Serum levels of interleukin-8 and gut-associated biomarkers in diagnosing necrotizing enterocolitis in preterm infants☆

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A R T I C L E   I N F O

Article history:
Received 19 December 2013
Received in revised form 13 March 2014
Accepted 13 March 2014

Key words:
Necrotizing enterocolitis, NEC
Interleukin-8, IL-8
Intestinal fatty acid binding protein, I-FABP
Liver fatty acid binding protein, L-FABP
Biomarker

A B S T R A C T

Background: In recent years several potential biochemical markers have been evaluated to facilitate a reliable diagnosis of necrotizing enterocolitis (NEC), but none have made progress to clinical routine. We performed a comparative assessment in premature infants to evaluate the diagnostic value of the routinely available cytokine interleukin (IL)-8, and two promising experimental biomarkers, the gut barrier proteins liver fatty acid binding protein (L-FABP) and intestinal fatty acid binding protein (I-FABP), respectively, for the diagnosis of NEC.

Methods: IL-8, L-FABP, and I-FABP concentrations were analyzed in the serum of 15 infants with NEC and compared with 14 gestational age-matched infants serving as a control group.

Results: Serum concentrations of I-FABP, L-FABP and IL-8 were significantly higher in infants with NEC compared with controls. IL-8 showed the highest diagnostic value with an area under the curve of 0.99, followed by L-FABP and I-FABP. In addition we found a significant correlation between IL-8 and both FABPs in infants with NEC.

Conclusion: Our results further advocate the possible role of IL-8 as a specific marker for NEC. The diagnostic value of IL-8 seems to be superior to L-FABP, and similar to L-FABP. The routinely availability facilitates IL-8 as a possible candidate for further clinical investigations.

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Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in premature infants, and remains one of the leading causes for morbidity and mortality [1]. Surviving patients of NEC are facing severe sequelae including feeding problems, failure to thrive, dependence on parenteral nutrition, short bowel syndrome and neurodevelopmental impairment [2].

The exact pathogenesis remains to be elucidated, but one unifying hypothesis states a deregulated inflammatory response by the neonatal intestine to luminal bacteria [3]. In addition, intestinal epithelial injury is caused by different initiating events including intestinal ischemia, formula feeding, and colonization by opportunistic pathogens, leading to activation of the mucosal innate immune system and further damage of the epithelial barrier [4].

Against this background, several serologic biomarkers for the diagnosis of NEC have been investigated in the last decade, including markers of epithelial damage and gut barrier function, inflammation and pathogen invasion [5].

Interleukin (L)-8 seems to play a pivotal role in the activation of the proinflammatory cascade in NEC [6,7]. Our group has recently demonstrated that IL-8 may serve as a potential predictive marker in diagnosing NEC. We were able to show that IL-8 detected in the serum of infants with NEC was significantly correlated with disease extent [8] and that IL-8 was able to significantly differentiate between infants with surgical and medical NEC in a large patient collective [9].

Fatty acid binding proteins (FABP) comprise a group of cytoplasmatic small molecular mass proteins (~15 kDa) with high organ sensitivity. Elevated levels of intestinal fatty acid binding proteins (I-FABP) were shown in the plasma [10–13] and in the urine [14–16] of premature infants with NEC. Liver fatty acid binding protein (L-FABP) seems to be elevated in NEC compared with healthy control infants and septicemic patients [11,13]. Preliminary data on the diagnostic properties of fatty acid binding proteins for the early diagnosis of NEC are very promising, but remain to be determined. One drawback is the limited availability of these novel biomarkers in a daily routine clinical laboratory.

To the best of our knowledge there are no data available to show the association between a proinflammatory cytokine and markers of gut wall integrity. The aim of the study is to evaluate the diagnostic value of the routinely available cytokine IL-8 compared with the two experimental gut-associated biochemical markers I-FABP and L-FABP.

☆ Conflicts of interest and source of funding: None declared.

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http://dx.doi.org/10.1016/j.jpedsurg.2014.03.012
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1. Materials and methods

1.1. Study design and population

This prospective study was conducted at the Department of Pediatric Surgery and Pediatrics and Adolescent Medicine, Medical University of Vienna, and with the approval of the local ethics committee. Informed written consent was obtained from parents of all study subjects. Infants with NEC were recruited from January 2010 until July 2013. Each of the NEC blood samples was obtained from neonates exhibiting clinical signs and radiographic findings of NEC, according to modified Bell's staging criteria [17]. Blood samples were collected at the onset of clinical presentation of NEC. IL-8 was analyzed immediately after blood collection. Plasma from blood samples for I-FABP and L-FABP analysis was separated by centrifugation (3500 × g for 5 min) at 4 °C and stored at −80 °C until pooled analysis could be performed.

