The pathophysiology of necrotizing enterocolitis in preterm infants
Heida, Fardou Hadewych

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CHAPTER 1

INTRODUCTION AND AIMS OF THE THESIS
## Contents

- Epidemiology 12
- Clinical presentation 13
- Treatment options 14
- Diseases outcomes 15
- Pathophysiology 15
- Intestinal barrier 17
  - Enterocytes 18
  - Tight junctions 19
  - Immune cells contributing to the intestinal barrier 19
  - Paneth cells 20
  - Goblet cells 21
- Hypoxic-ischemic mechanisms 22
  - NIRS 22
- Normal bacterial colonization? 23
- Bacterial colonization 23
  - Preterm versus term microbiota 24
  - Mother-child symbiosis 25
  - Influence of nutrition of the intestinal microbiota 26
  - Intestinal microbiota associated with NEC 26
  - Microbiota analysis via 16S rRNA sequencing 27
- Aims of the thesis 27
- References 30
Necrotizing enterocolitis (NEC) is a complex disease involving among others bacterial invasion, inflammation, and necrosis of (premature) intestinal tissue.¹ NEC is a devastating gastrointestinal disease occurring in preterm infants.² NEC has also been one of the most difficult diseases in neonatal care to eradicate, and thus has become a priority for research.³

The first described conditions closely resembling NEC go far back, but the disease was not widely recognized until after the development of modern neonatal intensive care.³⁻⁵ The incidence of NEC increased during the 1970s, due to higher neonatal survival rates resulting from seminal breakthroughs in neonatal care, such as Continuous Positive Airway Pressure and total parental nutrition.⁶ Despite improvements in neonatal care and extensive research regarding NEC, the disturbing reality is that the reported incidence and survival of infants with NEC has not changed in the past quarter of the century.⁶ One of the explanations for the unchanged incidence and survival of NEC infants is the fact that its etiology and pathophysiology are as yet incompletely understood. Therapeutic options therefore mainly consist of supportive measures, such as bowel rest and inotropics and/or mechanical ventilation. Therefore, the main goal of this thesis is 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 primarily affects preterm infants.⁷ When the gestational age (GA) and birth weight (BW) decreases, the incidence of NEC will increase.⁸ The lower the GA, the later this condition occurs after birth, with a peak incidence at a postmenstrual age (PMA) of 29-33 weeks.³

Cases of NEC appear to occur sporadically with reports of clusters of outbreaks.⁹ A few large scale NEC outbreaks occurring simultaneously at different sites have provided evidence of the potential clustering of cases.⁹ The occurrence of these temporal clusters provide support for a common etiological factor whose exposure may be involved in the etiology of NEC.⁹ However, there is currently no generally accepted operational definition of a NEC cluster.⁹ While most NEC cases occur sporadically, NEC ‘epidemics’ have been reported in the literature, with the number of cases, clinical presentations, and potentially causative agents differing greatly.⁹ Overall, large multicenter and population-based studies estimate the incidence of NEC in very low birth weight infants (birth weight <1500 grams) between 7 and 11%.¹⁰⁻¹³ As mentioned previously, the incidence of NEC vary across neonatal intensive care units (NICUs) and countries.¹⁴ We do not know if and how the
incidence of NEC in the Netherlands has evolved in the last decade. In this thesis we will therefore start with investigating the incidence of NEC in three pediatric surgical centers in the Netherlands.

**Clinical presentation**

The clinical presentation of NEC can range from non-specific signs that progress insidiously over several days to a fulminant onset of gastrointestinal signs, multi-organ dysfunction and shock within a few hours. Symptoms that indicate the presence of NEC can include both intestinal and systemic perturbations, such as abdominal distention, feeding intolerance, bloody stools, apnea, bradycardia, and temperature instability. Common laboratory findings are leukocytosis, thrombocytopenia, metabolic acidosis, and increased C-reactive protein levels. The fact that these symptoms and laboratory results can be observed in the presence of a variety of other diagnoses, such as sepsis, make a timely diagnosis of NEC challenging. The only signs that confirm the presence of NEC are pneumatosis intestinalis (representing submucosal gas possibly produced by bacterial fermentation) and/or portal venous gas on abdominal radiographic examination.

