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

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Contents

Abstract

Introduction: Bacterial involvement is believed to play a pivotal role in the development and disease outcome of NEC. However, whether bloodstream infections predispose to NEC (e.g. by activating the pro-inflammatory response) or result from the loss of gut wall integrity during NEC development is a longstanding question.

Objective: We hypothesize that the occurrence of a blood stream infection (BSI) plays a complementary role in the pathogenesis of NEC. The first aim of the study was to correlate the occurrence of a BSI during early phase of NEC with intestinal fatty acid-binding protein (I-FABP) levels, as a marker for loss of gut wall integrity due to mucosal damage, and interleukin (IL)-8 levels, as a biomarker for the pro-inflammatory cascade in NEC. The second aim of the study was to investigate the relation between the occurrence of a BSI and disease outcome.

Methods: We combined data from prospective trials from two large academic pediatric surgical centers. 38 neonates with NEC, 5 neonates with bacterial sepsis, and 14 controls were included.

Results: BSIs occurred in 10/38 (26%) Neonates at NEC onset. No association between the occurrence of BSIs and I-FABP levels in plasma (cohort 1: median 11 ng/mL (range 0.8–298), cohort 2: median 6.8 ng/mL (range 1.3–15)) was found in NEC patients (cohort 1: p=0.41; cohort 2: p=0.90). In addition, the occurrence of BSIs did not correlate with IL-8 (median 1,562 pg/mL (range 150–7,500); p=0.99). While the occurrence of a BSI was not correlated with Bell's stage (p=0.85), mortality was higher in patients with a BSI (p=0.005).

Conclusion: The low incidence of BSIs and the absent association of both the markers for loss of gut wall integrity and the pro-inflammatory response during the early phase of NEC, support the hypothesis that the presence of a BSI does not precede NEC.

Introduction

Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in newborn neonates.[1] Whether the occurrence of a blood stream infection (BSI) plays a causative role in the development of NEC (e.g. by activating the pro-inflammatory response) or is merely occurring as a result from the breech in gut wall integrity characterizing NEC is a longstanding issue.[2–5] In one theory, a BSI may lead to NEC by vasomotor and/or coagulation pathway responses; in a second theory, a BSI is the result from bacterial translocation from the gut preceded by a breech in gut wall integrity during NEC development.[2,5] Thereby, it remains challenging to discriminate neonates with NEC from neonates with bacterial sepsis during the early phase of the disease.

Intestinal fatty acid-binding protein (I-FABP) is a small protein located in the enterocytes, which is released immediately after enterocyte damage.[6–9] Recent studies introduced I-FABP as a promising marker for gut wall integrity in neonates with NEC.[6–9] However, the relationship between I-FABP levels and the occurrence of BSIs has until now never been investigated. The exaggerated inflammatory response of the immature gut immune system in preterm neonates is triggered by various factors. interleukin-8 (IL-8) seems to play an important role in the pro-inflammatory cascade during early NEC and is believed to be a reliable marker for NEC.[10,11]

According to the currently available literature, showing a low incidence of BSIs during NEC development, we hypothesize that the occurrence of a BSI is complementary to NEC development. To test this hypothesis we aimed to analyze the relationship between the occurrence of a BSI with the onset of NEC. Next we intended to assess relations between the occurrence of a BSI and the loss of gut wall integrity caused by mucosal damage (as expressed by I-FABP) together with the pro-inflammatory cascade (via IL-8) in neonates with NEC. Moreover, we compared the results in NEC patients with the results from neonates with bacterial septicemia and controls. Finally we studied the association between the occurrence of a BSI with disease outcome in NEC.

