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ARTICLE

Reference intervals for stool calprotectin in preterm neonates and their utility for the diagnosis of necrotizing enterocolitis

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Abstract

Objective Calprotectin is an antimicrobial protein found in stool when released by granulocytes. We sought to create stool calprotectin reference ranges in preterm neonates and to evaluate whether levels exceeding the upper reference interval are diagnostic for necrotizing enterocolitis (NEC).

Study design Stool calprotectin was measured in premature neonates without gastrointestinal pathology to create reference intervals. For comparison, levels from infants undergoing "rule out NEC" evaluations were plotted on these reference intervals.

Results Stool calprotectin reference intervals were created according to gestational age at birth and corrected gestational age. Levels during "rule out NEC" evaluations were more often above the upper reference interval with NEC vs. those without NEC.

Conclusions Stools from preterm neonates have a higher range of calprotectin than stools from healthy term neonates. In evaluating preterm neonates for NEC with stool calprotectin, a calprotectin upper reference interval that incorporates corrected gestational age best predicts the diagnosis of NEC.

Introduction

Calprotectin is an antimicrobial protein found in neutrophils, monocytes, and macrophages. High levels of calprotectin are detected in stools of children and adults with active inflammatory bowel disease [1–4]. Stool calprotectin levels are generally higher in neonates with necrotizing enterocolitis (NEC) compared to healthy term neonates. However, the latter comparison is faulty, because NEC typically occurs in growing premature neonates in a NICU, not in healthy term neonates [5–9]. Thus to utilize calprotectin as a marker for NEC, reference intervals are needed from the population most "at risk" for NEC. Only with that comparison would it be clear whether a level from a premature neonate suspected of having NEC was indeed elevated, or was actually within the reference interval of the proper comparison population.

We conducted this study in two sequential parts; first we developed reference intervals for calprotectin in stools of healthy growing preterm neonates over their first eight weeks after birth. Only stools from neonates with no obvious gastrointestinal disturbances, but cared for in NICUs, were included in the reference interval data set. We displayed values on the day of birth according to gestational age on one chart, and according to corrected gestational age on a second chart. Then we sought to use our newly created reference interval charts to assess normalcy of calprotectin levels measured in stools of neonates during "rule out NEC" evaluations.

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Materials and methods

Study design and populations

This was a two-part sequential study. The first was a prospective, de-identified, multi-NICU collection of stool from diapers of neonates with no obvious gastrointestinal pathology born <35 weeks gestation and between 1 and 8 weeks old. The only clinical data collected and correlated with the stool calprotectin levels were: gestational age at birth; age in days at the time the stool was collected; sex; and whether for the past three days the neonate was fed human milk only, cow's milk-based formula only, or human milk to which cow's milk-based fortifier or formula were added.

The sample size of 250 for the first study was determined primarily by the practical matter of the research budget available to cover the costs of the calprotectin assays and the study nurse time. This number was in keeping with the general recommendation of Horowitz, for creating reference intervals for laboratory studies, who suggested a minimum of 120 samples be obtained, and when dividing into subclasses, at least 120 samples in each subclass [10]. Stools were collected from infants in four Intermountain Healthcare NICUs in Utah; McKay-Dee Hospital in Ogden, Intermountain Medical Center in Murray, Utah Valley Regional Medical Center in Provo, and Dixie Regional Medical Center in St. George. The Intermountain Healthcare Institutional Review Board approved the protocol as a de-identified, not consented study, with proper privacy protection. Intermountain Healthcare is a not-for-profit system that owns and operates 22 hospitals in Utah and Idaho.

The second study was a retrospective review of all cases from the Intermountain Healthcare and University of Utah NICUs, where a stool calprotectin was measured at ARUP Laboratories in the 5-year period, July 2012–May 2017, for a "rule out NEC" evaluation. The sample size for the second study was determined by the actual number of calprotectin levels run by the ARUP Laboratories on neonates during that period. To detect a 50 µg/g stool difference in stool calprotectin levels between infants with NEC compared to infants without NEC, with a level of significance of 5% and power of 80%, 22 infants were needed in each group. Only cases originating from an Intermountain Healthcare or University of Utah NICU were included because we had IRB approval to review those records. Clinicians decided when to submit a stool sample for analysis. As a generalization, samples were not submitted when the diagnosis of NEC was reasonably certain (from X-rays or other features). The typical practice during the study period was to submit a stool sample from questionable cases. About one week or more after submitting the sample, clinicians charted whether they concluded that the neonate indeed had NEC, or did not have NEC, or that the diagnosis was still too uncertain to make a declaration. Additional chart review (by BCM) was performed to confirm the diagnosis according to the modified Bell's staging criteria for NEC [11]: stage 1—suspected or possible NEC, stage 2—definite mild/moderate NEC with the presence of radiologic findings, or stage 3—definite severe NEC. Infants with a diagnosis of spontaneous intestinal perforation were excluded from this study.

