Non-Invasive Markers for Early Diagnosis and Determination of the Severity of Necrotizing Enterocolitis

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Objectives: To improve diagnosis of necrotizing enterocolitis (NEC) by noninvasive markers representing gut wall integrity loss (I-FABP and claudin-3) and gut wall inflammation (calprotectin). Furthermore, the usefulness of I-FABP to predict NEC severity and to screen for NEC was evaluated.

Methods: Urinary I-FABP and claudin-3 concentrations and fecal calprotectin concentrations were measured in 35 consecutive neonates suspected of NEC at the moment of NEC suspicion. To investigate I-FABP as screening tool for NEC, daily urinary levels were determined in 6 neonates who developed NEC out of 226 neonates included before clinical suspicion of NEC.

Results: Of 35 neonates suspected of NEC, 14 developed NEC. Median I-FABP, claudin-3, and calprotectin levels were significantly higher in neonates with NEC than in neonates with other diagnoses. Cutoff values for I-FABP (2.20 pg/nmol creatinine), claudin-3 (800.8 INT), and calprotectin (286.2 µg/g feces) showed clinically relevant positive likelihood ratios (LRs) of 9.30, 3.74, 12.29, and negative LRs of 0.08, 0.36, 0.15, respectively. At suspicion of NEC, median urinary I-FABP levels of neonates with intestinal necrosis necessitating surgery or causing death were significantly higher than urinary I-FABP levels in conservatively treated neonates.

Of the 226 neonates included before clinical suspicion of NEC, 6 developed NEC. In 4 of these 6 neonates I-FABP levels were not above the cutoff level to diagnose NEC before clinical suspicion.

Conclusions: Urinary I-FABP levels are not suitable as screening tool for NEC before clinical suspicion. However, urinary I-FABP and claudin-3 and fecal calprotectin are promising diagnostic markers for NEC. Furthermore, urinary I-FABP might also be used to predict disease severity.

PAINTENTS AND METHODS

Patients and Sample Collection
To facilitate diagnosis and to predict severity of NEC, 35 consecutive neonates suspected of NEC were included in the neonatal intensive care units (NICU) at Maastricht University Medical Centre and Wilhemmina Children’s Hospital in Utrecht, between July 2005 and September 2008. Suspicion of NEC was defined as presence of abdominal distension, causing sufficient clinical concern to require abdominal x-ray (AXR), and/or to stop enteral feeding.

In addition, to examine I-FABP as screening tool for NEC, all neonates (n = 226) admitted to the NICU were included at Maastricht University Medical Centre, between September 2007 and September 2008. Samples were analyzed if there was a later suspicion of NEC.

Written consent was obtained from both parents, and the study was conducted with approval from local medical ethical committees.

Urine from all included neonates was obtained daily by placing a dental cotton roll (Henry Schein, Almere, The Netherlands) in the diaper of the neonate. Once the roll was filled with urine, it was placed in a sterile 5 mL syringe (Becton Dickinson, Oxford, United Kingdom), the urine was pressed into Micronic tubes (Micronic B.V., Lelystad, The Netherlands) and stored at –20°C until analysis. Feces was sampled from the diaper and frozen at –20°C until analysis. NEC was diagnosed with the current golden standard of AXR showing pneumatosis intestinalis (Bell stage II or higher).17 All analyses were performed after completion of patient inclusion. The analyses were performed by one person who was blinded for final diagnosis.

Markers for Systemic Inflammation
To compare the studied markers with the classic systemic inflammatory markers in the early diagnosis of NEC, platelet count, C-reactive protein (CRP), and white blood cell count (WBC), determined as routine patient care in the first blood sample upon suspicion of NEC by the clinical chemistry laboratory, were retrieved from patient records.

Urinary I-FABP Measurement
Urinary I-FABP was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) that selectively detects human I-FABP (standard: 20–5000 pg/ml), kindly provided by Hycult Biotechnology (Uden, the Netherlands). Values were expressed as ratio (pg/nmol creatinine) of I-FABP (pg/ml) to creatinine (μmol/l), to compensate for variations in urine concentration.

