CHAPTER 13

Necrotizing enterocolitis: a clinical review on diagnostic biomarkers and the role of the intestinal microbiota

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ABSTRACT

Necrotizing enterocolitis (NEC) remains one of the most frequent gastro-intestinal diseases in the neonatal intensive care unit, with continuing high mortality and morbidity rates. 20-40% of the infants with NEC will need surgical intervention. Although the exact pathophysiology is not elucidated yet, prematurity, us of formula feeding and an altered micro biome are supposed to induce an inflammatory response of the intestine.

The clinical picture of NEC has been well described. However, an early diagnosis and differentiation against sepsis is complicated. Besides, it is difficult to timely identify the NEC cases that will progress and need surgical intervention. This may interfere with optimal treatment of infants with NEC.

In this review we discuss the pathogenesis, diagnosis and treatment of NEC with a focus on the role of microbiota in the development of NEC. An overview of different clinical models and biomarkers is given. Some of these are promising tools in the diagnosis of NEC and timely identification of infants who need surgical treatment.

INTRODUCTION

Necrotizing enterocolitis (NEC) is a common gastrointestinal disease in premature born and low birth weight neonates, and one of the leading causes of morbidity and mortality at neonatal intensive care units.[1] The incidence of NEC in very low birth weight infants ranges between 3-15% with a high mortality rate varying between 15-30%.[2] The incidence rates of NEC have increased over the last decades, largely attributable to an increased survival of extremely low birth weight infants, who are at the highest risk of developing NEC. This devastating disease puts an enormous burden on healthcare system expenses, illustrated by the estimation that financial costs in the United States are up to $1 billion yearly.[3] This can be ascribed to prolonged hospitalization in the early phase and to long-term complications of survivors, including neurodevelopmental delay, short bowel syndrome, recurrent systemic infections and parenteral nutrition-associated liver disease. Early detection and initiation of treatment in NEC are key factors in its course and prognosis. In the diagnostic work-up of NEC, clinical and characteristic radiological findings remain the most important tools so far. Unfortunately, these signs are usually detectable in an advanced stage of disease. Therapeutic strategies of NEC consist of cessation of feeding, nasogastric decompression and broad systemic antibiotics and, in particular cases, surgery. These precautions are most effective when started at early stage. Hence, to reduce the disturbingly high mortality rate, there is an ongoing need for novel preventive strategies and non-invasive diagnostic biomarkers for early and accurate detection of NEC. Of preventive and diagnostic importance may be the gut microbiota. The indigenous gut microbiota has increasingly been described as key player in the pathogenesis of NEC. Therefore many studies focused on the development of biomarkers reflecting NEC-specific shifts in microbiota composition and on prophylactic strategies targeted at manipulation of the early neonatal microbiota colonization. In this paper, we review the microbiota-related factors involved in NEC and describe the potential of non-invasive biomarkers and future perspectives on their use in the (early) detection of NEC.

PATHOGENESIS

From an historical perspective, NEC was considered to result primarily from ischemic mucosal injury in an immature gut of preterm very-low-birth-weight infants.[4] More recent studies have shown that the development of NEC better fits a multifactorial model, including both antenatal and postnatal factors. Chorioamnionitis but also maternal antibiotic usage are

considered antenatal risk factors.[5] Postnatal factors include dysregulation of the immune system, altered gut motility, reduced enzymatic function, altered mucus production and composition, reduced innate defense mechanisms, rapid introduction and advancement of enteral feeding next to intestinal hypoxia-ischemia-reperfusion, formula and not human milk, and disturbance of normal neonatal gut colonization.[6,7] All these factors may provoke an inappropriate inflammatory response in an immature intestine,[8] inducing activation of (inflammatory) cytokines, decreased epidermal growth factor, increased platelet activating factor, and progressive mucosal damage by free radical production, which may subsequently lead to development of NEC.

