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Acute Drop in Blood Monocyte Count Differentiates NEC from Other Causes of Feeding Intolerance

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Abstract

Objective—Necrotizing enterocolitis (NEC) is characterized by macrophage infiltration into affected tissues. Because intestinal macrophages are derived from recruitment and *in situ* differentiation of blood monocytes in the gut mucosa, we hypothesized that increased recruitment of monocytes to the intestine during NEC reduces the blood monocyte concentration, and that this fall in blood monocytes can be a useful biomarker for NEC.

Patients and methods—We reviewed medical records of very low birth weight (VLBW) infants treated for NEC, and compared them with a matched control group comprised of infants with feeding intolerance but no signs of NEC. Clinical characteristics and absolute monocyte counts (AMC) were recorded. Diagnostic accuracy of AMC values was tested using receiver-operator characteristics (ROC).

Results—We compared 69 cases and 257 controls (median 27 weeks, range 26–29 in both groups). In stage II NEC, AMC decreased from median 1.7×10^9/L (interquartile range (IQR)

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0.98–2.4) to 0.8 (IQR 0.62–2.1); p <0.05. In stage III NEC, monocyte counts decreased from median 2.1 × 10^9/L (IQR 0.1.5–3.2) to 0.8 (IQR 0.6–1.9); p <0.05. There was no change in AMC in control infants. ROC of AMC values showed a diagnostic accuracy (area under the curve) of 0.76. In a given infant with feeding intolerance, a drop in AMC of >20% indicated NEC with sensitivity of 0.70 (95% CI 0.57–0.81) and specificity of 0.71 (95% CI 0.64–0.77).

Conclusions—We have identified a fall in blood monocyte concentration as a novel biomarker for NEC in VLBW infants.

Keywords

Absolute monocyte counts; feeding intolerance; diagnostic test; monocytopenia; diagnosis; neonate

INTRODUCTION

Necrotizing enterocolitis (NEC), an inflammatory bowel necrosis of premature infants, remains a major cause of mortality in infants born before 32 weeks of gestation or with a birth weight <1500 grams.[1, 2] The diagnosis of NEC is currently based on radiological findings such as *pneumatosis intestinalis*, fixed bowel loop(s), or portal venous air,[3] characteristic abdominal signs and clinical progression,[4] and/or histopathological findings such as coagulative necrosis, *pneumatosis*, and inflammation.[5] Unfortunately, these signs of NEC have low sensitivity and are often recognized late in the course of NEC[3, 6, 7] when many infants already have advanced disease requiring surgical resection of affected bowel.[8] Cognizant of these difficulties in diagnosis and in the absence of a reliable biomarker of early NEC, clinicians frequently provide presumptive treatment to all at-risk infants with abdominal signs, an approach that is costly and undesirable.[9] The lack of a reliable biomarker is also a major limitation in clinical trials; new treatment(s) may not be effective late in the clinical course of NEC when the affected tissue has already lost viability, whereas enrollment of patients before the onset of pathognomonic signs may result in erroneous inclusion of patients who never had NEC in the first place, thereby diluting the effect of the treatment.

Histopathologically, NEC is characterized by the presence of macrophage-rich leukocyte infiltrates,[10] which contrasts with other causes of bowel dysfunction in neonates such as dysmotility, sepsis-related ileus, and ischemia-reperfusion.[11, 12, 13] We have previously shown that gut macrophage populations are normally maintained through continuous recruitment and *in situ* differentiation of circulating monocytes in the *lamina propria*.[14, 15] Because preterm infants have a limited circulating monocyte pool[16] and lack significant reservoirs of mature monocytes in the bone marrow or elsewhere,[17] we hypothesized that a massive influx of circulating monocytes into intestinal tissue during NEC in a preterm infant will result in an acute drop in peripheral blood monocyte counts and may help differentiate early NEC from other causes of feeding intolerance. To investigate this hypothesis, we compared absolute monocyte counts (AMC) in peripheral blood obtained at the time of onset of feeding intolerance in all patients treated for confirmed NEC at our center during the last 10 years and compared these with counts from matched controls with feeding

intolerance due to causes other than NEC. We then validated our findings in a small cohort of infants with NEC from another center.

