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Complete List of Authors:	Elabiad, Mohamad; University of Tennessee Health Science Center, Department of Pediatrics, Division of Neonatology Harsono, Mimily; University of Tennessee Health Science Center, Department of Pediatrics, Division of Neonatology Talati, Ajay; University of Tennessee Health Science Center, Department of Pediatrics, Division of Neonatology Dhanireddy, Ramasubbareddy; University of Tennessee Health Science Center, Department of Pediatrics, Division of Neonatology
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Effect of Birth Weight on the Association between Necrotizing Enterocolitis and Red Blood Cell Transfusions in ≤1500 g Infants

Mohamad Tammam Elabiad, MD, MSc; Mimily Harsono, MD; Ajay J. Talati, MD; and Ramasubbareddy Dhanireddy, MD

Affiliations: Department of Pediatrics, Division of Neonatology, University of Tennessee Health Science Center, Memphis, Tennessee

Address correspondence to: Mohamad Elabiad, College of Medicine, Department of Pediatrics, Division of Neonatology, 853 Jefferson Avenue, 2nd floor, Memphis, TN 38163, Tel: 901-448-4751, Fax: 901-4481691, Email: melabiad@uthsc.edu

Short title: Blood Transfusions and NEC: A retrospective cohort

Abbreviations: AA- African American, VLBW - very low birth weight, NEC - necrotizing enterocolitis, PDA - patent ductus arteriosus, PMA- post menstrual age, PRBC - packed red blood cells, UAC - umbilical arterial catheter

Keywords: necrotizing enterocolitis, prematurity, packed red blood cells, transfusions

Word count: 2515

Abstract

Context
Reports evaluating a possible association between necrotizing enterocolitis (NEC) and blood transfusion have been predominantly case-control studies. As possible associations of disease with any variable on which cases and controls have been matched cannot be explored, a cohort study would offer a solution to this problem.

Objective
Our objective was to evaluate the association between exposure to a packed red blood cell (PRBC) transfusion and development of NEC in a cohort where biases of matching are omitted.

Design
In a retrospective cohort, exposed infants were defined as those who received a transfusion and did not develop NEC or developed NEC within 48 h of the transfusion. All others were considered unexposed.

Setting
A single regional perinatal center in Memphis, Tennessee, USA

Patients
Three thousand sixty infants ≤1500g birth weights (BW) were included.

Outcome measures
The relative risk of developing NEC after exposure to a PRBC transfusion was measured.

Results
3060 infants were identified. 174 infants (5.7%) developed NEC; 116 of 174 (67%) were exposed. NEC infants had a significantly lower BW (924g vs. 1042g) and required a longer stay on a ventilator (7 vs. 2 days). Divided into groups, infants with a BW ≤750g, 751-1000g, 1001-1250g, and 1251-1500g (n=52, 51, 46, 25 respectively) had a relative risk of 0.14, 0.46, 1.83, and 1.78 (P<0.01, 0.02, 0.07, 0.17), respectively, to develop NEC after exposure. Infants with longest ventilator days were also significantly less likely to develop NEC after exposure, relative risk = 0.11, (P<0.01).

Conclusion
Exposure to transfusions was less likely associated with NEC in ≤1000g infants and remained a risk factor in 1001-1500 infants. Birth weight has to be factored in any study evaluating the association between PRBC transfusions and NEC.

Article Summary

Article focus

- Reports evaluating a possible association between necrotizing enterocolitis (NEC) and blood transfusion have been predominantly case-control studies.
- Our objective was to evaluate the association between exposure to a packed red blood cell (PRBC) transfusion and development of NEC in a cohort where biases of matching are omitted.

Key messages

- Exposure to transfusions was less likely associated with NEC in ≤1000g infants and remained a risk factor in 1001-1500 infants.
- Birth weight has to be factored in any study evaluating the association between PRBC transfusions and NEC.

Strengths and limitations of this study

- A large single center cohort over a 16 year period and the new findings of different associations between transfusions and NEC based on birth weight groups were among the main strengths.
- Retrospective nature and unavailability of hematocrit data were among the main limitations.

Funding This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent Waived.

Ethics approval The study protocol was approved by the Institutional Review Board at the University Of Tennessee Health Science Center.

Data sharing statement No additional data are available.

Introduction

Recent evidence supports an increased risk for the development of necrotizing enterocolitis (NEC) after a transfusion with packed red blood cells (PRBC)[1-3]. Transfusion related acute gut injury or TRAGI has been proposed [4] as one of many terms to describe this process. Difficulties in evaluating any association between NEC and blood transfusions stem from two main facts. The first pertains to the low incidence of NEC; the second relates to NEC's unclear multifactorial pathogenesis [5]. These are probably the reasons all previous reports studying this association could only be carried out as retrospective case-control studies as it is more practical to find the NEC cases and their respective controls[1-4,6-8].

One major disadvantage of case-control studies concerns matching. The possible association of disease with any variable on which cases and controls have been matched cannot be explored. For example, birth weight and gestational age have been a common denominator to find controls in case-control studies. Large cohort studies do not have this problem. Hence, our objective was to use an established database to study the association between exposure to a PRBC transfusion and development of NEC in all very low birth weight infants (VLBW) over an extended period of time without the need for matching.

Methods

This was a retrospective cohort study. Data were obtained for all infants discharged between January 1, 1996, and December 31, 2011, at the Neonatal Intensive Care Unit (NICU) at The Regional Medical Center, Memphis, Tennessee. The study protocol was approved by the Institutional Review Board at the University Of Tennessee Health Science Center.

Patients

All infants with a birth weight of ≤ 1500 g were included for further evaluation. To decrease effects from perinatal factors, infants who died or were transferred out within the first 7 days of life were excluded. Infants who died after 7 days of life were included.

Transfusion practices

There were no written guidelines on transfusion thresholds during the study period. Transfusion was a clinical decision by the care team. The default PRBC unit used was O negative, irradiated, leukocyte depleted, and cytomegalovirus negative. The preservative used was citrate-phosphate-dextrose-adenine-1. Patients were not assigned to receive blood from a dedicated unit. We used one or two "active" units that served as the source of all transfusions for every recipient in the NICU, until this unit is depleted. A unit was typically received within 3-4 days from collection and was exhausted in a week. Feedings were maintained and advanced the same way in infants receiving transfusions as others. Erythropoietin was not used.

NEC

Only infants meeting Bell's stage II criteria [9] or above for the diagnosis of NEC were included. To reduce potential bias from infants receiving transfusions at a time point when NEC incidence was rare, we defined a period beyond which infants' data was not included in the final analysis. The upper limit of this period was set at 2 standard deviations from the average timing of all NEC cases based on post menstrual age (PMA). This would avoid dampening possible positive association between transfusions and NEC.

Definition of Exposure

Most of the previous reports have concluded that transfusion-associated NEC is likely the result of transfusions given within the 48 h prior to development of NEC[3,7,8]. With this as a premise, infants were considered exposed if they developed NEC within 48 h following a transfusion. Infants that developed NEC and had received a transfusion within a period longer than 48 h were considered not exposed. Data from infants after they developed NEC were excluded. In all, there were six groups of patients (Table 1).

Table 1 Exposure and outcome infant groups

Exposure/ Outcome	Developed NEC	Did not develop NEC
Transfused	1. Transfused within 48 hours of NEC onset	4. Transfused in critical period
Not transfused	2. Never transfused 3. Transfused > 48 hours prior to NEC onset	5. Never transfused 6. Transfused after critical period

Data collection

For the purpose of our study, collected data included birth weight (BW), gestational age, gender, race, 5-min Apgar score, small for gestational age (SGA) status defined as birth weight below 10% for gestational age, pharmacologic treatment of a patent ductus arteriosus (PDA), ventilator days (high frequency plus conventional mechanical ventilation), NEC, umbilical arterial catheter (UAC) insertion days, and survival outcome.

Statistics

SAS 9.1.3 was used. A chi-square test was used to measure the degree of association between categorical variables. A relative risk with the 95% confidence interval was calculated for each test. A Wilcoxon rank-sum test was used to compare continuous variables between the NEC and no-NEC groups. All tests were two-sided; $P<0.05$ was considered statistically significant. Data

were presented as mean ± standard deviation and as median, interquartile range (IQR). A simple logistic regression model was initially run between NEC as the outcome and all independent variables of interest including exposure to blood transfusions. When $P<0.1$, interactions and collinearity among variables were evaluated before progressing with the model. If collinearity was present, the independent variable was divided in groups and quartiles. The association between transfusion exposure and NEC was then analyzed in each of these groups. Tables 3-6 have been adjusted for gender, race, and SGA status. Extended Mantel-Haenszel test was used to evaluate linear trend using OpenEpi version 3.0.1. Pharmacologic treatment of a PDA and 5-min Apgar score were excluded due to covariance with birth weight. The final number of patients in these tables was slightly less than 3060 as some non NEC cases were lost due to incomplete data in the multivariable analyses. To address potential variations in secular rates of NEC over time, the sixteen-year study duration was divided in two 8 year periods. The time NEC occurred in either period was entered as another variable in the analysis.

Results

A total of 3462 VLBW infants were admitted in the study period; 397 infants, including 3 with NEC, were excluded as they died or were transferred out by day 7 of life. The mean PMA for developing NEC was 30.4 ± 2.6 weeks; median was 30.4 (3.4) (Figure 1). Using this mean, the upper limit of the NEC period was thus defined at 35.6 weeks. Five infants were also excluded as they developed NEC after this period. Three thousand sixty infants were available for analysis. The median number of transfusions received by each infant was 2 (6). The incidence of NEC remained constant over this time with a mean 6.0 ±1.9% (Figure 2).

