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Urinary intestinal fatty acid–binding protein concentration predicts extent of disease in necrotizing enterocolitis

Nicholas J. Evennett*, Nigel J. Hall, Agostino Pierro, Simon Eaton

Department of Surgery, Institute of Child Health, WCIN IEH London, UK

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Abstract

Purpose: Urinary intestinal fatty acid–binding protein (i-FABP), a marker of intestinal mucosal cell damage, has recently been proposed as a clinically useful measure in the early detection of necrotizing enterocolitis (NEC). However, there are no data on urinary i-FABP in more advanced (Bell stage II /III) NEC. The aim of this study was to test the use of urinary i-FABP in surgical NEC.

Methods: Urine was collected every 24 hours from infants with Bell stage II/III NEC admitted to a surgical Neonatal Intensive Care Unit. Clinical, laboratory, and surgical data were collected concurrently. Urinary i-FABP was quantified by enzyme-linked immunosorbent assay and expressed as picograms per nanomole creatinine (median [range]). Results are presented as median (range) and compared by Mann-Whitney test and by linear regression.

Results: There was a trend toward an increase in i-FABP:Cr in infants with NEC (controls, 1.0 [0.4-1.3], vs NEC, 2.1 [0.39-35.1], $P = .055$). Urinary i-FABP:Cr was significantly higher in infants with extensive disease (7.4 pg/mmol [2.1-35.0 pg/mmol]) than in those with focal disease (1.1 pg/mmol [0.3-1.7 pg/mmol]), $P = .002$. In addition, i-FABP:Cr was less than the previously suggested 2 pg/mmol cutoff in 6 of 16 infants with NEC, 5 of whom had focal disease. Urinary i-FABP:Cr decreased during both successful nonoperative management ($P < .0001$) and after surgery in the operated group.

Conclusions: In this pilot study, urinary i-FABP was associated with extensive disease in infants with NEC requiring surgery. Further work, in a larger number of patients, is required to investigate the applicability of urinary i-FABP as a marker of intestinal damage and as an adjunct to current indications for surgical intervention in infants with NEC.

Necrotizing enterocolitis (NEC) is a severe inflammatory disease of the neonatal gastrointestinal tract and the most common abdominal surgical emergency among this population [1]. Although Bell et al [2] published staging criteria for NEC more than 20 years ago, which were subsequently modified by Walsh and Kliegman [3], and continue to see widespread use today, the diagnosis of NEC remains a challenge to neonatologists and surgeons. Accordingly, there has been interest in developing improved diagnostic tests for NEC [4-9].

Intestinal fatty acid–binding protein (i-FABP) is a small (14-15 kd) and abundant protein, which constitutes up to 2% of the cytoplasmic protein content of the mature enterocyte [10]. Upon death of the enterocyte, its cytoplasmic contents

* Corresponding author. Tel.: +44 20 7095 2733.
E-mail address: nevennett@mac.com (N.J. Evennett).

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are liberated into the circulation, and a rise in plasma i-FABP concentration has been demonstrated both in animal models [11] and in a variety of human intestinal diseases [12-14] including NEC [12,15,16]. Because of its small size, i-FABP can pass through the glomerular apparatus and be detected in urine [13]. The use of urinary i-FABP concentration in the diagnosis of NEC among infants with abdominal signs has recently been demonstrated [17]. However, there is no information available on urinary i-FABP and disease severity or outcomes in infants with Bell stage II or III NEC.

The aim of this study was to examine, in infants with established NEC, the relationship between urinary i-FABP and (i) need for surgery and the (ii) extent of disease found at laparotomy.

1. Methods

1.1. Patients

The study was approved by the institutional review board of Great Ormond Street Hospital, study number 02SG26. Two groups of neonates who were treated in the Neonatal Intensive Care Unit (NICU) at Great Ormond Street Hospital, London, UK between January 2002 and December 2003 were enrolled in a prospective, ethically approved observational cohort study. The study group consisted of neonates with definite NEC (Bell stage II or III; all had pneumatosis intestinalis). A control group consisted of corrected gestational age- and weight-matched patients who were admitted to NICU without NEC, sepsis or septic shock, systemic inflammatory response syndrome, or an inborn error of metabolism. Treatment, including the decision as to whether to perform a laparotomy, was decided on the basis of clinical criteria, and urinary i-FABP levels were not used in this process because i-FABP levels were measured after all patients were discharged. Clinical and demographic data were collected from all patients enrolled in the study, including details relating to surgical procedures performed, the extent of disease noted at laparotomy, and the clinical outcome. The extent of disease was classified as focal when a single segment was affected, multifocal if 2 or more segments were affected, and panintestinal if more than 50% of the small and large bowels were affected [18].

