Noninvasive measurement of fecal calprotectin and serum amyloid A combined with intestinal fatty acid–binding protein in necrotizing enterocolitis

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Abstract

Background: Diagnosis of necrotizing enterocolitis (NEC), prevalent in premature infants, remains challenging. Enterocyte damage in NEC can be assessed by intestinal fatty acid–binding protein (I-FABP), with a sensitivity of 93% and a specificity of 90%. Numerous markers of inflammation are known, such as serum amyloid A (SAA) and fecal calprotectin.

Purpose: The aim of the present study was to evaluate which combination of noninvasive measurement of inflammatory markers and I-FABP improves the diagnostic accuracy in neonates suspected for NEC.

Methods: In 62 neonates with clinical suspicion of NEC (29 with final diagnosis of NEC), urinary I-FABP, urinary SAA, and fecal calprotectin levels were determined quantitatively. Diagnostic accuracy was calculated for the combinations I-FABP–SAA and I-FABP–fecal calprotectin, using a multivariable logistic regression model.

Results: The combination of SAA and I-FABP did not increase the diagnostic accuracy of I-FABP. However, the combination of fecal calprotectin and I-FABP improved accuracy significantly. The combination of urinary I-FABP and fecal calprotectin measurement produced a sensitivity of 94%, a specificity of 79%, a positive likelihood ratio of 4.48, and a negative likelihood ratio of 0.08.

Conclusion: The combination of noninvasive measurement of I-FABP and fecal calprotectin seems promising for diagnosing NEC at an early time point. Prospective analysis is required to confirm this finding and to evaluate better treatment strategies based on noninvasive measurement of I-FABP and calprotectin.
cell damage [1]. Diagnosis of NEC remains challenging because the initial presentation is nonspecific and often hard to distinguish from other gastrointestinal disorders and sepsis. Although most laboratory tests and imaging techniques lack diagnostic accuracy [2], measurement of intestinal fatty acid–binding protein (I-FABP), a marker of intestinal cell damage, has been reported to discriminate NEC from other diseases with high sensitivity and specificity [3-5]. To determine the proper treatment strategy, it is of great importance to differentiate NEC at an early time point. Noninvasive measurement of diagnostic markers is preferred, to avoid the risk of anemia caused by blood sampling. This study evaluated whether noninvasive measurement of the inflammatory markers serum amyloid A (SAA) and fecal calprotectin could improve the diagnostic accuracy of I-FABP in neonates with suspected NEC.

Serum amyloid A is an acute-phase protein (11.5 kD), synthesized by the liver upon induction by proinflammatory cytokines [6]. Serum amyloid A was recently reported by Ng and colleagues [7] to be a promising serum biomarker of both NEC and sepsis in neonates. However, differentiation of NEC from other diseases is important because several treatment aspects are different, including surgical intervention in cases of persistent NEC [8].

Calprotectin, a heterodimeric peptide (36 kD), is released from the cytosol of neutrophils upon activation. Fecal calprotectin is a specific marker for neutrophil infiltrate in bowel mucosa. In intestinal inflammation, calprotectin is readily detectable in feces and plasma, making fecal calprotectin a suitable marker for NEC [4,9,10].

We studied whether noninvasive measurement of the inflammatory markers SAA (in urine) and calprotectin (in feces) improves the diagnostic accuracy of urinary I-FABP in neonates with gastrointestinal symptoms suspected of NEC.

1. Patients and methods

1.1. Patients and sample collection

Sixty-two consecutive patients with clinical suspicion of NEC in the neonatal intensive care units at Maastricht University Medical Center and Wilhelmina Children’s Hospital in Utrecht were studied between July 2005 and August 2010. Data on 34 (13 NEC cases) neonates have been previously published [4]. Suspected NEC was defined as the presence of abdominal distension causing sufficient clinical concern to require an abdominal radiograph and/or to stop enteral feeding. Final diagnosis of NEC was made with the current criterion standard of abdominal radiographic evidence of pneumatosis intestinalis (Bell stage ≥II) [11]. Written informed consent was obtained from both parents, and the study was conducted with approval from local medical ethical committees and according to the revised version of the Declaration of Helsinki (October 2008, Seoul). The principles of good clinical practice were followed during this study.