One hundred seventy four infants (5.7%) developed NEC; 116 of 174 (67%) infants had NEC within 48 h after exposure to a blood transfusion. In univariate analysis, NEC was associated with birth weight, gestational age, UAC insertion days, number of days on a ventilator, and number of transfusions (Table 2).

Table 2 Univariate analysis with NEC as outcome

Risk factors	NEC n=174	No NEC n=2886	RR (95% CI)	*P* value
Apgar score 5 min	7 (2)	7 (2)	0.997 (0.913-1.088)	0.7
Gender, male as percent	55.8%	48.3%	1.32 (0.99-1.77)	0.057
Race, AA as percent	85.1%	80.3%	1.37(0.91-2.06)	0.12
Birth weight (g)	924(429)	1042(492)	0.9998 (0.9988-0.9998)	<0.01
Gestational age (wks)	27(4)	28(5)	0.89 (0.85- 0.94)	<0.01
Small for gestational age	35.1%	37.4%	0.90(0.67-1.23)	0.54
Umbilical artery catheter days	4(10)	2(8)	1.031 (1.009-1.054)	<0.01
Days on Ventilator	7(16)	2(13)	1.012(1.004- 1.020)	<0.01
Treatment for PDA	25.30%	24.60%	1.033 (0.74-1.44)	0.85
Study period 2004-2011 (n)	87	1473	1.021 (0.88-1.19)	0.79
Number of transfusions	6(8)	5(8)	1.060 (1.039- 1.080)	0.017
Exposure to blood transfusion, %	66.7%	59.8%	1.32 (0.97-1.80)	0.073

AA: African American, NEC: necrotizing enterocolitis, PDA: patent ductus arteriosus

Evaluating for collinearity, gestational age and number of transfusions were dropped as birth weight and exposure, respectively, covaried with and represented both variables. Birth weight, UAC insertion days, ventilator days, and exposure to blood transfusions were highly collinear with each other. The correlation coefficients between any two of these four variables ranged

from 0.45-0.64 (P<0.0001 for all coefficients). These coefficients were much stronger than each with NEC, where the correlation coefficients were lower, 0.052-0.077 (P<0.0001-0.006). These markers likely represented the same phenomenon, mainly the severity of illness. The transfusion-NEC association was thus evaluated in the context of each variable alone. Exposure to blood transfusions was protective in infants with a birth weight ≤ 1000g, those who stayed longer on a ventilator, and those who required a longer UAC insertion period. However, exposure to blood transfusions carried a risk for developing NEC in infants with less severity of illness markers. These infants with a birth weight of 1001-1500g and who did not need mechanical ventilation or did not require UAC insertion had a higher risk of developing NEC after a transfusion exposure (Tables 3-5).

Table 3 Multivariate risk of developing NEC by weight subgroup

Weight Group (g)	N	Cases(exposed)	RR[a,b]	95% CI	*P*	NEC day	NEC PMA
≤750	662	52 (39)	0.14	0.07-0.30	<0.01	20(18)	28(4)
751-1000	747	51 (37)	0.46	0.24-0.89	0.021	20(8)	30(3)
1001-1250	810	46 (31)	1.83	0.95-3.5	0.071	16(13)	31(2)
1251-1500	828	25 (9)	1.78	0.77-4.19	0.17	16(9)	32(2)

NEC: necrotizing enterocolitis, PMA: post menstrual age
[a] Adjusted by gender, race, and SGA
[b]Extended Mantel-Haenszel for linear trend, p<0.01

Table 4 Multivariate risk of developing NEC by ventilator day periods

Vent. days	N	Cases (exposed)	RR[a,b]	95% CI	*P*	Mean BW
0	839	8 (3)	3.5	0.82-15.15	0.09	1262±191
1-2	797	31(17)	1.04	0.50-2.14	0.92	1107±216
3-13	650	72 (49)	0.29	0.7-0.51	<0.01	947±255
>13	761	63 (47)	0.11	0.06-0.23	<0.01	739±196

BW: birth weight
[a] Adjusted by gender, race, and SGA
[b]Extended Mantel-Haenszel for linear trend, p<0.01

Table 5 Multivariate risk of developing NEC by UAC insertion day periods

UAC days	N	Cases(exposed)	RR[a,b]	95% CI	*P*	Mean BW
0	1352	52 (28)	2.11	1.2-3.69	<0.01	1173±253
1-2	184	12 (8)	1.44	0.41-5.12	0.31	1052±259
3-7	707	53 (37)	0.81	0.44-1..49	0.49	989±243
>7	804	57 (43)	0.2	0.1-0.39	<0.01	796±234

BW: birth weight, **UAC**: umbilical arterial catheter
[a] Adjusted by gender, race, and SGA
[b]Extended Mantel-Haenszel for linear trend, p<0.01

The association of exposure and the development of NEC after 28 days of life was also examined, (Table6). Smaller infants were again less likely to develop NEC after exposure to a transfusion.

Table 6 Multivariate risk of (late) onset NEC after day 28 by BW group

Weight Groups (g)	N	Cases(exposed)	RR[a,b]	95% CI	*P*
≤750	629	19 (10)	0.057	0.021-0.15	<0.01
751-1000	711	15 (8)	0.17	0.058-0.49	<0.01
1001-1250	771	7 (6)	4.32	0.49-37	0.19
1251-1500	810	1 (0)	-	-	-

[a] Adjusted by gender, race, and SGA
[b]Extended Mantel-Haenszel for linear trend excluding last weight group, p=0.003

In the 174 infants who developed NEC, exposed infants (n=116) were of a lower postmenstrual age at the time of NEC, median=30.2(3.2) compared to 31.1(3.1) weeks in the non exposed, p=0.02. Evaluating for differences between two birth weight groups at a 1000g cutoff, infants with a birth weight ≤1000g had significantly more UAC insertion days, median 7(9) vs. 0(4) d, more ventilation days, median 15(22) vs. 3(5), and more transfusions 8(12) vs. 3(4), p<0.01 for all, when compared to infants with a birth weight >1000 g.

Discussion

This is the first study showing that the association between NEC and transfusion is not similar among different birth weight groups. We expected to see a much higher association in the group that received transfusions the most: the extremely low birth weight infants with a BW ≤1000 g. Our findings were completely the reverse. Exposure to transfusions was significantly less likely to be associated with NEC in ≤1000 infants and more likely associated with NEC in larger infants with a BW of 1001-1500 g.

Because of their critical condition and finite blood volume, VLBW infants tend to have transfusion thresholds that are inversely proportional to their birth weight. In a systemic review of prospective randomized studies evaluating hematocrit thresholds with morbidity outcomes, infants who were transfused more and maintained a higher hematocrit were less likely to develop NEC [10]. Retrospective studies evaluating the association between anaemia, exposure, and NEC also had similar conclusions[4,6,7]. Infants with a lower hematocrit were more likely to develop NEC following a transfusion. In our practice, VLBW infants were initially transfused when their hematocrit dropped below a predefined level in their first weeks of life. This level varied slightly between care teams. With age, these predefined levels changed from a preset hematocrit level to presence of clinical signs combined with oxygen demands. Our practice has remained in parallel with the universal approach [11]. In a recent survey, 1018 neonatologists from 11 countries responded with minimal variability that in the first week of life higher hematocrit thresholds were targeted irrespective of the degree of respiratory support. At 4 weeks of life, however, decisions were more variable, with transfusion hematocrit thresholds being affected by degree of

oxygen requirements [12]. By that time, variable levels of gut injury may already have been ongoing potentiating the development of NEC. Clinical signs like tachycardia and increased frequency of apnea could well represent preclinical NEC and not the severity of anaemia.

In some studies, infants with exposure associated NEC were reported as older by an average of 2-3 weeks compared to non exposed NEC infants; they were also "stable" with no clinical signs of illness [4,7,8]. Being older may reflect a longer period of anaemia and lower hematocrit level. In our cohort, exposed infants with NEC were older by only 5 days as compared to non exposed NEC infants, much in contrast to the 2- to 3-week period previously mentioned. We also did not observe different results when only infants with late onset NEC were evaluated.

Severity of illness has been proposed as another factor affecting the association between exposure to PRBC and the development of NEC. Exposed infants were more likely to be ventilated [2,4,7,8], have a PDA [4,7,8], and have a lower birth weight [1,2,7,8]. As all variables exhibit some interaction, interpretation of these results becomes difficult. We initially arrived at similar conclusions. In unadjusted analysis, longer ventilator days and longer UAC placement days increased the likelihood for developing NEC. A prolonged UAC period likely reflected poor intravascular access, frequent blood draws, and/or hemodynamic instability. When both variables were stratified into four periods based on quartiles, infants who had longer periods on a ventilator and UAC placement were less likely to develop NEC. The linear change in the RR was very significant. These infants were also in the lowest birth weight group at ≤1000g. Thus, and in

contrast to current reports, severity of illness, represented here by infants with lowest birth weights and longest ventilator days, was not a risk for exposure associated NEC.

Recently, a meta-analysis of reported case studies on the association of NEC and blood transfusion concluded that recent exposure to transfusion was associated with NEC [13]. Case studies can produce an exaggerated effect size as compared to cohort studies [14]. Kirpalani et al[15] has recently reviewed the effects of study design on the outcome of the association between transfusions and NEC. Randomized control trials showed an opposite effect to observational trials; higher transfusions were associated with a lower incidence of NEC. Most observational trials had the problem of confounding or small sample size. Since there wasn't any matching done in our study, we were able to evaluate birth weight as an independent variable and show its significance.