Material & methods

Patients

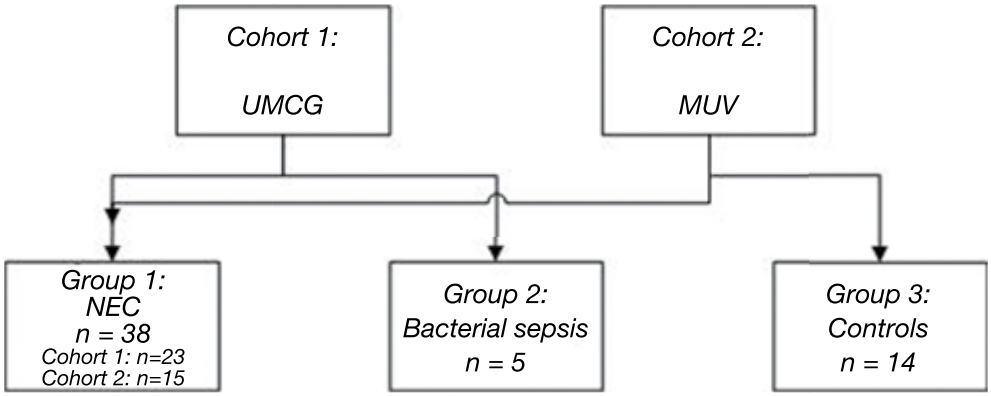
This study combines data from two prospective studies conducted at the University Medical Center of Groningen (UMCG), The Netherlands, and the Medical University of Vienna (MUV), Austria. Patient cohorts from both medical centers were termed cohort 1 and cohort 2, respectively (Figure 1). In cohort 1 all (parents of) patients with suspected NEC admitted to the NICU were asked to participate in a prospective

study (registered under trial number NTR3239 in the Dutch Trial Register). In cohort 2 all (parents of) patients with NEC admitted to the NICU and premature neonates with a birth weight <2000 grams were asked to participate in a prospective study (registered under trial number EK875/2009 in the Austria Clinical trial Register). Both prospective trials focused on the diagnostic value of several biomarkers in NEC and were carried out between October 2010 - October 2012 and January 2010 - July 2013, respectively. Both studies have been approved by the Institutional Review Board of the UMCG and the Ethics Committee of the MUV, respectively, and were conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from the parents of all participants.

study design

In both cohorts, suspected NEC was defined as the decision to obtain an abdominal X-ray because of non-specific NEC symptoms, e.g. distended abdomen or bloody stools. Suspected NEC was defined as Bell's stage I with only non-specific symptoms, such as gastric retention, abdominal distension and mild ileus present.[8,12] When suspicion of NEC arose, all patients were treated by nil per os, gastric decompression and broad-spectrum antibiotics after having obtained blood cultures (BCs). Diagnostic laboratory and radiological studies were performed every 6-8 hours. Severity of disease was defined using Bell's stage. Length of NICU stay and mortality were used as additional parameters for disease outcome. When pneumatosis intestinalis was present, the time of that X-ray was used as the time of diagnosis (Bell's stage ≥ II).[13]

FIGURE 1:



Patients were categorized in three patient groups. Group 1 consisted of patients with NEC (Bell's stage ≥ II) from cohort 1 and 2. The second group included neonates from cohort 1 with bacterial sepsis and suspected NEC, who did not progress to definite NEC (Bell stage ≥ II). Bacterial sepsis was defined as confirmed symptomatic bacteremia. The third group consisted of neonates from cohort 2 with a birth weight of less than 2000 grams without clinical or laboratory signs of infection. This group was considered the control group. Neonates with spontaneous intestinal perforation (i.e. without pathologic evidence of NEC) and/or neonates with congenital bowel defects were excluded from both study cohorts.

Blood cultures

In both cohorts results from all available blood cultures were retrieved from the digital hospital information system. In both trial centers, blood cultures were obtained by peripheral puncture or through central venous catheters and further processed with the BactAlert system using Pediatric Fan blood cultures bottles (Biomerieux). Blood sample volumes of 1.0 mL were preferred and only blood cultures with a minimum of 0.5 mL blood were assessed. Blood cultures were considered positive when blood cultures were obtained from within 24 hours prior until 24 hours after onset of NEC symptoms and before the start of antibiotic treatment, thereby meeting the criteria for a laboratory confirmed BSI from the Center for Disease Control and Prevention.[14] In the case of a BSI, microorganisms were identified using standard microbiological procedures.