Control blood samples were collected from infants with a birth weight of less than 2000 g, and of younger than 6 months of age. Exclusion criteria were: any history of surgical intervention in the medical history, culture-proven or clinically suspected infection, chronic inflammatory diseases or congenital malformations. Signs for clinically suspected infection were at least one of the following symptoms: respiratory distress, feeding intolerance, abdominal distension, lethargy, irritability, or temperature instability.

1.2. Treatment groups

Infants with NEC were divided into two different treatment groups, medical NEC and surgical NEC. All infants assigned to the medical treatment group had been conservatively treated, and did not receive any surgical intervention. In the surgical treatment group, the medical treatment group had been managed conservatively, and did not receive any surgical intervention. The decision to perform surgery was made by the attending pediatric surgeon and was not influenced by this study whatsoever.

1.3. Demographic and clinical parameters

Demographic parameters recorded and evaluated for all patients included birth weight, gestational age, 1 min Apgar score, 5-min Apgar score, and age at diagnosis of NEC. Important clinical data pertaining to NEC were: frequencies of intraventricular hemorrhage, persistent ductus arteriosus, use of medication (steroids, antibiotics and vasopressors) and mechanical ventilation as well as hematologic indices and acute-phase markers like white blood cell count, platelet count and C-reactive protein.

1.4. Determination of IL-8

A sequential chemiluminescent immunoassay was used with the threshold set to 70 pg/ml as recommended by the manufacturer (Siemens Immulite; DPC, Los Angeles, CA). Median serum IL-8 levels of 27 (20–1213) and 29 (20–778) pg/ml are documented in the literature for a total of 351 healthy neonates over two consecutive time spans [19].

1.5. Immunoassays for I-FABP and L-FABP

Serum concentrations of I-FABP and L-FABP were determined by using commercially available ELISA kits (Hyclud Biotech, Uden, the Netherlands). Sample workup was done according to the manufacturer’s recommendation. Plasma samples were diluted 1:20 and 1:100 for I-FABP and L-FABP, respectively. Absorption was determined on a microplate reader (Statfax 3200, Awareness Technology Inc., Palm City, FL) at 450 nm.

1.6. Statistical analysis

Values of patient groups are presented as median values. Continuous variables were tested for normal distribution by applying the Kolmogorow–Smirnow test. When test failed (significant result \( p < 0.05 \)) Mann–Whitney U test was used for testing significance otherwise Student's t-test was used. Spearman rank-order correlation was used to evaluate correlation between parameters. Correlation coefficients \( (r_{\text{rho}}) \) of 0.7–1.0 or −0.7 to −1.0 were considered as strong correlations 0.5 to 0.7 or −0.5 to −0.7 as moderate correlations. Variables with lower correlation coefficients were not considered even when they were significant. The reported \( p \)-values are the results of two-sided tests. A \( p \)-value <0.05 was considered statistically significant. Statistical calculations were done using SPSS 17.0 software (IBM; Armonk, NY). SigmaPlot 11.0 (Systat Software GmbH, Erkrath, Germany) software was used to perform the ROC analysis. The method of DeLong et al. [20] implemented to the SigmaPlot software ROC option was used to compare AUCs. The report shows results for all pairs of data sets. The difference of each area pair, its standard error, 95% confidence interval, and chi-square statistic and its associated \( p \)-value for the area comparison were calculated. The heat map was drawn by using Excel 2007 software (Microsoft, Redmond, WA).

2. Results

2.1. Characterization of the study groups

During the study period a total of 15 infants with NEC were included and compared with 14 gestational age, birth weight and age at diagnosis-matched infants serving as a control group (details are shown in Table 1). Clinical characteristics including laboratory parameters of NEC and control infants are summarized in Table 2. No significant differences were found for the following parameters: 1-min and 5-min Apgar score, frequency of male sex and the frequency of twin pregnancies.

In the NEC group comprising 15 infants, 10 received surgical and 5 received medical treatment, respectively.

2.2. Significant increase of IL-8 and gut barrier markers in serum of NEC patients

Serum concentrations of IL-8, I-FABP and L-FABP were significantly higher in patients with NEC when compared to the control group (IL-8, 46 pg/ml vs 1562 pg/ml, \( p < 0.001 \); I-FABP, 1.4 ng/ml vs 2.8 ng/ml, \( p = 0.010 \); L-FABP, 35.0 ng/ml vs 122.4 ng/ml, \( p < 0.001 \)). Fig. 1a illustrates the concentrations of all the three serum concentrations.
parameters. The median value of IL-8 was 34-fold, I-FABP was 2.0-fold and L-FABP was 3.5-fold elevated compared to the control group.