MICROBIOTA AND NEC

Studies describing microbial composition in neonates with NEC have rapidly expanded in the last decade, by the availability of DNA-based high-throughput detection techniques, instead of culture-based methods. This has made it feasible to unravel the highly complex microbiota composition of the human intestinal tract. The currently assumed role of the intestinal microbiota in the development of NEC results from a wide range of observations. Protective effects on the development of NEC of oligosaccharides in breast milk, by stimulation of protective bifidobacteria and inhibition of the growth of coliforms and other pathogenic organisms, have already been described several decades ago.[9,10] Interestingly, outbreaks of NEC are reported in clusters and also seasonal variation in presentation has been observed, suggesting an infectious origin.[11,12] Moreover, NEC is typically diagnosed beyond the first week of age. From this age on, anaerobic bacteria have commenced to colonize the infant gut, including pathogens triggering inflammatory processes. Furthermore, in animal studies on experimental NEC, bacteria have been ascribed a pivotal role, since NEC does not occur in a germ-free environment, but can only develop following exposure to microbes.[13] The efficacy of antibiotics in the treatment of NEC and reduction in incidence following prophylactic administration of probiotics both indirectly link NEC to the intestinal microbiota. That not all bacteria act similarly is shown in a neonatal (preterm) pig model where some strains in probiotic mixtures are increasing the risk of NEC.[14]

Dysbiosis, defined as a disturbance in the precarious balance between beneficial, protective species versus harmful intestinal bacteria, was already described in 1975 as a provocative factor in NEC pathogenesis.[15] Later studies reported that mainly Clostridial species (including *Clostridium perfringens*, *Clostridium butyricum*, and *Clostridium neonatale*) were involved

in the etiology of NEC.[16-19] In 2004, Cochetierre and colleagues reported an association between early colonization with *Clostridia Perfringens* and development of NEC.[20] However, this assumed role for Clostridia was not confirmed in more recent studies, in which differences in microbial colonization between children with NEC and controls were mainly observed within the phylum Proteobacteria.[21-25] A predominance of Proteobacteria was observed in fecal samples of NEC cases up to two weeks before the clinical development of NEC, suggesting that dysbiosis predate the onset of NEC.[24] Interestingly, in one study describing the microbiota composition at two different time points before diagnosis of NEC, a lower proportion of Proteobacteria was observed in infants one week prior to the diagnosis of NEC compared with controls, with an excessive increase of Proteobacteria in the following days.[21] According to the authors, insufficient colonization with Proteobacteria early in preterm life may lead to a disturbed immune tolerance, provoking an exaggerated intestinal inflammation response and subsequently NEC, when exposed to an excessive increase of Proteobacteria later in life. Others reported more detailed NEC-specific differences in fecal microbiota composition, like colonization with Citrobacter-like sequences and Sphingomonas spp. Reports on overall diversity and abundance of enterococcal populations are contradictory, some studies have described reduced diversity and depletion of enterococcal microbes in NEC samples, a finding that could not be confirmed in other studies.[24,26,27] In only few studies no differences in microbial composition were noticed, like in a recent study from Normann and colleagues.[28] Comparing microbial communities in fecal samples from 10 children with NEC and 10 controls by means of 454 pyro-sequencing, no statistically significant differences were observed between the two groups. In the same study, the two groups could also not be differentiated based on microbiota analysis in mothers milk and fecal samples of mothers of the subjects.

In summary, the majority of studies on intestinal microbiota composition in NEC described disease-specific abnormalities as compared to controls. This enforces microbiota profiling, and biomarkers reflecting compositional disturbances, as a potential future diagnostic tool to detect (early) NEC. Reported findings differ highly across studies and no single causative bacterial agent or NEC-specific microbiotal signature has been identified yet. This might be explained by differences in applied (logistical) procedures and used detection techniques, and by sampling during different institutional outbreaks, in which different pathogens might be involved.

