

Fecal Phagocyte-Specific S100A12 for Diagnosing Necrotizing Enterocolitis

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Objective To determine whether longitudinal measurements of fecal S100A12, a fecal marker of intestinal inflammation, can identify very low birth weight infants at risk for necrotizing enterocolitis (NEC).

Study design This prospective study included 145 preterm infants with birth weight <1500 g. Meconium and stool samples (n = 843) were collected prospectively on alternate days for 4 weeks, and fecal S100A12 and calprotectin were measured by enzyme-linked immunosorbent assay.

Results Eighteen patients (12.4%) developed NEC. Gestational age and birth weight were significantly lower in the patients with NEC compared with unaffected reference infants. Fecal S100A12 levels were significantly higher in patients with severe NEC at onset of disease and also, in contrast to fecal calprotectin, at 4-10 days before onset of NEC compared with unaffected reference infants (ideal cutoff value, 65 μ g/kg; sensitivity, 0.76; specificity, 0.56).

Conclusions Fecal S100A12 level may be a helpful marker for predicting disease severity and early risk assessment for subsequent development of NEC. However, the use of fecal S100A12 as a predictive biomarker for NEC in very low birth weight infants may be limited due to a high interindividual and intraindividual variability in S100A12 fecal excretion. (*J Pediatr* 2012;161:1059-64).

Necrotizing enterocolitis (NEC) is a serious gastrointestinal disorder of preterm infants,¹ with an incidence of 10%-15% and a mortality rate of 10%-30% in very low birth weight (VLBW) infants (birth weight <1500 g), as well as a high rate of long-term complications.²⁻⁴ The pathogenesis of NEC remains largely unknown but is suspected to be multifactorial, involving such factors as gastrointestinal ischemia, enteral alimentation, and microorganisms in combination with immature gastrointestinal functions and host defense mechanisms. Early detection of NEC and adequate intervention could possibly improve the prognosis; however, initial clinical manifestations of NEC are nonspecific and are difficult to distinguish from other gastrointestinal disorders and neonatal sepsis. Thus, diagnosis is often delayed and limited by insufficient serum-based laboratory tests and current imaging modalities. Conventional fecal inflammatory markers (eg, calprotectin, lactoferrin) or urinary markers for enterocyte damage (eg, intestinal fatty acid binding protein) have not met the initially high expectations for diagnosing NEC.^{1,5}

More recently, fecal S100A12 (calgranulin C) has been identified as a fecal marker of intestinal inflammation. S100A12 belongs to a novel group of proinflammatory molecules that play important roles in the mechanisms of innate immunity. In contrast to pathogen-associated molecular patterns proteins as exogenous factors initiating inflammation, S100A12 is one of the damage-associated molecular pattern proteins, endogenous molecules released by activated or damaged cells under conditions of cell stress. Phagocyte-specific damage-associated molecular pattern proteins of the S100 family are released from neutrophils or monocytes, followed by proinflammatory activation of pattern recognition receptors.⁶⁻⁹ Thus, in intestinal inflammation, S100A12 is readily detectable in feces and plasma.^{10,11} Furthermore, S100A12 is remarkably resistant to degradation by fecal bacteria, making fecal S100A12 a suitable marker for gut wall inflammation, as has been reported for inflammatory bowel disease.¹² The small amount of feces (50-100 mg) required for the measurement of fecal S100A12 by enzyme-linked immunosorbent assay (ELISA) makes this test potentially useful in preterm infants. We analyzed fecal S100A12 levels in VLBW infants to obtain baseline expression characteristics and to evaluate this protein's role as biomarker of intestinal inflammation in the screening for NEC and prediction of disease severity.

| | |
|-------|-----------------------------------|
| CRP | C-reactive protein |
| GA | Gestational age |
| ELISA | Enzyme-linked immunosorbent assay |
| IL-6 | Interleukin 6 |
| NEC | Necrotizing enterocolitis |
| ROC | Receiver operating characteristic |
| VLBW | Very low birth weight |

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Methods

Patients were recruited from 5 German tertiary neonatal intensive care units (Wuppertal, Schwerin, Erfurt, Münster, and Krefeld) between April 2008 and December 2009. Ethical approval was obtained from the Ethics Committee of Witten/Herdecke University for all participating hospitals, and fully written informed consent was obtained from all legal guardians. All preterm infants with a birth weight <1500 g were included. Meconium and stool samples were collected prospectively on alternate days for at least 28 days. On admission, baseline characteristics and maternal information for enrolled infants were recorded. In addition, postnatal age, daily feeding regimen, respiratory support, laboratory and radiograph results, and clinical findings were recorded at the collection of each stool sample throughout follow-up.