Only one IL-8 value of the 14 control samples overlapped with the 0th to 25th percentile range of the corresponding NEC samples. One I-FABP value within the control group exceeded the median value of the corresponding NEC group. One L-FABP value of the control group exceeded the 25th percentile of the NEC group.

The serum levels of all three parameters were significantly lower in control patients than in surgically treated NEC patients (IL-8, 46 pg/ml vs 3754 pg/ml, \( p < 0.001 \); I-FABP, 1.4 ng/ml vs 4.2 ng/ml, \( p = 0.009 \); L-FABP, 35.0 ng/ml vs 131.5 ng/ml, \( p < 0.001 \)) (Fig. 1b). The serum levels of IL-8 and L-FABP were also significantly lower in controls than in medically treated NEC patients (IL-8, 46 pg/ml vs 447 pg/ml, \( p = 0.002 \); L-FABP, 35.0 ng/ml vs 110.4 ng/ml, \( p = 0.008 \)) (Fig. 1b).

None of these three parameters showed significant differences between both medical treated NEC (\( n = 5 \)) and surgically treated NEC patients (\( n = 10 \); IL-8, 447 pg/ml vs 3754 pg/ml, \( p = 0.098 \); I-FABP, 2.2 ng/ml vs 4.2 ng/ml, \( p = 0.298 \); L-FABP, 110.4 ng/ml vs 131.5 ng/ml, \( p = 0.326 \)).

![Table 2](image)

**Table 2**
Clinical characteristics of study patients.

<table>
<thead>
<tr>
<th></th>
<th>Control (( n = 14 ))</th>
<th>NEC (( n = 15 ))</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IVH, n (% of total)</strong></td>
<td>4 (29%)</td>
<td>8 (53%)</td>
<td>0.264</td>
</tr>
<tr>
<td><strong>PDA, n (% of total)</strong></td>
<td>8 (57%)</td>
<td>9 (60%)</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Medical therapy of PDA, n (% of total)</strong></td>
<td>4 (29%)</td>
<td>7 (47%)</td>
<td>0.450</td>
</tr>
<tr>
<td><strong>Use of steroids, n (% of total)</strong></td>
<td>4 (29%)</td>
<td>5 (33%)</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Use of antibiotics, n (% of total)</strong></td>
<td>0 (0%)</td>
<td>10 (67%)</td>
<td>–</td>
</tr>
<tr>
<td><strong>Mechanical ventilation, n (% of total)</strong></td>
<td>0 (0%)</td>
<td>4 (27%)</td>
<td>–</td>
</tr>
<tr>
<td><strong>Use of vasopressors, n (% of total)</strong></td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>–</td>
</tr>
<tr>
<td><strong>Positive blood cultures, n (% of total)</strong></td>
<td>0 (0%)</td>
<td>4 (27%)</td>
<td>–</td>
</tr>
<tr>
<td><strong>WBC (( \times 10^9/\text{mm}^3 )), median 25%–75% quartile</strong></td>
<td>10 (8–12)</td>
<td>5 (3–9)</td>
<td>0.010*</td>
</tr>
<tr>
<td><strong>PLT (( \times 10^9/\text{mm}^3 )), median 25%–75% quartile</strong></td>
<td>359 (234–453)</td>
<td>167 (118–358)</td>
<td>0.011*</td>
</tr>
<tr>
<td><strong>CRP (mg/dl), median 25%–75% quartile</strong></td>
<td>0.2 (0.10–0.50)</td>
<td>12.0 (2.93–18.20)</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

IVH, intraventricular hemorrhage; PDA, patent ductus arteriosus; WBC, white blood count; PLT, platelet count; CRP, C-reactive protein.

* Significant differences.

