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The relationship between reticulated platelets, intestinal alkaline phosphatase, and necrotizing enterocolitis

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ABSTRACT

Background: Necrotizing enterocolitis (NEC) affects up to 10% of extremely-low-birthweight infants, with a 30% mortality rate. Currently, no biomarker reliably facilitates early diagnosis. Since thrombocytopenia and bowel ischemia are consistent findings in advanced NEC, we prospectively investigated two potential biomarkers: reticulated platelets (RP) and intestinal alkaline phosphatase (iAP).

Methods: Infants born ≤32 weeks and/or ≤1500 g were prospectively enrolled from 2009 to 2012. Starting within 72 hours of birth, 5 weekly whole blood specimens were collected to measure RP and serum iAP. Additional specimens were obtained at NEC onset (Bell stage II or III) and 24 hours later. Dichotomous cut-points were calculated for both biomarkers. Non-parametric (Mann-Whitney) and Chi-square tests were used to test differences between groups. Differences in Kaplan-Meier curves were examined by log-rank test. The Cox proportional hazards model estimated hazard ratios.

Results: A total of 177 infants were enrolled in the study, 15 (8.5%) of which developed NEC (40% required surgery and 20% died). 14 (93%) NEC infants had "low" (≤2.3%) reticulated platelets, and 9 (60%) had "high" iAP (>0 U/L) in at least one sample before onset. Infants with "low" RP were significantly more likely to develop NEC [HR = 11.0 (1.4–83); P = 0.02]. Infants with "high" iAP were at increased risk for NEC, although not significant [HR = 5.2 (0.7–42); P = 0.12]. Median iAP levels were significantly higher at week 4 preceding the average time to NEC onset by one week (35.7 ± 17.3 days; P = 0.02).

Conclusion: Decreased RP serves as a sensitive marker for NEC onset, thereby enabling early preventative strategies. iAP overexpression may signal NEC development.

Necrotizing enterocolitis (NEC) is a severe inflammatory disorder of the intestine that affects up to 10% of very low birth weight infants (<1500 g at birth) with a mortality of up to 30% [1–3]. NEC most commonly presents with abdominal distention with or without abdominal wall discoloration, abdominal tenderness, bloody stools, increased/bilious residuals, apnea, lethargy, metabolic acidosis, and thrombocytopenia [4].

Despite improvement in the care of extremely premature infants over the past few decades the morbidity and mortality caused by NEC remain high [3,5]. While the pathophysiology of NEC remains unclear, inflammation, ischemia, immature mucosal immunity, and endothelial injury are thought to play a role, prompting the search for biomarkers [2,6]. While many biomarkers (such as, acute-phase reactants, cytokines, chemokines, and cell surface antigens) have been sought in order to allow for early diagnosis or prevention, none have been found to identify specifically NEC [7].

Reticulated platelets represent newly produced platelets and are expected to be prevalent during periods of thrombocytopenia [8]. Intestinal alkaline phosphatase (iAP) is a brush border enzyme in the intestinal mucosa with unclear activity levels in preterm infants. Intestinal alkaline phosphatase is a controversial biomarker that in some studies have shown an association with elevated levels and bowel ischemia; yet, in other studies a protective role is suggested [6,9]. Given the consistent findings of thrombocytopenia and bowel ischemia with advanced NEC, these two biomarkers were prospectively studied to ascertain the role each plays in the development of NEC.

1. Methods

Loyola University Medical Center is a perinatal referral center with a fifty-bed level III NICU that admits both inborn and outborn infants. Infants born at ≤32 weeks and/or ≤1500 g were prospectively enrolled. Enrollment extended from May 2009 through July 2012.

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An initial whole blood sample (0.5 ml) was drawn within 72 hours of birth and then repeated weekly for the next 4 weeks, for a total of 5 per patient. If the infant developed NEC (as defined by Bell Stage II or III; Table 1), a sample was drawn at the onset of NEC and again 24 hours later [10,11].

Each blood sample was analyzed for both reticulated platelets and intestinal alkaline phosphatase (iAP). Percentage of reticulated platelets was determined by flow cytometry. Serum iAP levels were determined by gel electrophoresis. Data were also collected regarding maternal blood group, infant blood group, gestational age, birthweight, date of birth, date of discharge or death, transfusion history, complete blood count data at the time of sample specimen (if available), and feeding history for each infant enrolled in the study.

Feeding practice of all infants in our unit was left to the discretion of the attending neonatologist on service. Infants typically begin with trophic feeds (about 5 ml/kg) within the first few days of life, if stable, and then are slowly advanced by 5–10 ml/kg daily until reaching goal feeds of approximately 150 ml/kg/day. Breast milk is preferentially used for feeding, but preterm formula is used if insufficient breast milk is available. Human milk fortifier is generally added to the breast milk when volume of enteral feeds reaches100 ml/kg/day.

The study was approved by the IRB (#20124102110). All infants born at ≤32 weeks and/or ≤1500 g were eligible for the study and the parents of eligible infants were invited to participate following admission to the unit.