PATIENTS AND METHODS

A retrospective chart review was performed on very-low-birth-weight (VLBW) infants with a diagnosis of NEC at the University of Illinois Hospital during Jan 2001–Jun 2011, after approval by the Institutional Review Board. We used a nested case-control format, where infants with a diagnosis of NEC (Bell stages II or III)[4] and for each case, 3–4 controls were identified based on the date of admission (±3 months), gestational age (±1 week), birth weight (±200 g), and the presence of feeding intolerance but lack of suggestive clinical features (tenderness, abdominal wall erythema, or abdominal mass) and radiological signs (*pneumatosis*, fixed bowel loop, bowel wall thickening, and/or portal venous air) or histopathological evidence (coagulative necrosis, *pneumatosis*, bacterial overgrowth, and inflammation) of NEC. Feeding intolerance was defined as the presence of ≥2 of the following criteria: abdominal distension, pre-feeding residuals ≥30% of the feeding volume, emesis, diarrhea, or bloody stools, resulting in radiological evaluation and temporary cessation of feedings. Infants with major congenital anomalies and spontaneous bowel perforations in the 1st postnatal week were excluded.

Demographic characteristics including birth weight, gestational age, gender, ethnicity (African-American, Caucasian, Latino, or other), and mode of delivery were noted. We also recorded clinical information including Apgar scores, blood culture-proven sepsis prior to onset of feeding intolerance, central line, patent *ductus arteriosus* (PDA), indomethacin therapy, intraventricular hemorrhage (IVH), and age of onset of NEC or feeding intolerance. Data retrieved from complete blood counts (CBC) included the date of the test, white cell counts (WCC), absolute neutrophil counts (ANC), absolute lymphocyte counts (ALC), and the AMC. These data were obtained from the day of onset of feeding intolerance, from the last available CBC drawn prior to the onset of feeding intolerance, and from 3 follow-up CBCs. All CBCs were performed at the clinical laboratory of the UI hospital using Siemens-Bayer Advia 2120 automated hematology counters (Siemens Medical Solutions, Hoffman Estates, IL).

Statistical Analysis

Statistical analysis was performed using the Sigma Stat 3.1.1 software (Systat, Point Richmond, CA). Data were classified as parametric if 4 conditions were met: (1) continuous scale; (2) equal difference between consecutive data points; (3) normality, evaluated by Shapiro-Wilk test; and (4) equality of variance, evaluated by Levene's test.[18] Clinical characteristics were compared by the Mann-Whitney *U* test,[19] whereas the frequency of risk factors in various groups was compared by the Fisher's exact test.[20] We normalized the WCC, ANC, ALC, and AMC values recorded at onset of feeding intolerance against the last available value prior to the onset of feeding intolerance. Serial blood counts were compared using the Wilcoxon's signed rank test[21] or the Friedman's repeated measures analysis of variance on ranks.[22, 23] AMC data were depicted using Tukey-Koopman box-whisker plots.[24] All statistical tests were 2-sided and considered significant at $p < 0.05$. A compound-

symmetry form was assumed for repeated measurements.[25] Model-based results were accepted as unbiased if missing data were randomly distributed.

We next computed receiver-operating characteristics (ROC) of AMC values by plotting sensitivity *vs*. 1 – specificity.[26] To identify a 'cut-off' value for normalized AMC with the best diagnostic effectiveness, we picked the normalized AMC point with the highest sum of sensitivity and specificity (Youden's *J* statistic).[27] The ability of this cut-off value to discriminate between infants with NEC *vs*. those with feeding intolerance from other causes was determined by computing sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios from a 2 × 2 confusion matrix.[28] Finally, to determine whether the diagnostic accuracy of normalized AMC could be improved by including other clinical characteristics in the ROC model, we performed logistic regression and compared full (with all variables) *vs*. reduced models to determine whether these predictors were associated with NEC.

Validation of findings in an independent cohort of patients

To test the diagnostic accuracy of AMC as a test for NEC, we reviewed the medical records of infants treated for confirmed NEC at Loyola University Medical Center, Maywood, IL during the period Jan 2007 – Jan 2011 and retrieved data on clinical characteristics and blood counts. We then tested our ROC model in this cohort to determine the percentage of patients who were correctly identified as having NEC.