There were several limitations to our study. Bias due to its retrospective nature limited our ability to differentiate between the overlapping preclinical signs of NEC and those of anaemia. Understandably, this problem cannot be resolved in a retrospective study. Information about hematocrit levels was not usable as central, peripheral, and blood gas hematocrit were used interchangeably to drive transfusion decisions. A change in the hospital laboratory data base mid study period made hematocrit data for the first half of the period inaccessible. These factors limited our ability to test for anaemia as a plausible explanation. Information about the use of antenatal steroids was also not available. Treatment with antenatal corticosteroids has been shown to be associated with an overall reduction in necrotizing enterocolitis[16]. Lower weight infants may have received antenatal steroids more frequently than larger infants thereby lowering

the risk for development of NEC. Another limitation pertains to the days to total feeds and formula vs. breast milk use. Historically in our unit, breast milk use was less than 20% and only increased above 60% in the last 2 years of the study period. There are also concerns about the lack of increased superior mesenteric artery blood flow in response to a feeding, following a blood transfusion [17], suggesting that feeds should be held after a transfusion. The fact that hundreds of infants received transfusions while feeding without an increased risk of NEC post transfusion makes this association unlikely.

A likely explanation of our findings would be that as an infant's birth weight increases, there is a threshold crossed that changes the polarity of the association between exposure and NEC. This is nicely demonstrated in the progressive increase in the relative risk value with each weight group. The threshold could be a change in clinical practice like using a lower transfusion limit. It could also be some other factor related to gut maturity, to feeding practice, or altogether still to be discovered.

In summary, our findings show that exposure to transfusions does not increase the risk of NEC. Specifically, after exposure to a PRBC transfusion, ≤1000g BW infants were less likely to develop NEC than were infants born 1001-1500g BW. These larger infants were likely to have lower transfusion thresholds as they were less ill and probably more anaemic. We speculate that anaemia could be the cause of their exposure associated NEC. Future studies evaluating this association should be prospective, should incorporate birth weight as a risk factor, and should incorporate newer modalities such as near-infrared spectroscopy to evaluate hypoxia at the intestinal level[18].

Acknowledgements

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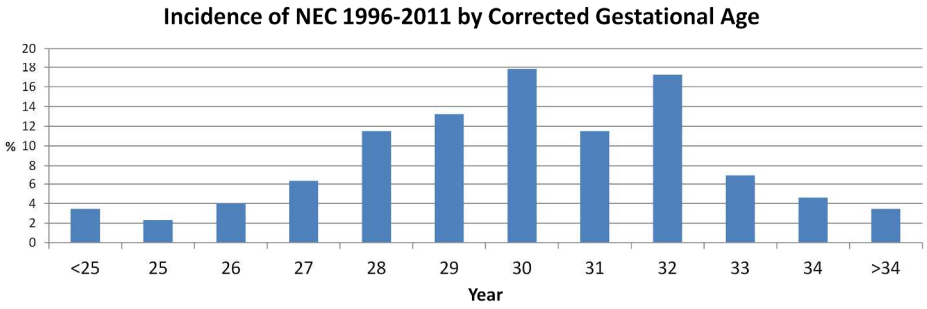
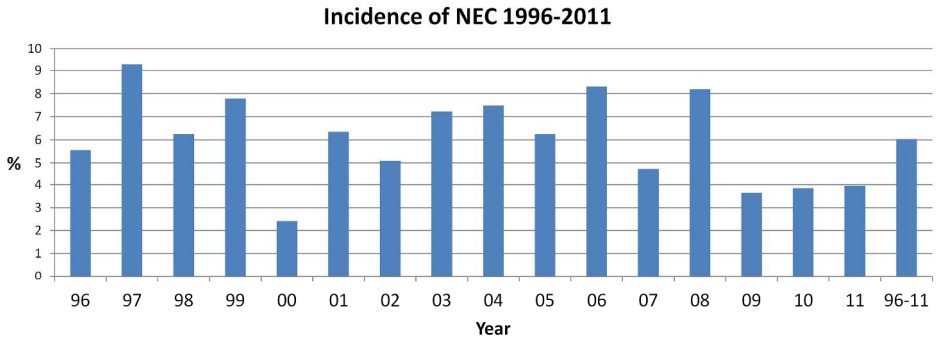
Figure legends

Figure 1 Incidence of NEC by post menstrual age

Figure 2 Incidence of NEC over the study period



Incidence of NEC by post menstrual age
254x190mm (300 x 300 DPI)



Incidence of NEC over the study period
254x190mm (300 x 300 DPI)

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	**4**
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	**4,5**
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	**4,5**
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	5
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	6
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6,7
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	N/A
Bias	9	Describe any efforts to address potential sources of bias	6, 7
Study size	10	Explain how the study size was arrived at	5-7
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6, 7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6, 7
		(*b*) Describe any methods used to examine subgroups and interactions	6, 7
		(*c*) Explain how missing data were addressed	7
		(*d*) If applicable, explain how loss to follow-up was addressed	N/A
		(*e*) Describe any sensitivity analyses	N/A

Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	7
		(b) Give reasons for non-participation at each stage	7
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8
		(b) Indicate number of participants with missing data for each variable of interest	8-10
		(c) Summarise follow-up time (eg, average and total amount)	N/A
Outcome data	15*	Report numbers of outcome events or summary measures over time	8-10
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	8-10
		(*b*) Report category boundaries when continuous variables were categorized	8-10
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	9-10
Discussion			
Key results	18	Summarise key results with reference to study objectives	14
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12-13
Generalisability	21	Discuss the generalisability (external validity) of the study results	12-13
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	N/A

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

Effect of Birth Weight on the Association between Necrotizing Enterocolitis and Red Blood Cell Transfusions in ≤1500 g Infants

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Effect of Birth Weight on the Association between Necrotizing Enterocolitis and Red Blood Cell Transfusions in ≤1500 g Infants

Mohamad Tammam Elabiad, MD, MSc; Mimily Harsono, MD; Ajay J. Talati, MD; and Ramasubbareddy Dhanireddy, MD

Affiliations: Department of Pediatrics, Division of Neonatology, University of Tennessee Health Science Center, Memphis, Tennessee

Address correspondence to: Mohamad Elabiad, College of Medicine, Department of Pediatrics, Division of Neonatology, 853 Jefferson Avenue, 2nd floor, Memphis, TN 38163, Tel: 901-448-4751, Fax: 901-4481691, Email: melabiad@uthsc.edu

Short title: Blood Transfusions and NEC: A retrospective cohort

Abbreviations: AA- African American, VLBW - very low birth weight, NEC - necrotizing enterocolitis, PDA - patent ductus arteriosus, PMA- post menstrual age, PRBC - packed red blood cells, UAC - umbilical arterial catheter

Keywords: necrotizing enterocolitis, prematurity, packed red blood cells, transfusions

Word count: 2664

Abstract

Context

Reports evaluating a possible association between necrotizing enterocolitis (NEC) and blood transfusion have been predominantly case-control studies. As possible associations of disease with any variable on which cases and controls have been matched cannot be explored, a cohort study would offer a solution to this problem.

Objective

Our objective was to evaluate the association between exposure to a packed red blood cell (PRBC) transfusion and development of NEC in a cohort where biases of matching are omitted.

Design

In a retrospective cohort, exposed infants were defined as those who received a transfusion and did not develop NEC or developed NEC within 48 h of the transfusion. All others were considered unexposed.

Setting

A single regional perinatal center in Memphis, Tennessee, USA

Patients

Three thousand sixty infants ≤1500g birth weights (BW) were included.

Outcome measures

The relative risk of developing NEC after exposure to a PRBC transfusion was measured.

Results

3060 infants were identified. 174 infants (5.7%) developed NEC; 116 of 174 (67%) were exposed. NEC infants had a significantly lower BW (924g vs. 1042g) and required a longer stay on a ventilator (7 vs. 2 days). Divided into groups, infants with a BW ≤750g, 751-1000g, 1001-1250g, and 1251-1500g (n=52, 51, 46, 25 respectively) had a relative risk of 0.14, 0.46, 1.83, and 1.78 (P<0.01, 0.02, 0.07, 0.17), respectively, to develop NEC after exposure. Infants with longest ventilator days were also significantly less likely to develop NEC after exposure, relative risk = 0.11, (P<0.01).

Conclusion

Exposure to transfusions was less likely associated with NEC in ≤1000g infants and remained a risk factor in 1001-1500 infants. Birth weight has to be factored in any study evaluating the association between PRBC transfusions and NEC.

Article Summary

Article focus

- Reports evaluating a possible association between necrotizing enterocolitis (NEC) and blood transfusion have been predominantly case-control studies.
- Our objective was to evaluate the association between exposure to a packed red blood cell (PRBC) transfusion and development of NEC in a cohort where biases of matching are omitted.

Key messages

- Exposure to transfusions was less likely associated with NEC in ≤1000g infants and remained a risk factor in 1001-1500 infants.
- Birth weight has to be factored in any study evaluating the association between PRBC transfusions and NEC.

Strengths and limitations of this study

- A large single center cohort over a 16 year period and the new findings of different associations between transfusions and NEC based on birth weight groups were among the main strengths.
- Retrospective nature and unavailability of hematocrit data were among the main limitations.

Funding This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent Waived.

Ethics approval The study protocol was approved by the Institutional Review Board at the University Of Tennessee Health Science Center.

Data sharing statement No additional data are available.

Introduction

Recent evidence supports an increased risk for the development of necrotizing enterocolitis (NEC) after a transfusion with packed red blood cells (PRBC)[1-3]. Transfusion related acute gut injury or TRAGI has been proposed [4] as one of many terms to describe this process. Difficulties in evaluating any association between NEC and blood transfusions stem from two main facts. The first pertains to the low incidence of NEC; the second relates to NEC's unclear multifactorial pathogenesis [5]. These are probably the reasons all previous reports studying this association could only be carried out as retrospective case-control studies as it is more practical to find the NEC cases and their respective controls[1-4,6-8].

