmenstrual age at first diagnosis of NEC was 30 days (interquartile range [IQR] 21-28.5 days) and 30 weeks (IQR 28-33weeks), respectively. Eighty-three percent (33/40) of infants with NEC received a RBC transfusion before diagnosis and 58% (23/40) received an RBC transfusion within 48 hours of diagnosis. Thirty-eight percent (15/40) of infants with NEC required surgical treatment, with the majority of infants (13/15) receiving an exploratory laparotomy.

Clinical characteristics

A total of 54% (291/539) of infants received one or more RBC transfusions. Among infants who received RBC transfusion, the median number of transfusion exposures was 3 (IQR 2-7) and the median number of donor exposures was 2 (IQR 1-4) **(Table 3)**. The frequency of any episode of severe anemia (hemoglobin ≤ 8 g/dL) was 32% for infants who received at least 1 RBC transfusion compared to only 2% for infants who did not receive a RBC transfusion. After including only transfusion exposures prior to the onset of NEC, infants who developed NEC

received a higher number of RBC transfusions compared to infants without NEC **(Table 4).** Twelve of 40 (30.0%) infants with NEC died, whereas 18/499 (3.6%) infants without NEC died. The frequency of any breastfeeding among this cohort was high, with 39/40 (97.5%) infants with NEC having received breast milk during hospitalization compared to 428/499 (85.8%) infants without NEC.

Risk factors for NEC

For every 100 gram increase in birthweight, the risk of NEC decreased by 23% (hazard ratio (HR) 0.77; 95% confidence interval (CI) 0.67-0.87; P<0.0001) **(Table 5)**. Similarly, for every one week increase in gestational age, the risk of NEC decreased by 16% (HR 0.84; 95% CI 0.73-0.96; P=0.01). We also detected a significant association between center and NEC, with the risk of NEC over two times greater at one center compared to the other (P=0.008). We did not detect any association between sex, race, 5 minute Apgar score or baseline SNAP score and NEC. When we compared infants who were exposed to a RBC transfusion in a given week to those who were unexposed, the risk of NEC was over 6 times greater (HR 6.62; 95% CI 3.45-12.70; P<0.0001). Similarly, infants with severe anemia (hemoglobin ≤ 8g/dL) in a given week, compared to infants without severe anemia, had a 10 times greater risk of NEC (HR 10.34; 95% CI 5.03-21.25; P<0.0001). When RBC transfusion and number of episodes of severe anemia were evaluated as fixed covariates instead of time-varying covariates, both of these factors remained significant risk factors for NEC **(Table 5)**.

In multivariable analysis, including adjustment for birthweight and center, both severe anemia and RBC transfusion in a given week were independently associated with an increased risk of NEC **(Table 6)**. The risk of NEC for infants with severe anemia in a given week was over 5 times greater than those infants without severe anemia, after controlling for birth weight, center and the receipt of transfusion (HR 5.55; 95% CI 2.44-12.64; P<0.0001). Similarly, exposure to RBC transfusion in a given week was an independent risk factor for NEC (HR 2.37; 95%CI 1.06-

5.30; P=0.036). Inclusion of SNAP score, 5 min Apgar score and/or receipt of breast milk did not significantly change the estimates for the parameters of interest (RBC transfusion and severe anemia) and, therefore, were not included in the final model. As 18/539 (3.3%) infants died without developing NEC, we accounted for this competing risk using an alternative analysis where we estimated the risk of severe anemia and RBC transfusion if all competing deaths would have developed the outcome of interest (composite outcome of NEC or death). In this model, the risks of severe anemia (HR 4.39; 95% CI 2.21-8.71) and RBC transfusion (HR 2.61; 95% CI 1.33-5.12) on NEC were similar to models fitted to only the cause-specific hazard of NEC. This approach was favored over a competing risks model due to the low number of non-NEC deaths and limited ability to estimate the cause-specific hazard ratio of death for parameters of interest.