Urine from all included neonates was obtained at the time of clinical suspicion of NEC by placing a dental cotton roll (Henry Schein, Almere, the Netherlands) in the diaper of the neonate. The rolls containing urine were placed in a sterile 5-mL syringe (Becton Dickinson, Oxford, UK), and the urine was pressed into Micronic tubes (Micronic BV, Lelystad, the Netherlands). Urine was stored at −20°C until batch analysis. Stool samples were obtained and stored immediately at −20°C until batch analysis. All laboratory analyses were performed by 1 person after completion of patient inclusion.

1.2. Power analysis

The number of patients needed for this study was calculated using the difference in SAA levels between controls and neonates with NEC/sepsis [7] (α = .05, 1 − β = 0.95). This produced a minimum number of 9 patients per group.

1.3. Urinary I-FABP measurement

Urinary I-FABP was measured using an in-house enzyme-linked immunosorbent assay (ELISA) that selectively detects human I-FABP (lower detection limit, 12.5 pg/mL). Values are expressed as a ratio (in picograms per nanomole of creatinine) of I-FABP (in picograms per milliliter) to creatinine (in nanomoles per milliliter), to compensate for variations in urine concentration (I-FABP/Cr).

1.4. Urinary SAA measurement

Urinary SAA was measured using a commercially available ELISA kit (lower detection limit, 15.0 ng/mL), kindly provided by Hycult Biotechnology (Uden, the Netherlands). Values are expressed as ratio (in picograms per nanomole of creatinine) of SAA (in nanograms per milliliter) to creatinine (in nanomoles per milliliter) * 1000, to compensate for variations in urine concentration (SAA/Cr).

1.5. Fecal calprotectin measurement

After thawing of feces, 100 mg was weighed and 4.9 mL extraction buffer (0.1 M Tris, 0.15 M NaCl, 1.0 M urea, 10 mM CaCl₂·2H₂O, 0.1 M citric acid, 0.5% bovine serum albumin, pH 8.0) was added [12]. After 30 minutes of shaking, 1 mL of suspension was centrifuged at 10,000 rpm for 20 minutes at 4°C, and supernatant was aliquoted and stored at −20°C. Calprotectin concentration was measured in lysate using the commercially available calprotectin ELISA (lower detection limit, 625 ng/mL), kindly provided by Hycult Biotechnology. Fecal calprotectin concentration is given in micrograms of calprotectin per gram of feces.
1.6. Statistical analyses

Normality was tested using the Kolmogorov-Smirnov test. Mann-Whitney U test was used for between-group comparisons for continuous data. Dichotomous variables were compared using the Fisher exact test. All data are presented as median and range.

To determine the accuracy of I-FABP combined with an inflammatory marker in the diagnosis of NEC, 2 combinations of markers were tested: I-FABP–SAA and I-FABP–calprotectin. Predicted probabilities for the presence of NEC were calculated for both combinations using logistic regression analysis and then plotted in receiver operating characteristic (ROC) curves. Overall diagnostic accuracy of I-FABP–SAA and I-FABP–calprotectin was represented by the area under the curve (AUC). The best cutoff point of predicted probabilities \( P \) was defined as the cutoff point with the maximum sum of sensitivity and specificity. To calculate the linear function describing all combinations of ideal cutoff values for I-FABP–SAA and I-FABP–calprotectin in the diagnosis of NEC, the cutoff point \( P \) was used in the following equation:

\[
\ln\left(\frac{P}{1-P}\right) = B_0 + B_1X_1 + B_2X_2,
\]

in which \( B_0 \) represents the constant of the logistic regression analysis and \( B_1 \) and \( B_2 \) represent the logistic regression coefficients of I-FABP and SAA or I-FABP and calprotectin, respectively. By calculating coordinates of intersections with the \( x \) - and \( y \)-axes, the linear functions describing the cutoff lines of I-FABP–SAA and I-FABP–calprotectin could be determined.

Statistical analyses were performed using Prism 5.0 for Windows (GraphPad Software Inc, San Diego, CA) and SPSS 15.0 for Windows (SPSS Inc, Chicago, IL). Standards for Reporting of Diagnostic Accuracy (STARD) statement for reporting studies of diagnostic accuracy was used in this study [13].