One major disadvantage of case-control studies concerns matching. The possible association of disease with any variable on which cases and controls have been matched cannot be explored. For example, birth weight and gestational age have been a common denominator to find controls in case-control studies. Large cohort studies do not have this problem. We thus hypothesized that including such variables like birth weight and gestational age in the analysis would lead to new information that may improve our understating of the relationship between blood transfusions and NEC. Hence, our objective was to use an established database to study the association between exposure to a PRBC transfusion and development of NEC in all very low birth weight infants (VLBW) over an extended period of time without the need for matching.

Methods

This was a retrospective cohort study. Data were obtained for all infants discharged between January 1, 1996, and December 31, 2011, at the Neonatal Intensive Care Unit (NICU) at The

Regional Medical Center, Memphis, Tennessee. The study protocol was approved by the Institutional Review Board at the University Of Tennessee Health Science Center.

Patients

All infants with a birth weight of ≤ 1500 g were included for further evaluation. To decrease effects from perinatal factors, infants who died or were transferred out within the first 7 days of life were excluded. Infants who died after 7 days of life were included.

Transfusion practices

There were no written guidelines on transfusion thresholds during the study period. Transfusion was a clinical decision by the care team. The default PRBC unit used was O negative, irradiated, leukocyte depleted, and cytomegalovirus negative. The preservative used was citrate-phosphate-dextrose-adenine-1. Patients were not assigned to receive blood from a dedicated unit. We used one or two "active" units that served as the source of all transfusions for every recipient in the NICU, until this unit is depleted. A unit was typically received within 3-4 days from collection and was exhausted in a week. Feedings were maintained and advanced the same way in infants receiving transfusions as others. Erythropoietin was not used.

NEC

Only infants meeting Bell's stage II criteria [9] or above for the diagnosis of NEC were included. To reduce potential bias from infants receiving transfusions at a time point when NEC incidence was rare, we defined a period beyond which infants' data was not included in the final analysis. The upper limit of this period was set at 2 standard deviations from the average timing of all

NEC cases based on post menstrual age (PMA). This would avoid dampening possible positive association between transfusions and NEC.

Definition of Exposure

Most of the previous reports have concluded that transfusion-associated NEC is likely the result of transfusions given within the 48 h prior to development of NEC[3,7,8]. With this as a premise, infants were considered exposed if they developed NEC within 48 h following a transfusion. Infants that developed NEC and had received a transfusion within a period longer than 48 h were considered not exposed. Data from infants after they developed NEC were excluded. In all, there were six groups of patients (Table 1).

Table 1 Exposure and outcome infant groups

Exposure/ Outcome	Developed NEC	Did not develop NEC
Transfused	1. Transfused within 48 hours of NEC onset	4. Transfused in critical period
Not transfused	2. Never transfused 3. Transfused > 48 hours prior to NEC onset	5. Never transfused 6. Transfused after critical period

Data collection

For the purpose of our study, collected data included birth weight (BW), gestational age, gender, race, 5-min Apgar score, small for gestational age (SGA) status defined as birth weight below 10% for gestational age, pharmacologic treatment of a patent ductus arteriosus (PDA), ventilator days (high frequency plus conventional mechanical ventilation), NEC, umbilical arterial catheter (UAC) insertion days, and survival outcome.

Statistics

SAS 9.1.3 was used. A chi-square test was used to measure the degree of association between categorical variables. A relative risk with the 95% confidence interval was calculated for each test. A Wilcoxon rank-sum test was used to compare continuous variables between the NEC and no-NEC groups. All tests were two-sided; $P<0.05$ was considered statistically significant. Data were presented as mean ± standard deviation and as median, interquartile range (IQR). A simple logistic regression model was initially run between NEC as the outcome and all independent variables of interest including exposure to blood transfusions. When $P<0.1$, interactions and collinearity among variables were evaluated before progressing with the model. If collinearity was present, the independent variable was divided in groups and quartiles. The association between transfusion exposure and NEC was then analyzed in each of these groups. Tables 4-7have been adjusted for gender, race, and SGA status. Extended Mantel-Haenszel test was used to evaluate linear trend using OpenEpi version 3.0.1. Pharmacologic treatment of a PDA and 5-min Apgar score were excluded due to covariance with birth weight. The final number of patients in these tables was slightly less than 3060 as some non NEC cases were lost due to incomplete data in the multivariable analyses. To address potential variations in secular rates of NEC over time, the sixteen-year study duration was divided in two 8 year periods. The time NEC occurred in either period was entered as another variable in the analysis.

Results

A total of 3462 VLBW infants were admitted in the study period; 397 infants, including 3 with NEC, were excluded as they died or were transferred out by day 7 of life. The mean PMA for developing NEC was 30.4 ± 2.6 weeks; median was 30.4 (3.4) (Figure 1). Using this mean, the

upper limit of the NEC period was thus defined at 35.6 weeks. Five infants were also excluded as they developed NEC after this period. Three thousand sixty infants were available for analysis. The median number of transfusions received by each infant was 2 (6). The incidence of NEC remained constant over this time with a mean 6.0 ±1.9% (Figure 2).

One hundred seventy four infants (5.7%) developed NEC; 116 of 174 (67%) infants had NEC within 48 h after exposure to a blood transfusion. In univariate analysis, NEC was associated with birth weight, gestational age, UAC insertion days, number of days on a ventilator, and number of transfusions (Table 2).

Table 2 Univariate analysis with NEC as outcome

Risk factors*	NEC n=174	No NEC n=2886	RR (95% CI)	*P* value
Apgar score 5 min	7 (2)	7 (2)	0.997 (0.913-1.088)	0.7
Gender, male as percent	55.8%	48.3%	1.32 (0.99-1.77)	0.057
Race, AA as percent	85.1%	80.3%	1.37(0.91-2.06)	0.12
Birth weight (g)	924(429)	1042(492)	0.9998 (0.9988-0.9998)	<0.01
Gestational age (wks)	27(4)	28(5)	0.89 (0.85- 0.94)	<0.01
Small for gestational age	35.1%	37.4%	0.90(0.67-1.23)	0.54
Umbilical artery catheter days	4(10)	2(8)	1.031 (1.009-1.054)	<0.01
Days on Ventilator	7(16)	2(13)	1.012(1.004- 1.020)	<0.01
Treatment for PDA	25.30%	24.60%	1.033 (0.74-1.44)	0.85
Study period 2004-2011 (n)	87	1473	1.021 (0.88-1.19)	0.79
Number of transfusions	6(8)	5(8)	1.060 (1.039- 1.080)	0.017
Exposure to blood transfusion, %	66.7%	59.8%	1.32 (0.97-1.80)	0.073

AA: African American, NEC: necrotizing enterocolitis, PDA: patent ductus arteriosus

***Presented as the percentage of the number of infants or as Median (Inter quartile range)**

Evaluating for collinearity, gestational age and number of transfusions were dropped as birth weight and exposure, respectively, covaried with and represented both variables. Birth weight, UAC insertion days, ventilator days, and exposure to blood transfusions were highly collinear with each other. The correlation coefficients between any two of these four variables ranged from 0.45-0.64 (P<0.0001 for all coefficients). These coefficients were much stronger than each with NEC, where the correlation coefficients were lower, 0.052-0.077 (P<0.0001-0.006). These markers likely represented the same phenomenon, mainly the severity of illness. The transfusion-NEC association was thus evaluated in the context of each variable alone. Birth weight was stratified into four clinical groups, table 3.

Table 3 Clinical characteristics by birth weight group

Risk factors*	≤ 750g	751-1000 g	1001-1250 g	>1250 g
Number of Infants	665	748	813	834
Apgar score 5 min	6(3)	7(2)	7(2)	8(2)
Gender, male as percent (%)	41.5	52.0	51.0	49.3
Race, AA as percent (%)	80.5	83.4	79.1	79.5
Birth weight (g)	626(137)	877(133)	1128(131)	1381(114)
Gestational age (wks)	25(2)	27(2)	29(3)	31(3)
Small for gestational age (%)	55.3	39.0	30.3	27.6
Umbilical artery catheter days	10(12)	5(9)	0(5)	0(0)
Days on Ventilator	21(28)	6(16)	1(4)	0(1)
Treatment for PDA (%)	50.5	37.7	13.2	3.6
Number of transfusions	12(9)	5(5)	2(3)	1(2)
Exposure to blood transfusion (%)	93.5	84.8	51.0	20.5
NEC (%)	7.7	6.8	5.7	3.0

AA: African American, NEC: necrotizing enterocolitis, PDA: patent ductus arteriosus

***Presented as the percentage of the number of infants or as Median (Inter quartile range)**

Exposure to blood transfusions was protective in infants with a birth weight ≤ 1000g, those who stayed longer on a ventilator, and those who required a longer UAC insertion period. However, exposure to blood transfusions carried a risk for developing NEC in infants with less severity of illness markers. These infants with a birth weight of 1001-1500g and who did not need mechanical ventilation or did not require UAC insertion had a higher risk of developing NEC after a transfusion exposure (Tables 34-6).