Diagnosis of NEC was established based on typical clinical features and radiologic and laboratory findings. Disease stage (I, II, or III) was determined using the modified Bell classification scheme.^{3,13} In brief, this classification scheme differentiates infants with NEC stage I (suspect), stage IIa (definite, mildly ill), stage IIb (definite, moderately ill), stage IIIa (advanced, severely ill with intact bowel), and stage IIIb (advanced, severely ill with bowel perforation).

The reference group consisted of all infants without signs and symptoms of intestinal distress. To obtain clear case definitions, patients without signs of NEC but with signs of other gastrointestinal disorders (eg, spontaneous intestinal perforation) were excluded from this study. Patients with an insufficient amount of stool to allow measurements of S100 proteins were excluded as well. Meconium was defined as the first stool passed after birth but no later than 72 hours after birth.

All stool samples were stored at -80°C before being analyzed within 24 hours of collection. S100A12 concentrations were determined by double-sandwich ELISA, as described previously.¹⁴ Fecal calprotectin concentrations were determined by ELISA (Immundiagnostik, Bensheim, Germany). Analyses were performed by investigators in Münster, Germany who were blinded to the diagnosis and disease stage. All analyses were performed in triplicate.

Statistical Analyses

Statistical comparisons of the data between groups (unaffected control vs NEC) were performed using the Mann-Whitney *U* test. Data are presented as median and range except when stated otherwise. To determine the accuracy of S100A12 measurements, receiver operating characteristic (ROC) curves were drawn by plotting sensitivity against 1 - specificity. Overall accuracy of the markers in detecting NEC is represented by the area under the ROC curve with 95% CI. The best cutoff point is defined as the maximum sum of sensitivity and specificity. These cutoff points were used to calculate sensitivity, specificity, and positive and negative predictive values. All tests of significance were 2-tailed. A *P* value of <.05 was considered significant. All calculations

were performed using SPSS version 14 (SPSS Inc, Chicago, Illinois).

Results

NEC in Patients

We enrolled 145 infants, including 18 (12.4%) who subsequently developed NEC and 127 without NEC or any other gastrointestinal distress (reference group) (Table). We excluded 5 patients with spontaneous intestinal perforation and 8 patients with insufficient stool samples. A total of 843 meconium and postmeconium stool samples were collected and analyzed. Gestational age (GA) and birth weight were significantly lower in the patients with NEC. According to the modified Bell scheme, 5 patients were classified as NEC stage IIa, 3 as stage IIb, 5 as stage IIIa, and 5 as stage IIIb. Eleven patients with NEC (61%) required surgery (all laparotomy), which confirmed the diagnosis of NEC. The mortality rate was 25% ($n = 2$) for patients in NEC stage II, 30% ($n = 5$) for those in NEC stage III, and 0.7% ($n = 1$) for those without NEC or gastrointestinal disorders. Causes of death were sepsis (*Klebsiella oxytoca*) with multiorgan failure, septic shock (*Clostridium difficile*) during surgery, and total intestinal necrosis (duodenum to rectum) with respiratory insufficiency in the patients with NEC stage III; septic shock without detectable pathogens and severe sepsis (*Candida glabrata*) in those with NEC stage II; and severe cerebral and pulmonary hemorrhage in the reference group.

S100A12 in Meconium

S100A12 concentrations were higher in meconium samples from VLBW infants before the onset NEC stage III (median, 398 $\mu\text{g}/\text{kg}$; range, 70-94 000 $\mu\text{g}/\text{kg}$; $n = 10$; $P < .05$) and lower