To evaluate potential effect modification of the risk of transfusion on NEC by severe anemia, we performed an alternative, pre-planned analysis using logistic regression. We detected significant heterogeneity in the risk of NEC between RBC transfused and non-transfused patients, depending on the presence of severe anemia (interaction P=0.008). Among patients with severe anemia, the risk of NEC was higher among RBC transfused patients compared to non-transfused patients (odds ratio (OR) 4.56; 95%CI 1.30-15.97) **(Table 7)**. By contrast, among patients without severe anemia, we did not detect a significantly increased risk of NEC when comparing RBC transfused to non-transfused patients (OR 2.02; 95% CI 0.56-7.21).

In analysis of potential risk factors for NEC among a subset of transfused VLBW infants (n=291), the number of RBC transfusions, presence of severe anemia and age of blood transfused in a given week were all associated with an increased risk of NEC **(Table 8)**. After categorizing irradiation storage time (IST) and total RBC storage age using pre-specified cut-points and adjusting for center, severe anemia and birthweight, we did not detect any association between IST or total RBC storage age and NEC **(Table 9)**. However, we found close correlation between IST and center, with the median IST at center 1 of 5 days (IQR 3-7) and at center 2 of 0 days

(IQR 0-0). Further, there were limited numbers of transfusions with IST beyond 4 days. Therefore, we performed a *post-hoc* analysis removing center, due to strong correlation with IST, and categorizing IST using a cut-point of 1 day, to determine if any irradiation storage was potentially harmful. In this *post-hoc* analysis, we did detect a potential association between IST > 1 day and NEC (HR 2.29; 95% CI 1.03-5.07; P=0.042) **(Table 10)**.

DISCUSSION

Our study is the first prospective study to evaluate the association between RBC transfusion and NEC in VLBW infants. This study demonstrates that both RBC transfusion and severe anemia are independent risk factors for NEC and that severe anemia may be an important effect modifier of the risk of transfusion on the occurrence of NEC. This study confirms prior retrospective reports demonstrating an association between RBC transfusion and NEC (4). Furthermore, this is the first study to identify potential interaction between severe anemia and RBC transfusion, which was not detected in the study by Singh and colleagues (24). This finding is clinically relevant as RBC transfusion is a therapy used to prevent or treat severe anemia in VLBW infants. Although we were not fully able to evaluate for interaction between RBC transfusion and severe anemia in our Cox regression models, our alternative logistic regression analysis suggests that the risks of RBC transfusion may be greater for those infants with severe anemia.

In addition, we have identified a novel and modifiable potential candidate risk factor for NEC among infants who receive RBC transfusion: the duration of RBC storage following irradiation. Currently, there is no data available regarding the risks of prolonged RBC storage after gamma irradiation and additional studies are needed to better understand the effects that RBC irradiation, a widespread practice in neonatal transfusion medicine, has on the VLBW recipient. Of note, because of the strong correlation between IST and center, our finding of an association between IST and NEC may be confounded by center and requires further validation in additional studies. In addition, we did not find an association between total RBC storage age and the risk of NEC, which is consistent with the results of the ARIPI trial, a randomized controlled trial comparing the effects of transfusion of fresh versus old blood (42). However, the ARIPI trial did not study the timing of irradiation and the population of infants were more liberally transfused (median of 4 transfusions) than infants enrolled in our study. Thus, patients enrolled in the ARIPI study likely did not experience severe anemia as commonly as in our study, and this may limit some comparisons between the two studies.

Strengths and minimization of bias

The strengths of our study include the prospective and systematic collection of data. We were able to evaluate time-varying exposures, which allowed us to utilize data from the numerous episodes of transfusion and anemia that a VLBW infant may experience during hospitalization. Further, we were able to systematically evaluate each transfusion exposure with donor RBC characteristics. We also included only definite NEC cases (Bells stage II or greater), determined by active surveillance by research nurses with adjudication of staging by a neonatologist not directly involved with the study to minimize ascertainment bias. We minimized detection bias of NEC cases by following all enrolled infants for the primary outcome. We minimized selection bias by utilizing relatively broad inclusion criteria and limited exclusion criteria. Finally, we had minimal loss to follow-up by having 99% of enrolled infants complete the study.