RESULTS

We reviewed the medical records from all 1654 VLBW infants admitted to the level III NICU at the University of Illinois hospital between Jan 2001- Jun 2011 and identified 72 patients with a diagnosis of NEC. Three infants who developed bowel perforation within the 1st postnatal week were excluded after chart review because a diagnosis of spontaneous intestinal perforation was considered more likely than NEC in these infants. Thus, in our final analysis, we included 69 infants with confirmed NEC. These were compared with 257 control infants, who had a total of 261 episodes of feeding intolerance that were not due to NEC.

Demographic characteristics

The demographic characteristics of infants in the NEC and control groups are summarized in Table 1. Infants in the NEC group were more likely to have been delivered vaginally (59.1 *vs*. 41% in NEC and control groups, respectively; $p = 0.006$), transferred from an outside hospital (30.4 *vs*. 17.5% in NEC and control groups, respectively; p = 0.027), but were less likely to have had a central line (59.4 *vs*. 73.9% in NEC and control groups, respectively; p = 0.025) or a history of a positive blood culture during hospital stay (17.4 *vs*. 33.5% in NEC and control groups, respectively; p = 0.012). Infants in the control group developed feeding intolerance at an earlier postnatal age than those with NEC (median 12, range 7–23 days in control group *vs*. a median of 20, range 12–31 days in infants with NEC; $p < 0.001$).

Clinical characteristics

In the NEC group, 25 (36.2%) and 44 (63.8%) infants were classified as Bell stage II and III, respectively. In the NEC group, survivors had a longer length of hospital stay than controls (Table 2). As anticipated, there were more deaths in the NEC group ($p < 0.001$). Pre-feed residuals were recorded more often in the feeding intolerance group (76.6 *vs*. 60.9% in feeding intolerance and NEC groups, respectively; $p = 0.004$). The NEC group had a higher frequency of respiratory distress, apnea, and acidosis. Frank bleeding per rectum was recorded in 34.8% NEC patients but not in controls ($p < 0.0001$).

Blood counts

In our NEC group, 59/69 (85.5%) patients had a CBC in the chart that was performed median 3.5 days [inter-quartile range (IQR) 1–6 days] prior to the onset of symptoms. Sixty of the 69 (86.9%) cases had a CBC drawn on the day of onset of symptoms. Patients with a missing prior CBC had been transferred from another hospital following onset of NEC. Sixty-seven (97.1%) had a follow-up CBC drawn after median 1 day (IQR 1–1.75 days). A 2nd follow-up CBC was available in 61 (88.4%) patients drawn at median 2 days (IQR 2–3 days) after onset of NEC, whereas 53 (76.8%) had a 3rd follow-up CBC drawn at median 3 days (IQR 3–4 days). In the control group, 258/261 (98.8%) patients had a CBC from median 2 days (IQR 1–4 days) prior to the onset of symptoms. One hundred ninety-five (74.7%) had a CBC drawn on the day of onset of symptoms, whereas 253 (96.9%) had another CBC drawn after median 1.5 days (IQR 1–3 days). A 2nd follow-up CBC was available in 224 (85.8%) infants drawn at median 3.7 days (IQR 2.2–6.5 days) after onset of symptoms, whereas 53 (76.8%) had a 3rd CBC drawn at median 5.7 days (IQR 3.7–9 days).

Compared to the pre-symptomatic AMC, monocyte counts were significantly lower in patients with both Bell stage II and III disease on the day of onset of NEC and in the 1st follow-up CBC (Fig. 1). In patients with stage II disease, AMC decreased from median 1.7×10^9 cells/L (IQR 0.98 to 2.4) to median 0.8 (IQR 0.62 to 2.1); $p < 0.05$, whereas in those with stage III NEC, the AMC decreased from median 2.1×10^9 cells/L (IQR 0.1.5 to 3.2) to median 0.8 (IQR 0.6 to 1.9); $p < 0.05$. The WCC, ANC, and ALC did not change significantly (*not depicted*). In the control group, there were no significant changes in the AMC or the WCC, ANC, and ALC. Within the control group, there was no difference in these counts between infants with positive blood cultures and presumed ileus *vs*. others with 'idiopathic' feeding intolerance.