Table. Patient characteristics

| | Control | NEC | <i>P</i> value |
|---|------------------|------------------|----------------|
| Patients, n (%) | 127 (87.6) | 18 (12.4) | NA |
| GA, weeks, median (range) | 28.4 (23-35) | 25.5 (23-33) | <.0005 |
| Birth weight, g, median (range) | 1082 (436-1490) | 773 (354-1300) | <.0005 |
| Sex ratio, M/F | 0.91 | 1.71 | NS |
| Maternal age, years, median (range) | 30 (17-45) | 31 (22-41) | NS |
| Apgar score at 1 minute, median (range) | 6 (1-9) | 5 (1-8) | NS |
| Apgar score at 5 minutes, median (range) | 7 (2-10) | 6 (4-9) | NS |
| Apgar score at 10 minutes, median (range) | 9 (5-10) | 8 (6-10) | NS |
| Umbilical artery pH, median (range) | 7.34 (7.04-7.50) | 7.34 (7.20-7.47) | NS |
| Vaginal birth, % | 7.4 | 5.6 | NS |
| Stool samples, n | 671 | 172 | NA |
| NEC stage IIa/IIb/IIIa/IIIb, n | NA | 5/3/5/5 | NA |
| Weight at diagnosis, g, median (range) | NA | 834 (506-1520) | NA |
| Age at diagnosis, days, median (range) | NA | 13 (2-28) | NA |

NA, not applicable; NS, nonsignificant.

in samples from VLBW infants before the onset of NEC stage II (median, 5 $\mu\text{g}/\text{kg}$; range, 5-175 $\mu\text{g}/\text{kg}$; $n = 7$; $P < .01$) compared with reference infants (median, 86 $\mu\text{g}/\text{kg}$; range, 5-91 000 $\mu\text{g}/\text{kg}$; $n = 128$). No statistically significant correlations with S100A12 level were found in meconium and neonatal factors (ie, birth weight, sex, GA, Apgar score, pH at birth, enteral feeding regimen, corticosteroid use, and C-reactive protein [CRP]) or maternal factors (ie, preeclampsia, diabetes, amnionitis, premature rupture of membranes, and mode of delivery).

S100A12 Levels in Postmeconium Stool

In unaffected infants, fecal S100A12 levels decreased with increasing postnatal age. Median levels were 89 $\mu\text{g}/\text{kg}$ within the first week after birth, 33 $\mu\text{g}/\text{kg}$ during the following 3 weeks, and 15 $\mu\text{g}/\text{kg}$ after 28 days of life (Figure 1, A). During the second to fourth weeks after birth, overall fecal S100A12 levels were significantly higher in VLBW infants with suspected NEC who subsequently developed NEC (median, 116 $\mu\text{g}/\text{kg}$; range, 5-93 000 $\mu\text{g}/\text{kg}$; $n = 104$; $P < .0001$) compared with those with no gastrointestinal disease (Figure 1, A). In contrast, S100A12 levels in postmeconium stool of neonates with NEC did not differ during the first week after birth (median, 90 $\mu\text{g}/\text{kg}$; range, 5-59 000; $n = 32$; $P =$ not significant) or after 4 weeks of life (median, 18 $\mu\text{g}/\text{kg}$) (Figure 1, A). Furthermore, CRP and interleukin 6 (IL-6) did not differ significantly between neonates with NEC and those without NEC (data not shown).

Fecal S100A12 at Onset of NEC and Correlation with Severity of NEC

Fecal S100A12 levels were significantly higher in patients with NEC at disease onset (median, 510 $\mu\text{g}/\text{kg}$; range, 5-93 000 $\mu\text{g}/\text{kg}$; $n = 18$) compared with unaffected reference infants (median, 100 $\mu\text{g}/\text{kg}$; range, 3-31 950 $\mu\text{g}/\text{kg}$; $n = 590$; $P < .05$). Furthermore, S100A12 levels were specifically increased in patients with NEC stage III (median, 11 200 $\mu\text{g}/\text{kg}$; range, 5-93 000 $\mu\text{g}/\text{kg}$; $n = 10$; $P < .01$). S100A12 levels were also elevated in patients with NEC stage II (median, 241 $\mu\text{g}/\text{kg}$; range, 5-5500 $\mu\text{g}/\text{kg}$; $n = 8$), but this difference was not statistically significant (Figure 1, B).