Plasma sampling

In both cohorts plasma samples were obtained and I-FABP levels measured. First samples were obtained at a median of 7.5 (range 1-24) hours after first symptoms. A technician blinded to the clinical data analyzed all samples. We used commercially available Enzyme-Linked Immuno Sorbent Assay (ELISAs) for 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. Absorption was determined on a microplate reader (Statfax 3200, Awareness Technology Inc., Palm City, FL, US) at 450nm.

In samples obtained from cohort 2, IL-8 levels were measured. IL-8 levels were defined as the first IL-8 levels available immediately after onset of NEC. The protocol of IL-8 determination was previously described. In brief, 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).[11] Median IL-8 levels in plasma of 27 (range 20-1213) and 29 (range 20-778) pg/mL are documented in the literature for a total of 351 healthy neonates over two consecutive time spans.[15]

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. For testing differences between categorical variables, the $\chi 2$ (chi-square), or Fisher Exact analysis was used. For testing differences between continuous variables, the Student's t-test, or Mann Whitney U test were used as appropriate. Associations between BSIs and I-FABP levels were calculated per cohort separately, because different ELISAs were used in both study centers. To test this association for the whole population a logistic regression analysis was performed with the correction for differences between the cohorts (different ELISA systems). Risk factors for mortality were tested both by univariate and multivariate analyses and presented as odds ratios (OR) with 95% confidence interval (CI). In multivariate analyses, a change of more than 10% of the OR for mortality was considered collinear.

Results

Patient characteristics

We included 57 neonates diagnosed with either NEC (n=38) or suspected NEC (Bell's stage I) with bacterial sepsis without progression to NEC (n=5), or functioning as controls (n=14) (Figure 1). Patient characteristics are presented in Table 1. The median gestational age of all neonates was 28 (24-40) weeks with a median birth weight of 1143 (550-3200) grams. Of the 38 neonates with NEC, 20 were classified as having NEC Bell's stage II and 18 as NEC Bell's stage III.

TABLE 1:

Patient characteristics (n=57)

	Group 1: NEC n = 38	Group 2: Bell's stage I with an end diagnosis as bacterial sepsis n = 5	Group 3: controls n = 14	p-values
Male sex	27 (71%)	4 (80%)	8 (57%)	0.15
Gestational age (weeks)	28 (24-40)	28 (25-29)	29 (24-33)	0.68
Extreme prematurity (i.e. <33 weeks)	27 (72%)	5 (100%)	12 (86%)	0.50
Birth weight (grams)	1143 (550 – 3200)	1000 (900 – 1300)	1128 (560 – 1994)	0.34
Very low birth weight (i.e. <1500 grams)	27 (72%)	5 (100%)	10 (71%)	0.31
Caesarean section	22 (57%)	3 (60%)	14 (100%)	**0.01***
Antibiotic treatment post-partum	30 (78%)	(80%)	14 (100%)	0.12
Age at time of NEC diagnosis (Bell's stage II; days)	15 (3-85)	NA	NA	-
Antibiotic treatment at start of symptoms (Bell's stage I or above)	38 (100%)	5 (100%)	NA	-
Presence of BSIs	10 (26%)	5 (100%)	NA	**0.01***
IL-8 cohort 2 (pg/mL)	1,563 (147-15,000)	-	46 (20-197)	**<0.001***
I-FABP levels (ng/mL)				
- cohort 1	29 (1.2-3,748)	10 (0.8-91)	-	**0.03***
- cohort 2	2.8 (1.1-15)	-	1.4 (0.3-9.1)	**0.006***
NICU stay (days)	25 (8-102)	42 (22-79)	15 (3-30)	0.56
Surgical intervention in NEC patients (n)	12 (31%)	NA	NA	-
Mortality(n)	10 (26%)	2 (40%)	0 (0%)	0.14