Table 4 Multivariate risk of developing NEC by weight subgroup

Weight Group (g)	N	Cases(exposed)	RR[a,b]	95% CI	*P*	NEC day	NEC PMA
≤750	662	52 (39)	0.14	0.07-0.30	<0.01	20(18)	28(4)
751-1000	747	51 (37)	0.46	0.24-0.89	0.021	20(8)	30(3)
1001-1250	810	46 (31)	1.83	0.95-3.5	0.071	16(13)	31(2)
1251-1500	828	25 (9)	1.78	0.77-4.19	0.17	16(9)	32(2)

NEC: necrotizing enterocolitis, PMA: post menstrual age
[a] Adjusted by gender, race, and SGA
[b]Extended Mantel-Haenszel for linear trend, p<0.01

Table 5 Multivariate risk of developing NEC by ventilator day periods

Vent. days	N	Cases (exposed)	RR[a,b]	95% CI	*P*	Mean BW
0	839	8 (3)	3.5	0.82-15.15	0.09	1262±191
1-2	797	31(17)	1.04	0.50-2.14	0.92	1107±216
3-13	650	72 (49)	0.29	0.7-0.51	<0.01	947±255
>13	761	63 (47)	0.11	0.06-0.23	<0.01	739±196

BW: birth weight
[a] Adjusted by gender, race, and SGA
[b]Extended Mantel-Haenszel for linear trend, p<0.01

Table6 Multivariate risk of developing NEC by UAC insertion day periods

UAC days	N	Cases(exposed)	RR[a,b]	95% CI	*P*	Mean BW
0	1352	52 (28)	2.11	1.2-3.69	<0.01	1173±253
1-2	184	12 (8)	1.44	0.41-5.12	0.31	1052±259
3-7	707	53 (37)	0.81	0.44-1..49	0.49	989±243
>7	804	57 (43)	0.2	0.1-0.39	<0.01	796±234

BW: birth weight, **UAC**: umbilical arterial catheter
[a] Adjusted by gender, race, and SGA
[b]Extended Mantel-Haenszel for linear trend, p<0.01

The association of exposure and the development of NEC after 28 days of life was also examined, (Table7). Smaller infants were again less likely to develop NEC after exposure to a transfusion.

Table 7 Multivariate risk of (late) onset NEC after day 28 by BW group

Weight Groups (g)	N	Cases(exposed)	RR[a,b]	95% CI	*P*
≤750	629	19 (10)	0.057	0.021-0.15	<0.01
751-1000	711	15 (8)	0.17	0.058-0.49	<0.01
1001-1250	771	7 (6)	4.32	0.49-37	0.19
1251-1500	810	1 (0)	-	-	-

[a] Adjusted by gender, race, and SGA
[b]Extended Mantel-Haenszel for linear trend excluding last weight group, p=0.003

In the 174 infants who developed NEC, exposed infants (n=116) were of a lower postmenstrual age at the time of NEC, median=30.2(3.2) compared to 31.1(3.1) weeks in the non exposed, p=0.02 (Figure 3). When stratified by birth weight, there was no statistically significant difference in the incidence of NEC by PMA between exposed and non exposed infants based on the four birth weight groups. Evaluating for differences between two birth weight groups at a 1000g cutoff, infants with a birth weight ≤1000g had significantly more UAC insertion days,

median 7(9) vs. 0(4) d, more ventilation days, median 15(22) vs. 3(5), and more transfusions 8(12) vs. 3(4), p<0.01 for all, when compared to infants with a birth weight >1000 g.

Discussion

This is the first study showing that the association between NEC and transfusion is not similar among different birth weight groups. We expected to see a much higher association in the group that received transfusions the most: the extremely low birth weight infants with a BW ≤1000 g. Our findings were completely the reverse. Exposure to transfusions was significantly less likely to be associated with NEC in ≤1000 infants and more likely associated with NEC in larger infants with a BW of 1001-1500 g.

Because of their critical condition and finite blood volume, VLBW infants tend to have transfusion thresholds that are inversely proportional to their birth weight. In a systemic review of prospective randomized studies evaluating hematocrit thresholds with morbidity outcomes, infants who were transfused more and maintained a higher hematocrit were less likely to develop NEC [10]. Retrospective studies evaluating the association between anaemia, exposure, and NEC also had similar conclusions[4,6,7]. In our practice, VLBW infants were initially transfused when their hematocrit dropped below a predefined level in their first weeks of life. With age, these predefined levels changed from a preset hematocrit level to presence of clinical signs combined with oxygen demands. Our practice has remained in parallel with the universal approach [11]. In a recent survey, 1018 neonatologists from 11 countries responded with minimal variability that in the first week of life higher hematocrit thresholds were targeted irrespective of the degree of respiratory support. At 4 weeks of life, however, decisions were more variable, with transfusion

hematocrit thresholds being affected by degree of oxygen requirements [12]. By that time, variable levels of gut injury may already have been ongoing potentiating the development of NEC. Clinical signs like tachycardia and increased frequency of apnea could well represent preclinical NEC and not the severity of anaemia. This consensus among neonatologists of accepting lower hematocrit levels for larger infants and our findings of an increased risk of NEC post exposure only in the larger two birth weight groups strongly suggests that the severity of anaemia may have a significant impact on the development of NEC post transfusion.

In some studies, infants with exposure associated NEC were reported as older by an average of 2-3 weeks compared to non exposed NEC infants; they were also "stable" with no clinical signs of illness [4,7,8]. Being older may reflect a longer period of anaemia and lower hematocrit level. In our cohort, exposed infants with NEC were older by only 5 days as compared to non exposed NEC infants, much in contrast to the 2- to 3-week period previously mentioned. We also did not observe different results when only infants with late onset NEC were evaluated.

Severity of illness has been proposed as another factor affecting the association between exposure to PRBC and the development of NEC. Exposed infants were more likely to be ventilated [2,4,7,8], have a PDA [4,7,8], and have a lower birth weight [1,2,7,8]. As all variables exhibit some interaction, interpretation of these results becomes difficult. We initially arrived at similar conclusions. In unadjusted analysis, longer ventilator days and longer UAC placement days increased the likelihood for developing NEC. A prolonged UAC period likely reflected poor intravascular access, frequent blood draws, and/or hemodynamic instability. When both variables

were stratified into four periods based on quartiles, infants who had longer periods on a ventilator and UAC placement were less likely to develop NEC. The linear change in the RR was very significant. These infants were also in the lowest birth weight group at ≤1000g. Thus, and in contrast to current reports, severity of illness, represented here by infants with lowest birth weights and longest ventilator days, was not a risk for exposure associated NEC.

Recently, a meta-analysis of reported case studies on the association of NEC and blood transfusion concluded that recent exposure to transfusion was associated with NEC [13]. Case studies can produce an exaggerated effect size as compared to cohort studies [14]. Kirpalani et al[15] has recently reviewed the effects of study design on the outcome of the association between transfusions and NEC. Randomized control trials showed an opposite effect to observational trials; higher transfusions were associated with a lower incidence of NEC. Most observational trials had the problem of confounding or small sample size. Since there wasn't any matching done in our study, we were able to evaluate birth weight as an independent variable and show its significance.

There were several limitations to our study. Bias due to its retrospective nature limited our ability to differentiate between the overlapping preclinical signs of NEC and those of anaemia. Information about hematocrit levels in the first half of the study period were inaccessible. This limited our ability to test for anaemia as a plausible explanation. Information about the use of antenatal steroids was also not available. Treatment with antenatal corticosteroids has been shown to be associated with an overall reduction in necrotizing enterocolitis[16]. Lower weight infants may have received antenatal steroids more frequently than larger infants thereby lowering

the risk for development of NEC. Another limitation pertains to the days to total feeds and formula vs. breast milk use. Historically in our unit, breast milk use was less than 20% and only increased above 60% in the last 2 years of the study period. There are also concerns about the lack of increased superior mesenteric artery blood flow in response to a feeding, following a blood transfusion [17], suggesting that feeds should be held after a transfusion. These results could be attributed to using older blood as it has been shown that by two weeks of shelve life, stored PRBC lose the ability to cause regional vasodilation and to increase oxygen uptake by the tissue when compared to fresh PRBC stored for 3-7 days[18,19]. In our practice, PRBC units are usually exhausted within 4-10 days post collection. Using fresh blood for transfusions may explain our study findings but with data not available, this would be a speculation.

A likely explanation of the study findings would be that as an infant's birth weight increases, there is a threshold crossed that changes the polarity of the association between exposure and NEC. This is nicely demonstrated in the progressive increase in the relative risk value with each weight group. The threshold could be a change in clinical practice like using a lower transfusion limit. It could also be some other factor related to gut maturity, to feeding practice, or altogether still to be discovered.

In summary, using cohort approach in the investigation eliminated the need for matching by birth weight and lead to new information about different levels of association between blood transfusions and NEC in different weight groups. Our findings show that exposure to transfusions does not increase the risk of NEC. Specifically, after exposure to a PRBC transfusion, ≤1000g BW infants were less likely to develop NEC than were infants born 1001-

1500g BW. These larger infants were likely to have lower transfusion thresholds as they were less ill and probably more anaemic. We speculate that anaemia could be the cause of their exposure associated NEC. Future studies evaluating this association should be prospective, should incorporate birth weight as a risk factor, and should incorporate newer modalities such as near-infrared spectroscopy to evaluate hypoxia at the intestinal level[20].

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Contributorship Statement

All authors have participated in the conception and design of the study and then in the analysis and interpretation of the data. They all contributed to the drafting of the article. They all gave final approval of the version submitted.

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Figure legends

Figure 1 Incidence of NEC by post menstrual age

Figure 2 Incidence of NEC over the study period

Figure 3 Effect of exposure on the incidence of NEC by post menstrual age

Effect of Birth Weight on the Association between Necrotizing Enterocolitis and Red Blood Cell Transfusions in ≤1500 g Infants

Mohamad Tammam Elabiad, MD, MSc; Mimily Harsono, MD; Ajay J. Talati, MD; and Ramasubbareddy Dhanireddy, MD

Affiliations: Department of Pediatrics, Division of Neonatology, University of Tennessee Health Science Center, Memphis, Tennessee

Address correspondence to: Mohamad Elabiad, College of Medicine, Department of Pediatrics, Division of Neonatology, 853 Jefferson Avenue, 2nd floor, Memphis, TN 38163, Tel: 901-448-4751, Fax: 901-4481691, Email: melabiad@uthsc.edu

Short title: Blood Transfusions and NEC: A retrospective cohort

Abbreviations: AA- African American, VLBW - very low birth weight, NEC - necrotizing enterocolitis, PDA - patent ductus arteriosus, PMA- post menstrual age, PRBC - packed red blood cells, UAC - umbilical arterial catheter

Keywords: necrotizing enterocolitis, prematurity, packed red blood cells, transfusions

Word count: ~~2515~~2664

Abstract

Context
Reports evaluating a possible association between necrotizing enterocolitis (NEC) and blood transfusion have been predominantly case-control studies. As possible associations of disease with any variable on which cases and controls have been matched cannot be explored, a cohort study would offer a solution to this problem.