The differences between patients with NEC and control patients were not influenced by differences in GA. In patients with a GA of 24-27 weeks, fecal S100A12 levels did not differ between those without NEC (median, 92.5 $\mu\text{g}/\text{kg}$) and those who subsequently developed NEC (median, 92.5 $\mu\text{g}/\text{kg}$). The majority of the 18 patients with NEC developed NEC at a GA of 28-31 weeks (median, 28.1 weeks; range, 25.9-31.7 weeks). Consequently, at this GA range, fecal S100A12 levels were significantly higher in patients with NEC (median, 110.0 $\mu\text{g}/\text{kg}$; range, 5-44 000 $\mu\text{g}/\text{kg}$; $n = 65$) compared with controls (median, 35.0 $\mu\text{g}/\text{kg}$; range, 5-10 500 $\mu\text{g}/\text{kg}$; $n = 224$; $P < .005$). In patients with a GA of 32-37 weeks, fecal S100A12 levels remained unchanged in control patients without NEC (median, 35.0 $\mu\text{g}/\text{kg}$; range 5-2795 $\mu\text{g}/\text{kg}$; $n = 179$), and dropped to similar levels in patients with NEC (median, 25.2 $\mu\text{g}/\text{kg}$; range, 5-1600 $\mu\text{g}/\text{kg}$; $n = 13$, $P =$ not significant).

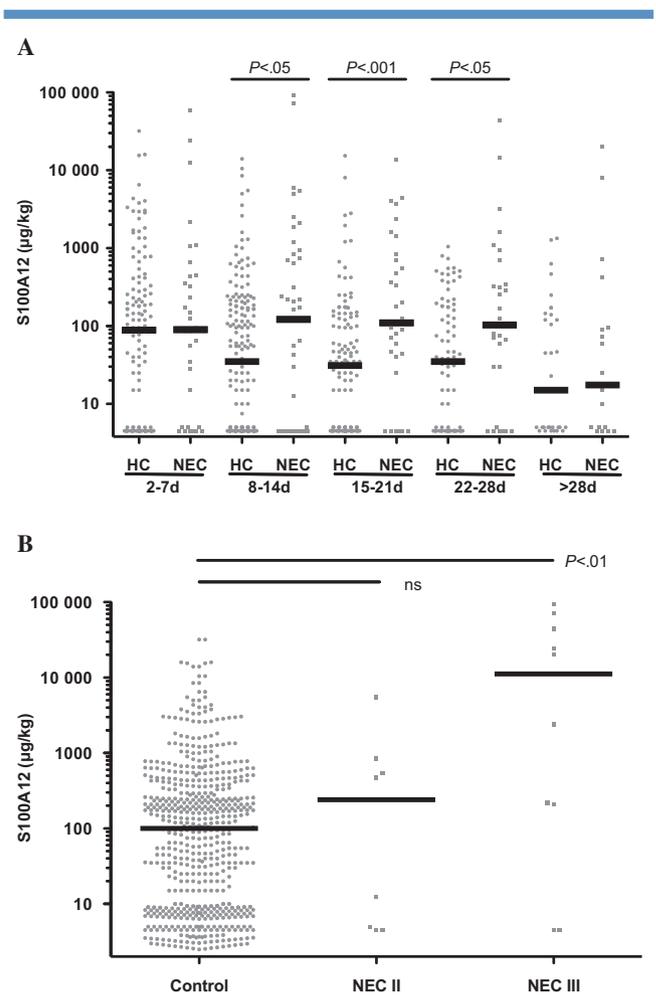


Figure 1. A total of 843 meconium and postmeconium stool samples were analyzed. The scatterplots show the median (central horizontal line) of fecal S100A12 levels. P values are shown (*ns*, not significant). **A**, Fecal S100A12 levels of 127 VLBW infants without gastrointestinal disease (unaffected controls [HCs]) were compared with 18 VLBW infants with NEC. The graph shows the S100A12 levels for both groups during the first, second, third, and fourth weeks of life and beyond. The decline in fecal S100A12 levels with increasing postnatal age in the control (reference) group was significant for 2-4 weeks after birth ($P < .05$) and after the fourth postnatal week ($P < .05$) compared with levels during the first week of life. **B**, Fecal S100A12 levels of 18 VLBW infants with NEC at the time of disease onset compared with fecal S100A12 levels of 590 stool samples of VLBW infants of the same GA without gastrointestinal disease (control). Patients with NEC are divided into those with NEC stage II ($n = 8$) and those with NEC stage III ($n = 10$) using the modified Bell classification scheme.