Calprotectin assay

The PhiCal fecal calprotectin immunoassay (Genova Diagnostics, Inc. Ashville, NC) is an enzyme-linked immunoassay system with colorimetric detection, and was performed at ARUP laboratories (Salt Lake City, UT) according to the manufacturer's instructions. This was the case for both the first and second parts of the study. Per the manufacturer's instructions, 1–5 g of stool is obtained in a clean vial. No preservative is necessary. Stool can be stored refrigerated or at room temperature for up to 11 days or frozen at −20 °C for up to 1 year. Stool samples from this study were received in the laboratory within 24 h of collection and always processed within 96 h of collection. The test uses a polyclonal antibody against calprotectin. Calprotectin present in the diluted stool sample is bound by the antibody absorbed to the surface of the plastic well. Calprotectin binding to the enzyme-conjugated antibody activates the enzyme allowing for conversion of the colorimetric substrate to a colored product. The intensity of the color is directly proportional to the amount of captured calprotectin bound. Concentration of calprotectin in the stool samples is calculated using a calprotectin standard curve generated in parallel and provided in the test kit.

Statistical analysis

Study data were collected and managed using REDCap electronic data capture tools hosted at Intermountain Healthcare [12]. REDCap (Research Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies, providing: (1) an intuitive interface for validated data entry; (2) audit trails for tracking data manipulation and export procedures; (3) automated export procedures for seamless data downloads to common statistical packages; and (4) procedures for importing data from external sources. Data were managed and accessed by authorized data analysts. Means and standard deviations were used to express values in groups that were normally distributed, and medians and interquartile ranges to express values in groups that were not. Differences in categorical variables were assessed using the Fisher exact test or χ^2 for normally distributed data and Tukey's bi-weight estimator

for groups that were not. Statistical analysis used Statit (Midas, Tucson, AZ, USA). Statistical significance was set as $P < 0.05$. A two-sided *t*-test was used to compare the effects of sex, and the effect of human milk vs. cow's milk formula (+mixtures of human and formula) on calprotectin values.

In the second study, stool calprotectin as continuous variable was compared between three diagnostic categories; (1) neonates who had definite NEC, (2) those who definitely did not have NEC, (3) uncertain whether or not they had NEC. The statistical performance of stool calprotectin was also assessed by receiver operating characteristic curves (IBM SPSS Statistics, version 23.0, Armonk, NY, USA). Youden's index (*J*) was computed to evaluate the effectiveness of stool calprotectin as a biomarker for NEC.

Results

To create reference intervals, calprotectin levels were measured on 249 stool samples from 120 neonates between 1 and 8 weeks old. Demographics of these neonates are shown in Table 1. The average gestational age of the neonates at birth was 31.0 ± 2.7 (mean ± SD) weeks. We found no difference in stool calprotectin levels explainable by sex.

Figure 1 shows calprotectin in stool according to corrected gestational age with curves denoting the 10th percentile, median, and 90th percentile values. Stool calprotectin levels in the first week were significantly lower, as a group, than were those collected in weeks 2 to 8 (Table 2).

Table 1 Demographics of the neonates from whom stool samples were collected over the first 8 weeks after birth to create reference intervals for stool calprotectin in growing preterm neonates in a NICU

Demographic	Number (*n*)	Percent
Sex		
Male	118	47
Female	131	53
Race		
Asian	8	3.2
Black	8	3.2
Pacific Islander	2	0.8
White	223	89.6
Unknown	8	3.2
Gestational age at birth (weeks)		
24–26	21	8.4
27–29	49	19.7
30–32	100	40.2
33–34	79	31.7

Table 2 displays the stool calprotectin levels based on type of enteral feeding the infant was receiving at time of stool collection; human milk only, cow's milk-based formula only, or human milk with a cow's milk-based fortifier or human milk and cow's milk-based formula.

Figure 2 displays calprotectin levels from stools of 118 neonates as part of a "rule out NEC" evaluation according to corrected gestational age. Those eventually diagnosed with NEC ($n = 33$) vs. those judged not to have NEC ($n = 68$) are noted separately. Stool calprotectin levels were above the upper reference interval in 58% of those diagnosed with NEC compared to 13% of those judged not to have NEC. Of the 9 infants with calprotectin levels higher than the 90th percentile who did not have NEC, the presenting symptom in 7 of the 9 was hematochezia, and the other 2 had abdominal distension. Seven were receiving breast milk with a cow's milk-based fortifier or cow's milk-based formula, 2 were receiving human milk only. Absolute eosinophil counts measured at the time of the "rule out NEC" evaluation were less than 1000 per μl in all of these infants.