*Data are expressed as median (range) or as numbers unless specified otherwise. *Statistically significant. Abbreviations: NA- not applicable; NEC – necrotizing enterocolitis; NICU – neonatal intensive care unit; I-FABP – intestinal fatty acid-binding protein; IL-8 – interleukin-8.*

The association between BSIs and diagnosis of NEC

When focusing on above-mentioned NEC patients, a BSI was present in 10 (10/38: 26%) neonates at the onset of symptoms (i.e. within 24 hours prior and 24 hours after onset of symptoms). In one neonate with NEC a BSI was present after 24 hours after onset of symptoms and within seven days after NEC onset. *Coagulase-negative staphylococcus* (CNS) was the most prevalent bacterial species (n=4). *Staphylococcus aureus* (n=2), *Escherichia coli* (n=2) and *Klebsiella pneumonia* (n=2) were among the other bacterial species found.

The association between BSIs and gut wall integrity

The median I-FABP levels of all three patient groups are presented in Table 1. I-FABP levels were significantly increased in neonates with NEC as compared to

neonates with bacterial sepsis (p=0.03). A logistic regression analysis showed that in the cohorts combined, corrected for the differences between the two cohorts, I-FABP levels were still significantly associated with NEC (p=0.006). No association was found between the occurrence of a BSI and I-FABP levels in patients with NEC (cohort 1: median=11 pg/mL, range 0.8-298; cohort 2: median=6.8 ng/ml, range: 1.3-15) (cohort 1: p=0.41; cohort 2: p=0.90). Again, a logistic regression analysis showed that in the cohorts combined, corrected for their respective differences, no relation between the occurrence of a BSI and I-FABP levels in patients with NEC was found (p=0.70). Also, no association was found between the occurrence of a BSI and I-FABP levels (median=10 ng/ml; range: 0.8-91) in patients with bacterial sepsis (p=0.73).

The association between BSIs and the pro-inflammatory cascade as based on IL-8 levels

IL-8 levels measured in samples of patients with NEC and/or bacterial sepsis are presented in Table 1. In NEC patients, median IL-8 levels were not statistically different in patients with or without a BSI: 1,562 (150-7,500) pg/mL versus 1,243 (147-15,000) pg/mL (p=0.99).

The relation of BSIs with patient outcome

The association of the occurrence of a BSI within 24h prior and 24h after start of symptoms and patient outcome, as defined by severity of disease, length of NICU stay and mortality was analyzed. A BSI occurred in 5 (5/20: 25%) patients with NEC Bell's stage II and in 5 (5/18: 28%) patients with NEC Bell's stage III (p=0.85). No relation between Bell's stage and the occurrence of a BSI was found (p=0.85), nor with the presence of the most prevalent cultured bacteria (i.e. CNS) (p=0.46). There was no difference in length of NICU stay between neonates suffering from NEC with a BSI as compared with neonates without a BSI: 50 (12 – 87) days versus 23 (8-102) days (p=0.45). We did not observe a statistically significant difference in length of NICU stay in neither the bacterial sepsis group nor control group when based on the occurrence of a BSI (p=0.56 and p=0.68, respectively). Mortality was significantly higher in NEC patients with a BSI as compared with NEC patients without a BSI 6/10 (60%) versus 4/28 (14%), p=0.005. When mortality in NEC patients with a BSI was compared to overall mortality in the bacterial sepsis group, no statistically significant difference was found: 6/10 (60%) versus 2/5 (40%), p=0.18. Mortality was not significantly increased in NEC patients compared with patients having a bacterial sepsis (OR: 4.5; 95% CI: 0.89-23; p=0.05). However, mortality rates significantly increased in patients with NEC Bell's stage III (OR: 5.6; 95% CI: 1.5-121; p=<0.01) or when a BSI (OR: 13; 95% CI: 3.2-58; p<0.01) was present. In

the present study, mortality was not significantly related to a very low birth weight (<1500grams) or very preterm birth (<33weeks) (OR: 2.1; 95% CI: 0.40-10; p=0.38 respectively OR: 1.6; 95% CI: 0.02-14; p=0.67). In multivariate analyses, NEC and/or NEC Bell's stage III did not influence the association between the occurrence of a BSI and mortality rates. The presence of a BSI remained an independent significant risk factor for increased mortality (OR: 13; 95% CI: 1.2-170; p=0.04).

