The pathophysiology of necrotizing enterocolitis in preterm infants

Heida, Fardou Hadewych

CHAPTER 9

GENERAL DISCUSSION

Contents

Necrotizing enterocolitis (NEC) is the most lethal gastrointestinal disease in neonatal care.[1] NEC is a complex disease involving among others bacterial invasion, inflammation, and necrosis of immature intestinal tissue.[2] Despite improvements in neonatal care and extensive research regarding NEC, the disturbing reality is that the overall incidence and survival of infants with NEC has not changed in the past quarter of a century. Treatment options are still limited and, unfortunately, we are still unable to predict which infant will develop NEC. This statistic is most probably a reflection of an incomplete understanding of the etiology and pathophysiology of NEC.[2,3]

The contributing factors in the pathophysiology of NEC are extensively discussed in the introduction section of this thesis. In brief, the key factor contributing to NEC is prematurity.[1] The ontogenesis of the gastrointestinal wall and the immune system starts during the first trimester of pregnancy and is correlated with gestational age (GA).[4–15] In term infants, an ideal balance of the defense mechanisms of the immune system within a mature intestine should protect the host from invading pathogens across a broad range of conditions, such as NEC.[15,16] In preterm infants the gastrointestinal tract and immune system are not fully developed yet.[15,16] For example, preterm infants have looser/disrupted tight junctions (TJs), which increase the intestinal permeability. They have an exaggerated immune responses (via toll-like receptors (TLRs) and paneth cells (PCs)) resulting in an elaborate inflammation.[1,17] Also, the intestinal perfusion in preterm infants is impaired due to an immature vasculatory regulation, with higher chances on intestinal hypoxia and ischemia.[1,17] Finally, the intestinal microbiota differs in preterm infants compared to term infants, with dysbiosis (microbial imbalance), higher amounts of lactate producing bacteria, and a higher susceptibility of invasion with opportunistic and/or pathogenic bacteria.[1,17] Hence, the vulnerable preterm infant, with its immature intestine and immune system, is at high risk for invasion of pathogens contributing to the development of NEC.

Currently, strategies to predict, prevent and diagnose NEC are limited.[18] It would be unlikely that strategies to prevent NEC will be successful unless the pathophysiology of the disease is better understood. Understanding the intestinal barrier function, the intestinal perfusion and intestinal bacterial colonization in preterm infants (at risk for NEC) might lead to a better understanding of the underlying pathophysiology of NEC.[19] Therefore, the main goal of this thesis was to increase our knowledge about the underlying pathophysiology of NEC, focusing in particular on the role of the intestinal barrier function, intestinal perfusion, and the intestinal microbiota.

Epidemiology

NEC occurs primarily in preterm infants.[20] When the GA and birth weight (BW) decreases, the incidence of NEC will increase.[21] The lower the GA, the later this condition occurs after birth, with a peak incidence at a postmenstrual age (PMA) of 29-33 weeks.[22] Overall, large multicenter and population based studies estimate the incidence of NEC in very low BW infants (BW<1500 grams) between 7 and 11%.[17,23–25] However, the incidence of NEC vary across NICUs and countries.[26] In **chapter 2** we describe our epidemiological findings derived from a retrospective cohort study in three academic referral centres in the Netherlands between 2005 and 2013. Between 2005 and 2013 a total of 14.161 infants were admitted at one of the three participating (neonatal intensive care units) NICUs, of which 441 infants (3.1%) were diagnosed with NEC. We observed a significant increase in the incidence of NEC starting at 2.1% in the period of 2005-2007 to 3.9% in 2008-2010 and 3.4% in 2011-2013 of all NICU admissions. The increase was primarily the result of the increased incidence of NEC in the extreme preterm group (GA <28weeks): from 6.4% in 2005-2007 to 16% in 2008-2013. The percentage of infants admitted to the NICU at a GA of 24- and 25 weeks increased (from 1.7% to 3.4%) and we observed the highest increase of NEC cases in this group (from 2.5% in 2005-2007 to 5.3% in 2008-2010, to 6.8% in 2011-2013).

We offer two explanations for the increase of incidence of NEC during the last decade. First, the incidence of NEC might be increased due to the revised management of preterm infants in which infants born at 24 weeks of gestation are actively treated since 2010. Second, the increased incidence of NEC might be caused by an improved early survival of extremely preterm infants which otherwise would not have reached the age at which NEC typically presents (PMA of 29-33 weeks).[27] Either way, the increase in incidence of NEC as presented in **chapter 2** emphasizes the importance of research on NEC.

Treatment & mortality

We observed that 30-day mortality of NEC decreased between the period 2005-2007 (41%) and the period 2008-2010 (29%) in the three NICUs in the Netherlands (**chapter 2**). After 2010 the 30-day mortality of NEC did not significantly change (2011-2013: 33%; **chapter 2**) in these three centres. This is comparable with numbers on 30-day mortality for NEC stated in the literature.[1]

Despite the high mortality of NEC, there are still no adequate treatment strategies. We observed that after 2005 there were no major changes implemented in the non-surgical therapy of NEC in the three academic referral centres as described in **chapter 2**. We did observe that treatment using peritoneal drainage decreased in the last nine years (**chapter 2**). Peritoneal drainage is seen as a temporary intervention to stabilize the critically ill infant and as a definite therapy in a small amount of patients.[1,28] The last years there is an on-going debate about the use peritoneal drainage in infants with NEC.[27] In our cohort (**chapter 2**) we observed that peritoneal drainage was associated with increased 30-day mortality. We speculate that this increase in mortality is primarily the result of the use of peritoneal drainage as a temporally solution in patients who are too instable for surgery instead of the result of peritoneal drainage itself.

The need for surgical interventions decreased in our cohort. Between 2005 and 2007 53% of the patients needed surgery, compared to 29% between 2011 and 2013 (**chapter 2**). This percentage is comparable with the literature in which a percentage between 20 and 40% is stated for surgical interventions for NEC.[1] While we hypothesized that this trend was incited by an increase of extremely preterm infants with NEC – a population in which healthcare workers remain very reticent with surgical interventions – we did not observe a significant decrease of infants who underwent surgery in the group of extremely preterm infants in our cohort.

Pathophysiology

The main goal of this thesis was to increase our knowledge about the underlying pathophysiology of NEC, focusing in particular on the role of the intestinal barrier function, intestinal perfusion, and the intestinal microbiota. We investigated components of the intestinal barrier function by studying PCs, interleukin-8 (IL-8), and mucosal damage via intestinal fatty acid-binding proteins (I-FABPs). The intestinal perfusion was investigated via near infrared spectroscopy (NIRS) monitoring. The intestinal microbiota was investigated via the identification of bloodstream infections (BSIs), the intestinal microbiota prior to NEC development and bacterial invasion of the intestinal wall during surgical NEC. In Figure 1 and 2 we present the key findings of this thesis.