Two of the 118 babies who had stool calprotectin levels measured had surgical NEC with positive pathologic specimens. One infant was 13 days old with a corrected gestational age of 33 1/7 weeks, and the other was 19 days old with a corrected gestational age of 30 2/7 weeks. Prior to surgery, their stool calprotectin values were 834 and 1579 μg/g stool respectively which are both above the 90th percentile for corrected gestational age.

Figure 3 shows receiver operating characteristic analysis of stool calprotectin levels in the diagnosis of NEC. Figure 3a shows the receiver operating characteristic (ROC) curve containing all infants who had a stool calprotectin measured, and Fig. 3b shows only those infants less than 35 weeks-corrected gestational age at the time of sample collection. In infants of all corrected gestational ages, a stool calprotectin value of 226 μg/g stool was the value with the best Youden index ($J = 0.508$, sensitivity = 75%, specificity = 76%). In only infants less than 35 weeks-corrected gestational age, a stool calprotectin value of 299 μg/g stool was the value with the best Youden index ($J = 0.589$, sensitivity = 71%, specificity = 88%). Table 3 shows positive and negative predictive values in the diagnosis of NEC based on corrected gestational age.

Discussion

When preterm neonates develop abdominal distension or other signs of intestinal distress, NEC is generally considered in the differential diagnosis. At the onset of symptoms, it is sometimes difficult to know precisely whether the condition is indeed NEC, particularly when abdominal

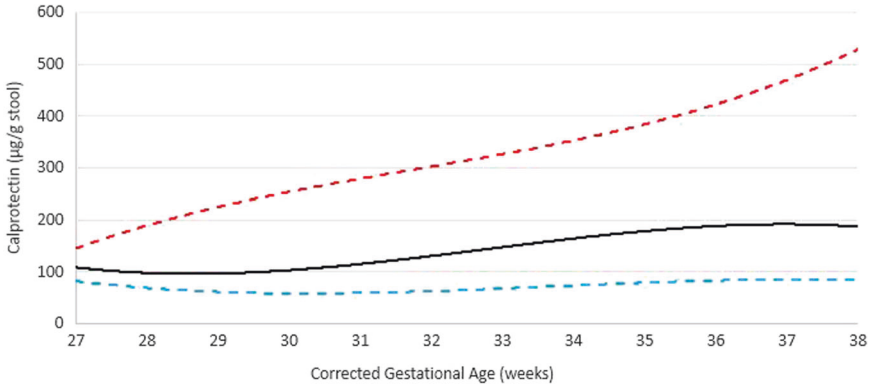

Fig. 1 Stool calprotectin reference intervals for preterm infants. Median, lower (10th percentile), and upper (90th percentile) reference intervals for calprotectin in stools of preterm infants (24–34 weeks) according corrected gestational age

X-rays are non-diagnostic. Several studies suggest that in these circumstances of uncertainty, a stool calprotectin level performs well as a diagnostic test for NEC [7, 9, 11, 13]. However, those reports typically compared levels from neonates with NEC to levels from healthy term neonates as controls. A more appropriate comparison group would be stools from healthy preterm neonates of similar postnatal age, data that we now provide.

Li et al. [14] reported that healthy infants younger than six months had higher stool calprotectin levels than did older children and adults; however, they defined no cutoff level at which a stool calprotectin is considered elevated for premature neonates. Moussa et al. [15] reported no correlation between gestational or postnatal age and stool calprotectin level, studying 26 control neonates and 26 with signs of feeding intolerance. In contrast, in the present study we found that the upper reference interval for stool calprotectin steadily increased with increasing corrected gestational age. There was a gradual upward trend of the median started at 30 weeks-corrected gestational age that plateaued around 36 weeks. This pattern suggests that many preterm infants might have a biologic shift in the gut around this time. Possibilities include changes in the innate immune response with increased intestinal granulocyte activation and/or alterations of the intestinal microbiome [16]. La Rosa et al. [17] studied the bacterial colonization from stools in a group of very low birth weight premature neonates and reported decreasing proportions of bacilli species and increasing proportions of clostridia with increasing corrected gestational age.