2. Results

2.1. Patients

Sixty-two neonates were included, of whom 32 were male. The median gestational age was 215 (175-289) days, and the median birth weight was 1328 (585-3570) g. Twenty-nine infants were diagnosed as having NEC (47%).

There were no significant differences in gestational age, birth weight, or sex between the groups (Table 1). Final diagnoses of premature infants without NEC are listed in Table 2.

2.2. Single-marker analyses

2.2.1. Ratio of I-FABP to Cr

Median I-FABP/Cr levels in neonates with NEC were significantly higher compared with infants with other diseases (11.4 [0.4-4878] and 1.2 [0.0-11.0] pg/nmol, respectively; \( P < .001 \)). An ideal cutoff value of 2.4 pg/nmol was found, with a sensitivity of 79%, a specificity of 85%, a positive likelihood ratio (LR+) of 5.23, and a negative likelihood ratio (LR−) of 0.24 (Fig. 1). The ROC curve yielded an AUC of 0.88 (95% confidence interval [CI], 0.80-0.97).

2.2.2. Ratio of SAA to Cr

Median SAA/Cr levels in neonates with NEC were significantly higher compared with infants with other diagnoses (44.4 [9.3-31,127] and 21.5 [12.0-1469] pg/nmol, respectively; \( P = .009 \)). The AUC of the ROC curve was 0.70 (95% CI, 0.56-0.83), with an ideal cutoff value of 42.2 pg/nmol (sensitivity 52%, specificity 91%, LR+ 5.69, LR− 0.53).

2.2.3. Calprotectin

Stool samples were only available in 35 of 62 neonates (16 with NEC [55%] and 19 with other final diagnoses [58%]). Median calprotectin levels in neonates with NEC were significantly higher compared with infants with other diagnoses (402.2 [107.6-847.6] and 79.6 [1.0-625.1] μg/g

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NEC (n = 29)</td>
</tr>
<tr>
<td>Gestational age (d)</td>
<td>215 (184-268)</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>1400 (860-1960)</td>
</tr>
<tr>
<td>Sex</td>
<td>11 M (38%)</td>
</tr>
</tbody>
</table>

Data are presented as median (range). M, male; F, female.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Diagnoses associated with abdominal symptoms in no-NEC group</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoNS sepsis</td>
<td>17</td>
</tr>
<tr>
<td>Gastrointestinal symptoms of unknown origin</td>
<td>10</td>
</tr>
<tr>
<td>Norovirus</td>
<td>2</td>
</tr>
<tr>
<td>Portal vein thrombosis</td>
<td>2</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>1</td>
</tr>
<tr>
<td>Enterococcus faecalis sepsis</td>
<td>1</td>
</tr>
<tr>
<td>Enterobacter cloacae sepsis</td>
<td>1</td>
</tr>
<tr>
<td>Constipation</td>
<td>1</td>
</tr>
<tr>
<td>Hypomotility</td>
<td>1</td>
</tr>
<tr>
<td>Morbus Hirschsprung</td>
<td>1</td>
</tr>
<tr>
<td>Gastrostomosis</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>38*</td>
</tr>
</tbody>
</table>

CoNS, coagulase-negative staphylococcus. * Five patients had 2 diagnoses associated with abdominal symptoms.
feces, respectively; \( P = .001 \). The AUC of the ROC curve was 0.82 (95% CI, 0.68-0.96), with an ideal cutoff value of 286.3 \( \mu \)g/g feces (sensitivity 81%, specificity 79%, LR+ 3.86, LR− 0.24).

### 2.3. Multiple-marker analyses

First, the combinations of I-FABP with SAA and I-FABP with fecal calprotectin were analyzed by combining the cutoff values found in the single-marker analyses. This implies that a positive test result is obtained when both values are above the cutoff value, that is, I-FABP greater than 2.4 pg/nmol and SAA greater than 42.2 pg/nmol. The combination of I-FABP/Cr and SAA/Cr yielded the following: sensitivity, 45%; specificity, 100%; LR+, infinite; and LR−, 0.55 (Table 3). The combination of I-FABP/Cr and fecal calprotectin produced the following: sensitivity, 63%; specificity, 100%; LR+, infinite; and LR−, 0.38 (Table 3). In this analysis, no ROC curve can be calculated.