Several staging systems have been developed to help and guide clinicians in diagnosing and treating NEC. In our NICU, the modified Bell’s staging system is used, as presented in Table 1 (page 14). The modified Bell’s staging system has shortcomings, since severe NEC requiring surgery can develop in patients even though pneumatosis intestinalis and/or portal gas has not been detected on imaging. These patients may only have abdominal distention, without intraluminal bowel gas, on presentation. Therefore, the ominous progression of the disease may be missed, with a failure to intervene early enough. A more reliable diagnostic approach that allows for aggressive preventive measures is needed. Such an approach might include biomarkers (such as intestinal fatty acid-binding protein (I-FABP), interleukin-8 (IL-8), and near infrared spectroscopy (NIRS) monitoring) that accurately predict the development and progression of NEC.
CHAPTER 1

Medical interventions may involve drain placement and/or surgical procedures due to the limited understanding of the pathophysiology of NEC. As treatment does not specifically target the underlying pathology it often proves inadequate. Surgical interventions typically include abdominal decompression, bowel rest, and broad-spectrum intravenous antibiotics. Medical interventions are generally required in infants with intestinal perforation and/or infants with clinical deterioration despite maximal medical treatment. Surgical procedures may involve drain placement and/or an

<table>
<thead>
<tr>
<th>Stage</th>
<th>Classification</th>
<th>System Signs</th>
<th>Intestinal signs</th>
<th>Radiological signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>Suspected NEC</td>
<td>Temperature, instability, apnea, bradycardia,</td>
<td>Increased gastric aspirates, mid abdominal distension,</td>
<td>Normal or intestinal dilation, mild</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ilethargy</td>
<td>emesis, occult blood in stool</td>
<td>ileus</td>
</tr>
<tr>
<td>1B</td>
<td>Suspected NEC</td>
<td>Same as above</td>
<td>Same as above, plus bright red blood from rectum</td>
<td>Same as above</td>
</tr>
<tr>
<td>2A</td>
<td>Proven NEC-mild</td>
<td>Same as above</td>
<td>Same as above, plus absent bowel sounds, with or</td>
<td>Intestinal dilation, ileus, pneumatosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>without abdominal tenderness</td>
<td>intestinalis</td>
</tr>
<tr>
<td>2B</td>
<td>Proven NEC-moderate</td>
<td>Same as above plus mild metabolic acidosis and mild thrombocytopenia</td>
<td>Same as above, plus absent bowel sounds, definite  abdominal tenderness, with or without abdominal cellulitis or right lower quadrant mass</td>
<td>Same as 2A, plus portal venous gas, with or without ascites</td>
</tr>
<tr>
<td>3A</td>
<td>Advanced NEC – severe, bowel intact</td>
<td>As 2B plus hypotension, bradycardia, severe apnea, combined respiratory and metabolic acidosis, disseminated intravascular coagulation and neutropenia</td>
<td>Same as above, plus signs of generalized peritonitis, marked tenderness, and distension of abdomen</td>
<td>Same as 2B, plus definite ascites</td>
</tr>
<tr>
<td>3B</td>
<td>Advanced NEC – severe, bowel perforated</td>
<td>Same as 3A</td>
<td>Same as 3A</td>
<td>Same as 2B, plus pneumoperitoneum</td>
</tr>
</tbody>
</table>