I-FABP values of the first 17 consecutive neonates were previously published.9

Urinary Claudin-3 Measurement
Urinary claudin-3 levels were analyzed by Western blotting. Equal amounts of each sample adjusted to urinary creatinine levels were separated by SDS-PAGE, transferred to PVDF-membrane and probed using primary antibody to claudin-3 (rabbit anticlaudin-3 (34–1700), Zymed Laboratories, San Francisco, CA). After incubation with goat anti-rabbit HRP-conjugated secondary antibody (Jackson, West Grove, PA), signal was detected by supersignal west pico chemiluminescence substrate (Pierce, Etten-Leur, the Netherlands). Band intensity was semi-quantitatively analyzed using Quantity One (Biorad, Hercules, CA).

Fecal Calprotectin Measurement
After thawing of feces, 100 mg was weighed and 4.9 mL extraction buffer (0.1M Tris, 0.15M NaCl, 1.0M urea, 10mM CaCl2, 2H2O, 0.1M citric acid, 0.5% BSA, pH 8.0) was added.18 After 30 minutes 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.19 Calprotectin concentration was measured in lysate using the commercially available calprotectin ELISA (standard 0.78–50 ng/mL), kindly provided by Hycult Biotechnology. Fecal calprotectin concentration is given in μg calprotectin per gram feces.

Statistical Analyses
Mann-Whitney U test was used for between group comparison. Sex and stool were compared using Fisher exact test. All data are presented as median and range.

To find cutoff points of blood WBC, urinary I-FABP, urinary claudin-3 and fecal calprotectin levels that most accurately discriminate patients with NEC from patients with other final diagnoses, receiver operating characteristics (ROC) curves were drawn by plotting sensitivity against 1-specificity for all possible thresholds. Overall accuracy of the markers in detecting NEC was represented by area under the curve (AUC). Best cutoff point is defined as the maximum sum of sensitivity and specificity. These cutoff points were used to calculate sensitivity, specificity, positive and negative likelihood ratios (LRs). LRs are used and considered most important for evaluation of a clinical test, because they summarize information on both sensitivity and specificity and provide discriminative power of the test.19,20 Furthermore, optimal cutoff points of I-FABP to discriminate in NEC severity were determined.

Statistical analyses were performed with Prism 4.0 for Windows (GraphPad Software Inc. San Diego, CA). STARD statement for reporting studies of diagnostic accuracy was used in this study.21

RESULTS

Patients Suspected of NEC
Fourteen of the 35 neonates suspected of NEC ultimately developed NEC Bell stage II or higher. The remaining 21 patients were finally diagnosed with sepsis (n = 13), constipation (n = 2), Hirschsprung disease (n = 2), viral enteritis (n = 1), and gastrointestinal problem of unknown origin (n = 3). The NEC group did not differ from the patient group with other final diagnoses in gestational age, birth weight and sex (Table 1).

All neonates produced urine at a minimal rate of 1.9 mL/kg/h. Urine samples from all neonates suspected of NEC were obtained before final diagnosis with the current golden standard being AXR showing pneumatosis intestinalis, with an average of 1.5 days (range, 0–6 days) before final diagnosis. Fourteen neonates with suspicion of NEC did not produce stool before final diagnosis and therefore were excluded in the analysis of fecal calprotectin. From the 14 neonates who did not stool before final diagnosis 8 developed NEC and 6 had another diagnosis, representing an equal distribution (P = 0.159).

<table>
<thead>
<tr>
<th>Table 1. Patient Characteristics of Neonates Suspected of NEC</th>
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<tr>
<td>NEC</td>
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<tr>
<td>Gestational age 30±7 wk (27±7–38±7)*</td>
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<tr>
<td>Birth weight 1465 g (860–1960)</td>
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<td>Sex M:V = 9:5</td>
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<td>Total 14</td>
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No significant differences were observed in gestational age, birth weight and sex between the neonates with NEC and neonates with other diagnoses.

*Of the 14 neonates, 12 had gestational age of <34 wk, 1 neonate had an gestational age of 34 1/7 wk, and only 1-term neonate with a gestational age of 38 2/7 wk was included.

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Conventional Blood Laboratory Tests

Laboratory tests currently in use to diagnose NEC in infants suspected of NEC are platelet count, CRP and WBC. Blood platelet count and CRP were not different between NEC patients \((213 \times 10^3/L [4.0–694.0])\) and neonates with other diagnoses \((107 \times 10^3/L [12.9–325.0], P = 0.362\) and \(16.5 \text{mg} / L [1.0–167.0], P = 0.142\), respectively) (Fig. 1A,B). In contrast, median blood WBC was significantly lower in the NEC group \((14.1 \times 10^3/L [4.3–40.5], P = 0.029\), although substantial overlap is observed in blood WBC values of individuals in the 2 groups (Fig. 1C).