Objective
Our objective was to evaluate the association between exposure to a packed red blood cell (PRBC) transfusion and development of NEC in a cohort where biases of matching are omitted.

Design
In a retrospective cohort, exposed infants were defined as those who received a transfusion and did not develop NEC or developed NEC within 48 h of the transfusion. All others were considered unexposed.

Setting
A single regional perinatal center in Memphis, Tennessee, USA

Patients
Three thousand sixty infants ≤1500g birth weights (BW) were included.

Outcome measures
The relative risk of developing NEC after exposure to a PRBC transfusion was measured.

Results
3060 infants were identified. 174 infants (5.7%) developed NEC; 116 of 174 (67%) were exposed. NEC infants had a significantly lower BW (924g vs. 1042g) and required a longer stay on a ventilator (7 vs. 2 days). Divided into groups, infants with a BW ≤750g, 751-1000g, 1001-1250g, and 1251-1500g (n=52, 51, 46, 25 respectively) had a relative risk of 0.14, 0.46, 1.83, and 1.78 (P<0.01, 0.02, 0.07, 0.17), respectively, to develop NEC after exposure. Infants with longest ventilator days were also significantly less likely to develop NEC after exposure, relative risk = 0.11, (P<0.01).

Conclusion
Exposure to transfusions was less likely associated with NEC in ≤1000g infants and remained a risk factor in 1001-1500 infants. Birth weight has to be factored in any study evaluating the association between PRBC transfusions and NEC.

Article Summary

Article focus

- Reports evaluating a possible association between necrotizing enterocolitis (NEC) and blood transfusion have been predominantly case-control studies.
- Our objective was to evaluate the association between exposure to a packed red blood cell (PRBC) transfusion and development of NEC in a cohort where biases of matching are omitted.

Key messages

- Exposure to transfusions was less likely associated with NEC in ≤1000g infants and remained a risk factor in 1001-1500 infants.
- Birth weight has to be factored in any study evaluating the association between PRBC transfusions and NEC.

Strengths and limitations of this study

- A large single center cohort over a 16 year period and the new findings of different associations between transfusions and NEC based on birth weight groups were among the main strengths.
- Retrospective nature and unavailability of hematocrit data were among the main limitations.

Funding This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent Waived.

Ethics approval The study protocol was approved by the Institutional Review Board at the University Of Tennessee Health Science Center.

Data sharing statement No additional data are available.

Introduction

Recent evidence supports an increased risk for the development of necrotizing enterocolitis (NEC) after a transfusion with packed red blood cells (PRBC)[1-3]. Transfusion related acute gut injury or TRAGI has been proposed [4] as one of many terms to describe this process. Difficulties in evaluating any association between NEC and blood transfusions stem from two main facts. The first pertains to the low incidence of NEC; the second relates to NEC's unclear multifactorial pathogenesis [5]. These are probably the reasons all previous reports studying this association could only be carried out as retrospective case-control studies as it is more practical to find the NEC cases and their respective controls[1-4,6-8].

One major disadvantage of case-control studies concerns matching. The possible association of disease with any variable on which cases and controls have been matched cannot be explored. For example, birth weight and gestational age have been a common denominator to find controls in case-control studies. Large cohort studies do not have this problem. We thus hypothesized that including such variables like birth weight and gestational age in the analysis would lead to new information that may improve our understating of the relationship between blood transfusions and NEC. Hence, our objective was to use an established database to study the association between exposure to a PRBC transfusion and development of NEC in all very low birth weight infants (VLBW) over an extended period of time without the need for matching.

Methods

This was a retrospective cohort study. Data were obtained for all infants discharged between January 1, 1996, and December 31, 2011, at the Neonatal Intensive Care Unit (NICU) at The

Regional Medical Center, Memphis, Tennessee. The study protocol was approved by the Institutional Review Board at the University Of Tennessee Health Science Center.

Patients

All infants with a birth weight of ≤ 1500 g were included for further evaluation. To decrease effects from perinatal factors, infants who died or were transferred out within the first 7 days of life were excluded. Infants who died after 7 days of life were included.

Transfusion practices

There were no written guidelines on transfusion thresholds during the study period. Transfusion was a clinical decision by the care team. The default PRBC unit used was O negative, irradiated, leukocyte depleted, and cytomegalovirus negative. The preservative used was citrate-phosphate-dextrose-adenine-1. Patients were not assigned to receive blood from a dedicated unit. We used one or two "active" units that served as the source of all transfusions for every recipient in the NICU, until this unit is depleted. A unit was typically received within 3-4 days from collection and was exhausted in a week. Feedings were maintained and advanced the same way in infants receiving transfusions as others. Erythropoietin was not used.

NEC

Only infants meeting Bell's stage II criteria [9] or above for the diagnosis of NEC were included. To reduce potential bias from infants receiving transfusions at a time point when NEC incidence was rare, we defined a period beyond which infants' data was not included in the final analysis. The upper limit of this period was set at 2 standard deviations from the average timing of all

NEC cases based on post menstrual age (PMA). This would avoid dampening possible positive association between transfusions and NEC.

Definition of Exposure

Most of the previous reports have concluded that transfusion-associated NEC is likely the result of transfusions given within the 48 h prior to development of NEC[3,7,8]. With this as a premise, infants were considered exposed if they developed NEC within 48 h following a transfusion. Infants that developed NEC and had received a transfusion within a period longer than 48 h were considered not exposed. Data from infants after they developed NEC were excluded. In all, there were six groups of patients (Table 1).

Table 1 Exposure and outcome infant groups

Exposure/ Outcome	Developed NEC	Did not develop NEC
Transfused	1. Transfused within 48 hours of NEC onset	4. Transfused in critical period
Not transfused	2. Never transfused 3. Transfused > 48 hours prior to NEC onset	5. Never transfused 6. Transfused after critical period

Data collection

For the purpose of our study, collected data included birth weight (BW), gestational age, gender, race, 5-min Apgar score, small for gestational age (SGA) status defined as birth weight below 10% for gestational age, pharmacologic treatment of a patent ductus arteriosus (PDA), ventilator days (high frequency plus conventional mechanical ventilation), NEC, umbilical arterial catheter (UAC) insertion days, and survival outcome.

Statistics

SAS 9.1.3 was used. A chi-square test was used to measure the degree of association between categorical variables. A relative risk with the 95% confidence interval was calculated for each test. A Wilcoxon rank-sum test was used to compare continuous variables between the NEC and no-NEC groups. All tests were two-sided; $P<0.05$ was considered statistically significant. Data were presented as mean ± standard deviation and as median, interquartile range (IQR). A simple logistic regression model was initially run between NEC as the outcome and all independent variables of interest including exposure to blood transfusions. When $P<0.1$, interactions and collinearity among variables were evaluated before progressing with the model. If collinearity was present, the independent variable was divided in groups and quartiles. The association between transfusion exposure and NEC was then analyzed in each of these groups. Tables ~~3~~4-~~6~~ 7have been adjusted for gender, race, and SGA status. Extended Mantel-Haenszel test was used to evaluate linear trend using OpenEpi version 3.0.1. Pharmacologic treatment of a PDA and 5-min Apgar score were excluded due to covariance with birth weight. The final number of patients in these tables was slightly less than 3060 as some non NEC cases were lost due to incomplete data in the multivariable analyses. To address potential variations in secular rates of NEC over time, the sixteen-year study duration was divided in two 8 year periods. The time NEC occurred in either period was entered as another variable in the analysis.

Results

A total of 3462 VLBW infants were admitted in the study period; 397 infants, including 3 with NEC, were excluded as they died or were transferred out by day 7 of life. The mean PMA for developing NEC was 30.4 ± 2.6 weeks; median was 30.4 (3.4) (Figure 1). Using this mean, the

upper limit of the NEC period was thus defined at 35.6 weeks. Five infants were also excluded as they developed NEC after this period. Three thousand sixty infants were available for analysis. The median number of transfusions received by each infant was 2 (6). The incidence of NEC remained constant over this time with a mean 6.0 ±1.9% (Figure 2).

One hundred seventy four infants (5.7%) developed NEC; 116 of 174 (67%) infants had NEC within 48 h after exposure to a blood transfusion. In univariate analysis, NEC was associated with birth weight, gestational age, UAC insertion days, number of days on a ventilator, and number of transfusions (Table 2).