DIAGNOSIS

NEC typically presents in the second week of life, when the introduction of enteral feedings (especially cow's milk based formula) has occurred. Age of presentation is inversely related to gestational age, especially extremely preterm infants may develop NEC at later time points. The peak age of presentation is at 29-31 weeks postmenstrual age.[7,29] A recent large cohort study showed a bimodal distribution of NEC, with presentation at 8 and 19 postnatal days.[2]

Clinically, NEC can present with both gastro-intestinal and non-specific systemic signs as delayed gastric emptying, abdominal distention or tenderness (or both), bloody stools, lethargy, apnea, respiratory distress, or poor perfusion. Progression of NEC typically leads to abdominal tenderness and guarding, abdominal wall erythema or ecchymosis with a shiny appearance, or palpable distended loops of bowel. Abdominal wall erythema is strongly predictive of NEC but present in only 10% of affected patients.[30] A blue or discolored abdomen may be a sign of bowel perforation.[31]

Because early clinical signs of NEC usually lack specificity, NEC might be difficult to differentiate from sepsis. The course of NEC also widely differs, non-specific signs can slowly progress over several days, but NEC may also present with a fulminant onset of gastro-intestinal signs and shock with multi-organ-failure within only several hours.

Laboratory findings in patients with NEC can include increased or decreased white blood cell count (with left shift of neutrophils), thrombocytopenia (a rapid drop in platelet count being a poor prognostic sign) metabolic acidosis, hypo- or hyperglycemia and electrolyte imbalance, but all have low specificity.[32,33] Bloodstream infection -mostly with gram-negative bacteria- is present in 43% of the NEC cases, which is remarkable as the mucosal barrier of the intestines is completely disrupted.[6,34]

Non-specific *radiographic signs* of NEC include thickened bowel walls, dilated bowel loops, paucity of bowel gas and a fixed dilated loop. The latter observation may indicate necrotic bowel. Dilated gas-filled loops central in the abdomen may be a sign of ascites of free peritoneal fluid. Paucity of intestinal air may be caused by intestinal compression. Although these signs lack specificity for NEC, they are non-reassuring and require further investigation.[35,36]

Pneumatosis intestinalis is a pathognomonic radiological finding of NEC, reflecting presence of gas within the bowel wall. Pneumatosis is commonly detected in the right lower abdominal quadrant. The magnitude of pneumatosis does not always relate to the severity of NEC,

and its disappearance does not necessarily implicate clinical improvement.[35-38] When the present gas gets absorbed into the mesenteric circulation it may result in portal venous gas, which can be recognized as a linear air dense area in the hepatic region on a plain X-ray. Alternatively, it can easily be detected by ultrasound. Portal venous gas is related to the severity of the disease[39] but not to mortality or to the need of surgical intervention.[40] The most relevant radiological finding of NEC is presence of pneumoperitoneum, which is caused by perforation of the bowel wall. On plain abdominal radiograph, pneumoperitoneum can be recognized as the aspect of a (American) football, with the appearance of a longitudinal strip of sutures from a football caused by the falciform ligament. A small amount of free air may present as a small triangular or rectangular lucencies. A cross-table lateral or left lateral decubitus film (the infant is placed on its left side with horizontal radiation beam) may help in establishing the diagnosis of free air.[35,41-43]

Abdominal ultrasonography may be helpful in the diagnostic process of NEC. It can detect pneumatosis, portal venous gas, pneumoperitoneum and decreased perfusion of the bowel. Moreover, ultrasonography can exclude other gastrointestinal abnormalities, like intestinal malrotation and volvulus, which clinical symptoms may resemble NEC. However, ultrasonography is highly operator dependent, limiting its use in clinical practice Other imaging studies like contrast studies, computed tomography and MRI have currently no significant value in the diagnostic work-up and follow-up of NEC.[35,44]

Notably, extremely preterm infants have a different set of clinical symptoms at presentation of NEC. They are more likely to present with abdominal distention, ileus and emesis. Abdominal tenderness and guarding may only be present in advanced stages but may also be completely absent.[3,29] On plain radiography, preterm infants are less likely to present with pneumatosis intestinalis and portal venous gas, but they are at increased risk to develop pneumoperitoneum.[29]

The modified Bells staging criteria by Walsh and Kliegman is used to construct a grading system, and incorporate clinical symptoms, laboratory indices and radiological findings (Table 13.1). This can be helpful to define severity of illness and to select adequate treatment in routine clinical practice.[45]

Table 13.1 Staging system of necrotizing enterocolitis (NEC) according to Walsh and Kliegman[45]