Limitations

Our study has several limitations. The observational design of our study prohibits the establishment of causality between RBC transfusion and NEC. Further, our study was underpowered to detect clinically significant differences in the subgroup of transfused VLBW infants. We were also unable to fully account for the effect of center in our subgroup analysis as there was a strong correlation between center and IST. Therefore, the effect of center on NEC may be through the differences in duration of RBC storage after irradiation. Alternatively, the association between IST and NEC may be confounded by center characteristics that are not related to the irradiation of donor RBCs. Finally, the external validity of our study may be limited to centers with differing transfusion practices.

Next Steps

The Transfusion of Prematures Trial (clinicaltrials.gov identifier NCT01702805) may provide experimental data regarding the risks of both anemia and RBC transfusion. This is a currently ongoing randomized controlled trial investigating the effect of two different levels of tolerated anemia to which extremely preterm infants are randomized. However, the primary outcome of the

trial is long-term neurologic outcome and blood banking practices will not be standardized as part of the study. In addition, the trial may be underpowered to detect clinically significant differences in NEC between treatment arms. Further studies investigating the specific biochemical characteristics of transfused blood on the risk of adverse outcomes in the neonate, including NEC, will provide new insight into potential biomarkers of blood that are associated with harm in the transfused recipient.

Although we attempted to evaluate for statistical interaction, we could not definitively conclude that the risk of NEC following exposure to RBC transfusion was only increased among infants with severe anemia. As both severe anemia and RBC transfusion are independent risk factors for NEC, prevention of severe anemia by administration of RBC transfusion may not be an optimal strategy. New strategies, such as the administration of darbepoetin alfa (48), a synthetic form of erythropoietin with a weekly dosing interval that can prevent both anemia and RBC transfusion exposure, are potentially exciting strategies that may reduce the risk of NEC. However, further studies are needed to determine the efficacy of such a strategy to prevent NEC and also evaluate risks, such as retinopathy of prematurity, that are associated with early erythropoietin therapy (49).

Conclusion

In conclusion, our prospective study demonstrates that both RBC transfusion and severe anemia are independent risk factors for NEC in VLBW infants. Further study is needed to determine if severe anemia is an important effect modifier of the risk of RBC transfusion.

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Table 1. Baseline characteristics of RBC transfused and non-transfused infants

Baseline patient characteristics	RBC Transfused (n=291)	RBC Non-Transfused (n=248)
Birth weight (g) - median (Q1, Q3)	820 (689, 1046)	1207 (1035, 1350)
Gestational age (wk) - median (Q1, Q3)	26.0 (25.0, 28.0)	29.0 (28.0, 31.0)
Male gender	145/291 (49.8%)	118/248 (47.6%)
Race		
Black	179/291 (61.5%)	134/248 (54.0%)
White	89/291 (30.6%)	90/248 (36.3%)
Other	23/291 (7.9%)	24/248 (9.7%)
Mode of delivery		
Caesarean	217/291 (74.6%)	201/248 (81.0%)
Vaginal	74/291 (25.4%)	47/248 (19.0%)
5 minute Apgar ≤ 5	41/291 (14.1%)	12/248 (4.8%)
SNAP score - median (Q1, Q3)	13.0 (9.0, 16.0)	8.0 (4.0, 12.0)

Data are presented as n (%) unless indicated otherwise.

Abbreviations: NEC, necrotizing enterocolitis; Q1, 25th percentile; Q3, 75 percentile; SNAP, Score for Neonatal Acute Physiology; Hgb, hemoglobin.