FIGURE 1:

Key findings of this thesis implemented in the multifactorial model of NEC

Abbreviations: Intestinal fatty acid-binding protein: I-FABP, human defensin: HD5, fractional tissue oxygen extraction: FTOE, necrotizing enterocolitis: NEC.

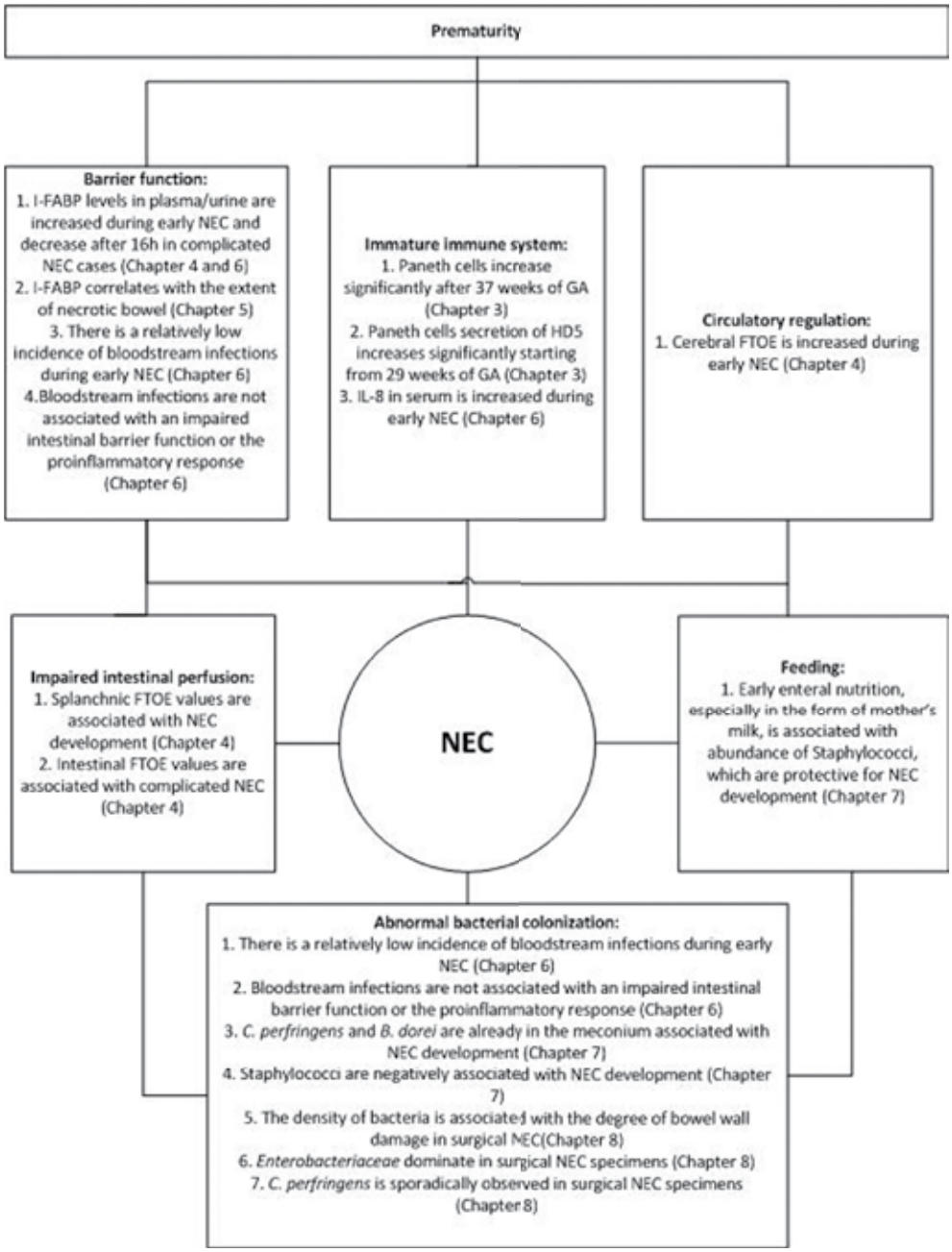

TABLE 2:

Key findings of this thesis regarding temporal aspects of NEC development

Abbreviations: Intestinal fatty acid-binding protein: I-FABP, human defensin: HD5, fractional tissue oxygen extraction: FTOE, necrotizing enterocolitis: NEC, Paneth cells: PCs.

Key findings		
Before NEC onset	**Early NEC**	**Severe NEC**
Abundance of PCs increase after 37 weeks GA. Starting from 29 weeks, the secretion of HD5 by PCs increases significantly.	Increased I-FABP levels are associated with NEC development	Splanchnic FTOE levels increase and I-FABP levels decrease in complicated NEC
C. perfringens and *B. dorei* are associated with NEC development already in the meconium	Increased IL-8 levels are associated with NEC development	Bacterial invasion is associated with the degree of affected surgical NEC tissue
Staphylococci protect the preterm infant against NEC development	The incidence of bloostream infections is relatively low (26%) during the first 24h of NEC	*Enterobacteriaceae* dominate in surgical NEC specimens
	Cerebral and splanchnic FTOE levels were higher during early NEC	C. *perfringens* is only occasionly found in surgical NEC samples

Intestinal barrier

The gastrointestinal system is unique because its close interaction between the single epithelial lining, the immune system and the intestinal microbiota.[29] The intestinal barrier of the preterm infant is inherently fragile with increased permeability and an immature immune system.[30] The preterm intestinal barrier with an increased intestinal permeability has the advantage to allow passage of important macromolecules from amniotic fluid or breast milk.[31] The disadvantage of this immature intestinal barrier is the increased vulnerability to environmental threats and/or internal stressors, such as hypoxia and/or ischemia and intestinal colonization with pathogenic bacteria, which could lead to NEC.[29,30]

Contrariwise, disruption of the intestinal epithelial barrier decreases intestinal barrier integrity and makes bacterial invasion possible. This is thought to be an early event in the pathogenic cascade of NEC.[29,32,33] The inflammatory response of the intestinal barrier can be triggered by either commensal, opportunistic, or pathogenic bacteria.[34] Whether loss of intestinal barrier function precedes NEC or is the consequence of intestinal injury in NEC remains unknown.[35]

In the present thesis we focused on the immature immune system and intestinal barrier integrity. First the main findings of the thesis will be discused and next we provide some future perspectives.

