The pathophysiology of necrotizing enterocolitis in preterm infants

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SUMMARY

Necrotizing enterocolitis (NEC) is a devastating gastrointestinal disease characterized by severe inflammation and necrosis. NEC is the most common acute gastrointestinal disease at the neonatal intensive care unit (NICU). The most vulnerable children, extreme premature infants and/or infants small–for-gestational age, are at highest risk for NEC development. NEC can depict a severe clinical course with mortality rates around 20-30%. If infants survive NEC, they often have to deal with debilitating long-term complications, such as developmental disorders (motor and cognitive) and/or short bowel syndrome. Despite the severity of the disease and decades of research, the underlying pathophysiology of NEC is not completely revealed yet. Unfortunately, both preventive and treatment options in NEC are limited due to the incomplete understanding of the underlying multifactorial pathophysiology of NEC. Therefore, treatment options are mainly supportive: aimed at the treatment of symptoms and possibly preventing progression of disease. Treatment initially consists of stopping (enteral) nutrition and starting antibiotic treatment, in combination with supportive therapy to stabilize vital functions. In cases of intestinal perforation or clinical deterioriation despite maximal conservative therapy surgical intervention can be indicated. During surgery, resection of the necrotic intestinal tissue is performed with the construction of an ostomy or a primary anastomosis when deemed possible by the surgeon. Gaining more insight in the underlying pathophysiology of NEC might offer further preventive or therapeutic strategies. Currently, three factors are believed to play a key role in the pathophysiology of NEC. All three factors are related to prematurity: 1) the intestinal barrier function, 2) intestinal circulation and perfusion, and 3) the intestinal microbiota. We aimed to gain insight in the role of these three factors in relation to the pathophysiology of NEC.

In **chapter 2**, we retrospectively analyzed the epimediology, clinical presentation, treatment strategies and disease outcome of NEC in three large tertiary referral centers in the Netherlands between 2005 and 2013. The urgency to elucidate the pathophysiology of NEC becomes even clearer given the observed increase in the incidence of NEC during the last decade.

The Dutch guidelines for active treatment of extremely preterm infants changed in 2006 from 26+0 to 25+0 weeks of gestation, and in 2010 to 24+0 weeks of gestation. The percentage of infants born at a gestational age (GA) of 24 and 25 weeks increased from 1.7% tot 3.4% of the total of infants admitted to the NICU in the last nine years after introducing these guidelines. The incidence of NEC increased from 2.1 to 3.4% of all NICU admissions, mostly in the group of infants born with

a birth weight <1500 grams. We suggest that the increase of incidence of NEC is related with the increase of the total admissions of children born at a GA of 24 and 25 weeks after the change in the guidelines for active treatment. The percentage of patients needing surgery decreased, while 30-day mortality did not change.

In the next part of this summary we outline the key findings of this thesis from **section 2**. We start with a brief introduction on several methods we used for the studies described in section 2, i.e measuring intestinal damage using **intestinal fatty acid-binding protein (I-FABP)**, the use of **near infrared spectroscopy (NIRS)** to measure tissue perfusion, **16S rRNA based sequencing** to identify the intestinal microbiota, and **fluorescent in situ hybridization (FISH)** to identify and quantify bacterial species colonizing the intestinal wall.

I-FABPs are cytoplasmic proteins 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 of these cells. In the context of progressive intestinal barrier failure in NEC, enterocytes are damaged and I-FABP is released in the circulation and subsequently secreted by the kidneys. In several studies I-FABP levels in urine and plasma have been identified as an early marker for NEC development and progression (including the need of surgical intervention) of NEC. In chapter 4, 5 and 6 we used I-FABP levels in plasma as a marker for mucosal damage.