![Fig. 1](image)

**Fig. 1.** (a) Serum concentrations of IL-8, I-FABP and L-FABP in 14 controls (con) and in 15 patients affected by different grades of NEC (NEC). Boxes indicate the 25th and 75th percentiles; errorbar-caps indicate the 5th and the 95th percentiles. The middle line and the values below indicate the median value of the corresponding group. The dots indicate the single corresponding concentrations of control patients and NEC patients, respectively. *Significant differences (\( p < 0.05 \)). (b) Serum concentrations of IL-8, I-FABP and L-FABP in controls (\( n = 14 \)), nonsurgically treated NEC (\( n = 5 \)) and surgically treated NEC (\( n = 10 \)) patients. Boxes indicate the range between the 25th and 75th percentiles; errorbar-caps indicate the 5th and the 95th percentiles. The middle line and the values below indicate the median values of the corresponding group. The dots indicate the single corresponding concentrations of control patients and NEC patients, respectively. *Significant differences (\( p < 0.05 \)).
2.3. Association of IL-8 with both FABPs

There were significant moderate and strong positive correlations between IL-8 and both FABP concentrations (I-FABP, \( r_{\rho} = 0.67 \) and L-FABP, \( r_{\rho} = 0.86 \)) and a moderate correlation between both FABP concentrations \( (r_{\rho} = 0.64) \) in all study patients. Only low correlations of IL-8 and both FABPs were found within control patients. Within NEC patients IL-8 was strongly correlated with both FABP concentrations (I-FABP, \( r_{\rho} = 0.76 \); L-FABP, \( r_{\rho} = 0.70 \)).

In order to visualize serum levels of IL-8 and both FABPs in individual subjects a heat map was created (Fig. 2). A clear positive trend between NEC and the plasma concentrations of the three parameters was shown. In all NEC patients at least one of the three parameters was elevated in the range of the 50th to the 100th percentiles (IL-8, 197–14,564 pg/ml; I-FABP, 1.8–15.4 ng/ml; L-FABP, 49.0–1426.0 ng/ml). Furthermore, it shows that samples with high IL-8 or high L-FABP separate clearly from control samples. Only control 14 clusters in the same group as the NEC group.

2.4. Diagnostic value of IL-8 compared to both FABPs

In order to evaluate the diagnostic value of the three serum markers a receiver operating characteristic (ROC) analysis was performed. The ROC analysis showed that serum levels of all three parameters are potentially useful diagnostic markers to differentiate between control and NEC patients (all three parameters had area under the curve (AUC) values of above 0.8; Fig. 3). In detail, IL-8 had an AUC value of 0.99, \( p < 0.001 \), followed by I-FABP (AUC = 0.95, \( p < 0.001 \)) and L-FABP (AUC = 0.81, \( p = 0.002 \)). The AUC of IL-8 was significantly higher compared to I-FABP (\( \Delta \text{AUC} = 0.176, p = 0.030 \)) but not to L-FABP (\( \Delta \text{AUC} = 0.040, p = 0.278 \)).

3. Discussion

The present study represents a comparison of the serum levels of the proinflammatory chemokine IL-8 and two epithelial gut barrier markers I-FABP and L-FABP in NEC patients and age-matched controls. We were able to show that all three evaluated serum markers were strongly affected by the disease. The diagnostic accuracy of IL-8 seems to be superior to I-FABP and similar to L-FABP. Our findings indicate a strong positive correlation between IL-8 and both gut-associated proteins in NEC infants, suggesting that IL-8 is a specific biomarker for bowel injury in NEC.

Current data further support the hypothesis that IL-8 might play a pivotal role in the initial event and in the perpetuation of the local inflammatory process of NEC. The excessive inflammatory response of the immature intestine to colonizing bacteria and endogeneous inflammatory stimuli is in part caused by the underexpression of inflammatory regulator genes (IkB) [21] and the overexpression of effector IL-8 genes in the immature gut [22]. Accordingly, excessively elevated concentrations of the proinflammatory cytokine IL-8 have been reported in infants with NEC [6–9,23–26]. Moreover, in critically ill infants with sepsis, IL-8 levels were consistently lower in cases without intestinal involvement, in contrast to cases with gut involvement [24].

We have reported in a comprehensive analysis of 11 cytokines in surgical NEC, that only IL-8, IL-6 and IL-10 were significantly influenced by the disease [6]. These findings were in accordance to results found by Chan et al. [7] who identified IL-8 as one of the most relevant proinflammatory cytokines in NEC upon a panel of 174 immunoregulatory molecules. Accordingly, the present study showed...
a 34-fold median increase of IL-8 in 15 infants with NEC compared to controls.

In the present study, we were able to demonstrate that IL-8 levels were higher in infants with surgical NEC compared to those infants with medical NEC, even though these differences did not reach statistical significance \((p = 0.098)\). In a previous larger retrospective study on 113 infants with NEC, we demonstrated that IL-8 was able to discriminate between infants who will respond to medical treatment and of those in whom surgical treatment is necessary \([9]\). Furthermore, our study group reported a significant correlation of IL-8 with intestinal involvement in 40 infants with surgical NEC \([8]\). The prognostic value of IL-8 is further underlined by its property to predict the 60-day mortality \([9]\). Taken the results together, current data indicate that the excessively high levels of IL-8 obtained in infants with NEC mirrors the degree of intestinal damage. The significant positive correlation between IL-8 and both gut barrier markers in infants with NEC, but not in the control group further supports the hypothesis that the detected IL-8 is directly derived from the affected tissue.