Second, a logistic regression analysis approach was used to analyze the combinations of I-FABP with SAA and I-FABP with fecal calprotectin.

#### 2.3.1. Combination of I-FABP/Cr and of SAA/Cr

When I-FABP/Cr and SAA/Cr were combined, a cutoff line was calculated with a sensitivity of 83%, a specificity of 82%, an LR+ of 4.55, and an LR− of 0.21 (Fig. 2), thereby not increasing diagnostic accuracy compared with measurement of I-FABP/Cr. The cutoff line was described by the linear function:

\[
[SAA/Cr (pg/nmol creatinine)] + 231.6 \cdot [I-FABP/Cr (pg/nmol creatinine)] = 862,
\]

which means that a positive test is obtained when any combination of SAA/Cr and I-FABP/Cr levels in this formula results in a value more than 862. The AUC of the ROC curve for discrimination of neonates with NEC from those with other final diagnoses was 0.88 (95% CI, 0.79-0.97).

### Table 3: Diagnostic markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cutoff value</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>LR+</th>
<th>LR−</th>
<th>AUC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-FABP/Cr</td>
<td>2.4 pg/nmol (creatinine)</td>
<td>79</td>
<td>85</td>
<td>5.23</td>
<td>0.24</td>
<td>0.88 (0.80-0.97)</td>
</tr>
<tr>
<td>SAA/Cr</td>
<td>42.2 pg/nmol (creatinine)</td>
<td>52</td>
<td>91</td>
<td>5.69</td>
<td>0.53</td>
<td>0.70 (0.56-0.83)</td>
</tr>
<tr>
<td>Calprotectin</td>
<td>286.3 ( \mu )g/g (feces)</td>
<td>81</td>
<td>79</td>
<td>3.86</td>
<td>0.24</td>
<td>0.82 (0.68-0.96)</td>
</tr>
<tr>
<td>I-FABP/Cr + SAA/Cr(^a)</td>
<td>2.4 + 42.2</td>
<td>45</td>
<td>100</td>
<td>Infinite</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>I-FABP/Cr + calprotectin(^a)</td>
<td>2.4 + 286.3</td>
<td>63</td>
<td>100</td>
<td>Infinite</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>I-FABP/SAA</td>
<td>83</td>
<td>82</td>
<td>4.55</td>
<td>0.21</td>
<td>0.88</td>
<td>(0.79-0.97)</td>
</tr>
<tr>
<td>I-FABP/Calprotectin(^c)</td>
<td>94</td>
<td>79</td>
<td>4.48</td>
<td>0.08</td>
<td>0.95</td>
<td>(0.89-1.00)</td>
</tr>
</tbody>
</table>

\(^a\) Combination of 2 markers in which a positive test result is obtained when both single-marker tests are positive.

\(^b\) \([\text{SAA/Cr (pg/nmol creatinine)}] + 231.6 \cdot [\text{I-FABP/Cr (pg/nmol creatinine)}] = 862.\)

\(^c\) \([\text{Calprotectin (}\mu\text{g/g feces)}] + 43.2 \cdot [\text{I-FABP/Cr (pg/nmol creatinine)}] = 486.\)

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**Fig. 1** Urinary I-FABP levels when NEC is clinically suspected can accurately distinguish between neonates with final diagnosis of NEC and those with other final diagnoses. A cutoff value of 2.4 pg/nmol creatinine is depicted by the dotted line.

**Fig. 2** Combination of urinary I-FABP levels and urinary SAA levels in neonates with final diagnosis of NEC (squares) and those with other final diagnoses (triangles) when NEC is clinically suspected. The ideal cutoff line for differentiating between NEC and other diagnoses is depicted by the dotted line.
3. Discussion

This study aimed to assess whether noninvasive measurement of the inflammatory markers SAA (in urine) and calprotectin (in feces) improves the diagnostic accuracy of the enterocyte damage marker I-FABP in neonates with gastrointestinal symptoms suspected of NEC. The data show that measurement of fecal calprotectin but not SAA improves the diagnostic accuracy of urinary I-FABP measurement in diagnosing NEC.