**TABLE 1:** Modified Bell’s staging criteria for NEC


**Treatment options**

Current NEC treatment options do not target the specific underlying disease processes due to the limited understanding of the pathophysiology of NEC. As treatment does not specifically target the underlying pathology it often proves inadequate. Symptomatic treatment of the infant with NEC begins with prompt recognition of the symptoms and medical stabilization. Medical interventions typically include abdominal decompression, bowel rest, and broad-spectrum intravenous antibiotics. Surgical interventions are generally required in infants with intestinal perforation and/or infants with clinical deterioration despite maximal medical treatment. Surgical procedures may involve drain placement and/or an
exploratory laparotomy with resection of diseased bowel followed by primary anastomosis or the construction of one or more ostomies.\textsuperscript{3,23} The assessment of intestinal necrosis and the timing of surgery, especially in the absence of perforation, remains a key problem in NEC.\textsuperscript{24} Furthermore, the decision of early surgical intervention might lead to an unnecessary laparotomy (including general anesthesia with its associated risks), while postponing surgery might lead to further disease progression with severe sepsis and eventually death.\textsuperscript{4,23} Consequently, novel diagnostic and prognostic tools are in great demand.

**Disease outcomes**
Internationally, mortality rates range between 20-40%, but vary with the severity and extent of intestinal necrosis.\textsuperscript{4} Mortality is inversely related to GA.\textsuperscript{3,4,12} We do not know how mortality rates of NEC in the Netherlands have evolved in the last decade. Approximately 27-63\% of affected infants may require surgery, and as many as 50\% infants may die in the peri-operative period.\textsuperscript{25,26} Subacute complications include intestinal strictures, recurrent NEC, dysmotility of the bowel, malabsorption, short bowel syndrome, and cholestatic liver disease.\textsuperscript{27–30} Severe NEC is associated with growth delay that can persist beyond infancy into childhood and with poor neurodevelopmental outcomes.\textsuperscript{25,31,32}

**Pathophysiology**
Current evidence supports a complex, multifactorial model of NEC (Figure 1).\textsuperscript{25}

**FIGURE 1:**
**Multifactorial model of NEC**

*The preterm infant has an immature intestine, immune system, and circulation which all make the preterm infant vulnerable for the development of NEC. Preterm infants have an immature intestine with loose and/or disrupted tight junctions (TJs), less paneth cells (PCs) and less goblet cells. The immune system is not yet fully developed, whereby preterm infants often suffer from an exaggerated excessive immune response. The preterm infant experiences stressors such as hypoxia, hypoperfusion, and enteral feeding that may induce an impaired intestinal perfusion of this vulnerable bowel. Lastly, the preterm infants have a*
different intestinal bacterial colonization pattern, which is in turn also influenced by enteral feeding. All factors mentioned above are considered to increase the risk of NEC development in the preterm infant.

The pathophysiology of NEC includes a complex interplay between components of the gut with its microbiota and the immune system (Figure 2). The intestine of a preterm infant is characterized by underdeveloped, uncontrolled immune defenses and a compromised intestinal barrier function. Preterm infants often experience prenatal stressors such as hypoxia, hypoperfusion, and postnatal stressors including enteral feeding that may induce an impaired intestinal perfusion by influencing the vascular resistance. Therefore, these events may predispose preterm infants to intestinal mucosal injury. An impaired intestinal perfusion might set the stage for further intestinal barrier breakdown and invasion of the intestinal wall by opportunistic/pathogenic bacteria, which incite an exuberant inflammatory response resulting in NEC. Elucidating this complex interplay of factors associated with the development of NEC might open new avenues to prevent the disease and/or to improve outcomes.

**FIGURE 2:**
Simplified model of the intestine in healthy term infants compared to preterm infants at risk for NEC

Healthy: The epithelium of a term and healthy infant includes an intact intestinal barrier function: solid tight junctions (TJs), adequate immune cells and receptors (e.g. goblet cells, paneth cells (PCs) and Toll-like receptors (TLRs)). Thereby, the intestine is well vascularized and colonized with commensal bacteria.
NEC: The epithelium of a preterm infant at risk for NEC development has often a decreased intestinal barrier integrity due to loose and/or disrupted TJs, immature immune cells and receptors (e.g. goblet cells, PCs and TLRs). Thereby, the immature intestine is vulnerable for ischemic and hypoxic stressors. These factors set the stage for further deterioration of the intestinal barrier breakdown and subsequent pathogenic bacterial invasion, which causes an exaggerated inflammatory response and in the most severe cases frank necrosis of the intestine.

