The pathophysiology of necrotizing enterocolitis in preterm infants

Heida, Fardou Hadewych

CHAPTER 5

INTESTINAL FATTY ACID-BINDING PROTEIN LEVELS IN NECROTIZING ENTEROCOLITIS CORRELATE WITH EXTENT OF NECROTIC BOWEL: RESULTS FROM A MULTICENTER STUDY

Heida FH, Hulscher JBF, Schurink M, Timmer A, Kooi EMW, Bos AF, Bruggink JLM, Kasper DC, Pones M, Benkoe T

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Contents

Abstract

Introduction: Intestinal fatty acid-binding protein (I-FABP) is considered as a specific marker for enterocyte damage in necrotizing enterocolitis (NEC). The purpose of this study was to evaluate the association of plasma and urinary I-FABP levels with the extent of macroscopic intestinal necrosis in surgical NEC.

Methods: We combined data from prospective trials from two large academic pediatric surgical centers. Nine and 10 infants with surgical NEC were included, respectively. Plasma and urinary of I-FABP at disease onset were correlated with the length of intestinal resection during laparotomy.

Results: Median length of bowel resection was 10cm (range 2.5–50) and 17cm (range 0 –51), respectively. Median I-FABP levels were 53ng/mL (range 6.3–370) and 4.2ng/mL (range 1.1–15.4) in plasma in cohort 1 respectively cohort 2 and 611ng/mL (range 3–23,336) in urine. The length of bowel resection significantly correlated with I-FABP levels in plasma (Rho 0.68; p=0.04 and Rho 0.66;p=0.04) and in urine (Rho 0.92; p=0.001).

Conclusion: This 'proof of concept' study demonstrates that plasma and urine I-FABP levels at disease onset was strongly associated with the length of intestinal resection in surgical NEC. This offers further evidence that I-FABP levels are a promising biomarker for assessing intestinal necrosis in infants with advanced NEC.

Introduction

Necrotizing enterocolitis (NEC) is a severe intestinal inflammatory disorder of newborns associated with high mortality rates.[1] Initial treatment consists of discontinuation of enteral feeding, nasogastric suction, intravenous administration of broad-spectrum antibiotics, and cardiopulmonary support.[2] In the case of perforation or clinical deterioration despite maximal conservative treatment, resection of the affected bowel is often the treatment of choice.[2,3] The fact that symptoms and laboratory results are often unspecific during the early stages of disease makes a timely diagnosis of NEC challenging.[4] The assessment of intestinal necrosis and the timing of surgery, especially in the absence of perforation, remain as key problems in NEC. Furthermore, the decision of early surgical intervention might lead to an unnecessary laparotomy (including general anesthesia with its associated risks), while postponing surgery might lead to further disease progression with severe sepsis and eventual death.[3,4] Consequently, novel diagnostic and prognostic tools are in great demand.

In recent years, several promising diagnostic and prognostic markers for NEC have been identified. One of these markers is intestinal fatty acid-binding protein (I-FABP). I-FABP is a cytoplasmic protein with high organ sensitivity found in the enterocytes located at the tip of the villi. I-FABP plays a central role in the fat-metabolism processes of these cells.[5–7] In the context of progressive gut wall barrier failure in NEC, enterocytes are damaged and I-FABP is released in the circulation with subsequent secretion by the kidneys. In several studies I-FABP levels have been identified as an early marker and also as a predictor for the severity (including the need of surgical intervention) of NEC.[5–7] These studies assume a correlation between I-FABP levels and the degree of intestinal involvement. However, no study confirming the correlation between I-FABP levels and the length of resected bowel is available. Therefore, we aimed to investigate the relation between I-FABP levels, as measured in plasma and urine, and the length of resected bowel (as a surrogate for the extent of tissue necrosis) in neonates with surgical NEC.

Methods

Patients

We conducted this multicenter study at the University Medical Center of Groningen (UMCG), termed as cohort 1, and at the Medical University of Vienna (MUV), termed as cohort 2. In the UMCG all parents of neonates with suspected NEC admitted to the NICU were asked to participate in a prospective study (registered under trial number NTR3239) focusing on the diagnostic value of several biomarkers in suspected NEC cases between October 2010 and October 2012. The Institutional Review Board approved this study.

In the MUV blood samples were collected from infants exhibiting clinical signs and radiographic findings of NEC between January 2010 and July 2013. This prospective observational study aimed to evaluate the diagnostic value of different biomarkers in infants with proven NEC. The Institutional Review Board approved this study (study number EK875/2009).

Only infants with proven NEC (Bell's stage ≥ II) who needed surgery were included in the present study. Infants with isolated intestinal perforations were excluded in both centers. Indications for surgery were bowel perforation (NEC IIIb) or lack of improvement despite optimal conservative therapy.[8] The decision to go to the operating theater was always a multidisciplinary decision by the neonatology and pediatric surgery team caring for the neonate. Those specialists were not aware of I-FABP levels during the clinical course of the disease.