1.2. Urinary i-FABP:Cr quantification

After written informed consent from the parents, urine was collected via an indwelling catheter (when present), via a urine bag, or from cotton wool swabs placed in the nappy and squeezed through a syringe barrel into a collection tube. Urine was continuously sampled throughout the day and stored on ice at the end of the cot before being transferred to a fridge at 4°C. At the end of each day, urine was pooled to form a representative 24-hour sample. Total urine volume was recorded before the samples were aliquoted and stored at −80°C. A single urine sample was taken at admission from controls. Before analysis, urine was centrifuged at 10,000 rpm for 10 minutes to remove the sediment. Because there was variation in the method of urine collection and uncertainty about completeness of a 24-hour collection, all i-FABP data were normalized to creatinine and expressed as a ratio between i-FABP and urinary creatinine (i-FABP:Cr, units in picograms per millimole). Urinary creatinine concentration was quantified by the Department of Chemical Pathology at Great Ormond Street Hospital.

Human i-FABP was quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) (HBT, Hycult Biotech, the Netherlands). Briefly, samples were diluted 2 times and incubated on a precoated ELISA plate for 1 hour at room temperature. The samples were discarded, the ELISA wells were washed 3 times, and a biotinylated secondary antibody was added for 1 hour. After further wash steps, the wells were incubated with streptavidin peroxidase for 1 hour. After a final 3-wash steps, tetramethylbenzidine was added for 30 minutes in the dark, before the reaction was stopped by the addition of citric acid. The plate was read at 450 nm, and i-FABP concentration was quantified by comparison with a set of predetermined standards.

1.3. Statistical analysis

Results are presented as median and range. Mann-Whitney tests were used to compare continuous data. Statistical analysis was performed using SPSS version 15.0 (SPSS, Chicago, Ill).

2. Results

Twenty neonates with NEC were enrolled into the study. Of these, 4 were excluded from analysis: 2 because only postoperative urine samples were available, and a further 2 with nonoperative disease were excluded because only a single urine sample from late in the disease process was available. Of the 16 remaining neonates with NEC, 4 were managed nonoperatively, 6 had focal disease, 3 had multifocal disease, and 3 had panintestinal disease. Six control infants were enrolled. Patient characteristics are outlined in Table 1. There was no clinical evidence of intestinal pathology in any of them, and all were either tolerating enteral feeds or had their enteral feeds stopped for anesthesia before patent ductus arteriosus (PDA) ligation. The infant with abdominal distension was not vomiting and had evidence of obstruction or other intestinal pathology, and the abdominal distension rapidly resolved spontaneously without any intervention. Admission weight was similar between the groups (NEC median, 1.68 kg [range, 1.19-3.26 kg] vs 1.91 kg [0.90-3.21 kg] in controls; $P = .33$). Corrected gestational age was also similar between the

Table 1 Characteristics of patients with NEC

Patient	Corrected Gestational Age (wk)	Admission weight (kg)	Bell stage	Extent of disease	Bowel resected (cm)	No. of urine samples	Initial i-FABP:Cr (illness day)		Peak i-FABP:Cr (illness day)		Survival
NEC 1	38	1.90	3b	Focal	21 colon, 7 ileum	6	0.53	(2)	0.72	(4)	Yes
NEC 2	40	3.20	3b	Focal	10 colon	2	0.39	(1)	0.39	(1)	Yes
NEC 3	33	1.70	2	Nonoperative		2	0.52	(3)	0.52	(3)	Yes
NEC 4	31	1.20	3a	Focal	4 colon, 2.2 ileum	3	2.16	(1)	2.16	(1)	Yes
NEC 5	41	3.26	3b	Focal	3.3 colon	3	0.28	(0)	1.09	(3)	Yes
NEC 6	29	1.19	3a	Focal	9 ileum	1	1.65	(1)	1.65	(1)	Yes
NEC 7	35	1.66	3a	Multifocal	19 ileum, 7 colon	3	5.36	(1)	5.36	(1)	Yes
NEC 8	37	2.28	3a	Panintestinal		1	4.93	(1)	4.93	(1)	No
NEC 9	36	1.88	3a	Nonoperative		3	1.38	(1)	1.38	(1)	Yes
NEC 10	35	1.70	3a	Panintestinal		1	2.08	(1)	2.08	(1)	No
NEC 11	31	1.28	3a	Panintestinal		1	35.13	(1)	35.13	(1)	No
NEC 12	28	1.19	2b	Nonoperative		5	4.34	(1)	4.34	(1)	Yes
NEC 13	31	1.56	3a	Multifocal	43 small bowel, 17 colon	4	2.36	(3)	2.36	(3)	Yes
NEC 14	32	1.25	3a	Multifocal	56 small bowel	2	14.60	(0)	14.60	(0)	Yes
NEC 15	31	1.19	3a	Nonoperative		6	5.33	(0)	5.33	(0)	Yes
NEC 16	32	1.88	3b	Focal	0.5 ileum 22.6 colon	5	1.48	(0)	1.48	(0)	Yes