Stage	I	IIa	IIb	IIIa	IIIb
Description	Suspected	Mild	Moderate	Severe	Severe
Systemic signs	Temperature instability, apnea, bradycardia	Similar to stage 1	Mild acidosis, thrombocytopenia	Respiratory and metabolic acidosis, mechanical ventilation, hypotension, DIC, oliguria	Further deterioration, shock
Intestinal signs	Increased gastric residuals, mild abdominal distention, occult blood in stool	Marked abdominal distention ± tenderness, absent bowel sounds, grossly bloody stools.	Abdominal wall edema and tenderness ± palpable mass.	Worsening wall edema with erythema and induration	Evidence of perforation
Radiographic signs	Normal or mild ileus	Ileus, dilated bowel loops, focal pneumatosis	Extensive pneumatosis ± PVG	Prominent ascites, fixed bowel loop.	Pneumoperitoneum

DIC = Diffuse Intravasal Coagulation; PVG = Portal Venous Gas.

TREATMENT

Therapeutic strategies of newborns with NEC mainly consist of two streams: patients who can be treated medically (non-surgical) and those patients who are in need of a surgical intervention.

Medical

The mainstay of the treatment of NEC is medical stabilization and conservation of general homeostasis. This regime typically consists of abdominal decompression, bowel rest, parenteral feeding with sufficient protein intake and administration of intravenous antibiotics. Abdominal decompression can be achieved by a double lumen gastric tube with continuous or intermittent suctioning. Significant aspirated volumes should be replaced.[41,43] Antibiotic therapy usually consists of ampicillin (or cephalosporin) combined with an aminoglycoside. In case of peritonitis or bowel perforation, the addition of metronidazole or clindamycin is warranted to cover anaerobic bacteria.[43] In the Netherlands, amoxicillin with clavulanic acid in combination with aminoglycoside, or alternatively meropenem, is administered in order to achieve immediate anaerobic coverage. Antibiotic therapy is guided by routine culture outcomes. Antibiotic treatment and bowel rest should be continued during 5-7 days in case of NEC stage II and 10 days in case of NEC stage III.[7] With NEC stage I, the necessity of starting and the duration of antibiotic treatment is based on the judgment of the team of treating physicians.[36] As the level of Urinary Intestinal Fatty Acid Binding Proteins (I-FAPB) at time of start of enteral feeding after NEC was correlated to adverse outcomes, this biomarker may help in timing of recommencing enteral feeding.[46]

Further supportive treatment consists of intubation in case of respiratory insufficiency, inotropic support when indicated, adequate fluid resuscitation and correction of anemia, thrombocytopenia and electrolyte imbalances. Therefore, blood counts and electrolytes should be closely monitored. To anticipate fluid loss to the third space, maintenance fluid has to be increased with 50% plus replacement of gastric output.[41] Since NEC is considered to be painful, adequate pain control and minimal handling are important aspects of medical management.[41]

During the course of NEC, the affected newborn must frequently be re-assessed to timely detect clinical deterioration and presence of pneumoperitoneum, which warrants surgical intervention. It must be kept into mind that by the time very preterm infants manifest

tenderness or abdominal mass, NEC has usually progressed to an advanced stage.[29] Therefore, serial abdominal radiographs (every 6-24h, and in case of clinical detoriaton or increase in abdominal girth)[35] contribute to optimize and guide treatment. Abdominal ultrasonography may also help in detecting intra-peritoneal air and bowel necrosis.[44] At last, serial C-reactive protein (CRP) measurements may be helpful for the detection of complications, like strictures or abscesses, or the need for surgical intervention.[47]

Surgical

About 20-40% of newborns with NEC eventually require surgical intervention.[7] Many aspects of surgical intervention are still subject to discussion.