The cutoff value for differentiating all patients with NEC from those without NEC at the time of NEC onset was 210 $\mu\text{g}/\text{kg}$ (Figure 2). Because the patients with severe NEC and rapid deterioration tended to have even lower S100A12 levels before the onset of NEC compared with the

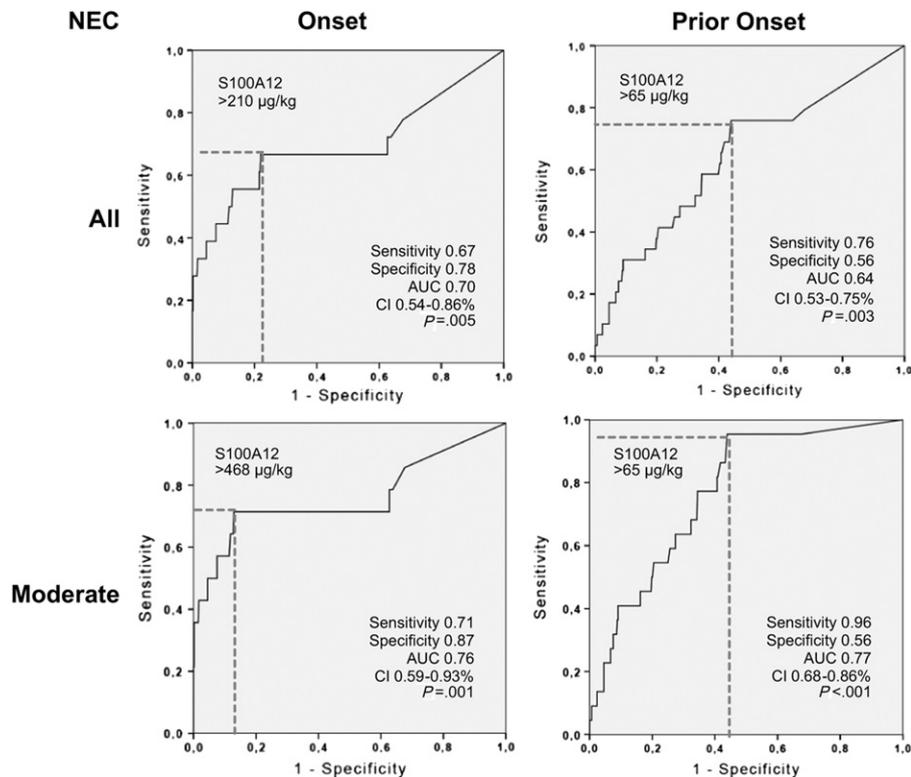


Figure 2. ROC curve analyses performed to analyze the sensitivity and specificity of fecal S100A12 in differentiating all infants with moderate/severe NEC (upper panels) or only moderate NEC (lower panels) from those without gastrointestinal disease (control) at disease onset (left panels) and within 1 week before onset (right panels). Area under the ROC curve (AUC), 95% CI, and P value are shown for each curve. The fecal S100A12 value that gives the best accuracy is shown.

reference population, ROC curves were also calculated for patients with moderate NEC, leading to a higher cutoff value and improved sensitivity and specificity (Figure 2). To assess whether fecal S100A12 can be used as a marker to predict whether a patient will need surgery, patients with NEC were divided into those with stage II disease and those with stage III disease. On suspicion of NEC, fecal S100A12 levels were higher in infants who subsequently developed bowel perforation than in those who did not (median, 2400 µg/kg [range, 5-93 000 µg/kg; n = 13] vs 122 µg/kg [range, 5-24 500 µg/kg; n = 17]; $P < .05$). CRP and IL-6 levels were not related to disease severity (data not shown).

Prediction and Monitoring of NEC

Time course analysis of fecal S100A12 at 1-3 weeks before and 1-3 weeks after onset of NEC showed significantly elevated S100A12 levels at 4-10 days before disease onset (median, 95 µg/kg; range, 5-94 000 µg/kg; n = 39; $P < .005$) and for up to 7-14 days after onset (median, 285 µg/kg; range, 5-8050 µg/kg; n = 42; $P < .0005$) compared with unaffected neonates whose stool specimens were collected at a similar GA and postnatal age (median, 33 µg/kg; range, 5-15 400 µg/kg; n = 381) (Figure 3, A). The ideal cutoff value (sensitivity, 0.76; specificity, 0.56) for identifying patients with NEC