Table 2. Baseline characteristics of infants with and without NEC

Baseline patient characteristics	NEC (n=40)	non-NEC (n=499)
Birth weight (g) - median (Q1, Q3)	778 (617, 995)	1038 (800, 1235)
Gestational age (wk) - median (Q1, Q3)	26.5 (25.0, 28.0)	28.0 (26.0, 30.0)
Male gender	19/40 (47.5%)	244/499 (48.9%)
Race		
Black	28/40 (70.0%)	285/499 (57.1%)
White	10/40 (25.0%)	169/499 (33.9%)
Other	2/40 (5.0%)	45/499 (9.0%)
Mode of delivery		
Caesarean	30/40 (75.0%)	388/499 (77.8%)
Vaginal	10/40 (25.0%)	111/499 (22.2%)
5 minute Apgar ≤ 5	7/40 (17.5%)	46/499 (9.2%)
SNAP score - median (Q1, Q3)	11.0 (7.0, 15.5)	11.0 (7.0, 14.0)

Data are presented as n (%) unless indicated otherwise.

Abbreviations: NEC, necrotizing enterocolitis; Q1, 25th percentile; Q3, 75 percentile; SNAP, Score for Neonatal Acute Physiology; Hgb, hemoglobin.

Table 3. Clinical characteristics of RBC transfused and non-transfused infants

Clinical Characteristics	RBC Transfused (n=291)	RBC Non-Transfused (n=248)
Number of RBC transfusions - median (Q1, Q3)	3.0 (2.0, 7.0)	-
Number of donor exposures - median (Q1, Q3)	2.0 (1.0, 4.0)	-
Anemia (nadir Hgb ≤ 8g/dL) during hospitalization	93/291 (32.0%)	5/248 (2.0%)
Age at first Hgb ≤ 8g/dL – median d (Q1, Q3)	28.0 (18.0, 40.0)	39.0 (33.0, 42.0)
Death	29/291 (10.0%)	1/248 (0.4%)
Patent ductus arteriosus	109/291 (37.5%)	29/248 (11.7%)
Intraventricular hemorrhage (≥ grade II)	60/291 (20.6%)	15/248 (6.0%)
Positive blood culture	56/291 (19.2%)	3/248 (1.2%)
Antibiotics use in 1st month (d) - median (Q1, Q3)	10.0 (4.0, 17.0)	3.0 (1.0, 4.0)
Ever fed breast milk	246/291 (84.5%)	221/248 (89.1%)
Age at first feed – median DOL (Q1,Q3)	3.0 (2.0, 6.0)	2.0 (1.0, 3.0)

Data are presented as n (%) unless indicated otherwise.

Only includes transfusion, outcome and medication data prior to NEC for NEC patients.

Abbreviations: NEC, necrotizing enterocolitis; RBC, red blood cell; Q1, 25th percentile; Q3, 75 percentile; Hgb, hemoglobin; DOL, day of life.

Table 4. Clinical characteristics of infants with and without NEC

Clinical Characteristics	NEC (n=40)	non-NEC (n=499)
Ever given RBC transfusion	33/40 (82.5%)	258/499 (51.7%)
Number of RBC transfusions - median (Q1, Q3)	3.0 (1.0, 5.0)	1.0 (0.0, 3.0)
Number of donor exposures - median (Q1, Q3)	1.5 (1.0, 3.0)	1.0 (0.0, 2.0)
Anemia (nadir Hgb ≤ 8g/dL) during hospitalization	16/40 (40.0%)	88/499 (17.6%)
Age at first Hgb ≤ 8g/dL – median d (Q1, Q3)	36.0 (28.0, 45.0)	27.5 (17.0, 40.5)
Death	12/40 (30.0%)	18/499 (3.6%)
Patent ductus arteriosus	13/40 (32.5%)	123/499 (24.6%)
Intraventricular hemorrhage (≥grade II)	6/40 (15.0%)	68/499 (13.6%)
Positive blood culture	7/40 (17.5%)	47/499 (9.4%)
Antibiotics use in 1st month (d) - median (Q1, Q3)	9.0 (4.0, 17.5)	4.0 (3.0, 10.0)
Ever fed breast milk	39/40 (97.5%)	428/499 (85.8%)
Age at first feed - median DOL (Q1,Q3)	3.0 (2.0, 5.0)	2.0 (2.0, 4.0)

Data are presented as n (%) unless indicated otherwise.