NIRS is a non-invasive method whereby the perfusion of the underlying tissue can be measured by differentiating between oxygenated hemoglobin and deoxygenated hemoglobin. The ratio between oxygenated hemoglobin and total hemoglobin determines the oxygen saturation in tissue (rSO_2). When transcutaneous arterial oxygen saturation (SpO_2) is measured simultaneously, fractional tissue oxygen extraction (FTOE) can be calculated with the following formula: FTOE = (SpO_2 - rSO_2)/SpO_2. FTOE is thought to reflect the balance between tissue oxygen supply and consumption and might therefore be an early indicator of impaired tissue perfusion. In chapter 4 we used NIRS measurements of the cerebral and splanchnic tissue.

16S rRNA based sequencing allows us to identify the bacterial composition, the so-called microbiota, including the accurate measurement of unculturable bacterial species. 16S rRNA genes are presented in cells of bacteria only. The differences in the 16S rRNA genes can be used to identify certain bacterial species. We used 16S rRNA based sequencing (chapter 7) to identify the composition of the intestinal microbiota in infants at risk for NEC development.

FISH is a cytogenetic technique that uses fluorescent probes to visualize and identify microorganisms within their environment. FISH uses oligonucleotide probes

containing a sequence of genetic material complementary to the known sequence of the bacterial RNA. Consequently, the material is labeled with fluorochromes, making the microorganisms visible under the fluorescence microscope. In chapter 8 of this thesis FISH was used for the identification and localization of bacteria invading the intestinal wall in surgical NEC specimens.

In **section 2** of this thesis we zoomed in on the pathophysiology of NEC.

In **chapter 3** we studied the development of Paneth cells (PCs) in the developing gut. PCs are important cells of the immune system located in the crypts between the villi of the intestine. PCs work as 'the guardians of the crypts' by protecting the intestine from harmful bacteria.

We used intestinal tissue derived from autopsy material from fetuses and infants without gastrointestinal abnormalities (GA between nine and 40 weeks). The abundance of PCs increased significantly when infants reached a GA >37 weeks. The abundance of immune competent PCs (expressing human defensin-5) increased significantly starting at a GA of 29 weeks. This corresponds with the time of the peak incidence of NEC (GA 29-33 weeks). The significant increase of immune competent PCs starting from a GA of 29 week mimics the rise in incidence of NEC during a similar postmenstrual age in preterm infants.

In **chapter 4** we provide a 'proof of concept' that FTOE (as an indicator for an impaired perfusion) and I-FABP levels (as a marker for mucosal damage) can be used to reveal the underlying pathophysiological chain of events during NEC. Our results demonstrated a strong association between cerebral and splanchnic FTOE values and I-FABP levels during the first hours after NEC onset. When time progressed, we observed distinct patterns of FTOE values and I-FABP levels for uncomplicated and complicated (surgery, death) NEC cases. The initial FTOE started much higher for the complicated NEC cases, suggesting a compromised intestinal circulation, and stayed high for at least 24 hours before decreasing. Uncomplicated NEC cases started with lower FTOE (indicating a non-compromised intestinal circulation), with an increase during the following 24-36 hours. I-FABP levels decreased over time, presenting either tissue recovery or an absent venous return from the necrotic bowel/ complete destruction of the mucosa respectively. Therefore, the combination of high FTOE values and decreasing I-FABP levels over time suggests progression of intestinal ischemia. Combination of FTOE values and I-FABP levels gives insight in the pathological events resulting in progression- or recovery of intestinal ischemia.

In **chapter 5** we investigated whether I-FABP levels in plasma were associated with the length of the necrotic segment in surgical NEC patients. The study showed that, indeed, I-FABP levels were strongly correlated with the length of intestinal necrosis.

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. The data offer additional evidence that I-FABP can be considered a marker for mucosal damage.

In **chapter 6** we aimed to get more insight in the longstanding question whether a bloodstream infection (BSI) precedes NEC or that BSIs are the result of loss of intestinal barrier integrity during NEC. Therefore we investigated the relation between the occurrence of an early BSI (within 24h prior until 24h after onset of NEC symptoms) during NEC, the pro-inflammatory response (Interleukin-8), the loss of intestinal barrier integrity due to mucosal damage (I-FABP), and the severity of NEC. Our data showed a relatively low incidence of 26% of early BSIs. Furthermore, there was no relation between the occurrence of a BSI and Interleukin-8, nor between the occurrence of a BSI and I-FABP levels. These findings suggest that BSIs do not precede NEC. However, it is still plausible that bacterial translocation into the bowel wall occurs during early NEC.