Discussion

In this study the occurrence of a BSI and its relation with loss of gut wall integrity caused by mucosal damage and the pro-inflammatory response during early NEC development was investigated. We aimed to gain knowledge about whether a bacterial infection predisposes to NEC or results from the loss of gut wall integrity due to mucosal damage, which characterizes NEC. No association between the occurrence of a BSI and I-FABP levels (reflecting the loss of the gut wall integrity) in the earlier phases of disease development was observed. Furthermore, we were not able to find a positive association between the occurrences of a BSI and the exaggerated pro-inflammatory cascade as measured by IL-8 secretion in early NEC. Most likely, the occurrence of a BSI does not predispose for NEC.
Only limited data are available concerning the occurrence of BSIs in the early phase of NEC.

A low incidence of a BSI in patients with NEC has recently been reported in a large retrospective study: in 410 patients with NEC, the authors observed 69 (17%) patients with a BSI within 72 hours after onset of the disease.[3] Another similar large retrospective study found a BSI in 231/854 (27%) patients within seven days after onset of disease.[16] While these studies include a longer time span after NEC onset, these numbers are comparable to our results (occurrence of a BSI in 26% of NEC patients). The finding that a minority of patients suffered from a BSI in the early phase of NEC strengthens the hypothesis that NEC is not caused by the occurrence of a BSI and that BSIs only play a minor role in the initial NEC development. However, the occurrence of a BSI later during the course of the disease could result from disease progression and increased enterocyte damage with a breech in gut wall integrity.

CNS was the most prevalent pathogen at onset of NEC in our study. While Bizzarro et al.[3] reported a predominance of gram-negative bacteria during early NEC, Cole et al.[16] found similar results with a predominance of CNS. The predominance of CNS in these patients might be due to either skin colonization and contamination associated with prolonged use of intravascular devices or increased intestinal permeability.[16–18] Although not considered an enteric bacterium, CNS has been known to colonize

the gastrointestinal tract of neonates in western countries during the first month of life as early as the 3rd day of life.[16,18,19] In these studies, they are the predominant intestinal bacteria identified initially in fecal samples from preterm neonates.[16,18,19] Therefore, with only the detection of CNS in the bloodstream one could not rule out the possibility of an intestinal origin of CNS.

If bacteria derived from the gastrointestinal tract move to the systemic circulation because gut wall integrity is diminished, one would expect that the occurrence of a BSI would correlate with I-FABP levels at onset of NEC. Our data did not show such an association. Therefore one could suggest that failure of the gut wall barrier during early NEC is not the most relevant mechanism to allow for bacterial translocation from the intestinal lumen to the systemic circulation. In line of the results of this study, in which I-FABP levels did not correlate with the presence of a BSI, allows further speculation whether I-FABP levels are the most promising candidates to identify neonates with NEC from those with intestinal manifestations secondary to sepsis. While we suggest that in the early phase of NEC the assumed loss of gut wall integrity is not severe enough to allow bacterial translocation to the systemic circulation, this could be true during the later phase of the disease. Enterocyte damage and the integrity of the gut wall seem to be mirrored by circulating I-FABP levels. Recently our study group was able to show a positive correlation of I-FABP levels with the extent of transmural necrosis in neonates with surgical NEC.[9] These findings further underline the role of I-FABP as a marker for gut wall integrity in NEC. However, bacterial translocation can also occur (during the early phase of NEC) via the loss of tight junctions between the enterocytes,[6] but this evaluation was beyond the scope of the present study.