Enterocytes

Enterocytes constitute the most abundant epithelial cell type.[32] As current evidence supports, enterocyte damage is not primarily causing NEC, but is part of a vicious cycle of inflammation-inflicted epithelial damage.[36] Opportunistic and/or pathogenic bacteria can interact with specific apical surface receptors on the enterocytes. This interaction triggers a response that induces overexpression of inflammatory cytokines, causing an (exaggerated) inflammatory response in the preterm gut resulting in NEC.[37] This inflammatory response damages the vulnerable enterocytes during NEC, causes decreased intestinal barrier integrity resulting in progression of the disease.[37] Intestinal fatty acid-binding proteins (I-FABPs) are small proteins released immediately after enterocyte damage from the enterocytes. I-FABPs are a marker for loss of intestinal barrier integrity due to mucosal damage in infants with NEC.[38,39] **Chapter 4** describes an increase of I-FABP levels during early NEC (the first 48hours after onset of disease), suggesting that loss of intestinal barrier integrity is already present in the early phase of NEC. We observed, however, in **chapter 6** that intestinal barrier integrity was not severe enough to allow bacterial translocation during the first 24 hours after NEC onset. When NEC progresses we observed that I-FABP levels correlated with the extent of mucosal damage **(chapter 5),** suggesting that intestinal barrier integrity plays a pivotal role in NEC progression. We speculate that loss of intestinal barrier integrity is already present during early NEC, but is not severe enough to allow bacterial translocation into the bloodstream. However, it is plausible that loss of intestinal barrier integrity is severe enough to allow bacterial translocation into the intestinal wall itself, triggering the intestinal immune system to an exaggerated immune response in the intestine. Loss of intestinal barrier integrity progresses concurrently with NEC progression.

Tight junctions

The intestinal epithelium comprises tight junctions (TJs), that seal the intercellular spaces between the enterocytes, thus forming a barrier for bacteria and most macromolecules.[40] TJs get steadier by term gestation.[40,41] Thereby, fermentation products of commensal bacteria have been shown to enhance the intestinal barrier function by facilitating the assembly of TJs through the activation of AMP-activated protein kinase.[42] Preterm infants have a lower bacterial diversity compared to term infants.[40,41] A lower bacterial diversity means a lower amount of fermentation products of bacteria which normally strenghten the TJs, therefore resulting in looser TJs between the enterocytes. Looser TJs results in the loss of intestinal barrier integrity. Thuijls et al.[43] observed increased claudin-3 levels, a marker for loss of TJs, during early NEC compared with infants without NEC. Therefore, we speculate that loss of intestinal barrier integrity due to immature TJs could play an initial role

in allowing bacterial translocation contributing to NEC development. During NEC an excessive inflammatory reaction can cause intestinal damage aggravating the intestinal barrier loss.

TJ functioning is influenced by the intestinal microbiota.[35,44] For example, an abnormal intestinal microbiota (such as colonization with gram-negative bacteria) triggers lipopolysaccharide (LPS) expression which affects the TJ protein function, resulting in loose and/or disrupted TJs.[35,44] Contrariwise, lactobacilli and bifidobacteria are examples of bacteria that up-regulate the expression of TJ proteins, improving the intestinal mucosal barrier function significantly.[45]

The immune system contributing to the intestinal barrier

The immune system plays an important role in maintaining the intestinal barrier function, and possibly contributes to NEC development. Little is known when each of the respective aspects of the immune system matures normally in utero in humans and what the consequences of preterm birth are on these processes.[15] Fetuses have limited exposure to antigens in utero, so their adaptive immunity is hardly induced.[46] Therefore they are heavily dependent on their innate immune system.[46] However, preterm infants also display markedly impaired innate immune functions.[15] The fetal innate immune response progressively matures in the last three months in utero.[47] The innate immune system of preterm infants have a smaller pool of monocytes and neutrophils, impaired ability of these cells to kill pathogens, and lower production of cytokines which limits T-cell activation and reduces the ability to fight bacteria and detect viruses in cells, compared to term infants.[47] Contrariwise, observations in animal models of NEC and in human fetal-cell cultures have suggested that the fetus and preterm infant have an excessive inflammatory response to luminal microbial stimuli.[22] This excessive inflammatory response can cause damage to the intestinal barrier. Via this thesis we cannot answer the question whether the incapacity of the immature immune system to provide an adequate immune response results in infection of the intestine or whether the excessive inflammatory response results in inflammation of the intestine. The inflammatory response in NEC should be further investigated to answer the exact relation between the immune system and NEC.

Recently, the knowledge about several signaling pathways has been expanded, including Notch, Wnt/ß-catenin- and TLR signaling, regulating the differentiation of the intestinal epithelium (such as enterocytes, PCs, and goblet cells).[48–51] TLR4, PCs, and goblet cells are important components of the immune system involved in maintaining the intestinal barrier function. TLR4 detect pathogens close to the intestinal surface and produce pro-inflammatory mediators.[30,52] PCs are unique to

the small intestine and secrete abundant quantities of antimicrobial proteins, such as human defensin-5 (HD5).[3,32,53,54] Goblet cells secrete mucin glycoproteins and trefoil factors that assemble to form a thick mucus layer overlying the epithelium.[32]

There are – generally speaking - two hypotheses why cell differentiation is important in the pathophysiology of NEC. In the first hypothesis an altered differentiation of epithelial stem cells leads to an abnormal composition of the epithelial lining of the intestine prone to increased intestinal wall permeability and an altered intestinal immune response, making the preterm infant prone for development of NEC.[44,55] In the second hypothesis the developmental role for TLR4 switches to a pro-inflammatory role due to its interaction with colonizing microbes, leading to the development of NEC.[49,51,56] Arguments pro and contra these hypotheses cannot be derived from our study results and should be a topic of interest for future research.

Toll like receptor 4 (TLR4)

TLR4 is a toll-like receptor which is responsible for the proliferation and differentiation of epithelium out of intestinal epithelial stem cells (by influencing the Notch and Wnt/ß-catenin signaling pathways) and for the activation of the innate immune system by recognizing, among others, LPS.[47,48,50,51,57] The expression of TLR4 in the intestinal lining increases during embryonic development and decreases significantly after (term) birth.[51] This mechanism might explain the preponderance of NEC in preterm infants, as the expression of TLR4 is still high in preterm infants.

TLR4 expression has been suggested to play a crucial role in the development of NEC.[57] TLR4 are involved in maintaining the intestinal barrier function, and exaggerated TLR4 signaling – as is observed in mouse and human NEC – could lead to disease development by disrupting the intestinal barrier.[57] TLR4 detects pathogenic bacteria nearby the intestinal surface. When TLR4 is activated by pathogenic bacteria it induces different inflammatory mediators, such as the inflammatory cytokine IL-8.[30,52] IL-8 cause increased production of acute-phase proteins in the intestine as seen in NEC and finally induce ischemia and necrosis.[58–60] TLR4 activation leads to a significant loss of intestinal barrier integrity due to a profound increase in enterocyte apoptosis, which leads to mucosal disruption.[48,57,61,62] Expression of TLR4 within the intestine normally drops precipitously at the time of full term birth, while factors that are associated with NEC development – including prematurity, hypoxia and the exposure to high concentrations of LPS – significantly increase TLR4 expression within the intestinal epithelium.[50] While we did not focus on the TLR4 expression in this particular thesis, we observed an increase in IL-8 expression during early NEC **(chapter 6).** This suggests an increased TLR4 expression during early NEC. IL-8 has earlier been identified by Benkoe et al.[58]

as an useful marker for the excessive inflammatory response specific for NEC induced by TLR4. Numerous (animal) studies focus on the role of TLRs in the pathophysiology of NEC and the possibility of developing methods to reduce TLR4 signaling as method to prevent NEC.[63–66]