In **chapter 7** we investigated the composition of the intestinal microbiota in premature infants with high risk for NEC. To this end we used 16S rRNA sequencing. Most importantly, we discovered that the presence and abundance of *C. perfringens* and *B. dorei* in meconium was associated with the development of NEC. This implies that a NEC-associated microbiota can already be identified within the first days after birth. This finding also suggests that (yet unrevealed) factors during the first days of life or even in utero could play an important role in the formation of a more healthy or a more NEC-associated microbiota. In post-meconium fecal samples, the presence and abundance of lactate-producing bacteria was negatively associated with NEC development. Abundances of lactate-producing bacteria were positively related with early enteral nutrition, especially in the form of mother's milk. Our findings therefore confirm the hypothesis that early enteral nutrition after birth, especially in the form of mother's milk, is important in protecting the intestine against development of NEC. Early commencement of mother's milk likely stimulates lactate-producing bacteria in infants with a low GA in a similar way as breast milk normally stimulates bifidobacteria in infants with a normal GA.

In **chapter 8** we moved our attention from the intestinal microbiota before NEC development to the bacterial colonization during NEC. We investigated the bacterial location, bacterial density, and bacterial differentiation adhering to and within the intestinal wall in NEC tissue samples and compared this with controls. Using FISH we detected bacterial invasion in the intestinal wall in 51% of the

most affected NEC tissue samples. In the least affected NEC tissue samples we observed bacterial invasion in the intestinal wall in 37%. In control samples this was 16%. *Enterobacteriaceae* dominated in the NEC tissue samples. Surprisingly, only in sporadic cases we observed *C. perfringens*, a type of bacteria commonly associated with NEC. We offer the following hypothesis for this observation. As a result of inflammation, oxidative stress is induced by reactive oxygen species (ROS), resulting in an environment in which *C. perfringens* cannot survive. *Enterobacteriaceae* include versatile species that derive energy for growth from aerobic or anaerobic nitrate respiration or from fermentation. *Enterobacteriaceae* are therefore resistant against nutrient limitation as well as oxidative stress and can survive in a highly inflamed and necrotic intestine, which is seen during NEC.

In conclusion, this thesis provides new insights in the underlying pathophysiology of NEC. With our findings we offer the following hypothesis regarding the pathophysiology of NEC: alterations of the intestinal microbiota can be already present in the first meconium of infants. When this microbiota consists of a NEC-associated microbiota (*C. perfringens* and *B. dorei)* amplification of pro-inflammatory mediators will be stimulated. The amplification of pro-inflammatory mediators will in turn cause oxidative stress. Oxidative stress harms, via free radicals, the surrounding cells and DNA. When the infant experiences more prenatal stressors – such as hypoxia, hypoperfusion, formula feeding – the amplification of pro-inflammatory mediators continues. These events alter the balance between vasoconstriction and vasodilatation of the intestinal circulation and may lead to a relatively hypoxic-ischemic state, predisposing to loss of intestinal barrier integrity and later to irreversible necrosis of the intestine. When the disease progresses, intestinal perfusion could diminish even further, oxidative stress will increase and the affected tissue will be further colonized with *Enterobacteriaceae* leading to further aggravation of the inflammatory response.

To date, the underlying pathophysiology remains incompletely understood. Although we shed some light on the underlying pathophysiology of this devastating disease, our findings only suggest – and did not prove – a causal relation between our observations. The findings in the present thesis should be confirmed in large multicenter trials. This could be followed by targeted antibiotics and probiotic trials, aimed at development of preventive and therapeutic strategies against NEC.