L-FABP is expressed in the small and large intestine, liver, kidney and epithelial cells \([27]\). Data on plasma L-FABP and NEC are very limited. Only two studies evaluated the role of plasma L-FABP in the diagnosis of NEC \([11,13]\) but did not involve any cytokines. Guthmann and coworkers \([11]\) reported that L-FABP was significantly elevated in infants with suspected NEC, indicating to be a sensitive marker for early detection of the disease. Ng and colleagues \([13]\) very recently demonstrated that plasma concentrations of L-FABP, I-FABP and trefoil factor 3 (TFF3) were significantly higher in NEC infants than in septicemia and healthy control patients. The authors proposed that these markers could serve as specific biomarkers for NEC. In our study cohort, the diagnostic accuracy of L-FABP to discriminate NEC form control infants exceeded those of I-FABP. The ROC analysis revealed the highest AUC values for IL-8 (AUC = 0.99, \(p = 0.001\)) followed by L-FABP (AUC = 0.95, \(p < 0.001\)) and I-FABP (AUC = 0.81, \(p = 0.004\)). In addition, the ratio between infants with NEC and controls was much higher for IL-8 (up to a 34-fold increase) than for the epithelial markers (L-FABP 3.3-fold; I-FABP 1.8-fold). In accordance to the study by Ng et al. \([13]\), we observed higher L-FABP concentrations in infants with surgical NEC compared with medical NEC, but this elevation did not reach statistical significance \((p = 0.326)\).

This is the first report on the association between IL-8 and L-FABP in neonates with NEC. The expression of I-FABP is solely restricted to the intestinal mucosa, with its maximum at the distal jejunum \([28]\). Plasma concentrations of I-FABP are significantly increased in advanced stages of NEC compared with healthy controls and septicemic patients \([10–13,29]\). It is of interest to note that one study reported that I-FABP was not detectable in the majority of infants with NEC \([29]\). In the present study we were able to detect I-FABP in all infants, and demonstrated a significant increase in I-FABP concentrations in infants with NEC compared with controls. The diagnostic value of I-FABP is controversially reported in the literature. Previous studies \([11,12,29]\) suggested that I-FABP was not able to discriminate suspected from definite NEC. In contrast, Ng et al. \([13]\) recently showed that I-FABP was able to discriminate between surgical and medical NEC. In line with their study, we observed higher I-FABP concentrations in infants with surgical NEC compared with medical NEC, but this elevation did not reach statistical significance \((p = 0.298)\). Previous reports on plasma FABP's on different patient cohorts, which either did not differentiate between surgical and nonsurgical NEC \([11,12,29]\), or state at what time point I-FABP samples were collected in relation to surgical intervention \([10]\), indicate a possible predictive value of I-FABP that requires further investigation.

We are aware that the study has limitations. Despite the relatively small number of NEC patients preventing any specific treatment recommendations, our findings still demonstrate that in direct comparison to L-FABP and I-FABP, the potential diagnostic value of IL-8 seems to be superior to I-FABP and similar to L-FABP. In addition to our previous data \([8,9]\), results derived from this study cohort underline the role of IL-8 as a specific biomarker for bowel injury and NEC. The question whether IL-8 is superior to gut wall markers in distinguishing medical from surgical NEC warrants further clarification in a larger patient collective.

In contrast to novel biomarkers such as L-FABP and I-FABP, IL-8 offers the advantage of being routinely available as a certified in vitro diagnostic (CE-IVD) parameter in many (pediatric) clinical laboratories. Potential novel biomarkers that have been reported recently are not standardized and certified, thus are not allowed to be implemented for clinical routine including therapeutic decision making. IL-8, as an acute parameter, is of interest because of the availability of a routinely reported result within an hour after blood sampling. This circumstance offers unique possibilities regarding assessment of disease extent and therapeutic monitoring. We assume that longitudinal monitoring of IL-8 could substantially influence conservative management in order to limit antibiotic treatment according to disease activity and facilitate early initiation of oral feedings. In critically ill infants with NEC IL-8 may prove especially useful in difficult therapeutic decision making whether to prolong conservative management or to proceed to surgical intervention.

References