within 7 days before disease onset was 65 µg/kg, regardless of whether all patients with NEC or only patients with NEC with moderate course of the disease were considered (Figure 2). This resulted in an overall sensitivity of 60%, a specificity of 55%, a positive predictive value of 25%, and a negative predictive value of 84%. Sensitivity, specificity, positive predictive value, and negative predictive value were dependent on the underlying observation period: meconium: 67%, 41%, 14%, and 90%, respectively; 2-7 days after birth: 59%, 45%, 21%, and 84%; 8-14 days after birth: 52%, 58%, 23%, and 84%; 15-21 days after birth: 70%, 68%, 37%, and 89%; 22-28 days after birth: 68%, 59%, 37%, and 84%; more than 28 days after birth: 42%, 66%, 42%, and 66%.

Fecal calprotectin concentrations of identical samples were analyzed in parallel, and time course analyses of fecal calprotectin levels at 1-3 weeks before and 1-3 weeks after onset of NEC were performed. Although fecal calprotectin levels were also elevated at the onset of NEC (median, 349 mg/kg; range, 1-850 mg/kg; n = 18; $P < .05$) and 48 hours before onset (median, 83 mg/kg; range, 1-807 mg/kg; n = 9; $P < .05$), the differences at 48-240 hours before onset and after onset did not reach statistical significance (Figure 3, B). In addition, fecal calprotectin levels were not correlated with disease severity (data not shown). Likewise, CRP and IL-6 are not suitable

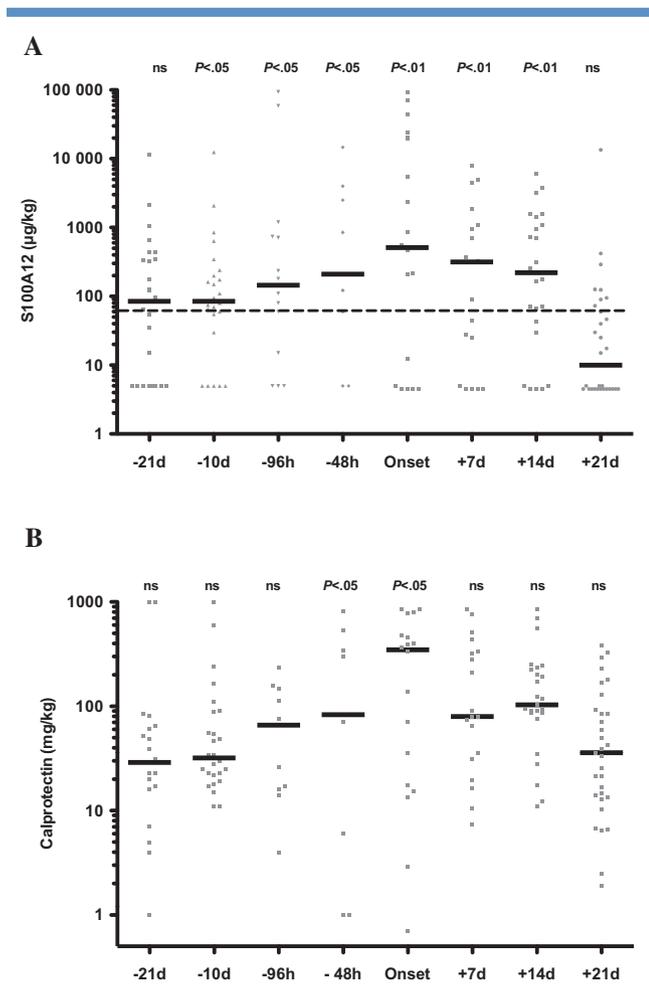


Figure 3. Time course analysis of fecal S100A12 and calprotectin levels in infants with NEC. A total of 159 stool samples of 18 VLBW infants with NEC were analyzed at different time points before disease onset (12-48 hours, 48-96 hours, 5-10 days, and 11-21 days), after disease onset (1-7 days, 8-14 days, and 15-21 days), and at the time of disease onset. The scatterplots show the median levels (*central horizontal line*) of **A**, fecal S100A12 and **B**, fecal calprotectin. The ideal S100A12 cutoff point for differentiating neonates with and without NEC (65 µg/kg) is represented by the *dotted line*. *P* values are shown.

for screening for NEC before clinical suspicion is raised (data not shown).