At first, indication and timing of surgical intervention is challenging. Bowel perforation and necrotic bowel are absolute indications for surgery. Bowel perforation can be diagnosed by detection of pneumoperitoneum on abdominal radiographs. Bowel necrosis is suggested by persistent metabolic acidosis or thrombocytopenia, together with lack of improvement following medical treatment.[48] Also palpable fixed abdominal mass and abdominal wall erythema are highly specific signs of bowel necrosis, but with limited sensitivity.[30] Besides, a fixed bowel loop on abdominal radiograph is suggestive of necrotic bowel. Although portal venous gas was related to disease severity, it should not lead to surgical intervention when this is the only feature present.[36] Pneumoperitoneum and bowel necrosis may be difficult to diagnose, especially in extremely preterm infants. For example, pneumoperitoneum can be missed in 20% of the cases of bowel perforation and negative laparotomy is just as detrimental as failure to early recognize a perforation.[35,43]

When surgical intervention is warranted, two different kinds of primary surgery are currently available: primary peritoneal drainage (PPD) and laparotomy. Two recent randomized controlled trials have compared PPD versus laparotomy.[49,50] A Cochrane meta-analysis of these two studies concluded that there were no significant differences between the two groups, with respect to mortality and total duration of parental nutrition.[51] However, these studies did not distinguish between NEC and focal intestinal perforation (FIP). The European Necrotizing Enterocolitis Trial (NET)[50] included infants < 1000 grams. Of the infants who were treated with primary PPD, 74% still needed rescue laparotomy. In another non-randomized study, worse survival rates and neurodevelopmental outcomes at 18 and 22 months were found in the infants who received PPD as primary therapy. It remains questionable whether there is a role for PPD in affected infants who are considered too instable for laparotomy.[52]

The classic surgical approach during laparotomy is to remove all areas of necrotic intestine and to exteriorize the bowel to allow adequate time for healing before restoring the intestinal continuity at later age.[41] However stomas – especially jejunostomas – are poorly tolerated by preterm infants as they predispose to nutritional and metabolic disturbances and poor growth as a consequence of fluid and electrolyte disturbances. Therefore, some surgeons advocate a primary anastomosis.[53] Currently, a RCT trial studying resection and stomas versus primary anastomosis is recruiting patients for inclusion (ISRCTN01700960).

TOWARDS BETTER DIAGNOSIS AND TREATMENT OF NEC: PREDICTIVE MODELS AND BIOMARKERS

As stated previously, two major dilemmas are encountered in daily clinical practice concerning diagnosis of NEC. Firstly, differentiation between early NEC and sepsis can be difficult or even impossible, which may lead to a delay in adequate diagnosis and treatment. Secondly, the optimal timing for surgical intervention remains unclear.

Cohort studies and predictive clinical models

Different authors tried to identify which (early) parameters are predictive for severe NEC. Christensen et al. identified bloody stools (32%), increased abdominal girth (66%) and elevated pre-feeding gastric residuals or emesis (48%) as the most frequent specific antecedents for presence of severe NEC (grade III).[54] In another study from the same group, a rise in CRP, immature to total neutrophils and mean platelet volume, a low pH value, antecedent blood transfusion, and first feeding not being colostrum were identified as factors related to the severity of NEC.[55]

Moss et al. investigated which clinical factors were related to progression to severe NEC, requiring surgical intervention or leading to death. They found that 12 factors were related to progressive NEC (Table 13.2). However, it was not possible to develop a proper clinical model to predict progression of NEC.[56]

In order to optimize the timing for surgical intervention, Tepas et al. developed a scoring system incorporating seven items: metabolic acidosis (pH < 7.25), severe thrombocytopenia (< $50x10^9$/L), hypotension requiring volume expansion or drug therapy, hyponatremia < 130 mmol/L, neutropenia, left shift of neutrophils (I/T ratio < 0.2) and positive blood culture. In a retrospective study, a medical center with surgical intervention based on a score ≥ 3