Only includes transfusion, outcome and medication data prior to NEC for NEC patients.

Abbreviations: NEC, necrotizing enterocolitis; RBC, red blood cell; Q1, 25th percentile; Q3, 75 percentile; Hgb, hemoglobin; DOL, day of life.

Table 5. Univariable analysis of risk factors for NEC

A. Fixed potential risk factors	**HR for NEC**	**95% CI**	**P**
Gestational Age (per 1 week increase)	0.84	0.73-0.96	0.01
Birth weight (per 100 gram increase)	0.77	0.67-0.87	<0.0001
Male gender	0.94	0.51-1.75	0.85
Race			
Black (reference)	1.00	-	0.27
White	0.62	0.30-1.29	
Other	0.45	0.11-1.80	
Center (reference: center 1)	2.42	1.26-4.65	0.008
5 min Apgar ≤ 5	1.86	0.82-4.20	0.14
SNAP score (per 1 point increase)	1.02	0.96-1.09	0.48
Any RBC transfusion	3.66	1.62-8.27	0.002
Episodes of severe anemia (per 1 occurrence)	1.76	1.29-2.40	0.0004
B. Time-varying potential risk factors	**HR for NEC**	**95% CI**	**P**
RBC transfusion in a given week	6.62	3.45-12.70	<0.0001
Severe anemia (Hgb ≤ 8g/dL) in a given week	10.34	5.03-21.25	<0.0001

Abbreviations: NEC, necrotizing enterocolitis; HR, hazard ratio; CI, confidence interval; d, day; RBC, red blood cell; Hgb, hemoglobin.

Table 6. Multivariable analysis of risk factors for NEC

Risk Factor	HR for NEC	95% CI	P
Birth weight (per 100 gram increase)	0.83	0.72-0.95	0.008
Center (reference: center 1)	2.22	1.12-4.41	0.02
Severe anemia (Hgb ≤ 8g/dL) in a given week[a]	5.55	2.44-12.64	<.0001
RBC transfusion in a given week[a]	**2.37**	**1.06-5.30**	**0.036**

Primary exposure of interest indicated in boldface. Data includes 539 infants, of whom 40 developed NEC.

Abbreviations: NEC, necrotizing enterocolitis; HR, hazard ratio; CI, confidence interval; Hgb, hemoglobin.

[a] Time-varying covariate evaluated in weekly intervals.

Table 7. Effect modification of the risk of RBC transfusion on NEC by severe anemia

Risk Factor	OR for NEC	95% CI	OR for NEC or Death	95% CI
Birth weight (per 100 gram increase)	0.78	0.66-0.92	0.70	0.60-0.81
Center (reference: center 1)	2.09	1.01-4.30	1.61	0.87-2.97
Receipt of RBC transfusion[a]				
Severe anemia (Hgb ≤ 8g/dL)	4.56	1.30-15.97	5.05	1.51-16.94
Absence of severe anemia	2.02	0.56-7.21	1.62	0.41-2.89

Includes 539 infants, of whom 40 developed NEC.

Abbreviations: OR, odds ration; NEC, necrotizing enterocolitis; CI, confidence interval; RBC, red blood cell; Hgb, hemoglobin.

[a] Interaction P = 0.008 for OR of NEC and P = 0.002 for OR of NEC or death.

Table 8. Univariable analysis of risk factors for NEC among RBC transfused patients

Potential Risk Factor	HR for NEC	95% CI	P
Birth weight (per 100 gram increase)	0.81	0.68-0.96	0.01
Center (reference: center 1)	1.84	0.87-3.89	0.11
Number of RBC transfusions in given week[a]	1.50	1.15-1.96	0.003
Severe anemia (Hgb ≤ 8g/dL) in a given week[a]	5.68	2.63-13.07	<0.0001
Age of blood in given week (per 5d increase)[a]	1.27	1.04-1.55	0.02
IST in given week (per 5d increase)[a]	**1.64**	**0.93-2.91**	**0.09**

Includes 291 RBC-transfused infants, of whom 33 developed NEC.