Some of the changes in stool calprotectin levels in preterm infants appear to be on the basis of diet. Sullivan et al. [18] and Cristofalo et al. [19] reported premature neonates receiving an exclusive human milk diet had a lower incidence of NEC than did those whose diet included cow's milk-based formulas or fortifiers. In our population, stool calprotectin levels were higher in asymptomatic growing premature infants receiving a mixture of human milk plus cow's milk-based fortifier or formula. Panczuk et al. [20] from Toronto reported a similar finding, but Yoon et al. [13], in Korea, found no such dietary association. In our present data set, calprotectin levels in preterm infants fed breast milk did not differ from those fed formula only. This could be due to an effect of gestational age on the inflammatory response to cow's milk components, as the infants fed formula only were of older gestational age, or there could be an increase in inflammation if babies are fed a mixture of cow's milk and human milk, vs. only human milk or only cow's milk.

It is not clear to us why cow's milk-based fortifiers in human milk sometimes result in elevated stool calprotectin levels in neonates without NEC. We previously reported that the calprotectin in NEC-affected bowel is associated with neutrophil extracellular traps (NETs) [9]; nuclear chromatin ejected by activated neutrophils to trap and kill microorganisms [21]. It is likely that asymptomatic preterm infants with elevated stool calprotectin levels are experiencing an inflammatory process involving granulocyte activation. A potential non-NEC cause of increased neutrophil activation in this population is cow's milk protein-induced enterocolitis [22, 23]. The use of stool calprotectin as it relates to cow's milk protein-induced enterocolitis needs to be further studied.

NEC that occurs in term neonates likely has pathogenic elements that are distinct from the usual variety of NEC that occurs in premature neonates [24]. Our ROC analysis, done to assess the statistical performance of stool calprotectin in diagnosing NEC, was better when the neonates at and above 35 weeks-corrected gestational age were removed. Although this was a post hoc analysis based on a small sample size, it might suggest that stool calprotectin is not as good of a biomarker for term babies with NEC than for preterm babies with NEC.

Our study has several other limitations. First, although we collected almost 250 stool samples to create reference intervals, subsets based on specific corrected gestational ages result in a small number of patients at each point. Second, the population used to create reference intervals

Table 2 Stool calprotectin levels collected in the first week of life compared to those collected in weeks 2–8, and stool calprotectin levels based on type of feeding at the time of stool sample collection

	Number	Stool calprotectin median (IQR)	Postnatal age (days) mean ± SD	Corrected gestational age (weeks) mean ± SD
Postnatal age at collection (weeks)				
Week 1	38	123 (51,193)		
Week 2–8	211	148 (92,246)		
P value week 1 vs. weeks 2–8		0.003		
Feeding type				
Human milk only	34	119 (82,187)	9 ± 9	32 5/7 ± 2 6/7
Formula only	24	108 (77,215)	24 ± 10	35 5/7 ± 2 0/7
Human milk with fortifier or human milk and formula	191	153 (96,268)	25 ± 13	34 3/7 ± 2 3/7
P value human milk only vs. formula only		0.455		
P value human milk only vs. human milk with fortifier or human milk and formula		<0.001		

Values are either shown as median (1st interquartile range, 3rd interquartile range) or mean ± standard deviation.

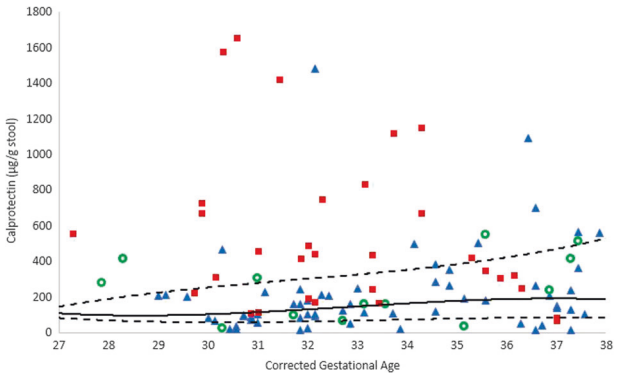

Fig. 2 Calprotectin levels in stools ($n = 118$) of neonates undergoing "rule out NEC" evaluations. Values where NEC was excluded as the diagnosis are shown by blue triangles. Values where the diagnosis of NEC remained uncertain (Bell's stage 1) are shown by green open circles. Values from stools where NEC was the final diagnosis are shown by solid red squares (Bell's stage 2 or 3). Reference intervals from stools of preterm NICU patients from study 1 are shown by the hatched (10th and 90th) and solid (median) lines. Values are displayed according to corrected gestational age