Paneth cells (PCs)

PCs might be important in the underlying pathophysiology of NEC. In the preterm gut, the immature PCs with their secretion of defensins might contribute to an excessive inflammatory response as observed in NEC.[67] Despite the hypothesis that PCs are involved in the pathophysiology of NEC, little is known about PC presence and functioning in the developing human intestine and the exact relation with NEC. In **chapter 3** we focus on when PCs arise and when they become immune competent during human gestation. We observed that the number of PCs increase rapidly when infants became term (GA 37 weeks). However, starting from 29 weeks of gestation we observed a rapid increase in immune competent PCs (HD5 expression), which corresponds with the peak incidence of NEC at a PMA of 29-33 weeks. While we analyzed fetal and neonatal ileum tissue without gastrointestinal abnormalities, the findings do emphasize the possible role for PCs in the underlying pathophysiology of NEC.

The current hypotheses on the role of PCs in NEC development PCs are based on either depletion of PCs, increased immune activity of PCs, or dysfunctioning of PCs.[21,67–69] In the first hypothesis it is suggested that there is a relative deficiency of (immune competent) PCs at a low GA.[53,56] This deficiency could lead to a limited protection against opportunistic bacteria involved in NEC development.[53,56] The results described in **chapter 3** suggest that we have to reject this hypothesis, because we observed a significant increase of immune competent PCs during gestation and not depletion. In the second hypothesis the secretion of antimicrobial peptides by the PCs might be over-activated in the immature immune system leading to an overwhelming inflammatory response.[3,57,70] This inflammatory aggravation could lead to increased intestinal damage, bacterial dysbiosis and reduced epithelial repair which could lead to the development of NEC.[3,57,70] With our results one could speculate that the rapid increase of defensin expression around a GA of 29 weeks (which mimics the rise of incidence of NEC during a similar PMA), colonization with opportunistic pathogens, and a still immature intestine could trigger NEC development. The last hypothesis includes dysfunction of PCs by environmental stressors, i.e. dysfunction of PCs may be an early event that predisposes the preterm infant to NEC by inducing bacterial dysbiosis.[3,71,72] This hypothesis was not tested in this thesis and should be of interest in future research.

Hypoxic-ischemic mechanisms

An impaired intestinal perfusion can be measured using near infrared spectroscopy (NIRS).[73] NIRS measurements represent the oxygen uptake in tissue and is referred to as regional tissue oxygen saturation (rSO_2).[74] When the transcutaneous arterial oxygen saturation (SpO_2) is measured simultaneously, the fractional tissue oxygen extraction (FTOE) can be measured.[73] FTOE reflects the balance between tissue oxygen supply and tissue oxygen consumption and might therefore be an early indicator of impaired tissue perfusion.[73,74] Since the technical aspects of NIRS are beyond the scope of this thesis, we refer to other articles discussing the techniqual aspects of NIRS in detail.[75–86] Limitations of intestinal NIRS measurements are the influence of peristalsis, gut movements, air and stools, which complicate the interpretation of intestinal NIRS values.[79,87] Therefore, research focusing on the feasibility of NIRS should be continued.

Impaired intestinal perfusion, resulting in intestinal ischemia, seems important in the development of NEC.[73,88] Yet the timing of this ischemic insult remains unclear. An ischemic insult could be the primary inciting factor for NEC develolopment.[73] Conversely, intestinal ischemia could play an secondary role if it emerges after the inflammatory cascade in NEC.[73] While it is not possible to clearify the timing of the ischemic insult with the results of this thesis, a previous study conducted in our center did. Schat et al.[74] demonstrated that preterm infants who developed NEC had higher intestinal FTOE values two days prior to NEC onset, suggesting that impaired intestinal perfusion is present before the clinical onset of NEC. This could plead for an impaired intestinal perfusion as a contributing factor for the development of NEC. Wether an impaired intestinal perfusion was an inciting factor for NEC development still remains unrevealed.

We offer the following explanation for the contribution of an impaired intestinal perfusion for the development of NEC. The infant experiences multiple prenatal stressors – such as hypoxia, hypoperfusion, enteral feeding – resulting in an increase of the pro-inflammatory response and an impaired endothelial functioning. This pro-inflammatory response and an impaired endothelial functioning results, in turn, in an impaired intestinal perfusion. An impaired intestinal perfusion may result in an hypoxic-ischemic state of the tissue, which may further decrease intestinal motility, increase overgrowth of (opportunistic/pathogenic) bacteria, further predisposing the infant to intestinal damage and irreversible necrosis.[28,89]

In **chapter 4** of this thesis we gave a proof of concept that an impaired intestinal perfusion is present during the whole course of the disease. We simultaneously

studied I-FABP levels, the marker for intestinal damage, and FTOE values, which reflects the balance between tissue oxygen supply and tissue oxygen consumption. We observed in **chapter 4** that the initial FTOE starts much higher for the complicated NEC cases and stayed high for at least 24 hours before going down. Uncomplicated NEC cases start with a lower FTOE which increased during the first 24-36 hours. I-FABP levels decreased in the uncomplicated and complicated cases, representing – most likely - healing of the mucosa and complete destruction of the mucosa, respectively (**chapter 4**).[90,91] In **chapter 5** we support the hypothesis that initially high I-FABP levels are associated with the extent of intestinal necrosis via the observation that initial I-FABP levens correlated with the extent of necrotic bowel in the surgically treated infants. The combination of FTOE and I-FABP values thus gives insight in the pathological events, representing a progression or recovery of the intestinal ischemia during early NEC with progression of the disease severity in the complicated NEC cases.

Preterm infants are more vulnerable to intestinal ischemia because their system for regulating vascular resistance is immature. Vascular resistance is an important factor involved in the autoregulation of blood flow, which is the ability of an organ (such as the kidneys, cerebrum, and heart) to maintain a consistent blood pressure despite negative or positive influencing factors.[92–95] At the same moment, the blood flow is preferentially diverted to the most vital organs, such as the heart and brain, rather than less vital organs (including the intestine), also causing hypoxia in the intestine. In **chapter 4** we observed strong associations between cerebral FTOE values and mucosal damage (I-FABP levels), which might be a reflection of an impaired autoregulation and/or an unequally distributed blood flow to the organs.[92–94] We were not able to differentiate whether an impaired cerebral perfusion is the initiating mechanism in the pathophysiology (via cerebrovascular autoregulation and/or an unequally distributed blood flow) or is the result of a critical disease in the preterm infant.