Intestinal barrier

The gastrointestinal system is unique because of its close interaction between the single epithelial lining, the immune system, and the intestinal microbiota. The epithelium is a key host defense mechanism critical for confining pathogenic bacteria to the intestinal lumen, but it must also allow the passage of nutrients from the intestine into the circulation. Preterm infants have an increased intestinal permeability, perhaps to allow passage of important macromolecules from amniotic fluid or breast milk. However, this same increased permeability can lead to an increased bacterial translocation. Maintenance of an intact intestinal epithelium is crucial to maintain intestinal barrier integrity. Contrariwise, disruption of the intestinal barrier decreases intestinal barrier integrity and makes bacterial invasion possible. This is thought to be an early event in the pathogenic cascade of NEC. The inflammatory response of the intestinal barrier can be triggered by either commensal, opportunistic, or pathogenic bacteria.

Recently, knowledge has expended about several signaling pathways, including the Notch receptor, Wnt/β-catenin receptor, and Toll-like receptor (TLR) signaling pathways, regulating the differentiation of the intestinal epithelium (such as enterocytes, paneth cells (PCs), and goblet cells). We discuss these pathways more clearly because of its potential impact on the impaired intestinal barrier in preterm infants contributing to the pathophysiology of NEC. Sodhi et al. demonstrated that TLR4, present on the epithelial lining, influences Notch and Wnt/β-catenin signaling pathways. In the development of a healthy (term) infant the Notch and Wnt/β-catenin signaling pathways, regulated via (among others) TLR expression, provide a balanced intestinal epithelial integrity. This mechanism is controlled by a strictly regulated balance between proliferation and differentiation of epithelium for intestinal epithelial stem cells and cellular loss by apoptosis. The upstream mechanisms that initiate intestinal differentiation remain largely unknown, but TLR expression is discovered to be important. The expression of TLR4 in the intestinal lining increases during embryonic development and decreases significantly after (term) birth. This mechanism might explain the preponderance of NEC in preterm infants, as the expression of TLR4 is highly present in preterm infants.
Another role of TLR4 in the human intestine is to detect pathogenic bacteria close to the intestinal surface. Activation of TLR4 by pathogenic bacteria induce various inflammatory mediators, such as the inflammatory cytokine interleukin-8 (IL-8). IL-8 can increase the production of acute-phase proteins in the intestine as seen in NEC and finally induce ischemia and necrosis.

The intestinal barrier includes two components: the intrinsic barrier and the extrinsic barrier. The intrinsic barrier includes the epithelial cells (enterocytes/colonocytes), tight junctions (TJs) and (immune) cells derived from proliferation of stem cells (e.g. PCs and goblet cells). The extrinsic barrier includes mucus that coats the epithelial lining. Both the intrinsic- and extrinsic barrier contributes to maintain the intestinal barrier function.

**Enterocytes**

Enterocytes and colonocytes are the most abundant cell types in both small and large intestines and define the structure of the mucosa. The primary function of enterocytes is to absorb nutrients apically and export them basally. The apical surfaces of enterocytes consist of characteristic microvilli that comprise the brush border. Thereby, enterocytes separate the host from the community of commensal, opportunistic and pathogenic microorganisms in the lumen of the intestine, which form the microbiota. As current evidence supports, enterocyte damage is not primarily causing NEC, but is part of a vicious cycle of inflammation-inflicted epithelial damage. 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. This exaggerated inflammatory response damages the vulnerable enterocytes during NEC, and causes decreased intestinal barrier integrity resulting in progression of the disease.