Receiver-operator characteristics

We next investigated whether AMC values obtained at onset of feeding intolerance could discriminate between NEC *vs*. feeding intolerance from other causes. To account for the variability in blood monocyte counts in neonates,[16] we normalized AMC at onset of symptoms against the last available pre-symptomatic AMC for each infant and then computed ROCs curves using normalized AMC (Fig. 2). The area under the curve was 0.76 [95% confidence interval (CI) 0.69 to 0.83; $p < 0.0001$], indicating "fair-to-good" diagnostic accuracy. To determine whether the inclusion of clinical characteristics could improve the diagnostic accuracy of our model, we performed logistic regression to identify covariates

associated with NEC. We identified birth weight, ethnicity, and sepsis as significant but the inclusion of these parameters in the ROC model did not improve its diagnostic accuracy.

To identify a 'cut-off' value with maximum diagnostic effectiveness, we computed (sensitivity + specificity) for each normalized AMC and picked normalized AMC = 0.8, which provided the highest summated value (Youden's *J* statistic) of 1.4 (Fig. 2). At this cut-off value, the sensitivity was 0.70 (95% CI 0.57–0.81), the specificity was 0.71 (95% CI 0.64 to 0.77), the positive predictive value was 0.43 (95% CI 0.33 to 0.54), and the negative predictive value was 0.88 (95% CI 0.81 to 0.92). Table 3 summarizes the clinical effectiveness of this test.

Validation of findings in an independent cohort of patients

We tested our diagnostic cut-off in an independent cohort of VLBW infants treated for confirmed NEC at Loyola University Medical Center. Twenty-three patients were identified, including 17 (74%) and 6 (26%) with Bell stages II and III, respectively. These patients had a median birth weight of 750 g (range 630–1319), gestation 26.5 weeks (range 24.5–30), and except for a higher number of Latino infants (10/23, 43.5%), had clinical characteristics similar to the primary cohort. A CBC was available from prior to onset of NEC in 21 (91%) and in all patients on the day of onset and at median 5 days (IQR 2.5 to 7 days). AMC decreased from median 1.5×10^9 cells/L (IQR 1.1 to 2.8) in the pre-symptomatic CBC to median 0.9×10^9 cells/L (IQR 0.5 to 1.9) at the time of NEC ($p = 0.03$). A diagnostic cut-off of normalized AMC <0.8 correctly identified 14/23 (66.7%) patients as having NEC, which was consistent with our ROC model.

DISCUSSION

We show that a fall in peripheral blood AMC can be a useful diagnostic marker of NEC in VLBW infants. At the time of onset of feeding intolerance, an acute drop in AMC (from the last available test) correctly discriminated between NEC *vs*. other causes of feeding intolerance with 76% accuracy. To our knowledge, this is the first study to show that blood monocyte counts can serve as an adjunctive diagnostic test for NEC. Although this information is already available in CBC reports generated from hematology analyzers, most neonatologists currently do not evaluate monocyte counts routinely in their practice. Previous studies have identified several candidate biomarkers of NEC such as the inter-alpha inhibitor protein, intestinal fatty acid-binding protein, hexosaminidase, proapolipoprotein CII, and serum amyloid A.[29, 30, 31, 32, 33, 34] However, despite its modest diagnostic accuracy, the AMC is an attractive marker of NEC because (1) the information is already available to the clinician at no extra cost; and (2) its high negative predictive value (88%) can help exclude the diagnosis of NEC in infants with feeding intolerance due to other causes. Because most clinical laboratories now use automated hematology counters, there are additional advantages of rapid turnaround times, a high degree of consistency, and the ease of extrapolation of findings to other centers.