AUC for WBC in diagnosis of NEC was 0.76 (95% confidence interval [CI]= 0.56–0.95, \(P = 0.027\)). The cutoff value, evaluated with ROC curves for WBC \((12.1 \times 10^3/L)\), showed a positive LR of 3.19 and a negative LR of 0.42 (data not shown).

New Urinary and Fecal Tests for Diagnosis of NEC

Median urinary I-FABP:creatinine ratio, urinary claudin-3 and fecal calprotectin levels were significantly higher in neonates suspected of NEC who finally developed NEC (respectively, 7.93 pg/nmol creatinine [2.05–448.1]; 1385.0 INT [267.9–2021.0]; 589.2 μg/g feces [146.1–847.6]), than in those with other final diagnoses (respectively, 0.94 pg/nmol creatinine [0.06–3.11], \(P < 0.001\); 477.0 INT [1.0–1677.0], \(P = 0.017\); 69.6 μg/g feces [1.0–556.4], \(P = 0.001\)) (Fig. 2). Ideal cutoff values to discriminate neonates with NEC from neonates suspected of NEC with other final diagnoses, were 2.20 pg/nmol creatinine for urinary I-FABP, 800.8 INT for urinary claudin-3, and 286.2 μg/g feces for fecal calprotectin.

Specificity of 90%, 81%, 93% and sensitivity of 93%, 71%, 86% were shown. This resulted in positive LRs of 9.30, 3.74, 12.29 and negative LRs of 0.08, 0.36, and 0.15, respectively. The AUC for I-FABP, claudin-3 and calprotectin in diagnosis of NEC was 0.98 (0.94–1.0), 0.76 (0.59–0.94), 0.94 (0.85–1.0), respectively (Table 2).

I-FABP as Predictor of Disease Severity

To assess whether I-FABP levels are related to disease severity, NEC patients were divided in 2 categories representing mild and severe NEC; ie, NEC conservatively treated and NEC necessitating surgery or causing death by intestinal necrosis. At suspicion of NEC, median urinary I-FABP levels of neonates with intestinal necrosis necessitating surgery or causing death were higher \((36.05 \text{pg} / \text{nmol creatinine} [6.56–448.1])\) than urinary I-FABP levels in the conservatively treated neonates \((5.02 \text{pg} / \text{nmol creatinine} [2.05–13.27], P = 0.002\) (Fig. 3).

To assess the cutoff point of urinary I-FABP levels that most accurately discriminate patients with NEC necessitating surgery or causing death from patients with conservatively managed NEC, ROC curves were drawn for all I-FABP thresholds. The ideal cutoff value, yielding the maximum sum of sensitivity (1.0) and specificity (0.86), found was 6.38 pg/nmol creatinine. AUC for I-FABP in the differentiation of NEC was 0.96 (0.86–1.0), \(P = 0.004\) (data not shown).

Urinary claudin-3 levels and fecal calprotectin levels were not related to disease severity (data not shown).

I-FABP as Possible Screening Tool

Assessment of urinary I-FABP levels to differentiate neonates with NEC from neonates with other diagnoses showed high accuracy and clinically relevant positive and negative likelihood ratios. Furthermore, measurement of I-FABP levels is done with a sensitive and rapid (<4 hours) ELISA. Therefore, we evaluated if urinary I-FABP levels can be used to predict the development of NEC in neonates before clinical suspicion. Of the 226 neonates included before clinical suspicion of NEC, 6 developed NEC. Five of the 6 neonates showed maximal I-FABP levels at the moment of suspicion of NEC. In 4 of the neonates I-FABP levels before suspicion were below 2.20 pg/nmol creatinine, the cutoff point to differentiate neonates with NEC from neonates with other diagnoses (Fig. 4). Urinary I-FABP levels are not suitable as screening tool for NEC before clinical suspicion was raised.