The pro-inflammatory cytokine IL-8 has already been found to be crucial in the pathogenesis of NEC by activation of the pro-inflammatory cascade.[10,11] Although IL-8 has been studied extensively in patients with sepsis, only limited data are available on the relation of BSIs and IL-8 in patients diagnosed with NEC. The activation of gut-wall associated macrophages by intraluminal bacteria or their products results in an exaggerated activation of the pro-inflammatory cascade in NEC, causing excessive IL-8 production.[5] The overwhelming activation of the pro-inflammatory cascade could explain why there is an association of IL-8 with disease severity and gut-wall barrier dysfunction,[20,21] but cannot explain an association of IL-8 levels with the occurrence of concomitant BSIs in neonates with NEC. Despite its association with disease severity and disease outcome, our data did not show any associations between IL-8 and the incidence of BSIs during the onset of NEC.

Our second aim was to investigate the association of the occurrence of a BSI with disease outcome. While late onset BSIs (>72 hours after NEC development) are thought to be associated with worse outcomes, we assumed this would not be the case for early onset BSIs.[3,22] Our study showed that during the initial phase of NEC, the occurrence of a BSI within 24h prior and 24h after start of symptoms was not associated with the severity of the disease as expressed by the patient's final Bell's stage. However, the occurrence of a BSI was associated with an increased mortality rate. This observation is shared by results published by Bizzarro et al.,[3] who found no significant association between severity of disease and length of NICU stay, but an increased mortality rate in patients with an early BSI (within 72h after onset of disease). In the study of Cole et al.[13] the occurrence of a BSI within the first 7 days after disease onset in NEC patients was not related with mortality. In the present study, parameters such as Bell's stage, birth weight or (low) gestational age tended to be associated with a higher mortality, even though without reaching statistical significance. Furthermore, these risk factors were not correlated with the occurrence of a BSI.

This study points to the clinical relevance of recently identified markers of gut wall integrity and the associated inflammatory process, with attention regarding their role in early discrimination between sepsis and NEC. Thereby, we assume that the occurrence of a BSI has no diagnostic value during the early phase of NEC, but has a prognostic value since the occurrence of BSI is associated with increased mortality. One limitation in this study is the usage of different ELISA kits for I-FABP measurements. The detected I-FABP levels in cohort 2, as measured with the Hycult ELISA, were systematically lower. This corresponds with the results of Matsumato,[22] who also reported lower I-FABP levels measured using the Hycult system compared with levels measured with other commercially available ELISAs. In a logistic regression analysis we combined data from both cohorts correcting for their respective differences, which showed no significant association between the occurrence of a BSI and I-FABP levels. This finding emphasizes that the differences in I-FABP levels due to the use of different ELISA systems did not invalidate our results. In order to confirm our hypothesis future research is warranted. In addition, while blood cultures are still considered the "gold standard" for diagnosing a BSI, many technical factors (e.g. a too small volume of blood to culture and/or the lack of an optimal growing medium) could affect the sensitivity of the results.[20,23–25] To get reliable results, 1-2 mL of blood per culture for neonates is recommended.[20,23–25] Despite the fact that some microorganisms grow poorly in conventional blood culture media, current media detect the most common microorganisms with a sensitivity of 93%.[20,23–25] New culture techniques, such as 16S rRNA-sequencing and real

time PCR assays, show an improved sensitivity and specificity for the detection of microorganisms. However, these methods have not yet been implemented in clinical routine. Further multi-institutional studies should aim to investigate the presence of BSIs during early NEC with these new culture techniques to elucidate our results.

Conclusion

Positive blood cultures are not associated with onset of NEC, decreased gut wall integrity, severity of inflammation, or severity of disease during the early stages of NEC. Therefore, we hypothesize that BSIs play a complementary role during these early stages. Moreover, the low incidence of BSI during early NEC suggests that they do not precede NEC. We hypothesize that loss of gut wall integrity based on mucosal damage during NEC is not severe enough to allow for measurable bacterial translocation to the systemic circulation.

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