In the present study, our hypothesis that NEC is associated with decreased blood monocyte concentrations emerged from our preclinical observations of macrophage-rich infiltrates in NEC.[10] Because macrophages in inflammatory gastrointestinal lesions are derived from

blood monocytes,[15] we reasoned that the rapid efflux of monocytes to NEC lesions is likely to deplete the limited circulating pool of monocytes in premature neonates.[16] Interestingly, decreased blood monocyte counts are likely to be a unique feature of NEC. Growth-restricted preterm infants may have low monocyte counts but most of these infants show a suppression of all leukocyte lineages and not isolated monocytopenia.[35] In the NICU, monocytosis may be seen more frequently than monocytopenia and may occur in association with extreme prematurity, repeated RBC transfusions, albumin infusions, and theophylline therapy.[36] Monocytosis can also be seen in congenital candidiasis and syphilis,[37, 38] and in immune-mediated neutropenia.[39, 40]

We normalized monocyte counts against the last available pre-symptomatic AMC to account for normal variability in blood monocyte counts in premature neonates. We recently developed reference ranges of AMC in neonates using data retrieved from over 62,000 CBCs,[16] where we showed that blood monocyte concentrations increase almost linearly between 22–42 weeks gestation.[16] Monocyte concentrations also increased during the first 2 postnatal weeks.[16] These data are consistent with kinetic studies in human fetuses, which show a similar maturational increase in monocyte precursors.[41, 42]

In our study, infants in the NEC group had a later onset of feeding intolerance compared to the control group. Control infants had a higher frequency of blood culture-positive sepsis and of having had a central line, suggesting that the abdominal signs in some of these infants may be explained by sepsis-related ileus, which may peak at an earlier postnatal age than NEC.[43, 44] Although monocytosis has been noted in neonatal infections, we did not detect a significant difference in monocyte counts in infants in the control group with feeding intolerance due to sepsis *vs*. other controls with feeding intolerance of unknown origin. Overall, the NEC group had a higher incidence of systemic signs, gastrointestinal bleeding, and mortality, indicating a higher acuity of illness than controls.

A major limitation of our study is its retrospective design, which increases the risk of bias.[45] Considering the limited sample size, our findings need further validation in larger/multi-centric cohorts and in larger infants with NEC. Further study is also needed to evaluate maternal/neonatal covariates known to be associated with NEC such as abnormal fetal umbilical Doppler signatures and chorioamnionitis, feeding practices, anemia, transfusions, and infections.[46, 47, 48] The relevance of AMC in spontaneous bowel perforations also remains uncertain. Although most neonatologists view spontaneous perforations and NEC as distinct entities, the two conditions may comprise a clinical continuum and may be difficult to distinguish from each other on the basis of clinical signs, histopathological findings, and even inflammatory markers.[49]

CONCLUSIONS

We have identified a fall in AMC as a novel biomarker for NEC in VLBW infants. When compared to the last available AMC from prior to onset of feeding intolerance, an acute drop in blood monocyte concentration can identify NEC with 76% accuracy. In a given infant with feeding intolerance, a fall in AMC by >20% indicated NEC with sensitivity of 0.70 (95% CI 0.57–0.81) and specificity of 0.71 (95% CI 0.64 to 0.77). This test offers a high

negative predictive value (88%), which can help exclude the diagnosis of NEC in infants with feeding intolerance due to other causes.

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Abbreviations

NICU	neonatal intensive care unit
AMC	absolute monocyte counts
NEC	necrotizing enterocolitis
PDA	patent *ductus arteriosus*
IVH	intraventricular hemorrhage
VLBW	very low birth weight
FI	feeding intolerance
IQR	inter-quartile range
CI	confidence interval