Intestinal fatty acid-binding protein (I-FABP), a marker for enterocyte damage, might be a possible biomarker for NEC. When enterocytes are damaged, I-FABPs are released from the enterocytes. I-FABP is a small cytoplasmic protein with high organ sensitivity found in the enterocytes located at the tip of the villi. In the context of progressive intestinal barrier breakdown in NEC, enterocytes are damaged and I-FABPs are released in the circulation with subsequent secretion by the kidneys. In this thesis we focus on the role of I-FABP as a marker for mucosal damage in NEC and its relation with an impaired intestinal perfusion.
Tight junctions (TJs)

TJ proteins that form the TJ in the intestinal tract are key molecules for the regulation of permeability of the epithelial lining.\textsuperscript{53–55} TJs seal the intercellular spaces at the apical parts of adjacent enterocytes, thus forming a barrier lattice that is impenetrable to bacteria and most macromolecules.\textsuperscript{56} TJs thereby contribute significantly to the defense against microbes.\textsuperscript{53–55} TJs develop early in the second trimester of pregnancy.\textsuperscript{56,57} They mature during the remainder of pregnancy. During this maturation the TJ proteins get more embedded in the epithelium.\textsuperscript{56,57} In case of prematurity, the TJ proteins might lay looser in the epithelium making the TJs more vulnerable compared to TJs in term infants.

The TJ has a surprisingly complex protein composition compared with other cell-cell junctions and is composed of at least 40 different proteins, including zonula occluding proteins (e.g. ZO-1, ZO-2 and ZO-3), and membrane-spanning proteins (e.g. occludin, junction adhesion molecules, and claudin).\textsuperscript{34,58,59} An abnormal intestinal microbiota (such as colonization with gram-negative bacteria), that activate the immune system via lipopolysaccharide (LPS), can affect the TJ protein function.\textsuperscript{50,53} Yang et al.\textsuperscript{60} observed that LPS initiates a decrease in the TJ protein expression, and thereby increase the intestinal wall permeability. Contrariwise, lactobacilli and bifidobacteria are examples of bacteria that up-regulate the expression of TJ proteins, improving the intestinal barrier function significantly.\textsuperscript{60} The abnormal microbial colonization patterns and lack of normal commensal bacteria in preterm infants can also result in a decrease in TJ proteins which might result in loose and/or disrupted TJs, further compromising the intestinal barrier. Loose- and disrupted TJs have been related to the pathogenesis of NEC.\textsuperscript{61} Thuijls et al.\textsuperscript{50} observed increased claudin-3 levels, a marker for loss of TJs, during early NEC compared with infants without NEC. Loose and/or disrupted TJs are involved in the pathophysiology of NEC by causing an increase in intestinal wall permeability. The increase in intestinal wall permeability might be either primary due to structural immaturity or secondary to an abnormal bacterial colonization and/or ischemic damage of the entire intestinal wall.\textsuperscript{54} In both cases this results in an increased permeability leading to invasion of (opportunistic/pathogenic) bacteria and toxins, which in turn could further diminish the intestinal barrier integrity and cause inflammation as seen in NEC.\textsuperscript{53,54,56,57} Contrariwise, the presence of commensal bacteria has been shown to maintain and improve intestinal barrier integrity by up-regulating the expression of TJ proteins.\textsuperscript{56,60,62,63}

**Immune cells contributing to the intestinal barrier**

The extrinsic barrier includes post-miotic differentiated cells, including PCs and goblet cells, derived from stem cells that reside near the base of the crypts under
the interaction of the Notch and Wnt/β-catenin signaling pathways. PCs and goblet cells both are related to the pathogenesis of NEC. Because PCs and goblet cells are related with NEC development, the mechanism of differentiation of both cells is of high interest for the present studies.

**Paneth cells**

PCs are specialized epithelial cells, located at the base of the crypts of Lieberkühn in the small intestine. PCs protect the intestinal stem cells from pathogens, stimulate stem cell differentiation, shape the intestinal microbiota, and assist in repairing the intestine. PCs secrete (among others) human α-defensins (HD5/HD6). These defensins show protective activity against bacterial agents, and are thought to be associated with initiating and adapting immunity. As such PCs can be considered the “guardians of the crypt.” According to current animal and laboratory studies (i.e. gene expression- and intestinal isograft studies), PCs first appear in the first trimester under influence of the Notch and Wnt/β-catenin signaling pathways. They subsequently mature in antimicrobial expression at 22-24 weeks of gestation and increase in numbers by term gestation. We do not fully understand the process of PC development and maturation during human gestation. This will therefore be one of the topics of the present thesis.