			Diagnosis	i-FABP:Cr	Survival
Control 1	40	2.37	Delayed meconium	0.42	Yes
Control 2	36	3.21	Abdominal distension	0.87	Yes
Control 3	34	1.82	PDA, intraventricular hemorrhage	1.28	Yes
Control 4	32	0.90	Chest wall necrosis	1.12	Yes
Control 5	39	2.00	Bilateral hernias	0.75	Yes
Control 6	35	1.71	PDA	1.23	Yes

groups (NEC, 32.5 weeks [28-41] vs 35.5 [32-40] in controls; $P = .2$).

In all control infants, urinary i-FABP:Cr ratio was below the 2 pg/mmol cutoff previously suggested to distinguish infants with NEC from premature infants with no gastrointestinal necrosis [17]. On admission to the surgical NICU, urinary i-FABP:Cr was above 2 pg/mL in 9/16 infants with NEC but below 2 pg/mL in the other 7 infants (Fig. 1). There was a trend toward an increase in urinary i-FABP:Cr ratio at admission in infants with NEC, but this just failed to reach significance (controls, 1.0 [0.4-1.3] vs 2.1 [0.39-35.1]; $P = .055$). There was, however, a significant difference between control infants and the peak i-FABP:Cr level (which was preoperative in infants that required surgery) (2.1 [0.4-35.1], $P = .033$). There was no significant difference in initial urinary i-FABP:Cr between those infants with NEC who were successfully managed conservatively and those in whom surgery was performed (Fig. 1) (nonoperative, 2.9 [0.52-5.3]; operative, 2.1 [0.3-35.1]; $P = .8$).

Infants who received operative intervention were classified as having either focal or severe (multifocal or panintestinal disease) disease. Infants with more severe NEC had significantly higher preoperative urinary i-FABP:Cr (7.4 pg/mmol [2.1-35.0 pg/mmol]) compared with those infants with focal disease (1.1 pg/mmol [0.3-1.7 pg/mmol], $P = .002$) (Fig. 2, note the logarithmic scale). The length of resected bowel would be expected to correlate with i-FABP. However, this measurement is not available from those patients with panintestinal disease in whom care was withdrawn, so too few data points were available to undertake a linear regression analysis of urinary i-FABP vs length of resected bowel (Table 1). Interestingly, those patients with small bowel involvement appeared to have a higher i-FABP than those with isolated or mainly colonic involvement (Table 1).

In those infants who had focal or multifocal disease, there was a significant decrease in i-FABP:Cr after surgery (Fig. 3) ($P \leq .0001$, $r^2 = 0.66$); postoperative samples were not available in those infants who had panintestinal disease

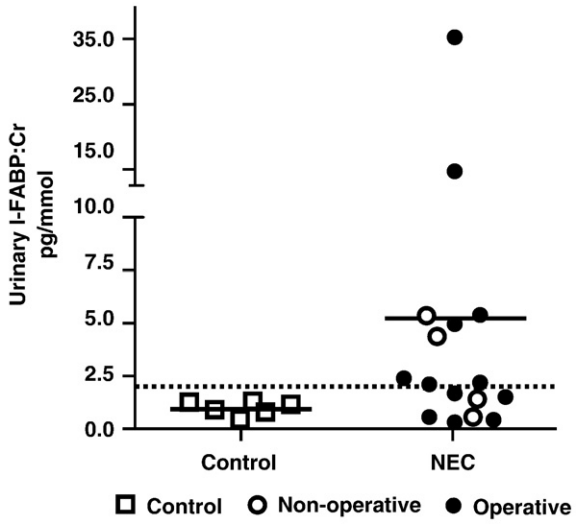

Fig. 1 Urinary i-FABP in infants with NEC at admission to surgical intensive care unit and in control infants. Dotted line represents 2 pg/mL, the cutoff previously suggested to differentiate between control premature infants with no gastrointestinal necrosis and those with NEC 17. Horizontal lines represent medians. Controls vs NEC, $P = .055$ (Mann-Whitney test).

where no attempt at resection was made, and further urine sampling was considered inappropriate.