Table 2 Univariate analysis with NEC as outcome

Risk factors*	NEC n=174	No NEC n=2886	RR (95% CI)	*P* value
Apgar score 5 min	7 (2)	7 (2)	0.997 (0.913-1.088)	0.7
Gender, male as percent	55.8%	48.3%	1.32 (0.99-1.77)	0.057
Race, AA as percent	85.1%	80.3%	1.37(0.91-2.06)	0.12
Birth weight (g)	924(429)	1042(492)	0.9998 (0.9988-0.9998)	<0.01
Gestational age (wks)	27(4)	28(5)	0.89 (0.85- 0.94)	<0.01
Small for gestational age	35.1%	37.4%	0.90(0.67-1.23)	0.54
Umbilical artery catheter days	4(10)	2(8)	1.031 (1.009-1.054)	<0.01
Days on Ventilator	7(16)	2(13)	1.012(1.004- 1.020)	<0.01
Treatment for PDA	25.30%	24.60%	1.033 (0.74-1.44)	0.85
Study period 2004-2011 (n)	87	1473	1.021 (0.88-1.19)	0.79
Number of transfusions	6(8)	5(8)	1.060 (1.039- 1.080)	0.017
Exposure to blood transfusion, %	66.7%	59.8%	1.32 (0.97-1.80)	0.073

AA: African American, NEC: necrotizing enterocolitis, PDA: patent ductus arteriosus

***Presented as the percentage of the number of infants or as Median (Inter quartile range)**

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Evaluating for collinearity, gestational age and number of transfusions were dropped as birth weight and exposure, respectively, covaried with and represented both variables. Birth weight, UAC insertion days, ventilator days, and exposure to blood transfusions were highly collinear with each other. The correlation coefficients between any two of these four variables ranged from 0.45-0.64 (P<0.0001 for all coefficients). These coefficients were much stronger than each with NEC, where the correlation coefficients were lower, 0.052-0.077 (P<0.0001-0.006). These markers likely represented the same phenomenon, mainly the severity of illness. The transfusion-NEC association was thus evaluated in the context of each variable alone. Birth weight was stratified into four clinical groups, table 3.

Table 33 Clinical characteristics by birth weight group

Risk factors*	≤ 750g	751-1000 g	1001-1250 g	>1250 g
Number of Infants	665	748	813	834
Apgar score 5 min	6(3)	7(2)	7(2)	8(2)
Gender, male as percent (%)	41.5	52.0	51.0	49.3
Race, AA as percent (%)	80.5	83.4	79.1	79.5
Birth weight (g)	626(137)	877(133)	1128(131)	1381(114)
Gestational age (wks)	25(2)	27(2)	29(3)	31(3)
Small for gestational age (%)	55.3	39.0	30.3	27.6
Umbilical artery catheter days	10(12)	5(9)	0(5)	0(0)
Days on Ventilator	21(28)	6(16)	1(4)	0(1)
Treatment for PDA (%)	50.5	37.7	13.2	3.6
Number of transfusions	12(9)	5(5)	2(3)	1(2)
Exposure to blood transfusion (%)	93.5	84.8	51.0	20.5
NEC (%)	7.7	6.8	5.7	3.0

AA: African American, NEC: necrotizing enterocolitis, PDA: patent ductus arteriosus

***Presented as the percentage of the number of infants or as Median (Inter quartile range)**

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Exposure to blood transfusions was protective in infants with a birth weight ≤ 1000g, those who stayed longer on a ventilator, and those who required a longer UAC insertion period. However, exposure to blood transfusions carried a risk for developing NEC in infants with less severity of illness markers. These infants with a birth weight of 1001-1500g and who did not need mechanical ventilation or did not require UAC insertion had a higher risk of developing NEC after a transfusion exposure (Tables 3-4-5-6).

Table 4-3 Multivariate risk of developing NEC by weight subgroup

Weight Group (g)	N	Cases(exposed)	RR[a,b]	95% CI	*P*	NEC day	NEC PMA
≤750	662	52 (39)	0.14	0.07-0.30	<0.01	20(18)	28(4)
751-1000	747	51 (37)	0.46	0.24-0.89	0.021	20(8)	30(3)
1001-1250	810	46 (31)	1.83	0.95-3.5	0.071	16(13)	31(2)
1251-1500	828	25 (9)	1.78	0.77-4.19	0.17	16(9)	32(2)

NEC: necrotizing enterocolitis, PMA: post menstrual age
[a] Adjusted by gender, race, and SGA
[b] Extended Mantel-Haenszel for linear trend, p<0.01

Table 4-5 Multivariate risk of developing NEC by ventilator day periods

Vent. days	N	Cases (exposed)	RR[a,b]	95% CI	*P*	Mean BW
0	839	8 (3)	3.5	0.82-15.15	0.09	1262±191
1-2	797	31(17)	1.04	0.50-2.14	0.92	1107±216
3-13	650	72 (49)	0.29	0.7-0.51	<0.01	947±255
>13	761	63 (47)	0.11	0.06-0.23	<0.01	739±196

BW: birth weight
[a] Adjusted by gender, race, and SGA
[b] Extended Mantel-Haenszel for linear trend, p<0.01

Table 5-6 Multivariate risk of developing NEC by UAC insertion day periods

UAC days	N	Cases(exposed)	RR[a,b]	95% CI	*P*	Mean BW
0	1352	52 (28)	2.11	1.2-3.69	<0.01	1173±253
1-2	184	12 (8)	1.44	0.41-5.12	0.31	1052±259

3-7	707	53 (37)	0.81	0.44-1..49	0.49	989±243
>7	804	57 (43)	0.2	0.1-0.39	<0.01	796±234

BW: birth weight, **UAC**: umbilical arterial catheter
[a] Adjusted by gender, race, and SGA
[b]Extended Mantel-Haenszel for linear trend, p<0.01

The association of exposure and the development of NEC after 28 days of life was also examined, (~~Table6~~Table7). Smaller infants were again less likely to develop NEC after exposure to a transfusion.

Table ~~6~~ 7 Multivariate risk of (late) onset NEC after day 28 by BW group

Weight Groups (g)	N	Cases(exposed)	RR[a,b]	95% CI	*P*
≤750	629	19 (10)	0.057	0.021-0.15	<0.01
751-1000	711	15 (8)	0.17	0.058-0.49	<0.01
1001-1250	771	7 (6)	4.32	0.49-37	0.19
1251-1500	810	1 (0)	-	-	-

[a] Adjusted by gender, race, and SGA
[b]Extended Mantel-Haenszel for linear trend excluding last weight group, p=0.003

In the 174 infants who developed NEC, exposed infants (n=116) were of a lower postmenstrual age at the time of NEC, median=30.2(3.2) compared to 31.1(3.1) weeks in the non exposed, p=0.02 (Figure 3). When stratified by birth weight, there was no statistically significant difference in the incidence of NEC by PMA between exposed and non exposed infants based on the four birth weight groups. Evaluating for differences between two birth weight groups at a 1000g cutoff, infants with a birth weight ≤1000g had significantly more UAC insertion days, median 7(9) vs. 0(4) d, more ventilation days, median 15(22) vs. 3(5), and more transfusions 8(12) vs. 3(4), p<0.01 for all, when compared to infants with a birth weight >1000 g.

Discussion

This is the first study showing that the association between NEC and transfusion is not similar among different birth weight groups. We expected to see a much higher association in the group that received transfusions the most: the extremely low birth weight infants with a BW ≤1000 g. Our findings were completely the reverse. Exposure to transfusions was significantly less likely to be associated with NEC in ≤1000 infants and more likely associated with NEC in larger infants with a BW of 1001-1500 g.

Because of their critical condition and finite blood volume, VLBW infants tend to have transfusion thresholds that are inversely proportional to their birth weight. In a systemic review of prospective randomized studies evaluating hematocrit thresholds with morbidity outcomes, infants who were transfused more and maintained a higher hematocrit were less likely to develop NEC [10]. Retrospective studies evaluating the association between anaemia, exposure, and NEC also had similar conclusions[4,6,7]. ~~Infants with a lower hematocrit were more likely to develop NEC following a transfusion.~~ In our practice, VLBW infants were initially transfused when their hematocrit dropped below a predefined level in their first weeks of life. ~~This level varied slightly between care teams.~~ With age, these predefined levels changed from a preset hematocrit level to presence of clinical signs combined with oxygen demands. Our practice has remained in parallel with the universal approach [11]. In a recent survey, 1018 neonatologists from 11 countries responded with minimal variability that in the first week of life higher hematocrit thresholds were targeted irrespective of the degree of respiratory support. At 4 weeks of life, however, decisions were more variable, with transfusion hematocrit thresholds being affected by degree of oxygen requirements [12]. By that time, variable levels of gut injury may already have been

ongoing potentiating the development of NEC. Clinical signs like tachycardia and increased frequency of apnea could well represent preclinical NEC and not the severity of anaemia. This consensus among neonatologists of accepting lower hematocrit levels for larger infants and our findings of an increased risk of NEC post exposure only in the larger two birth weight groups strongly suggests that the severity of anaemia may have a significant impact on the development of NEC post transfusion.

In some studies, infants with exposure associated NEC were reported as older by an average of 2-3 weeks compared to non exposed NEC infants; they were also "stable" with no clinical signs of illness [4,7,8]. Being older may reflect a longer period of anaemia and lower hematocrit level. In our cohort, exposed infants with NEC were older by only 5 days as compared to non exposed NEC infants, much in contrast to the 2- to 3-week period previously mentioned. We also did not observe different results when only infants with late onset NEC were evaluated.

Severity of illness has been proposed as another factor affecting the association between exposure to PRBC and the development of NEC. Exposed infants were more likely to be ventilated [2,4,7,8], have a PDA [4,7,8], and have a lower birth weight [1,2,7,8]. As all variables exhibit some interaction, interpretation of these results becomes difficult. We initially arrived at similar conclusions. In unadjusted analysis, longer ventilator days and longer UAC placement days increased the likelihood for developing NEC. A prolonged UAC period likely reflected poor intravascular access, frequent blood draws, and/or hemodynamic instability. When both variables were stratified into four periods based on quartiles, infants who had longer periods on a

ventilator and UAC placement were less likely to develop NEC. The linear change in the RR was very significant. These infants were also in the lowest birth weight group at ≤1000g. Thus, and in contrast to current reports, severity of illness, represented here by infants with lowest birth weights and longest ventilator days, was not a risk for exposure associated NEC.