Table 13.2 Diagnostic models of surgical necrotizing enterocolitis

Author	Outcome	Clinical parameters	Statistical analysis
Tepas[57]	Surgical NEC	≥ 3 of the following parameters: • Positive blood culture within 96h • Acidosis pH < 7.25 • I/T > 0.2 • Sodium < 130 mmol/L • Platelets < 50x10e9/L • Hypotension • Neutrophil < 0.2 x10e9	N/A
Moss[56]	Progressive (surgical intervention or death) vs. non-progressive NEC	• Gram negative bacteraemia • Abdominal wall discoloration • Portal venous gas • Teenage mother • Pneumatosis • Cardial resuscitation (compressions and/or adrenaline) • Metabolic acidosis (pH 7.30 and or HCO3 < 16 mmol/L) • Male gender • Birth weight <1000 grams • No enteral feeding before diagnosis • Gram positive bacteriemia	Multivariable logistic regression. $R^2 = 0.46$
Christensen[54]	Parameters in 48 h before NEC stage III	• Blood in stools (32%) • Increased abdominal girth (66%) • Emesis and/or gastric residuals (48%)	N/A
Miner[55]	NEC III vs. NEC II	• CRP ↑ • I/T ratio ↑ • Mean platelet volume ↑ • pH value ↓ • Platelets ↓ • Blood transfusion • First feeding not colostrum	Multivariable logistic regression
Sylvester[59]	Surgical vs. Medical NEC	5 most relevant: • pH Value • Portal Venous Gas • Air/fluid levels • Thrombocytopenia • Ventilator dependency	Linear discriminant analysis
Ji[58]	Surgical vs. Medical NEC	5 most relevant • pH value • Portal venous gas • Abdominal wall discoloration • Thrombocytopenia • Pneumatosis	Linear discriminant analysis

was compared to a control center which did not use this system. The usage of this scoring system showed better outcomes.[57] However, this scoring system should always be used together with clinical evaluation and serial radiography.

More recently, Ji et al. used clinical and laboratory parameters at the time of diagnosis to develop a data driven algorithm which can automatically classify NEC Bell's stage and predict the risk of progression NEC to the need of surgical intervention (http://translationalmedicine.stanford.edu/cgi-bin/NEC/index.pl).[58] The variables which contributed most in the model are summarized in Table 13.2. Although the groups with low and high risk of disease progression could be identified with high confidence, the investigators still experienced problems with prognostication of the intermediate risk group.[58]

It was postulated that novel biomarkers may help with predicting the prognosis of NEC. Indeed, in a previously published study, a similar algorithm placed 40% of the infants with NEC in the intermediate risk group in whom disease progression could not be predicted accurately. Implementation of urine peptide biomarkers (fibrinogen peptides FGA1826, FGA1883 and FGA2659) dramatically improved the ability of the algorithm to predict progression of disease.[59]

Radiologic models of NEC

Recently the Dukes Abdominal Assessment Scale (DAAS) was developed, which uses a 10-point radiographic scoring scale for NEC. Zero points stands for "normal gas pattern" and 5 points stands for "fixed or persistent dilated bowel loops". A score of 10 is indicative for "pneumoperitoneum". A recent evaluation of this score showed that an increasing DAAS score is related to increased disease severity and need for surgical intervention.[39]

Biomarkers in NEC (Table 13.3)

Early diagnosis of NEC and especially differentiation from neonatal sepsis is a major challenge. Despite extensive research, no reliable (early) biomarker is currently available. Some specific properties are essential to be an ideal candidate biomarker in the detection of NEC. This biomarker should be unresponsive to sepsis, and should substantially increase in concentration in, for example, blood, urine or stools at the onset of NEC. Its level magnitude of increase should be proportional to the severity of intestinal tissue injury[60] Biomarkers can be divided in different categories. Non-specific biomarkers, which are mostly biomarkers of

Table 13.3 Accuracy of diagnostic tests used in necrotizing enterocolitis

Marker	Cut-off Value	Sensitivity (%)	Specificity (%)
CRP(84)	16.05 mg/L	58	33
Urinary SAA (66)	42.2 pg/nmol	52	91
Calprotectin (66)	286.3 µg/g	81	79
S100A12 (77)	65 µg/kg	76	56
LIT (60)	3	55	94
I-FABP (60)	4.4 ng/mL	55	90
Urinary I-FABP (66)	2.4 pg/nmol	79	85
IL-8 (85)	1783 pg/mL	91	59
IL-6 (85)	73.2 pg/mL	63	56

CRP = C-reactive Protein; SAA = Serum Amyloid A; ApoSAA = Apoliprotein CII and SAA score; IFABP = Intestinal Fatty Acid Binding Protein; LIT = score of L-Fatty Acid Binding Protein, I-Fatty Acid Binding Protein and Trefoil Factor 3; IL-8 = Interleukin-8; IL-6 = Interleukin-6. Numbers in ( ) referring to references.

inflammation and specific biomarkers, which are biomarkers for gastro-intestinal mucosal injury or NEC.[61] Non-specific biomarkers are not able to differentiate between sepsis, NEC and other causes of (intestinal) inflammation.