The maturation of immune competent PCs might be crucial for NEC development. The current – and contradictory - hypotheses on the role of PCs in NEC development are based on either depletion of PCs, increased immune activity of PCs or PC dysfunction. In the first hypothesis, it is suggested that there is a relative deficiency of (immune competent) PCs in the immature intestine. This deficiency could lead to a limited protection against opportunistic bacteria involved in NEC development. In the second hypothesis, the secretion of antimicrobial peptides by PCs might be over-activated in the immature immune system leading to an overwhelming inflammatory response. This exaggerated inflammatory response could lead to increased intestinal damage, bacterial dysbiosis (the disruption of a healthy, functional microbiota) and reduced epithelial repair which in turn contributes to the development of NEC. The last hypothesis assumes a dysfunction of PCs by environmental stressors: dysfunction of PCs may be an early event that predisposes the preterm infant to NEC by inducing bacterial dysbiosis. Despite the possible role for PCs in the pathophysiology of NEC, little is known about PC maturation and functioning in the immature intestine and its subsequent relation with development of NEC.

With the suggested relation between PCs, located in the crypts of the intestinal villi, and the development of NEC, another point of debate comes up, i.e. the location
within the bowel wall where NEC starts to develop. Does intestinal ischemia start at the top of the villi, or on the contrary, in the crypts? The classic hypothesis states that intra-luminal bacteria disrupt and invade the mucosa at the tip of the intestinal villi, where they induce an inflammatory response resulting in NEC. This is further aggravated as the tips of the villi are the most distant parts and therefore most vulnerable for ischemia. However, the validity of this hypothesis needs to be questioned. McElroy, et al. hypothesized that NEC begins at the crypts of the intestine. The close location of the crypts to the lamina propria and submucosal arterioles suggest that bacterial invasion through the crypts is a more plausible explanation for an inflammatory response in the mucosa, compared to pathogens entering the intestine at the tip of the villi. The inflammatory response in the mucosa at the crypts activates the coagulation system in major blood vessels nearby resulting in ischemia of the intestine as seen in NEC. Other possible evidence, which might plead for the crypts as start point of NEC, includes: 1. the central role of PCs in crypt-related homeostasis, 2. the anatomic location of pneumatosis intestinalis close to the crypts, and 3. the proximity of PCs to occluded blood cells that cause ischemia. According to this hypothesis, PCs can therefore be considered as a key player in the development of NEC.

Goblet cells
Goblet cells are part of the innate immune system and produce and secrete mucins. Mucins coat the epithelial lining and provide the extrinsic barrier function and protect, on this manner, against bacterial invasion. While the production of mucus starts early during the development of the intestine, and the production reaches its ‘adult level’ by a gestation of 27 weeks, the mucus from preterm infants differs from that of term infants. Mucus produced in the immature intestinal tracts has a different viscosity, buoyancy, and carbohydrate composition than mucus produced by adults. If the ‘preterm mucus’ is less effective in providing an adequate extrinsic intestinal barrier compared to the mucus in term infants, it may help explain why preterm infants are more susceptible to NEC due to a diminished protection against pathogenic bacterial invasion.