Bacterial colonization

Bacterial colonization prior to NEC development

The normal intestinal microbiota is necessary for the protection against infection, tolerance of feeding, and the contribution to nutrient digestion and energy harvest.[32] In addition, enteral feeding and colonization with the normal commensal bacteria are necessary for the maintenance of intestinal barrier integrity. Intestinal commensal bacteria also provide signals that foster normal immune system development and influence the ensuing immune responses.[32] It is not surprising that an altered intestinal microbiota contributes to the pathophysiology of NEC.

Preliminary studies using molecular methods to evaluate fecal microbiota suggest that NEC is associated with both unusual intestinal microbial species and overall reduction in the diversity of microbiota.[22] In **chapter 7** we investigated in a case-control study the intestinal microbiota prior to NEC development. We observed a lower bacterial diversity in both NEC cases and in controls. We suggest that a decreased microbial diversity in combination with alterations in microbial species may reduce the colonization resistance, which normally offers protection against invasion of possible pathogenic bacteria.[22,96,97] Hence, an inappropriate microbiota does not alone induce NEC, but possibly the presence and abundance of *C. perfringens* and *B. dorei* are involved in pathophysiology of NEC, together with intestinal insults such as ischemia and hypoxia.[97]

Despite decades of extensive research (even with the availability of DNA-based high-throughput detection techniques), no consistent single microbial species contributing to NEC development has been identified. *Enterobacteriaceae*, *Clostridium* spp, *Klebsiella* spp, *Bacteroides*, and enterobacter spp have been previously related to NEC.[1,98,99] The exact role of the bacterial involvement in NEC remains elusive. Thereby, the question whether an acute bacterial insult initiates NEC or an altered intestinal microbiota makes the infant more vulnerable for NEC is still questionable. **Chapter 7** sheds new light on this debate. In **chapter 7** we describe a NEC-associated microbiota (with the presence of *C. perfringens* and *B. dorei*) already in the meconium of infants who developed NEC later in life. Therefore, we speculate that NEC is not an acute bacterial insult, but rather it is an altered intestinal microbiota together with a low bacterial diversity that contributes to the development of NEC later in life.

While *C. perfringens* has been associated with NEC development before[100–104], only de La Cochetière et al.[105] also suggested a significant relationship between early colonization by *Clostridium* spp and development of NEC. *C. perfringens* harms the immature intestine by producing alpha-toxins. In addition, we are the first to describe the association between *B. dorei* in meconium and subsequent NEC development. Thereby, we found the striking association between abundances of *B. dorei* in meconium and mortality. *Bacteroides* generally behave as a normal anaerobic (beneficial) commensal bacteria in the human gut, but they are also known to cause severe pathology with their ability to influence the immune system.[106] Unfortunately, the exact mechanism behind colonization with *B. dorei* and NEC development remains elusive.

Whereas *C. perfringens* and *B. dorei* are NEC-associated bacteria, we observed staphylococci and, in a lesser extent, streptococci as protective for the development of

NEC **(chapter 7**). Staphylococci and streptococci were more abundant in patients who did not develop NEC when compared with patients who did develop NEC. Staphylococci and streptococci are both lactate producing bacteria. La Rosa et al.,[107] observed that these bacteria are primarily found in the earliest weeks of life.[108] Staphylococci and streptococci may have a similar protective mechanism as bifidobacteria and lactobacilli. Bifidobacteria and lactobacilli are stimulated by breast milk and are often thought to provide protection against NEC development. These bacteria provide protection by lowering the pH via the production of lactate and thereby hampering the growth of opportunistic and/or pathogenic bacteria, such as *C. perfringens*.

Still it is surprising why we did not find bifidobacteria and lactobacilli as protective bacteria against NEC development in our cohort. An explanation for this is because bifidobacteria and lactobacilli are usually only seen in fecal sampling after a GA greater than 33 weeks.[109–112] Their presence increases with GA, suggesting that there may be GA thresholds for colonization with certain microbes.[109–112] Our cohort, as described in **chapter 7,** was significantly younger than a gestation of 33 weeks, and we observed that lactate producing bacteria – instead of bifidobacteria and lactobacilli – were stimulated by breast milk and negatively associated with NEC development. Based on the present data we might therefore speculate that in infants with a GA below 33 weeks staphylococci in particular are capable of taking over the bacterial niche of the still largely absent bifidobacteria. As such staphylococci might also offer the same protection as bifidobacteria do later in life.

It is important to know when bacterial colonization in the intestine starts, to determine wherefrom the intestinal bacteria in the meconium originate. Previously it was thought that the in utero environment was largely sterile and that a fetus was not colonized with bacteria until the time of birth.[113–115] Recently it has been suggested that the colonization process can begin before delivery.[113,115–118] The mechanism behind this pre-delivery colonization is not yet revealed. With the present study described in **chapter 7**, we share the hypothesis that (yet unrevealed) factors during the first days of life or even in utero influence the formation of a healthier or a more NEC-associated microbiota. A future prospective study should collect maternal stool samples, placenta material/amniotic fluid, and mother's milk for analysis to identify the origins of the colonizing strains.

In **chapter 7** we also investigated whether several maternal- and neonatal factors are involved in the formation of the intestinal microbiota. We only found that the infant's feeding regime was associated with the intestinal microbiota in relation to NEC development. We observed an association between early enteral nutrition,

especially in the form of mother's milk, and increased abundances of (protective) lactate-producing bacteria in post-meconium samples. With the data in this thesis we support the hypothesis that both early enteral nutrition after birth and mother's milk play a pivotal role in protecting the intestine against development of NEC.[32,119–122] Early commencement of enteral nutrition and subsequently predominantly giving mother's milk likely stimulates lactate producing bacteria in infants with a low GA in a similar way as mother's milk normally stimulates bifidobacteria in term infants.[108–110]

Bacterial colonization during NEC

In **chapter 6** we investigated whether bloodstream infections (BSIs) during early NEC (24h prior and 24h after NEC) precede loss of intestinal barrier function, or whether BSIs occur when there already is a loss of intestinal barrier integrity. We observed a low incidence of BSIs in NEC patients during early NEC, which was in agreement with the studies of Bizzarro et al.,[123] and Cole et al.[124] Thereby, we did not find a relation between BSIs and the pro-inflammatory response induced during NEC (IL-8) nor with loss of intestinal barrier integrity due to mucosal damage (I-FABP). The absence of an association between BSIs and the pro-inflammatory response (measured via IL-8) suggests that BSIs do not initiate the pro-inflammatory response contributing to NEC. Secondly, we suggest that in the early phase of NEC the assumed loss of intestinal barrier integrity due to mucosal damage is not severe enough to allow bacterial translocation to the systemic circulation. However, this could still be true during the later phases of the disease. Hence, the bacterial translocation within the intestinal wall can be suspected during early NEC. It is important to note that loss of intestinal barrier integrity could also occur via the loss of TJs between the enterocytes. In this thesis we did not investigate other markers for loss of intestinal barrier integrity, such as claudin-3. We speculate that BSIs do not precede NEC and play a complementary role during early NEC development.