DISCUSSION

Early and reliable diagnosis of NEC is crucial to provide adequate treatment. Neonatologists and pediatric surgeons usually rely on clinical signs and symptoms to diagnose NEC, while laboratory tests (platelets, CRP, WBC) and imaging techniques are used to confirm the diagnosis. Diagnostic accuracy of NEC has only marginally improved in the last decades.3,4

FIGURE 1. Blood platelet count (A) and CRP (B) did not differ between neonates with NEC and neonates with other final diagnoses. Median blood WBC level (C) was significantly lower in neonates with NEC. Cutoff point to differentiate neonates with NEC from neonates with other diagnoses is represented by the dotted line.
Therefore, we aimed at improving diagnosis of NEC by using markers representing gut wall integrity loss and gut wall inflammation.\textsuperscript{1,5} Gut wall integrity loss was studied by measuring urine levels of I-FABP, indicating epithelial cell damage and claudin-3, representing tight junction loss. Fecal calprotectin was used to study gut wall inflammation.

Preceding studies investigating the usefulness of measuring plasma I-FABP levels for diagnosis of NEC showed higher levels of

\begin{figure}
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\includegraphics[width=\textwidth]{figure2.pdf}
\caption{Median urinary I-FABP: creatinine ratio (A), urinary claudin-3 level (B), and fecal calprotectin level (C) were significantly higher in neonates with NEC than in neonates with other diagnoses. Cutoff point to differentiate neonates with NEC from neonates with other diagnoses is represented by the dotted line.}
\end{figure}

\begin{table}
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\begin{tabular}{|l|c|c|c|c|c|}
\hline
Marker & Cutoff Point & Sensitivity & Specificity & LR+ & LR− & AUC (95% CI*) & P \\
\hline
I-FABP & 2.20 pg/nmol creatinine & 0.93 & 0.90 & 9.30 & 0.08 & 0.98 (0.94–1.0) & <0.001 \\
Claudin-3 & 800.8 INT & 0.71 & 0.81 & 3.74 & 0.36 & 0.76 (0.59–0.94) & 0.016 \\
Calprotectin & 286.2 μg/g feces & 0.86 & 0.93 & 12.29 & 0.15 & 0.94 (0.85–1.0) & 0.001 \\
\hline
\end{tabular}
\caption{Cutoff Points, With Corresponding Sensitivity, Specificity, Positive and Negative Likelihood Ratios (LRs), and Area Under the Curve (AUC) for I-FABP, Claudin-3 and Calprotectin to Differentiate Between Neonates With NEC and Neonates With Other Diagnoses}
\end{table}

*Confidence interval.
plasma I-FABP in neonates with NEC compared with healthy controls.\textsuperscript{8,22} Furthermore, these studies indicated that higher I-FABP plasma values were related to more severe NEC.\textsuperscript{22,23} Although, Lieberman et al showed in 5 neonates with severe NEC that only 4 had elevated plasma I-FABP levels. Plasma I-FABP of the 5th neonate was not detectable, while a totally necrotic intestine was found at operation.\textsuperscript{8} Late plasma sampling explains this finding: a totally necrotic gut may no longer result in uptake of released I-FABP into the circulation. Rapid clearance of plasma I-FABP by the kidneys makes it a suitable marker to detect acute intestinal damage.\textsuperscript{10} However, this rapid clearance can also lead to missing of enterocyte damage by measuring plasma levels of I-FABP, as a result of the transient character of the elevation following an acute event. Urinary I-FABP measurement could be a good alternative to plasma I-FABP detection to reveal enterocyte damage, because urinary I-FABP levels will reflect I-FABP release over a longer period of time. Furthermore, the use of urine is of great advantage compared with blood, currently in use, because blood withdrawal for diagnosis is the main cause of anemia in neonates.\textsuperscript{74,23}

Previously, we collected urine of 17 consecutive neonates suspected of NEC and reported that 5 of the infants who developed NEC showed significantly higher I-FABP levels than the infants without NEC.\textsuperscript{9} The current study prospectively identified 14 neonates with NEC among a population of 35 infants suspected of NEC. This study showed that median urinary I-FABP was significantly higher in neonates suspected of NEC who developed NEC compared with neonates with other diagnoses. Moreover, overall accuracy of the marker tested was high, and clinically relevant positive and negative LRIs were found. In most patients, urinary I-FABP levels were not elevated before clinical suspicion of NEC. The observation that urinary I-FABP levels are only elevated at the moment of suspicion indicates that NEC is a disease with an acute onset rapidly leading to clinical signs. Therefore, screening for NEC with the current tool seems not to be useful.