In the 4 patients who were successfully managed nonoperatively, conservative treatment was also associated with a decreasing i-FABP:Cr ratio, regardless of the initial level (Fig. 4) ($P < .0001$, $r^2 = 0.88$).

3. Discussion

i-FABP is liberated into the circulation after intestinal mucosal injury [11-14], such as that occurring during NEC. Previous studies have examined the use of plasma i-FABP

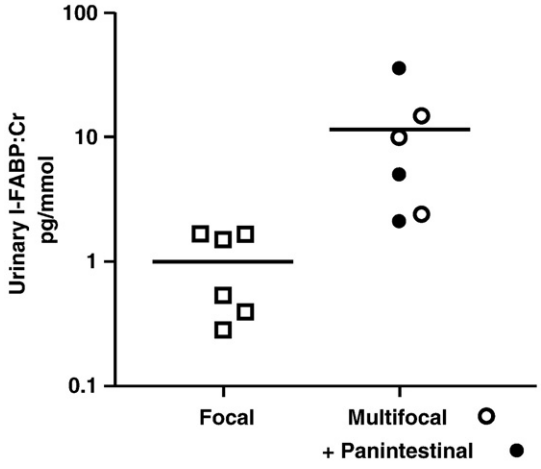

Fig. 2 Urinary i-FABP:Cr ratios in focal and extensive (multifocal and panintestinal) surgical NEC. Horizontal lines represent medians. Focal vs extensive NEC (multifocal plus panintestinal), $P = .0022$ (Mann-Whitney test).

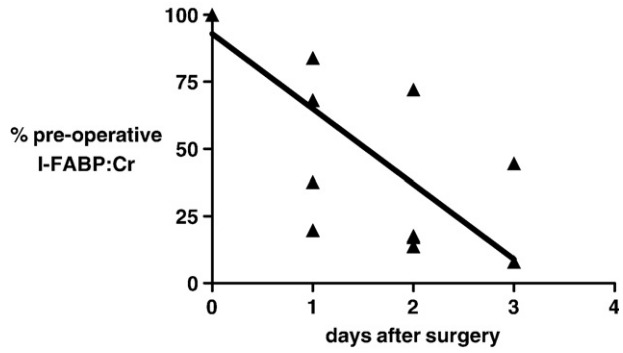

Fig. 3 Change in i-FABP:Cr ratio in patients with focal or multifocal NEC. i-FABP:Cr immediately preoperatively was given the value of 100% for each patient, and i-FABP:Cr on postoperative days calculated as a percentage of initial value. Linear regression $r^2 = 0.67$, $P = .0001$.

in distinguishing infants with NEC from premature controls without gut inflammation [12,15,16], and in a single study, a cutoff of 2 pg/mmol urinary i-FABP:Cr has been shown to distinguish, in Bell stage I infants, those who went on to develop Bell stage II or who had an ileal atresia with intestinal necrosis vs those who did develop NEC 17. In the present study, all control premature infants were below the 2 pg/mmol cutoff suggested in the previous study [17]. However, several infants with Bell stage 3 NEC also had urinary i-FABP:Cr ratios below this level, and the difference between infants with NEC and control infants was not significant. Thus, using 2 pg/mmol yields a specificity of 100% for NEC but a sensitivity of only 58%. Other cutoff values perform equally poorly. A receiver operating characteristic curve [19] gives an area under the curve of only 0.77 for admission i-FABP:Cr (Fig. 5; a perfect test would have an area under the curve of 1, whereas one performing no better than chance would have an area of 0.5. Although this is a worse performance than

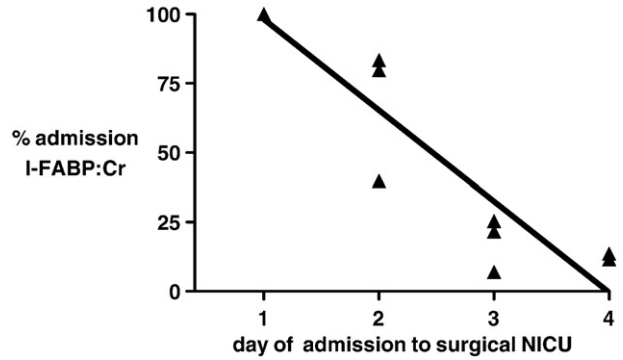

Fig. 4 Change in i-FABP:Cr ratio in patients with NEC who were successfully managed nonoperatively. i-FABP:Cr on the day of admission to the surgical intensive care unit was given the value of 100% for each patient, and i-FABP:Cr on subsequent days calculated as a percentage of initial value. Linear regression $r^2 = 0.88$, $P < .0001$.