Non-specific biomarkers

The most commonly used non-specific biomarker is C-reactive protein (CRP). CRP levels may increase 12-24 hours after onset of inflammation, the specificity is low and differentiation with sepsis is not possible.[62,63] Another non-specific biomarker is serum amyloid A (SAA). Serum-levels of SAA can increase up to 1000-fold in the 8 to 24 hours after the onset of inflammation. It can be used in the diagnosis and follow-up of NEC with a similar accuracy as CRP, as there is also a mild correlation with the height of SAA and the severity of NEC.[63] Recently, the measurement of ApoSAA has been investigated. It consists of plasma levels of SAA combined with apolipoprotein CII (apoC2). This combination of biomarkers is able to identify all NEC and sepsis cases at the onset of diagnosis. Furthermore, it can categorize sepsis/NEC children into different risk categories, thereby aiding in the decision making process regarding early cessation and withholding of antibiotic treatment. However, distinction between NEC and sepsis was not possible.[64,65] In another study, urinary SAA levels in neonates with NEC were investigated. Urinary SAA levels were significant higher in neonates with NEC and SAA levels could distinguish between medical and surgical

NEC.[65,66] The addition of another biomarker did not improve the accuracy of urinary SAA. However, the addition of a clinical parameter (thrombocytes) did improve accuracy in predicting surgical NEC.[66] Other non-specific biomarkers are procalcitonin,[63] arginine,[67] platelet-activating factor[68] and S100A8/A9.[69] All these biomarkers have been studied in small studies and their role in the diagnosis of NEC has yet to be confirmed by larger multicenter trials. Besides proteins, cytokines e.g. interleukin-6 (IL)-6[70] and chemokines e.g. IL-8[72] are considered early biomarkers for diagnosing neonatal sepsis and NEC, the combination of early and late-warning biomarkers can detect these conditions in almost all cases within 48 hours of disease onset.[61] Pro-inflammatory cytokines are essential for adequate immune response by the influx of activated leukocytes to sites of inflammation, but the excessive influx of potent pro-inflammatory mediators adhered to the leukocytes can in contrast lead to widespread damage and cell death. Anti-inflammatory cytokines, e.g. IL-10, can down-regulate the release and effect of pro-inflammatory mediators. Therefore both pro-inflammatory and anti-inflammatory cytokines are elevated in the context of sepsis and NEC. The combination of anti-inflammatory cytokines in a pro-inflammatory to anti-inflammatory cytokine ratio may reflect severity of illness and accurately predict complications of these diseases.[70,71]

Specific biomarkers

Feces contains valuable information regarding presence of gastrointestinal diseases and associated microbiotal profiles, however usage of feces for diagnostic properties has some limitations. In NEC, the intestinal mucosal permeability is increased, therefore proteins are secreted into the fecal stream.[61] These proteins are not equally secreted from the gastro-intestinal mucosa into the feces and different protein concentrations were found in one fecal sample.[61] Furthermore, feces is not always easily obtained, especially in NEC where constipation is common.[72]

One of the secreted proteins present in stools is calprotectin. Calprotectin is a calcium- and zinc-binding protein. For 60% it consists of soluble cytosol proteins in human neutrophil granulocyte. It is resilient to bacterial degradation and has been proposed as a useful laboratory marker in adults and children with inflammatory bowel disease.[73] In studies on NEC, many calprotectin values were outside the normal reference rates for adults.[74] However, there is also a wide variation in calprotectin levels in healthy neonates (1.0-625.1 µg/g) as there is for neonates with NEC (107.6-847.6 µg).[66,75,76] Moreover, differences have been found depending on gestational and postnatal age.[75] The wide variation and overlap

between healthy neonates and NEC, and the differences between studies, limits the use of this biomarker in daily practice.[61]

Another fecal biomarker is S100A12 (calgranulin C). It belongs to a novel group of pro-inflammatory molecules that play an important role in the innate immunity.[77] In small-scaled studies, its role has been evaluated in NEC. Although the values of S100A12 are significantly higher in neonates with NEC compared to healthy neonates, it has similar disadvantages as calprotectin: wide variation and high inter- and intravidual variability and significant differences depending on gestational age.[77] Like calprotectin, its use as a predictive biomarker in NEC is currently limited.