In line with previous studies, we found that significantly higher I-FABP values are related to NEC severity.\textsuperscript{22,23} Higher urinary I-FABP levels at the moment of suspicion of NEC were found in neonates with NEC necessitating surgery or resulting in death by intestinal necrosis compared with neonates with conservatively managed NEC. A cutoff value of 6.38 pg/nmol creatinine was found with a sensitivity of 1 and a specificity of 0.86. This cutoff point could be a guideline for making a decision to operate the neonate. Early surgery potentially lowers the extent of intestinal involvement at the time of operation which is related to improved survival rate and reduction in the devastating complication of NEC caused by surgery, the development of short bowel syndrome.\textsuperscript{26,27} However, future research aiming at finding a cutoff point at which operation is needed, should be performed in a larger patient population operated for NEC. It should also evaluate if early surgery leads to reduction of the extent of intestinal involvement and improves the prognosis of neonates.

In this study, samples were prospectively collected if there was a clinical suspicion of NEC and analyzed after completion of patient inclusion. Future analyses should and can be performed at the moment of NEC suspicion to be of clinical use.

The usefulness of fecal calprotectin in NEC diagnosis was apparent from a study that showed that fecal calprotectin differentiated between neonates with NEC and healthy age matched con-

**FIGURE 3.** Median I-FABP levels of neonates with intestinal necrosis necessitating surgery or causing death were higher than I-FABP levels of conservatively treated neonates. Cutoff point to discriminate patients with NEC necessitating operation or causing death from patients with conservatively managed NEC (6.38 pg/nmol creatinine) is represented by the dotted line.

**FIGURE 4.** In 4 of 6 neonates with NEC urine I-FABP levels were below cutoff level for NEC diagnosis until clinical suspicion of NEC. Levels in 5 of these neonates were maximal at suspicion. Cutoff point to differentiate neonates with NEC from neonates with other diagnoses is represented by the dotted line.
trols. In contrast, another prospective study showed that fecal calprotectin could not identify neonates with NEC in a population of neonates with a very low birth weight. Our study shows a high diagnostic accuracy and clinically relevant LRs of fecal calprotectin for the early diagnosis of NEC, with minimal overlap between individual values in patients with NEC and patients with other diagnoses. However, not all patients were included in this analysis, because only 21 neonates produced feces before diagnosis of NEC. Measurement of fecal calprotectin may have a limited utility in aiding in the diagnosis of NEC since stool samples cannot be obtained in almost half of the patients who developed NEC and a third of the patients without NEC.

This is the first study using noninvasive measurement of tight junction loss by urinary claudin-3 detection to diagnose NEC. The AUC of claudin-3 is high, although overlap is observed in urinary claudin-3 values of individuals in the group with NEC and without NEC. This could be explained by the fact that generalized inflammation which also occurs in other diseases, eg, during sepsis, causes tight junction loss.

Combination of the described markers can be useful to improve the accuracy of early noninvasive detection of NEC. In this study, we did not combine the markers to improve diagnostic accuracy because I-FABP already had very high accuracy and LRs. However, overestimation of LRs occurred because cutoff points and LRs were determined in the same patients. Therefore, measurement of the other markers may still contribute to the diagnosis of NEC, because inclusion of a new group of patients suspected of NEC could result in altered AUCs and LRs of the markers necessitating combination of the markers.

Term and preterm NEC are considered to be 2 different disease entities. In this study, we choose to include all patients suspected of NEC. Of the 14 neonates, 12 had a gestational age of <34 weeks, 1 neonate had a gestational age of 34 1/7 weeks, and only one term neonate with a gestational age of 38 2/7 weeks was included. This does not allow a valuable separation in term and preterm neonates. However, the NEC group did not differ from the patient group with other final diagnosis in gestational age or birth weight, preventing confounding on these parameters. A future study can elucidate if these markers are suitable for early diagnosis of term NEC.

In conclusion, this study showed that the urinary markers I-FABP and claudin-3, representing gut wall integrity loss, and the fecal marker calprotectin, representing gut wall inflammation, were significantly increased in neonates suspected of NEC who developed NEC compared with neonates with other final diagnoses, making them promising new noninvasive markers for early diagnosis of NEC. Furthermore, urinary I-FABP levels are increased immediately upon intestinal epithelial cell damage and are indicative of the extent of intestinal damage. Therefore, urinary I-FABP levels may also be of value to guide treatment strategies, as the necessity and timing of surgery. Screening for NEC before clinical suspicion does not seem useful, because I-FABP levels were not elevated before clinical suspicion of NEC, supporting the hypothesis that NEC is a disease with an acute onset which rapidly leads to clinical signs.

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