Surgery and resection specimens

All operations were performed by or under close supervision of the consultant pediatric surgeons in both centers. Only macroscopic necrotic tissue was resected, thereby saving as much as possible viable bowel. Resection material was measured two times in those neonates who underwent a laparotomy. During laparotomy the surgeon measured (using a tape-measure) or estimated the length of the affected bowel at its anti-mesenterial border before resecting it. The surgeon tried to avoid stretching of the tissue. After resection the specimen was fixed in 10% buffered formalin solution. To improve measurement objectivity, the length of the bowel was measured again by the pathologist with a tape-measure and any abnormalities were recorded. Representative sections of normal and diseased bowel were taken out and embedded in paraffin. Tissue sections of 4μm were stained using haematoxylin and eosin using standard staining protocols. The same pathologist, who was blinded for the I-FABP data, examined the slides.

Sampling for I-FABP

Initial I-FABP levels were defined as the first recorded I-FABP levels in plasma and urine available after the onset of NEC. In cohort 1, an extra sample of 100 μL was obtained for study purposes with every routine blood analysis after NEC diagnosis. In cohort 2, I-FABP levels in plasma were collected only at onset of disease, and no I-FABP levels in urine were collected.

Blood samples were fractionated by centrifugation for 10 minutes at 2000 x *g*. Plasma was then collected in a 0.5 mL Sarstedt tube and stored at -80°C. Approximately 1.5 mL of urine was transferred to a 2 mL Sarstedt tube and also stored at -80°C. Urine samples were collected at regular intervals by placing a cotton wool swab in the diaper of the patients. Once saturated with urine the cotton wool was gently

squeezed into a sterile syringe. In patients with an indwelling catheter, urine was collected directly from the catheter. A laboratory technician without knowledge of the clinical data performed plasma and urinary I-FABP measurements. We used commercially available ELISAs for both urine and plasma I-FABP measurements (in cohort 1: Human FABP2 kit from R&D Systems, Minneapolis, America and in cohort 2: Hycult Biotech, Uden, the Netherlands). Sample workup was done according to the manufacturer's recommendations. Plasma samples were diluted at 1:20. Absorption was determined on a microplate reader (Statfax 3200, Awareness Technology Inc., Palm City, FL, US) at 450nm.

Statistics

Statistical analysis was performed using the Statistical Package for the Social Sciences (IBM SPSS Statistics 21, IBM Corp., Armonk, New York, USA). All data are presented as median with range, unless otherwise specified. We compared baseline variables between the two centers using the Mann Whitney U test or the Chi squared test, where appropriate. For testing correlations between I-FABP levels and length of bowel resection the Spearman's correlation test was used. Testing correlations between I-FABP levels in plasma at onset of disease and the length of bowel resection in both cohorts were separated because the ELISAs used were not the same and therefore I-FABP levels might not be interchangeable. To test this correlation for the whole population a regression analysis was performed with the correction for differences between the cohorts. Two sided p-values less than 0.05 were considered statistically significant.

Results

Patients

TABLE 1:

Patient Characteristics

	UMCG	MUV
Patients (n)	9	10
Gender (m/f)	7/2	6/4
Gestational age (weeks + days)	26 +5 (25 – 34)	27 (24 – 40)
Birth weight (grams)	1000 (670 – 2280)	1130 (590 – 3200)
Time of surgery after first symptoms (hours)	79 (3 – 816)	36 (8 – 172)
Final Bell's stage (n)	NEC 3a: 2 NEC 3b: 7	NEC 3a: 7 NEC 3b: 3
Resection (n)	Small intestine: 3 Colon: 5 Both: 1	Small intestine: 2 Colon: 1 Both: 4 Covered perforation: 3
Length of resection (cm)	10.7 (2.5 – 50)	17 (0 – 51)
I-FABP at onset of disease (ng/mL)	Plasma: 53 (6.3 – 370) Urine: 1,132 (3.3 – 23,335)	Plasma: 4.2 (1.1 – 15.4)

** *Values are expressed as median (range) if applicable.*

We included 9 (m/f: 7/2) respectively 10 (m/f: 6/4) neonates with surgical NEC in both centers. All infants underwent a transverse laparotomy of which none were treated by percutaneous drainage. In cohort 1, median gestational age was 26+5 weeks (range 25 - 34) and median birth weight 1000 grams (range 670 – 2280). In cohort 2 this was 27+0 weeks (range 24-40) and 1130 grams (range 590 – 3200) respectively.