Abbreviations: NEC, necrotizing enterocolitis; HR, hazard ratio; CI, confidence interval; SNAP, score for neonatal acute physiology; RBC, red blood cell; Hgb, hemoglobin; IST, irradiation storage time.

[a] Time-varying covariate evaluated in weekly intervals.

Table 9. Multivariable analysis of risk factors for NEC among RBC transfused patients

Risk Factor	HR for NEC	95% CI	P
Birthweight (per 100 gram increase)	0.78	0.65-0.92	0.003
Severe anemia (Hgb ≤ 8g/dL) in a given week[a]	6.28	2.63-15.01	<.0001
Center (reference = center 1)	3.51	1.52-8.12	0.003
IST > 4 days[a]	**0.32**	**0.08-1.25**	**0.10**
Storage age > 7 days[a]	1.57	0.07-3.78	0.31

Includes 291 RBC transfused infants, of whom 33 developed NEC.

Abbreviations: NEC, necrotizing enterocolitis; HR, hazard ratio; CI, confidence interval; SNAP, score for neonatal acute physiology; RBC, red blood cell; IST, irradiation storage time.

[a] Time-varying covariate evaluated in weekly intervals.

Table 10. Sensitivity analysis of risk factors for NEC among RBC transfused patients with *post-hoc* IST cut-point and removal of center

Risk Factor	HR for NEC	95% CI	P
Birth weight (per 100 gram increase)	0.81	0.68-0.96	0.01
Severe anemia (Hgb ≤ 8g/dL) in a given week[a]	5.47	2.35-12.72	<0.0001
IST > 1 day[a]	**2.29**	1.03-5.07	**0.042**
Storage age > 7 days[a]	0.83	0.93-2.91	0.832

Abbreviations: NEC, necrotizing enterocolitis; HR, hazard ratio; CI, confidence interval; Hgb, hemoglobin; IST, irradiation storage time.

[a] Time-varying covariate evaluated in weekly intervals.

Figure 1. Causal pathway depicting relationship between exposures of interest, potential confounders and outcome.