Since differentiation from sepsis is the biggest challenge, recent research has focused on gut/NEC specific biomarkers. Two of these are intestinal fatty acid binding protein (I-FABP) and liver-fatty acid binding protein (L-FABP). These proteins are solely synthesized by enterocytes (I-FABP) or only by enterocytes and hepatocytes (L-FABP)1. Another protein is trefoil factor 3 (TFF3), which is predominantly expressed in the intestinal goblet cells and mucin-producing epithelial cells.[78] These plasma proteins are significantly higher in NEC patients than in septicemic patients. Also, these biomarkers can discriminate between surgical and non-surgical NEC (sensitivity 67%-75%, specificity 75-88%).[60] To investigate if the combination of these markers can increase the diagnostic sensitivity and specificity, the LIT score (**L**-FABP, **I**-FABP, **T**TF3) was formulated, with a scale of 0 to 9. This score improved the sensitivity and specificity to 83% and 100% for identifying surgical NEC cases.[60] These gut-specific biomarkers can aid neonatologists in the diagnostic and therapeutic work-up of NEC, especially in surgical NEC. These biomarkers have also been studied in urine samples, where they provide the same diagnostic abilities.[79]

In urine, with the aid of liquid chromatography-mass spectrometry (LC-MS), three more biomarkers have been identified and validated (fibrinogen peptides: FGA1826, FGA1883 and FGA2659) and successfully discriminated surgical NEC from non-surgical NEC.[59] Since LC-MS has no place in clinical practice because of time-consuming analysis and high costs, there is currently no role for these markers in routine clinical practice. A follow-up study of the same cohort also using urinary LC-MS revealed seven candidate biomarkers. When they were used in panels, they had strong diagnostic capacities, (NEC versus sepsis: sensitivity 89%, specificity 80%, medical NEC versus surgical NEC sensitivity 89%, specificity 90%).[80]

In a pilot study, the role of fecal volatile organic compounds (VOCs) in NEC has been studied. VOCs are volatile carbon based-molecules resulting from biochemical processes

in the body and are emitted from feces, urine and other body fluids. VOC analysis was performed by means of solid-phase micro-extraction and gas chromatography and mass spectrometry (GC-MS), In this small scaled study, (6 NEC cases versus 7 controls) decreased total number next to specific differences in VOC-composition was observed in the 4 days prior to onset of disease, therefore possibly serving as an early diagnostic marker.[81] However, GC-MS is an expensive and time-consuming technique, limiting its widespread use in clinical practice. Therefore, prospective studies on VOC-analysis as non-invasive biomarker for NEC, using high-throughput techniques, like electronic nose devices, are awaited. Other potential biomarkers could be markers of oxidative stress in the umbilical cord[82] or from genetic origin.[83] Analysis of fecal samples on microbiota face the limits at present as most biomarkers described above. This is not feasible in routine care as the determination of different patterns requires sophisticated analyses and are time consuming. Furthermore, as detailed out before, the prognostic value of bacterial species distribution next to the quantification of different strains are still unclear and require more research.

CONCLUSION AND FUTURE PERSPECTIVES

Diagnosis and course of NEC remains largely unpredictable based on the presently available clinical, biological and radiological markers. Current diagnostic tools use signs of already established or progressive disease. As early and accurate diagnosis is critical for therapy and outcome accurate predictive biomarkers of NEC are needed. Future research should focus on the biological and microbiotal processes underlying the development of NEC. There is a clear rationale to explore the usage of biomarkers based on the altered microbiotal profile in NEC. Measurement of VOCs emanating from feces may serve as such a rapid, non-invasive, low-cost and easy to use specific biomarker. A predictive model based on different (bio)markers may lead to better diagnostic possibilities, tailored probiotic-based feeding strategies as well as therapeutic interventions. As NEC remains to have an unacceptable high mortality and morbidity rate, we should proceed with the challenging search for better diagnostic and therapeutic strategies.

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