Surgery and length of specimens

Resections included: five neonates with a small intestine resection, six neonates with a colon resection, and five neonates with both a small intestine and colon resection. Three infants presented with characteristic morphologic changes of NEC (e.g. mosaic like pattern of affected intestine and pneumatosis intestinalis) and intestinal perforations in an otherwise non-necrotic bowel segment. These infants were treated with a diverting enterostomy proximal of the diseased intestine and the intestinal perforation was sutured, but not resected. The diagnosis of NEC was confirmed intra-operatively in all patients. The diagnosis of NEC was confirmed by histopathology in all patients who received bowel resections. Median length of bowel resection was 10.7 cm (range 2.5 - 50) in cohort 1, and 17 cm (range 0 – 51) in cohort 2. Measurements performed by the surgeon and then remeasured by the pathologist matched for all patients in both cohorts. In cohort 1 five enterostomies were performed, while in cohort 2 in all cases enterostomies were performed. No differences in length of resection were found in both centers (p=0.88). In addition we were not able to detect any differences in the length of the resected bowel: small intestine (p=0.20), colon (p=0.20) small intestine and colon together (p=0.21). Throughout all resection material in both cohorts ischemic necrosis was found. In most of the cases (>80%) the ischemia and necrosis reached into the resection margins. No differences in I-FABP levels were found between patients who were treated by small intestine resection and those treated by colon resection (p=0.68 respectively p=0.66).

I-FABP levels in plasma correlate with extent of intestinal necrosis at onset of disease

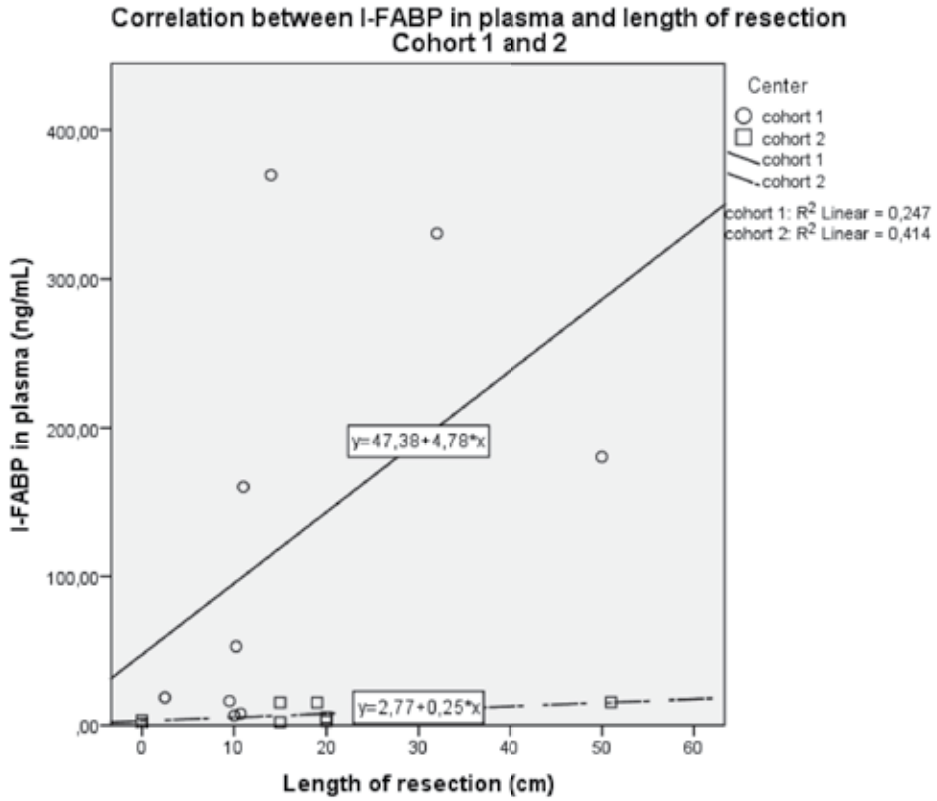
Median I-FABP levels in plasma were 53 ng/mL (range 6.3 – 370) in cohort 1 and 4.2 ng/mL (range 1.1 – 15) in cohort 2. Median urine I-FABP levels were 611 ng/mL (range 3.3 – 23,336) in cohort 1. In both cohorts, plasma I-FABP levels at onset of disease correlated significantly with the length of bowel resection (respectively, Spearman Rho 0.68; p=0.04 and Spearman Rho 0.66; p=0.04). A linear regression analysis showed that in the cohorts combined, corrected for their respective differences, I-FABP levels in plasma and the length of bowel resection were significantly correlated (p=0.02). These results are visualized in Figure 1. In addition,

urine I-FABP levels at onset of disease correlated significantly with the length of bowel resection in cohort 1 (Spearman Rho 0.92; p=0.001).

FIGURE 1:

Correlations between I-FABP in plasma and length of resection in cohort 1 and 2

This figure shows the correlation between I-FABP in plasma (ng/mL) and the length of bowel resection (cm) in both cohorts together with their regression line.