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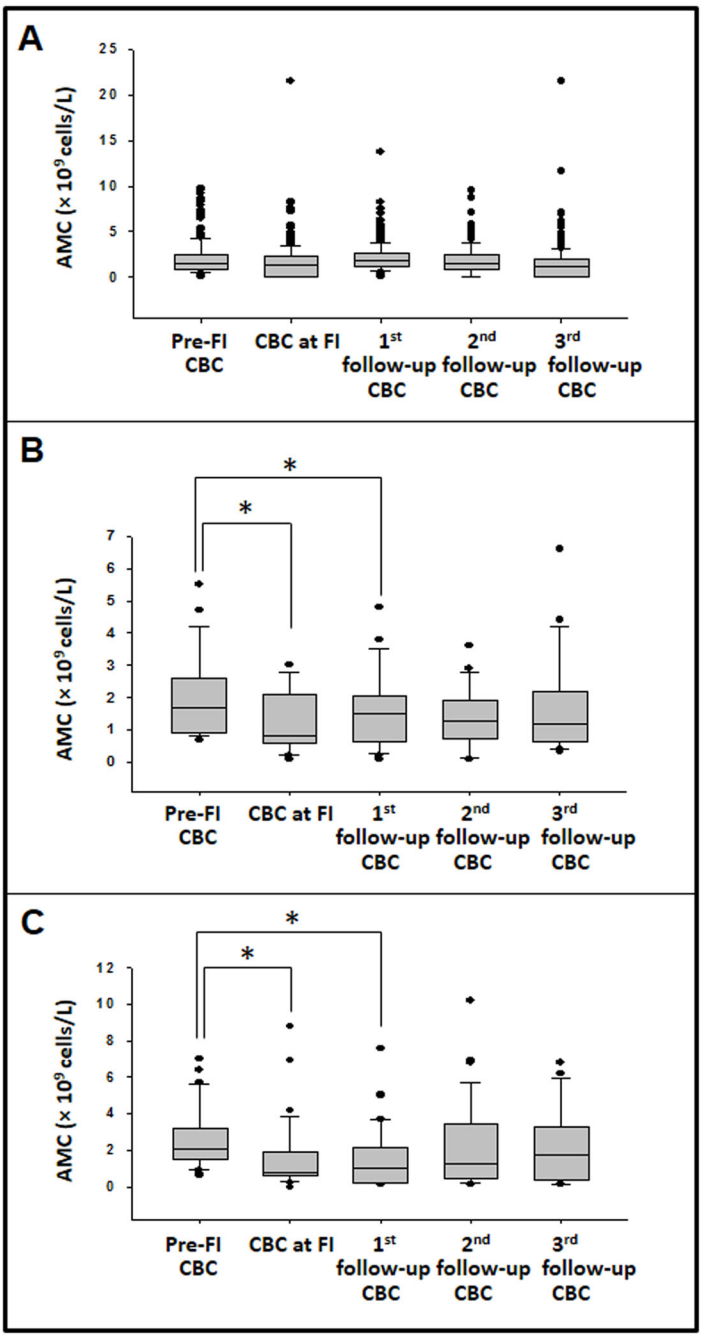

Figure 1. Longitudinal change in peripheral blood AMC in control and NEC groups

Box-whisker plots show AMC in (A) controls, (B) infants with NEC stage II, and (C) those with NEC stage III. Data were compared by repeated measures ANOVA on ranks with Dunnett's test using AMC prior to feeding intolerance as the comparison group.

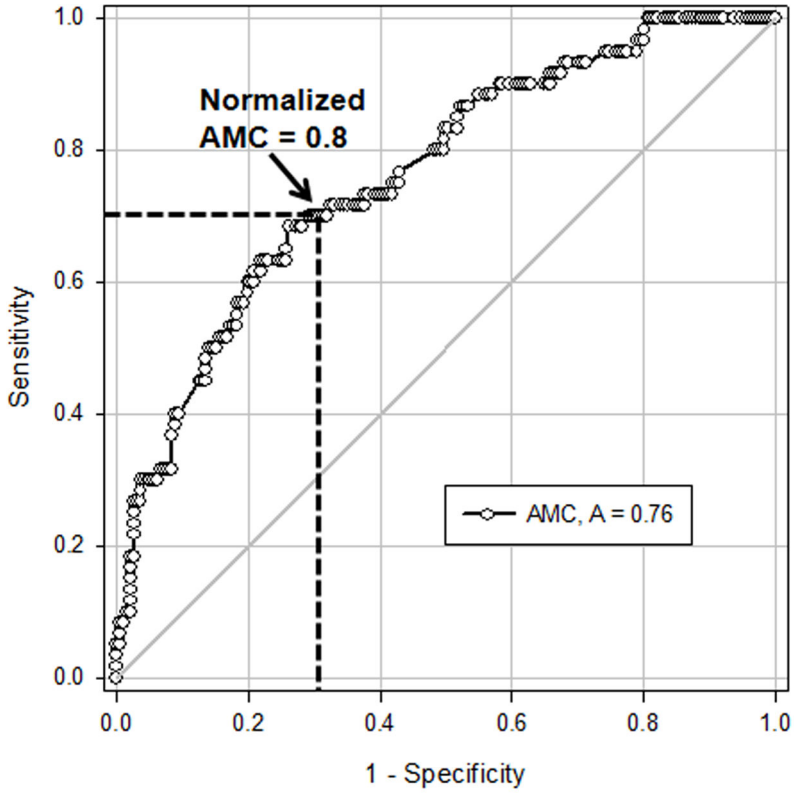

Figure 2. Diagnostic accuracy of decreased peripheral blood AMC as a test for NEC