In the current study, only noninvasive markers were investigated because blood sampling in neonates should be avoided [14] and because a clinical benefit of the markers has never been demonstrated. Classic parameters of infection (C-reactive protein, white blood cell count, platelets) are, therefore, not presented here. A limitation of noninvasive measurement of SAA is that little is known about the plasma clearance of SAA. Although urinary SAA levels were detected in a large part of the patients, further research is needed to elucidate the renal clearance of SAA.

Cetinkaya et al [15] showed that plasma SAA levels were elevated in neonates with NEC compared with neonates with sepsis at the moment of diagnosis. In contrast, however, Eras et al [16] reported higher plasma levels of SAA in children with sepsis, compared with neonates with NEC. In our cohort, measurement of urinary SAA alone was not useful as a diagnostic marker for NEC owing to the low sensitivity. In a recent study by Ng et al [7], SAA was reported to be a highly specific marker of both NEC and sepsis in low-birth-weight neonates. Importantly, the authors did not differentiate between NEC and sepsis. Although initial medical treatment is similar for both NEC and sepsis, resection of affected bowel is essential in NEC when signs of perforation occur or when symptoms do not improve on medical treatment. Here, we aimed to distinguish NEC from other diagnoses including sepsis at an early time point. Unfortunately, inclusion of urinary SAA levels did not substantially improve the diagnostic accuracy of urinary I-FABP measurement in diagnosing NEC. This might be caused by a lack of specificity of SAA being a marker of generalized inflammation.

Next, this study aimed to include intestinal inflammation and, more specifically, fecal calprotectin. It increased the diagnostic accuracy of I-FABP measurement. Elevated levels of fecal calprotectin in NEC are in line with several studies [4,9,17]. In a previous study from our institute, however, fecal calprotectin did not improve the diagnostic accuracy of I-FABP [4]. This could have been a consequence of the higher sensitivity and specificity of I-FABP than those found in the current cohort.

The diagnostic accuracy improved when measurement of fecal calprotectin was combined with I-FABP. The data demonstrated a substantial increase in sensitivity and a decrease in LR−, which is important in possibly lethal diseases like NEC. When applying Bayes theorem [18,19] with NEC prevalence of 47% in the studied cohort, a negative test result of I-FABP alone produces a posttest probability of 18% (95% CI, 10%-31%). This means that 18% of neonates with NEC are missed by the test (18% false negatives). When both I-FABP and fecal calprotectin are measured, the posttest probability of a negative test decreases to 7% (95% CI, 2%-22%), which means that only 7% of neonates with NEC are missed by the test. The posttest

![Fig. 3 Combination of urinary I-FABP levels and fecal calprotectin levels in neonates with final diagnosis of NEC (squares) and those with other final diagnoses (triangles) when NEC is clinically suspected. The ideal cutoff line for differentiating between NEC and other diagnoses is depicted by the dotted line. Note: stool samples were only available in 35 of 62 neonates.](image-url)
probability when a positive test result is acquired is 82% (95% CI, 67%-91%) for I-FABP and 80% (95% CI, 67%-89%) for I-FABP combined with fecal calprotectin, which is comparable. The benefit of the combination of I-FABP and calprotectin lies, thus, in a lower rate of missed NEC diagnoses.

The combination of 2 diagnostic markers allows us to calculate a cutoff line instead of a single cutoff value because we found that single markers or the combination of static cutoff points was not clinically relevant enough to differentiate NEC from other final diagnoses in neonates suspected for NEC. This cutoff line could be described as \[ \text{calprotectin} \left( \mu g/g \text{ feces} \right) + 43.2 \cdot \left[ \text{I-FABP/Cr} \left( \text{pg/nmol creatinine} \right) \right] = 486, \]
which means that a positive test is obtained when any combination of fecal calprotectin and urinary I-FABP levels in this formula results in a value more than 486. For clinical application, a table or graph should be used when quick decision making is required. Because feces was obtained in only 35 (56%) of 62 neonates, the combined method was only possible in 56% of the children. Further prospective studies are needed to confirm these results in a larger population and to evaluate the effects of treatment strategies based on calprotectin and I-FABP measurement.

This study shows that noninvasive measurement of SAA adds little to the diagnostic accuracy of urinary I-FABP in diagnosing NEC, whereas fecal calprotectin increases the diagnostic accuracy in children who produce feces. The combination of urinary I-FABP and fecal calprotectin may be a promising noninvasive method for diagnosing NEC at an early time point.

References