In **chapter 8** we investigated the identity and abundance of bacteria invading the intestinal wall in resection specimens of surgical NEC patients. We observed bacterial invasion within the intestinal in the most affected tissue samples in 51% of the cases, comparable with 36% in least affected NEC tissue samples. This finding suggests that the degree of bacterial invasion is associated with the degree of intestinal damage. While we scarcely observed bacteria potentially associated with NEC (*Clostridium* spp and *Bacteroides*), we did observe high densities of *Enterobacteriaceae*. While other pathogenic bacteria (such as *C. perfringens* and *B. dorei*) might be associated with NEC development, *Enterobacteriaceae* might have an important role in disease deterioration. These results - described in **chapter 8** - are in agreement with McMurtry et al.,[125] and Smith et al.[126] Both studies reported

a high abundance of proteobacteria (to which *Enterobacteriaceae* belong) and a lower abundance of *Clostridium* spp in severe NEC cases.[125,126] The finding of our study - low densities of bacteria associated with NEC development, such as *C. perfringens*, and high abundances of *Enterobacteriaceae* - argues for involvement of *Enterobacteriacea* in disease progression.

An explanation for the high abundances of *Enterobacteriaceae* in the surgical NEC specimens could lay in their resistance to antimicrobial peptides (AMPs). AMPs represent one of the most prominent and nonspecific components of the host response to certain pathogens with an anaerobic metabolism.[127,128] AMPs are produced in large quantities at sites of infection and/or inflammation and can have broad spectrum antibacterial and antisepsis properties.[129] One could hypothesize that *Clostridium* spp could induce NEC-specific intestinal inflammation and thereby activate AMP transcription, which offers resilience to these pathogenic bacteria. *Enterobacteriaceae* are often more unresponsive to AMPs and therefore have the ability to survive in highly inflamed tissue.[130] In addition, intensive antibiotic treatment in preterm infants could be corroborant. A more intensive antibiotic use reduces the diversity of bacterial species and increase the domination of *Enterobacteriaceae* because of their unresponsiveness to AMPs.[125,131] This hypothesis should be studied more exclusively in the nearby future.
Still the question remains why we only scarcely observed *C. perfringens* in **chapter 8**, which is known to be associated with NEC development. *C. perfringens* produce α-toxins – causing loss of intestinal barrier integrity and subsequently oxidative stress induced by reactive oxygen species (ROS).[132–134] *C. perfringens* can ultimately not survive this environmental oxidative stress. *Enterobacteriaceae* are resistant against nutrient limitation as well as oxidative stress and therefore survive properly in a highly inflamed and necrotic intestine.[135] This phenomenon is also described in the pathogenesis of Crohn's disease, where activated neutrophils infiltrate the intestinal wall and produce ROS, which leads to oxidative stress in which *Enterobacteriaceae* but not *C. perfringens* survive.[125,134,136]

Novel insights into the pathophysiology of NEC derived from this thesis

The preterm intestine is characterized by immature uncontrolled immune defenses and a compromised intestinal barrier function. We demonstrated that alterations of the intestinal microbiota are already present in the first meconium of infants with increased risk of NEC development**.** NEC-associated bacteria - such as *C. perfringens* and *B. dorei* - stimulate the elaboration of pro-inflammatory mediators (via PCs and TLR4s) and induce oxidative stress. When the infant experiences more

prenatal stressors – such as hypoxia, hypoperfusion, enteral feeding (in the form of formula feeding) – the stimulation of pro-inflammatory mediators increases and an impaired epithelial function continues. Thereby, hypoxia and hypoperfusion could alter the balance between vasoconstriction and vasodilatation of the intestinal circulation. Therefore, a NEC-associated microbiota, prenatal stressors, and an impaired circulatory regulation and perfusion contribute to a relatively hypoxic-ischemic state, predisposing to loss of intestinal barrier integrity and irreversible necrosis of the intestine. When disease progresses, an impaired intestinal perfusion and bacterial invasion within the intestinal wall might worsen. The composition of the intestinal microbiota will change: from a microbiota consisting *C. perfringens* and *B. dorei* before NEC development to a microbiota consisting mainly out of *Enterobacteriaceae* during advanced NEC. The extent of both ischemia and intestinal inflammation determine the symptoms observed and the clinical outcome for the patient.[137–139] Figure 2 presents a simplified overview.

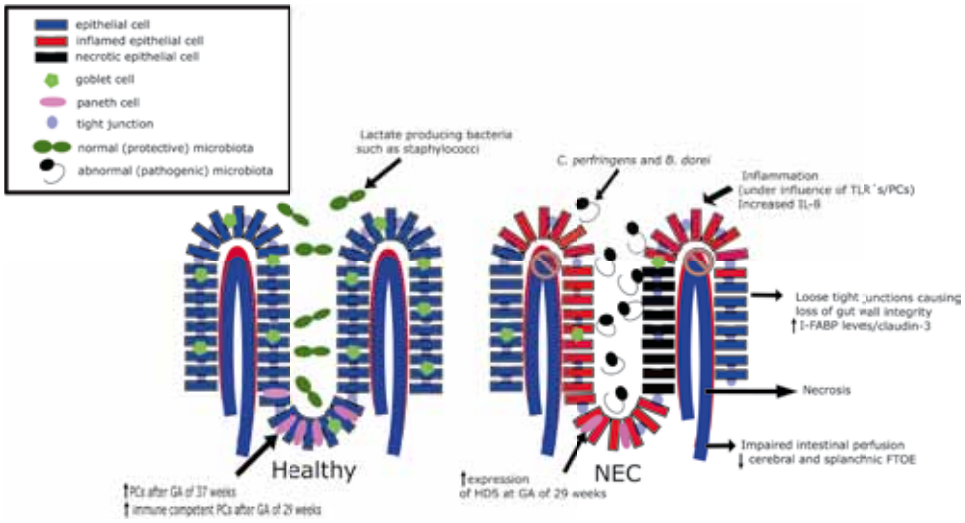

FIGURE 2:

legend measured via claudin-3, ipv measured visa claudin-3.

Healthy-The healthy (term) infant has an intact intestinal barrier function: solid tight junctions (TJs), an adequate amount of (immune competent) paneth cells (PCs), and goblet cells. Toll-like receptor (TLR) 4 expression and cytokine secretion upon lipopolysaccharide (LPS) stimulation, triggered by the intestinal microbiota, increases with gestational age. The intestinal microbiota in healthy infants is colonized with commensal bacteria, such as bifidobacteria and lactobacilli in term infants and other lactate-producing bacteria in preterm infants. Thereby the intestine is well vascularized and the infant is not exposed (a lot) to factors inducing hypoxia and ischemia (e.g. hemodynamic instability, inappropriate oxygenation).