Discussion

Previous studies investigating the role of fecal calprotectin in the diagnosis of NEC have reported divergent results^{1,2,5,15-19} and because of high intervariability and intravariability, fecal calprotectin is a poor marker for NEC. Several calprotectin thresholds for suspicion of NEC have been proposed (eg, 636 µg/g by Campeotto et al,¹⁹ 200 mg/L by Carroll et al,¹⁵ 2000 µg/g by Josefsson et al,² 286 µg/g by Thuijls et al⁵). More recently, fecal S100A12 level was reported to be

a more accurate fecal marker of intestinal inflammation (in, eg, inflammatory bowel disease).⁶ We found significantly elevated fecal S100A12 levels in postmeconium stool samples of VLBW infants at the onset of NEC, which help differentiate Bell stage II and III NEC (**Figure 1**, B). Thus, measurement of fecal S100A12 might provide important clinical information to pediatricians and pediatric surgeons. Fecal S100A12 levels were also elevated within 4-10 days before and 2 weeks after disease onset compared with reference infants without gastrointestinal disease (**Figure 3**, A). Sensitivity, specificity, and positive and negative predictive values for fecal S100A12 for the detection of NEC were 70%, 68%, 37%, and 89%, respectively. This limited sensitivity and specificity might be related to physiological high levels of fecal S100A12 during the first weeks of life (**Figure 1**, A), owing to the gut bacterial establishment stimulating transepithelial migration of granulocytes.¹⁸

In VLBW infants, we found significantly lower fecal S100A12 concentrations in meconium before the onset of NEC stage II and significantly higher concentrations before the onset of NEC stage III compared with reference infants. Whether these references reflect predispositions of individual patients is not known.^{18,20,21}

We observed an overlap in fecal S100A12 levels of infants with NEC and those without NEC, related to the wide range of fecal S100A12 levels in VLBW infants without gastrointestinal symptoms (**Figure 1**, A). This finding is in agreement with data on fecal calprotectin levels in VLBW infants, which also show wide variations in healthy individuals during the first month of life.^{2,15-18,22} This phenomenon may reflect true interindividual and intraindividual variability in S100A12 excretion in our patient population, as has been reported for fecal calprotectin excretion.^{2,18} In addition, we found that fecal S100A12 levels tend to decrease with increasing age and increasing enteral feeding volume by comparisons of levels at days of life 2-7, 8-28, and >28 (**Figure 1**, A). This is also suggested by previous data on fecal calprotectin in a small group of VLBW infants¹⁶ and may be explained by a simultaneously gradual decrease in intestinal mucosa permeability.¹⁸ Thus, the age dependence of fecal S100A12 levels may affect diagnostic accuracy. However, we found stable S100A12 levels in stool samples of reference infants obtained on days 8-28 after birth, and the majority of our patients with NEC experienced onset of disease during that time period.

We found no significant correlations between fecal S100A12 level and various neonatal and maternal factors apart from the diagnosis of NEC. We also found no correlation between CRP or IL-6 level and fecal S100A12 level in reference infants, suggesting that systemic infection does not affect fecal S100A12 level in the absence of severe gastrointestinal disease.

Overall, the increased fecal S100A12 levels in our patients with NEC suggests that monitoring fecal S100A12 might provide useful early warning signals for NEC. Several limitations of the present study must be taken into account, however.

First and foremost, the overlap between fecal S100A12 levels in patients with NEC and controls might limit this marker's utility in the clinical context. Furthermore, fecal S100A12 levels appear to be age-dependent. More generally, implementing large longitudinal studies in a population of preterm and/or VLBW infants is a challenge. In addition, NEC is a rare disease, with an incidence of only 0.1%, and with problematic and limited diagnostic options.

Further studies are needed to investigate fecal S100A12 levels in larger cohorts that include infants with other gastrointestinal symptoms or diseases apart from NEC, including feeding intolerance. Moreover, the fact that term and preterm NEC are considered 2 different disease entities must be taken into account.⁵ Our study cohort included only VLBW preterm infants with NEC and without gastrointestinal disease. Future studies are needed to examine whether fecal S100A12 is also suitable for early detection of NEC and possibly other gastrointestinal manifestations in term infants. Finally, the measurement of fecal inflammation markers may have limited utility in diagnosing NEC in VLBW infants, because stool samples are not necessarily available at all times. ■

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