was homogenous with 90% white infants, so our findings may not be applicable to a group with a different racial/ethnic distribution. Third, the data from which we created the reference intervals were de-identified so we don't know whether any of the infants were developing NEC when their stool was collected for calprotectin measurement. If a few were, this could explain why some "normal" growing preterm infants had high stool calprotectin values. Other potentially important clinical factors such as anemia or recent red blood cell transfusions, both of which have been reported to increase stool calprotectin levels [25], were not recorded in our de-identified data, but might explain some of the high levels. Fourth, for the second part of our study, the clinicians ordering calprotectin were aware of the calprotectin levels when they declared whether or not NEC was the correct diagnosis. The knowledge of this level might have influenced their diagnosis. Lastly, stool is not a homogeneous material. Perhaps the average stool calprotectin level from two specimens would result in less variability than in our study which included only one stool sample at each time point.

Several reports, including our present study, suggest that stool calprotectin levels can help decide whether to label an otherwise uncertain bowel condition as NEC [6, 7, 9, 11, 26]. We hypothesized that to judge whether a calprotectin level is elevated, it should be compared with levels from growing preterm infants of the approximate age as the patient. In applying this approach, we found a much wider range of calprotectin levels in stools of growing asymptomatic preterm infants than in those of healthy term neonates. We found that a level above the upper reference interval for age increased the likelihood that the condition would be called NEC, and this was most significant in infants <35 weeks-corrected gestational age. However the relationship was imperfect, in that some neonates with an elevated level apparently did not have NEC, and some with a level within the reference range did have NEC. As a consequence of this limitation, we suggest that, for diagnosing NEC, single stool calprotectin levels are of some value, but cannot be relied upon absolutely. Thus, when a neonate with bowel symptomatology has X-rays that are inconclusive for NEC, the diagnosis will still likely be guided by other factors, including hematological changes

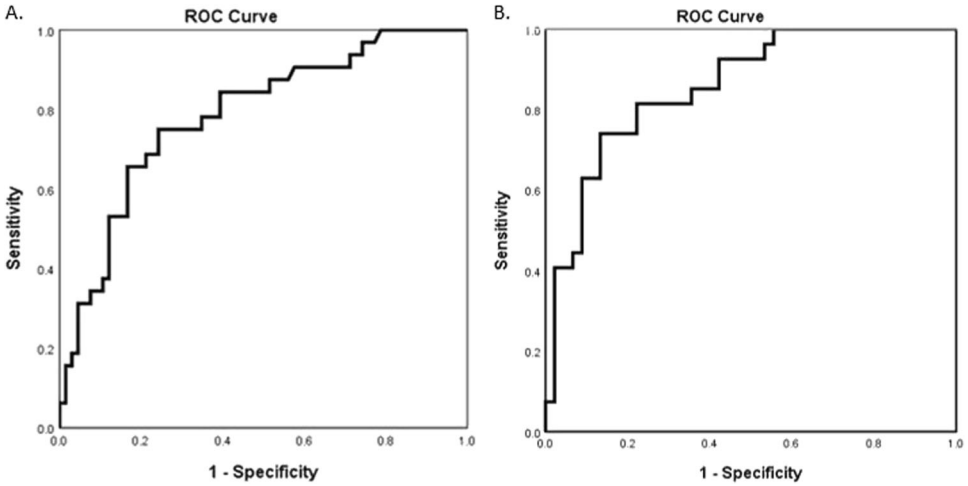

Fig. 3 Receiver operating characteristic (ROC) curves for calprotectin levels in stool. ROC curve for calprotectin levels in stools of all neonates undergoing "rule out NEC" evaluations in **a**. Area under the curve: 0.786 (95% confidence interval 0.690–0.881). ROC curve for calprotectin levels in stools of neonates <35 weeks-corrected gestational age undergoing "rule out NEC" evaluations in **b**. Area under the curve 0.865 (95% confidence interval 0.777–0.953)

Table 3 Positive and negative predictive values of stool calprotectin in the diagnosis of NEC based on corrected gestational age

Corrected Gestational Age at Collection	Positive predictive value (95% confidence interval)	Negative predictive value (95% confidence interval)
<35 weeks	77.3% (59.1–89.1%)	84.1% (73.7–91.0%)
≥35 weeks	36.4% (17.8–60.1%)	80.0% (68.2–88.2%)

[27], serial examinations, response to therapy, serum or urinary fatty acid-binding protein levels [28], ultrasonography [29], and perhaps also influenced by the stool calprotectin level.

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Compliance with ethical standards

Conflict of interest RS and JL are employees of ARUP laboratories where the calprotectin assays were run. The remaining authors declare that they have no conflict of interest.

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