Recently, a meta-analysis of reported case studies on the association of NEC and blood transfusion concluded that recent exposure to transfusion was associated with NEC [13]. Case studies can produce an exaggerated effect size as compared to cohort studies [14]. Kirpalani et al[15] has recently reviewed the effects of study design on the outcome of the association between transfusions and NEC. Randomized control trials showed an opposite effect to observational trials; higher transfusions were associated with a lower incidence of NEC. Most observational trials had the problem of confounding or small sample size. Since there wasn't any matching done in our study, we were able to evaluate birth weight as an independent variable and show its significance.

There were several limitations to our study. Bias due to its retrospective nature limited our ability to differentiate between the overlapping preclinical signs of NEC and those of anaemia. ~~Understandably, this problem cannot be resolved in a retrospective study.~~ Information about hematocrit levels ~~was not usable as central, peripheral, and blood gas hematocrit were used interchangeably to drive transfusion decisions. A change in the hospital laboratory data base mid study period made hematocrit data for~~ in the first half of the period were inaccessible. ~~These~~ This ~~factors~~ limited our ability to test for anaemia as a plausible explanation. Information about the use of antenatal steroids was also not available. Treatment with antenatal corticosteroids has

been shown to be associated with an overall reduction in necrotizing enterocolitis[16]. Lower weight infants may have received antenatal steroids more frequently than larger infants thereby lowering the risk for development of NEC. Another limitation pertains to the days to total feeds and formula vs. breast milk use. Historically in our unit, breast milk use was less than 20% and only increased above 60% in the last 2 years of the study period. There are also concerns about the lack of increased superior mesenteric artery blood flow in response to a feeding, following a blood transfusion[17], suggesting that feeds should be held after a transfusion. These results could be attributed to using older blood as it has been shown that by two weeks of shelve life, stored PRBC lose the ability to cause regional vasodilatation and to increase oxygen uptake by the tissue when compared to fresh PRBC stored for 3-7 days[18,19]. In our practice, PRBC units are usually exhausted within 4-10 days post collection. Using fresh blood for transfusions may explain our study findings but with data not available, this would be a speculation.

~~The fact that hundreds of infants received transfusions while feeding without an increased risk of NEC post transfusion makes this association unlikely.~~

A likely explanation of ~~our~~ the study findings would be that as an infant's birth weight increases, there is a threshold crossed that changes the polarity of the association between exposure and NEC. This is nicely demonstrated in the progressive increase in the relative risk value with each weight group. The threshold could be a change in clinical practice like using a lower transfusion limit. It could also be some other factor related to gut maturity, to feeding practice, or altogether still to be discovered.

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In summary, using cohort approach in the investigation eliminated the need for matching by birth weight and lead to new information about different levels of association between blood transfusions and NEC in different weight groups. Our findings show that exposure to transfusions does not increase the risk of NEC. Specifically, after exposure to a PRBC transfusion, ≤1000g BW infants were less likely to develop NEC than were infants born 1001-1500g BW. These larger infants were likely to have lower transfusion thresholds as they were less ill and probably more anaemic. We speculate that anaemia could be the cause of their exposure associated NEC. Future studies evaluating this association should be prospective, should incorporate birth weight as a risk factor, and should incorporate newer modalities such as near-infrared spectroscopy to evaluate hypoxia at the intestinal level[20].

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Figure legends

Figure 1 Incidence of NEC by post menstrual age

Figure 2 Incidence of NEC over the study period

Figure 3 Effect of exposure on the incidence of NEC by post menstrual age

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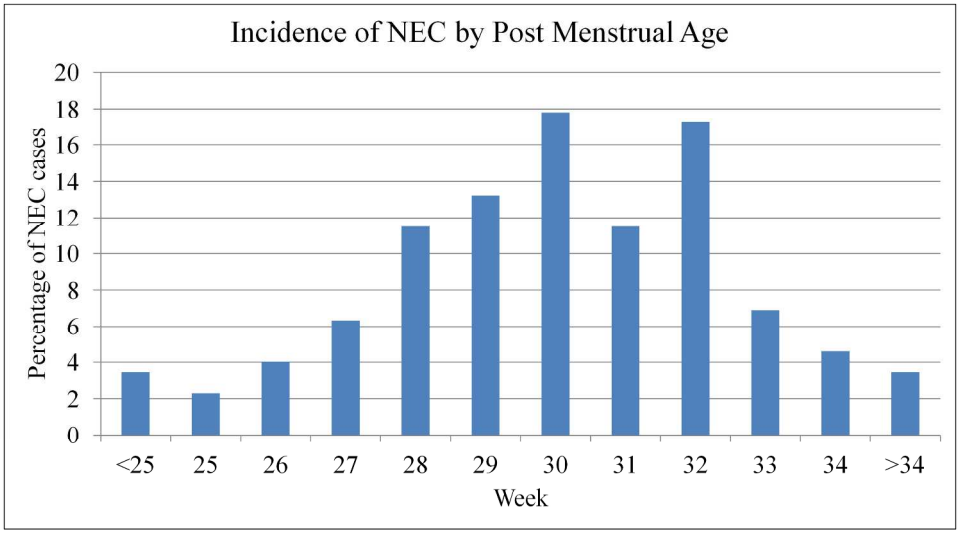
STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	**4**
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	**4,5**
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	**4,5**
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	5
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	6
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6,7
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	N/A
Bias	9	Describe any efforts to address potential sources of bias	6, 7
Study size	10	Explain how the study size was arrived at	5-7
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6, 7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6, 7
		(*b*) Describe any methods used to examine subgroups and interactions	6, 7
		(*c*) Explain how missing data were addressed	7
		(*d*) If applicable, explain how loss to follow-up was addressed	N/A
		(*e*) Describe any sensitivity analyses	N/A

Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	7
		(b) Give reasons for non-participation at each stage	7
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8
		(b) Indicate number of participants with missing data for each variable of interest	8-10
		(c) Summarise follow-up time (eg, average and total amount)	N/A
Outcome data	15*	Report numbers of outcome events or summary measures over time	8-10
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	8-10
		(*b*) Report category boundaries when continuous variables were categorized	8-10
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	9-10
Discussion			
Key results	18	Summarise key results with reference to study objectives	14
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12-13
Generalisability	21	Discuss the generalisability (external validity) of the study results	12-13
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	N/A

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.



Incidence of NEC by post menstrual age
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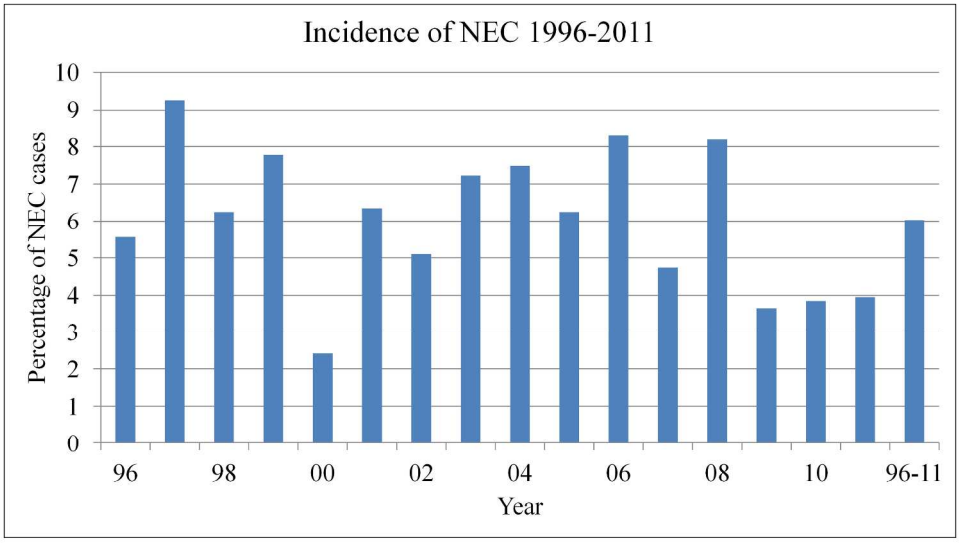

Incidence of NEC over the study period
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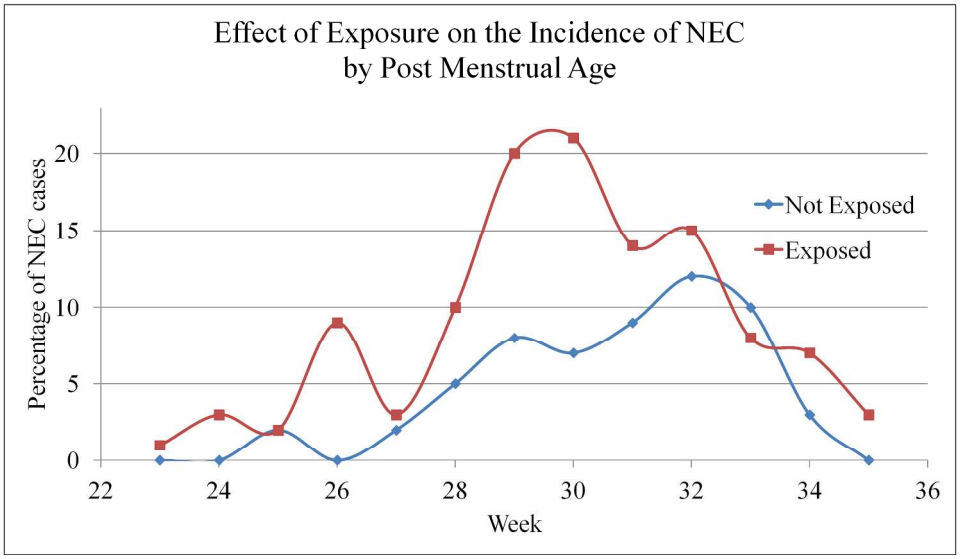

Effect of exposure on the incidence of NEC by post menstrual age
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