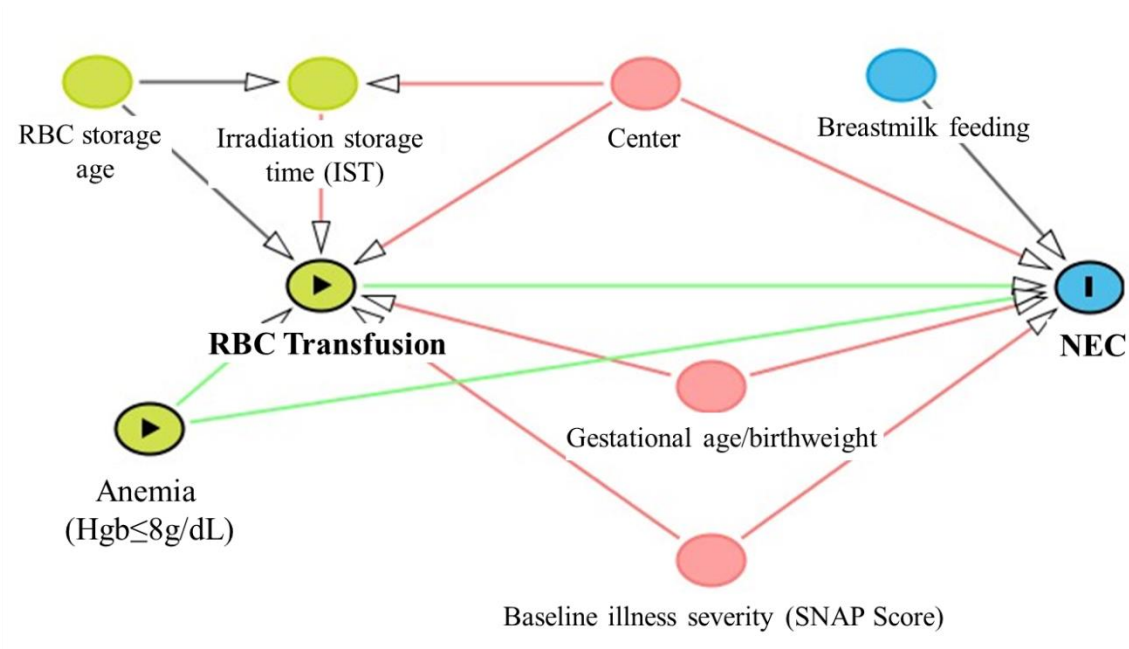

Figure legend: The green circles represent exposures of interest (with black triangles indicating the two primary exposures of interest). The pink circles indicate potential confounders and the primary outcome of interest is represented by a vertical line enclosed in a blue circle.

Figure 2. Study flow diagram

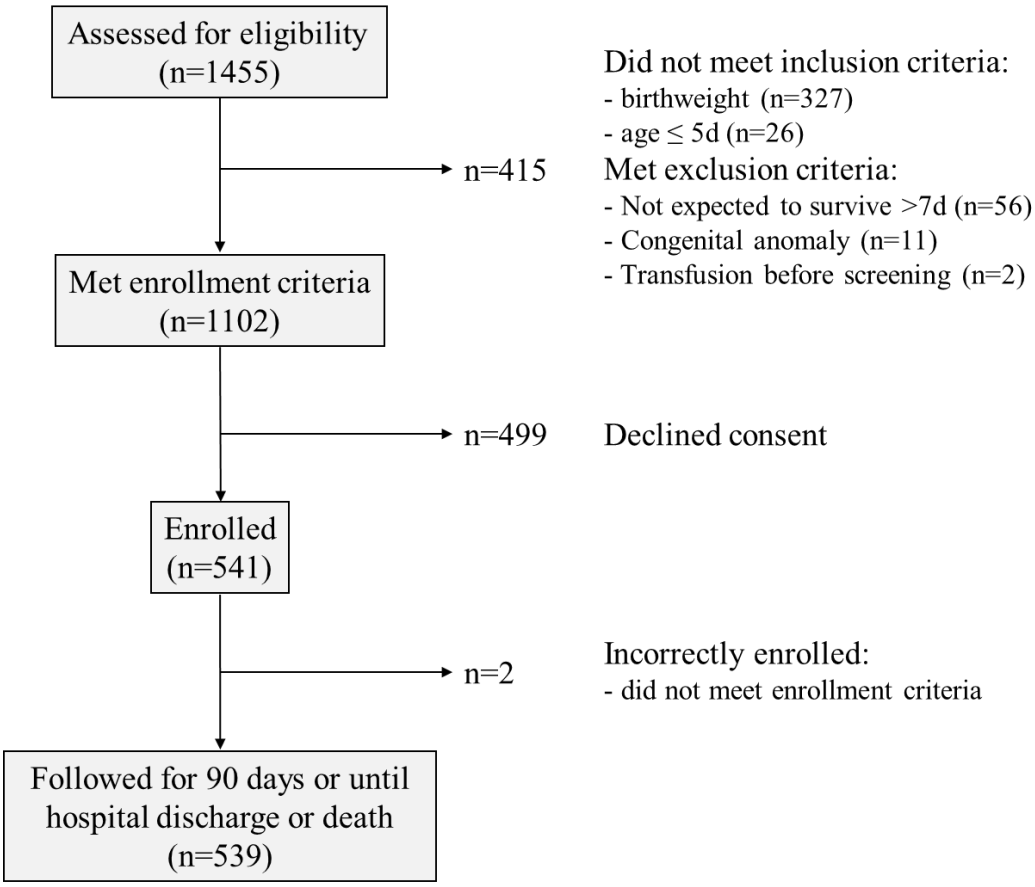

Figure 3. Cumulative incidence of NEC and death for all enrolled very low birth weight infants