Discussion

This multicenter study demonstrates that both plasma and urine I-FABP levels are strongly associated with the length of bowel resection in neonates with surgical NEC. The present data supports our hypothesis that elevated I-FABP levels correspond with the extent of necrotic tissue in infants with NEC. It further underlines the importance of I-FABP as a promising diagnostic tool regarding the prediction of disease severity in NEC. Despite the severity of disease for NEC patients being described by Bell in 1987 (Bell's stages I-III), the early signs and symptoms of NEC still remain elusive and are hard to interpret due to their non-specific character.[9] Therefore, specific markers to detect NEC and to predict the necessity to intervene surgically are highly sought after.[3] I-FABP is assumed to be a specific marker for determining the severity of NEC.[5–7,10–12] Even in the early stages of NEC, I-FABP levels are reported to possess diagnostic value to predict outcome and the need for surgery.[513] Recent studies demonstrated that urine I-FABP levels are useful in the early diagnosis of NEC and that urinary I-FABP may be of value to guide treatment strategies, such as deciding upon the need for and timing of surgery.[11,12]A similar study from our institution confirmed these findings showing the diagnostic value of I-FABP levels in plasma.[5]

Evennett et al.[14] reported the use of urinary I-FABP as a marker of mucosal damage in surgical NEC, where it predicts the extent of intestinal involvement. However, Derickx et al.[12] assumed that I-FABP levels could be used to estimate the extent of intestinal damage, but they were not able to prove their hypothesis. The present study is first to demonstrate the correlation between both plasma and urine I-FABP levels with respect to the length of resected bowel. With this study, we are able to show a very strong association between I-FABP levels in urine and the length of resected bowel. Previously it was reported that I-FABP in urine is not only a suitable biomarker for the presence of NEC, but could also be very useful in distinguishing surgical from conservatively treated NEC cases.[15] Importantly, these results further strengthen our hypothesis that I-FABP levels are indicative of enterocyte damage during NEC development.

While the pattern of intestinal involvement in NEC can be highly variable, in most cases the terminal ileum is affected. I-FABP is expressed in the entire intestinal tissue with the highest tissue contents detected in the jejunum.[15–17] Plasma levels of I-FABP have been shown to be valuable markers of intestinal epithelial compromise, but it is not possible to locate the site of diseased intestinal segment neither by detected I-FABP levels, nor by combining different fatty acid-binding proteins.[16]

We acknowledge several limitations of the present study. First, the population size is small and shows wide ranges. However, this "proof of concept" is comparable to other studies focusing on infants with surgical NEC. Secondly, the length of resected

bowel does not necessarily correspond to the actual extent of mucosal damage and is also susceptible to the surgeon's preference. All surgeons in the present series are senior consultants, who aimed to preserve as much bowel length as possible. This was confirmed by the pathologist who found throughout all resection material ischemic necrosis and in most cases, ischemic and necrotic lesions extending into the resection margins. Thereby, we acknowledge that the fact that we could not relate our data with the total bowel length/resection length ratio and body weight adjusted specimen weights is a limitation to our study. In addition, the time between first symptoms and surgery in cohort 1 was evidently longer than in cohort 2, which could have influenced our results. However, between both cohorts there was no significant difference between the lengths of resected bowel. Finally, different ELISA kits were used in both centers. The detected I-FABP levels in cohort 2, as measured with the Hycult system, were lower. This corresponds with the results of Matsumato[18], whose I-FABP levels measured using the Hycult system were also lower compared to levels found with other commercially available ELISA systems. In a linear regression analysis we combined data from both cohorts, which revealed a significant correlation between I-FABP levels in plasma and the bowel resection length. This finding further emphasizes that the differences in I-FABP levels due to the use of different ELISA systems did not invalidate our results. Future multi-institutional studies should aim at the prognostic value of urinary and plasma I-FABP levels not only to predict the severity of intestinal involvement, but also the location of NEC, and the amounts of (dead or alive) enterocyte mass in the specimens in relation to the remaining bowel.

Conclusion

This study represents the first 'proof of concept' study that I-FABP levels are correlated with the extent of necrotic tissue in infants with surgical NEC. The significant correlation of elevated plasma and urinary I-FABP levels with the length of resected bowel further underlines the possible clinical importance of I-FABP in the risk assessment of infants with surgical NEC. Our data offers additional evidence that I-FABP can be considered a marker for enterocyte damage.

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SECTION 3

BACTERIAL COLONIZATION AND NEC

Chapter 6 Bloodstream infections during the onset of necrotizing enterocolitis and their relation with the pro-inflammatory response, gut wall integrity and severity of disease in NEC

Chapter 7 A necrotizing enterocolitis-associated gut microbiota is already present in the meconium: results of a prospective study

Chapter 8 Identification of bacterial invasion within the intestine in NEC specimens using fluorescent in situ hybridization