Dichotomous cut-points for both biomarkers were sought to maximize sensitivity. Infants were considered to have low reticulated platelets if any one of their specimens measured ≤2.3% and high iAP if any specimens had a value >0. The Mann-Whitney U test was used to highlight differences in median biomarker levels between NEC and non-NEC infants. The chi-square or Fisher's exact test highlighted categorical differences. The Kaplan-Meier method and log-rank test were used to censor infants who did not develop NEC by the time of discharge to generate curves that estimate the probability of developing NEC. The Cox proportional hazards model was used to estimate hazard ratios. A P value of <0.05 was considered significant.

2. Results

A total of 177 infants were enrolled in the study, 15 (8.5%) of which developed NEC (Table 2).

Enteral feedings on the infants in the study were started at a mean of 6 (6.4 ± 0.5) days of life and full volume feeds were achieved at a mean 26 (26.7 ± 1.3) days. Of those infants who developed NEC, 14 (93%) had "low" reticulated platelets and 9 (60%) had "high" iAP in at least one sample collected before the onset of NEC (Figs. 1 and 2). Using the set cut-points for reticulated platelets and iAP, Kaplan-Meier plots (Fig. 3) and hazard ratios were calculated for the probability of developing NEC. They showed that infants with "low" reticulated platelets are significantly more likely to develop NEC [HR = 11.0 (1.4–83); P = 0.02]. Infants with "high" iAP show an increased risk (although not statistically significant) of developing NEC [HR = 5.2 (0.7–42); P = 0.12]. Of the 41 infants who had an iAP = 0, only 1 infant (2.4%) developed NEC. Those infants with "low" reticulated platelets or "high" iAP also had significantly higher gestational ages and birth weights, thereby bolstering their predictive impact within a conventionally less vulnerable neonatal population. While the percentage of reticulated platelets at admission was lower in infants with "high" iAP, reticulated platelets did not translate into a predictive biomarker within this subgroup (data not shown). The relationship of other, related biomarkers to NEC onset – including: platelet count and absolute RP count – were also investigated, but none were related to NEC onset in our study.

Weekly comparisons of median reticulated platelets and iAP levels over all infants were performed. By week 4, infants who would go on to develop NEC demonstrated significantly higher levels of iAP over their non-NEC counterparts (P = 0.02).

Table 1
Modified Bell Staging Criteria for necrotizing enterocolitis modified from Walsh, Kliegman, and Fanaroff [5].

Stage	Clinical signs	Radiologic signs
Stage I	Apnea, bradycardia, temperature instability	Normal gas pattern or mild ileus
Stage IIa	Same as I plus grossly bloody stools and abdominal distention	Ileus and pneumatosis intestinalis
Stage IIb	Same as IIa plus abdominal tenderness, mild metabolic acidosis and mild thrombocytopenia	Same as IIa plus portal venous gas ± ascites
Stage IIIa	Same as IIb plus hypotension, bradycardia, acidosis, coagulopathy, marked abdominal tenderness, abdominal wall erythema/induration	Same as IIb plus definite ascites
Stage IIIb	Same as IIIa plus shock	Same as IIb plus pneumoperitoneum

Table 2
NEC *versus* non-NEC infants (mean ± SE).

	NEC infants (mean ± SEM)	Non-NEC infants (mean ± SEM)	P-value
Gestational age (weeks)	26.5 ± 0.7	27.5 ± 0.2	0.14
Birth weight (g)	903 ± 109	1050 ± 32	0.15
Days to NEC	35.7 ± 4.5	N/A	N/A
	NEC infants (n)	Non-NEC infants (n)	P-value
Maternal blood type			
A	6	58	0.70
B	3	20	
AB	0	7	
O	6	74	
Maternal Rh			
+	14	139	0.55
−	1	20	
Infant blood type			
A	7	55	0.67
B	1	23	
AB	1	7	
O	6	77	
Infant Rh			
+	11	147	0.06
−	4	15	

3. Discussion

NEC is the most common surgical emergency in preterm infants in the neonatal intensive care unit [12]. NEC occurs in 1–3/1,000 live births with 90% of NEC cases occurring in the most premature and smallest infants [4,7]. With advances in neonatal care, these very low birthweight infants are surviving in greater numbers; thus, NEC has become a serious threat to the survival of these fragile infants. Unfortunately, treatment for NEC has not resulted in improved survival and there are no current methods available to predict which infants will develop NEC. Mortality for NEC is 10%–30% with extremely low birthweight infants and those with the lowest gestational age having the highest mortality [3]. Approaches to improve outcomes with NEC include prevention, early risk stratification, early detection and improved treatment modalities. Thus, our use of reticulated platelets and iAP as possible biomarkers may enable early identification of those infants at high risk for developing NEC.

Reticulated platelets are newly synthesized platelets that have a higher content of ribonucleic acid than platelets that have been circulating for several days [8]. As newly produced platelets, these reticulated platelets are considered to be more active [8]. Normal reticulated platelet count values are not well-defined. A study by