NEC- The epithelium of a preterm infant at risk for NEC development often has decreased intestinal barrier integrity: loose and/or disrupted TJs (measured via claudin-3), immature immune cells and receptors (goblet cells, PCs, and TLRs). For example, immature PCs secrete significantly more human defensin 5 (HD5) after 29 weeks of gestation, which results in either an exaggerated immune response or in an normal immune response which the preterm infant cannot adequate handle yet. The preterm infant is also at high risk of factors inducing hypoxia and ischemia (e.g. hemodynamic instability, inappropriate oxygenation), inducing intestinal barrier failure by intestinal ischemia and mucosal damage. In the preterm infant developing NEC an altered microbiota, including Clostridium perfringens and Bacteroides dorei, is found starting from the meconium. In the presence of the immature immune system and an impaired intestinal barrier function, the invasion of bacteria such as C. perfringens and B. dorei results in (again) an exaggerated immune response, leading to a high inflammatory state and mucosal damage.

Future perspectives

To date, the underlying pathophysiology of NEC remains incompletely understood. With this thesis we have shed some light on the underlying pathophysiology and offer some new insights which might aid in the understanding of this devastating disease, affecting the most vulnerable infants. However, the findings described in this thesis only suggest – and do not prove – causality. Still considerable work has to be done before we have the knowledge to provide a strong and confident hypothesis for the pathophysiology of NEC, and to provide adequate evidence-based strategies for NEC prevention, prediction, and treatment.

Pathophysiology

Further research should focus on all three major components in the pathophysiology of NEC: the intestinal barrier function, the intestinal perfusion and the intestinal microbiota.

First, research on perinatal and postnatal development of the epithelial lining (including the Notch and Wnt/ß-catenin signaling pathways) and the contributing immune system (including PCs, TLR4, and TJs) would increase our knowledge of the embryological and fetal development. Second, when we understand the normal human intestinal differentation we should identify the alterations in those patients who are at risk for NEC development.

Furthermore, an increased knowledge on the intestinal perfuson may shed light on the hypoxic-ischemic mechanisms contributing to NEC. Primarily we need to answer the question whether intestinal ischemia initiates NEC or is a secondary result from NEC. Factors which should be studied more exclusively are nitric

oxide, oxidative stress and autoregulation. Thereby, the external stressors (such as hemodynamic instability) causing ischemia should be identified and could be used for a prediction model for NEC.

While this thesis suggests causality between the intestinal microbiota and NEC development, this is far from proven. Our findings should be verified by testing for NEC induced infection with *C. perfringens* in animal models, possibly in combination with targeted antibiotics treatment. Thereby, the observation of a NEC-associated microbiota within the meconium suggests that (yet unrevealed) factors during the first days after birth or even in utero play an important role in the formation of a more healthy microbiota to a NEC-associated microbiota. This should be further investigated via a prospective study focusing on the analysis of maternal stool samples, placenta material/amniotic fluid, and mother's milk to identify the origins of the colonizing strains.

Prevention

Because of the incompletely understood pathophysiology of NEC, preventive options are still limited. New preventive options can interfere at different components in the pathophysiology of NEC. Research efforts focused mainly on the use of human milk feeding, additional nutritical supplements, timing of initial feeding, probiotics, and immunoglobins. Also, the avoidance of H2-blockers and avoidance of prolonged empirical antibiotic use has been recommended.[23,140]

Feeding

Mother's milk has immunoprotective properties and provide colonization resistance via commensal bacteria against pathogenic bacteria. Mother's milk is thus protective against the development of NEC.[141] Therefore, mother's milk is the feeding of choice for the vulnerable preterm infant at risk for NEC.[142] Unfortunately, not all preterm infants are fed with mother's milk, due to a yet insufficient production of mother's milk.[143] Infants often need supplements (formula feeding) added to their feeding schedule to guarantee a sufficient nutritional intake. Furtheremore, these infants need supplements to be added to their own mother's milk to meet the higher caloric and mineral requirements coinciding with their prematurity. These milk fortifiers and formula feeding decreases the protection of the intestine, making the infant vulnerable for the development of NEC. New studies should investigate whether other types of milk fortifiers, the use of donor human milk, and probiotics (live microorganism supplements) are effective preventive options against NEC development.

In **chapter 7** we emphasized the important protective role of mother's milk. We observed that early enteral nutrition, especially in the form of mother's milk, stimulated the colonization with lactate producing bacteria in post-meconium samples. This was found to be protective against NEC development. Formula feeding did not increase or decrease the risk of NEC development (**chapter 7**). We have to note that the population was small, and causality was not proven.
Formula feeding has a completely different composition than mother's milk, and therefore we emphasize that research focusing on the improvement of formula feeding is a research priority.[144] If we establish a type of formula feeding which is highly similar to mother's milk (e.g. forge of immunoglobulines, enzymes, and the present microbiota), the preterm infant can be protected against a various amount of diseases.

Another possibilty would be the avoidance of intestinal hypoperfusion. In animal studies the use of relaxin supplementation, a potent vasodilatory hormone, goes along with a decreased incidence of NEC by decreasing the intestinal hypoperfusion as observed in NEC.[145] This preventive method has not yet been tested in humans, and first possible complications and precise dosis-response needs to be evaluated prior to clinical application in human infants.[145]
Another, easily obtained, option seems to be human donor milk.[143,146] In a meta-analysis of 5 randomized controlled trials focusing on human donor milk a significantly higher incidence of NEC was reported in formula-fed infants compared to infants fed with human donor milk.[147] Donor human milk differs nutritionally from mother's milk mostly caused because of pasteurization processes.[146] For example, there is an increased chance that pathogenic microorganisms surive the pasteurization processes in pasteurized donor human milk.[146] Another problem with the use of human donor milk in the preterm infant is that human donor milk is often donated by term mothers, and thus contains less energy and proteins than preterm formulas or even the mother's milk of a preterm infant.[148] Therefore, future research, e.g. via a prospective randomized trial involving multiple feeding/fortification strategies are needed to optimalization the feeding regime for preterm infants.

Probiotics
Probiotics are probably the most promosing preventive treatment for NEC.[149] Probiotics might be valuable in the prevention of NEC and its associated morbidity by prevention of (opportunistic and/or pathogenic) bacterial invasion, prevention against dysbiotic conditions, and by enhancing the immune responses of the host.[116,150] The Cochrane review by Alfaleh, et al.[150] concluded that supplementation with probiotics reduced the risk of severe NEC and lowered the mortality in infants with a birth weight >1000 grams. This review also concluded that studies corning

on the use of probiotics in the population infants born with a birth weight <1000 grams were not available, and therefore, a reliable estimation of the safety and effectiveness of the use of probiotics cannot be made in this population. A large randomized controlled trial is needed to evaluate the use of probiotics in the infant born with a birth weight <1000 grams. Another point to mention in the perspective of probiotics is the composition of the probiotics. Previous studies suggested to use probiotics in the form of lactobacilli and bifidobacteria to prevent NEC.[149] However, we observed in **chapter 7** low numbers of lactobacilli and bifidobacteria, probably because lactobacilli and bifidobacteria are usually only seen in fecal sampling after a GA greater than 33 weeks, and their presence increases with GA.[110–112] In **chapter 7** we found that particularly lactate producing bacteria have a negative association with NEC, i.e. a lower incidence. Therefore, it would be interesting to focus on probiotic treatment with other lactate producing bacteria including staphylococci and streptococci.