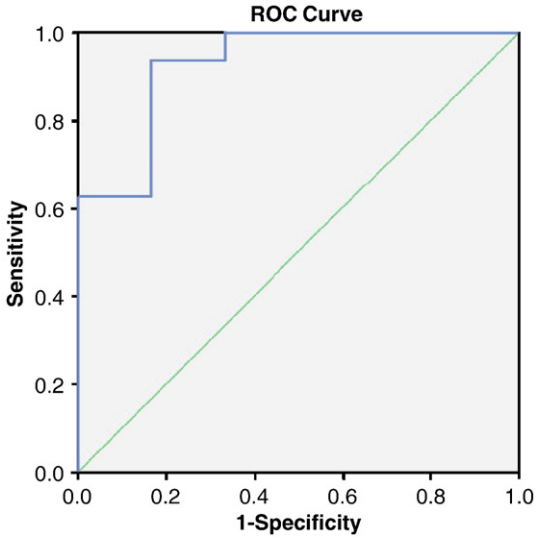

Fig. 5 Receiver operating characteristic curve of admission i-FABP:Cr.

reported in the previous study of urinary i-FABP [17], the accuracy is comparable with that reported in the previous studies using plasma i-FABP [12,15,16]. There are several possible reasons why urinary i-FABP may be low despite the presence of intestinal mucosal damage. i-FABP is mainly found in the mature enterocytes of the villus rather than the crypts [12]. If mucosal damage occurred several days previously in a single intestinal segment, all mature enterocyte contents may already have been lost to the circulation and i-FABP may have already been excreted in the urine. The plasma half life of the liver isoform of FABP (L-FABP) has been estimated to be approximately 11 minutes [20], and that of i-FABP would be expected to be similar, so that plasma or urinary i-FABP is only likely to be detectable if there is continued extension of the area of villus mucosal damage. It is interesting that i-FABP was less than 2 pg/mmol only in the NEC patients with focal disease, where the area of mucosal damage is small. In these patients, i-FABP could be low because the area of mucosal damage is so small that increases in i-FABP are either undetectable at any time-point or transient so that they were not detected in this study. If the villi have been completely lost and the area of damage extended to the muscle layers, i-FABP may no longer be detectable. It would be of interest to try to develop a marker of intestinal muscle injury and to measure i-FABP and a muscle marker in serial samples to address this issue.

The ELISA kit used for i-FABP is highly specific for the human intestinal isoform and does not cross-react with human L-FABP (manufacturer's data). Although i-FABP is exclusively present in intestine, L-FABP is predominant in the liver but also found in the intestine, so that any increase in L-FABP during NEC would not solely be caused by liver damage but could also be caused by release from damaged intestine.

Our results suggest that urinary i-FABP:Cr alone is not a useful marker to distinguish surgical and nonsurgical NEC. Presumably, this reflects that a significant intestinal mucosal injury can be sustained, liberating i-FABP into the circulation, without leading to the transmural intestinal necrosis that necessitates surgery. This again highlights the desirability of a test for intestinal muscle damage. However, our data do show that more extensive intestinal involvement is associated with higher urinary i-FABP:Cr ratios. This suggests that once the disease has reached surgical severity, the degree of mucosal injury as detected by i-FABP correlates with the unique view of the intestine that surgeon has at laparotomy. Thus, the receiver operating characteristic curve for the ability of i-FABP:Cr to distinguish severe NEC from focal disease has an area under the curve of 0.97, and the availability of such information preoperatively could be useful in planning operative strategies and anticipating perioperative management requirements. Conservative and surgical management were both associated with a decrease in i-FABP:Cr. However, as we have shown that i-FABP:Cr is also low in infants with focal disease who required surgery, an isolated low i-FABP:Cr value or a progressively decreasing i-FABP:Cr cannot be interpreted as an indication that surgical intervention is not indicated. We suggest that it could instead be interpreted as an indication that there is no further mucosal damage occurring. The site of involvement is, however, a confounding factor. Involvement of only or mainly the colon appears to be associated with a less marked increase in urinary i-FABP, and this is in keeping with the lower i-FABP levels in the colon compared with small bowel [21].

In conclusion, this study supports the use of urinary i-FABP:Cr as a marker of mucosal damage in surgical NEC, where it predicts the extent of intestinal involvement. Other markers can be difficult to interpret, and future studies should be aimed to determine whether urinary i-FABP is superior to the commonly available laboratory tests in predicting the extent of disease and useful in surgical decision making in NEC.

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