Receiver-operator characteristics of the ratio of AMC in infants at the time of feeding intolerance *vs*. AMC in the most recent CBC drawn prior to the onset of feeding intolerance show that a >20% drop in AMC correctly identified NEC in 76% cases (depicted by the area under the curve). A cut-off value of 0.8 (marked by broken lines in the figure) provided 70% sensitivity and 70.6% specificity.

Table 1

Demographic characteristics

Characteristic	Feeding Intolerance (n=257*)	Necrotizing Enterocolitis (n=69)	p-value
Birth weight (g); median (interquartile range)	968 (771–1186)	945 (718–1200)	
Gestational age (weeks); median (interquartile range)	27 (26–29)	27 (26–29)	
Male sex – n (%)	133 (51.8)	38 (55)	
Ethnicity – n (%)			
African-American	191 (74.3)	49 (71)	
Caucasian	17 (6.6)	7 (10.1)	
Latino	38 (14.8)	13 (18.8)	
Mode of delivery			
Cesarean section	152 (59.1)	28 (41)	0.006
Vaginal	105 (41)	41 (59.1)	0.006
Outborn (%)	45 (17.5)	21 (30.4)	0.027
5-min Apgar <6 – n (%)	36 (14)	9 (13.1)	
PDA – n (%)	125 (48.6)	33 (48)	
Indomethacin – n (%)	79 (30.7)	19 (27.5)	
IVH ≥Grade 2 – n (%)	24 (9.3)	11 (16)	
Central line† – n (%)	190 (73.9)	41 (59.4)	0.025
Positive blood culture‡ – n (%)	86 (33.5)	12 (17.4)	0.012
Onset of feeding intolerance (postnatal age in days); median (interquartile range)	12 (7–23)	20 (12–31)	<0.001

PDA, Patent ductus arteriosus; IVH, intraventricular hemorrhage.

†Placed before diagnosis of NEC.

‡Developed before diagnosis of NEC.

*261 cases of feeding intolerance in 257 patients

Table 2

Clinical characteristics

Characteristic	Feeding Intolerance (n=257)	Necrotizing Enterocolitis (n=69)	p-value
Length of Stay (days); median (interquartile range)	69 (47–93)	79.5 (34.7–124.7)	
Length of stay in survivors (days)	69 (47–93)	105 (34.7–142)	<0.01
Died - n (%)	17 (6.6)	23 (33.3)	<0.0001
Presentation			
Pre-feed Residuals - n (%)	197 (76.6)	42 (60.9)	<0.01
Abdominal Distention - n (%)	221 (86)	61 (88.5)	
Frank bleeding per Rectum - n (%)	0	19 (27.5)	<0.0001
Other (apnea, respiratory distress, acidosis) - n (%)	0	43 (62.3)	<0.0001
Radiological Signs			
Pneumatosis - n (%)	0	60 (87)	
Fixed Bowel Loop - n (%)	0	10 (14.5)	
Free Intraperitoneal Air - n (%)	0	11 (16)	
Portal Venous Gas - n (%)	0	11 (16)	
Surgery			
Exploratory laparotomy - n (%)	Not applicable	36 (52.1)	
Peritoneal drain - n (%)	Not applicable	16 (23.2)	

Table 3

Diagnostic value of decreased peripheral blood AMC as a test for NEC

	No NEC	NEC	Total
>20% drop in AMC	54	41	95
Increased or ≤20% drop in AMC	137	19	156
Total	**191**	**60**	**251**

	Value (95% CI)
Prevalence	0.24 (0.19–0.30)
Sensitivity	0.70 (0.57–0.81)
Specificity	0.71 (0.64–0.77)
Positive predictive value	0.43 (0.33–0.54)
Negative predictive value	0.88 (0.81–0.92)
Positive likelihood ratio	2.42 (1.82–3.21)
Negative likelihood ratio	0.44 (0.30–0.64)