Besides the use of probiotics to establish an appropriate protecting intestinal microbiota, it could also be used to maintain the intestinal barrier function via the stabilization of TJ-functioning.[35] Bergmann et al.[35] provided evidence for a beneficial role of bifidobacteria to stabilize claudins in TJs. Further research should investigate the effect of probiotics on TJ-funcitioning and should identify the benefits and disadvantages of the use of probiotics as a TJ-stabilizer in clinical practice.

Medication

The avoidance of H2-blockers and avoidance of prolonged empirical antiobic use has been recommended.[23,140] However, no study yet proved causality between H2-blockers and prolonged emprical antibiotic use and NEC development.[23,140] However, sometimes the use of H2-blockers and antibiotics is needed to protect the infant for the (progression of) other threats. Therefore, it is more interesting to investigate whether we can use medications to protect the infant to establish a 'healthy' intestinal microbiota by increasing the prevalance of lactate producers. Another possibility is the use of targated antibiotic therapies against NEC-associated bacteria, such as *C. perfringens* and/or *B. dorei*. Investigations on targeted antibiotics have only just started and should be studied extensively in the nearby future.

Larger trials are needed to further investigate the microbiota in both fecal samples and resection specimens in infants at risk for NEC and who develop NEC. Based on that data we could then outline an optimal and individual feeding strategy with probiotics and/or targeted antibiotics for infants at risk for NEC.

Predicting the development of NEC

As yet, clinically proven tools or tests are not available that predict the development of NEC. Currently available biomarkers lack accuracy to detect NEC in pre-clinical stages.[98] We offer two possible predictiors for the development of NEC which need more investigation in the nearby future: 1. cerebral and intestinal NIRS monitoring, 2. screening of the intestinal microbiota.

First, cerebral and intestinal NIRS monitoring might be a predictive tool for the development of NEC. While this thesis did not focus on NIRS measurments before the onset of NEC, Schat et al.[74] did shed light on this possibility. They observed in a prospective trial that infants with a cerebral tissue oxygen saturation <70% in the first 48 hours after birth had a nine-fold higher risk of development NEC.[74] Patel et al.[81] found lower intestinal tissue oxygen saturation values in the first week of life in preterm infants who later developed NEC compared to infants who did not develop NEC. Yet, the actual clinical utility of the findings of the study of Schat et al[74] and Patel et al[81] needs to be further investigated, whereas we keep in mind that NIRS technology has its limitations. There are different NIRS devices with each their own algorithm, making NIRS measurements heterogeneous.[151–153] Thereby, as mentioned before, intestinal NIRS measurements are influenced by peristalsis, gut movements, air and stools, complicating the interpretation of intestinal NIRS measurements.[79,87]

Secondly, we emphasize that screening of the intestinal microbiota starting from the first meconium could be an useful tool for predicting which infant is at significant risk to develop NEC. Analysis of feces via sequencing techniques as used in **chapter 7** is not feasible in routine care as the determination of different patterns requires sophisticated analyses. Thereby, feces analysing via sequencing techniques is costly and time consuming. Fecal volatile organic compounds (VOC) reflect microbial composition and could be measured using an electronic nose (eNose).[154] Therefore, VOC-profiling on the presence of *C. perfringens* and *B. dorei*, has potential as a non-invasive, predictive test to detect infants who are at significant risk for NEC development.

Diagnosis and detection of disease progression

Due to the often unspecific early onset of NEC, diagnosis of NEC is difficult. Clinical symptoms of NEC are usally indistinguishable from other diseases, such as sepsis, leading to delay in the start of therapy, thereby possibly worsening the prognosis of the infant. NIRS, I-FABP and IL-8 have been suggested markers for diagnosing NEC.[39,58,74,75] Schat et al.[74] reported that FTOE values measured using NIRS were higher in infants who developed NEC compared with controls before NEC onset. We observed that I-FABP and IL-8 levels are increased at NEC onset compared to controls. While NIRS, I-FABP, and IL-8 are all very promising markers, and will be

possibly used in the nearby future in the clinical setting, larger multicenter trials are needed to provide definite cut-off values for these three markers and to assess the clinical value to combine these three markers.

Further progression of NEC into complicated NEC can be predicted by the use of FTOE levels in combination with I-FABP levels, and the intestinal bacterial density. With this knowledge we suggest that FTOE- and I-FABP levels are regulary obtained in the infant suffering from NEC. Thereby, VOC-profiling during NEC could be useful. Also, the measurement of oxidative stress (via ROS measurements, measurement of the resulting damage to biomolecules, and/or detection of antioxidant levels) induced by *C. perfringens*, could be a useful tool for the prediction of the severity of NEC.

Conclusion

We observed that the incidence of NEC is still increasing, despite extensive research on this topic. One of the reasons that we are still unable to minimize this disease successfully is the not yet fully understood multifactorial pathophysiology. Therefore, the main goal of this thesis was to increase our knowledge about the underlying pathophysiology of NEC, focusing in particular on the role of the intestinal barrier function, intestinal perfusion, and the intestinal microbiota.

In the context of the intestinal barrier function we observed that a rapid increase in immune competent PCs occurs starting from 29 weeks of gestation, which corresponds with the peak incidence of NEC at a PMA of 29-33 weeks. This increase in immune competent PCs in a vulnerable preterm intestine could cause an (excessive) inflammatory reaction contributing to NEC development. During NEC we observed that mucosal damage and inflammation is seen in early NEC, but this is not severe enough to allow bacterial translocation to the bloodstream causing sepsis. However, intestinal barrier integrity could be severe enough to allow bacterial translocation locally within the intestinal wall. NEC progression is associated with the extent of mucosal damage.

In the context of intestinal perfusion earlier research reported that an impaired intestinal perfusion was associated with NEC development. In addition, we observed that the combination of FTOE and I-FABP values gives insight in the pathological events, representing a recovery or progression of the intestinal ischemia during early NEC in uncomplicated versus complicated cases. Lastly, in the context of microbiota we observed, most importantly, that *C. perfringens* and *B. dorei* within the meconium were already associated with NEC development later on. Staphylococci, via mother's milk, seemed protective. During NEC progression colonization of the intestinal wall with *Enterobacteriaceae* were present, while *Clostridium* spp and

Bacteroides were not observed anymore. While we unraveled some aspects of the complex multifactorial pathophysiology of NEC, a lot of questions still remain. We identified some aspects that make the infant prone for NEC development, but we still do not know what actually triggers NEC at which specific moment. With our results on the NEC-shaped microbiota, we opened options for new research on interventions aimed at restoring a more balanced microbiota and a less disrupted intestinal barrier.

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