The role of intestinal mucus in the pathophysiology of NEC is unknown. Recently a rat model of NEC showed decreased muc2-stained goblet cells in the intestine. In the complex role of goblet cells in NEC a couple of questions remain unanswered, namely 1. whether mucins are altered in infants who suffer from NEC, 2. whether this loss of mucins goes along with prematurity, and 3. whether this loss of mucins results from a decreased number and/or change of goblet cell function.
Hypoxic-ischemic mechanisms

Ischemia is an event involved in the pathophysiology of NEC which occurrence is proven by the pathological findings of NEC (necrosis of the intestine). Whether the hypoxic-ischemic insults incite the development of NEC or are a result of the local inflammatory response accompanying NEC is still a matter of debate. As mentioned before, preterm infants are more vulnerable for events causing hypoxia and intestinal ischemia (e.g. inappropriate oxygenation and hemodynamic instability). Preterm infants are also more vulnerable for intestinal ischemia because their system for regulating vascular resistance is immature. Vascular resistance is an important factor involved in the autoregulation of blood flow. Autoregulation 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. Nitric oxide (NO) and endothelin-1 (ET1) are important components of the vasculatory regulatory system contributing to autoregulation. NO causes vasodilatation, while ET1 causes vasoconstriction. The most explicit characteristic of the intestinal circulation of the preterm infant is the very low vascular resistance due to substantial generation of endothelial derived NO. Increased levels of NO in the preterm infant causes a defective splanchnic autoregulation in response to hypotension when the infants suffers from hemodynamic instability, causing hypoxia of the intestine. On top of that, the preterm infant is not able to adjust to the increased oxygen demands during hemodynamic instability, and hypoxia of the intestinal tissue can be a secondary event. 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. Also secondary factors, such as inflammatory mediators, can cause hypoxic-ischemic insults. In a neonatal rat model of NEC, which is currently the best accepted model, hypoxia-ischemia is one of the essential factors needed to generate NEC. In this model the authors suggested that a hypoxic-ischemic insult directly damages the intestinal barrier, causing bacterial inflammation. It still needs to be elucidated whether hypoxic-ischemic mechanisms in human infants have a primary inciting role in the development of NEC or occur merely secondary after inflammation.

NIRS

Impaired intestinal perfusion incited by hypoxic-ischemic events can be measured via near infrared spectroscopy (NIRS). NIRS is a non-invasive tool that can be used to continuously and non-invasively assess the cerebral and intestinal perfusion. NIRS uses light in the near-infrared range (wavelength between 700 and 1000nm), which can be effectively transmitted through biological tissue over longed
distances.\textsuperscript{84–95} Within the near-infrared range, the majority of the near-infrared light will be absorbed by oxygenated and deoxygenated hemoglobin, each of which have a distinct absorption spectrum.\textsuperscript{84–95} The residual of the light will be reflected or scattered. Via the use of NIRS we can measure the spectral absorption for oxygenated and deoxygenated hemoglobin separately.\textsuperscript{84–95} We are able to calculate the ratio of oxygenated hemoglobin to total hemoglobin. Via these NIRS measurements we are able to gain information on the regional tissue oxygen saturation (rSO\textsubscript{2}), which represents oxygen uptake in tissue. When at the same moment transcutaneous arterial oxygen saturation (SpO\textsubscript{2}) is measured, fractional tissue oxygen extraction (FTOE) can be calculated, with the following formula: FTOE = (SpO\textsubscript{2} - rSO\textsubscript{2}) / SpO\textsubscript{2}.\textsuperscript{90} FTOE reflects the balance between tissue oxygen supply and consumption.\textsuperscript{90} FTOE can be used to gain information on tissue perfusion and possible hypoxic-ischemic events.\textsuperscript{70} Splanchnic NIRS monitoring is of additive value for the prediction of the onset and development of NEC and its complications. Therefore, NIRS monitoring are part of the studies in this thesis. Since the technical aspects of NIRS are beyond the scope of this thesis, we refer to other articles discussing this issue more in detail.\textsuperscript{84–95}

**INVOS 5100C spectrometer**

We used the INVOS 5100C spectrometer (Covidien, Mansfield, MA, USA) with neonatal SomaSensors (Covidien) for our study on the intestinal perfusion during NEC. The SomaSensor has one light emitting diode that emits two wavelengths into underlying issue, i.e. 730 and 810 nm. A shallow and deep detector, at 30 and 40 mm distance from the emitter respectively, receive the light as a function of wavelength. The shallow detector provides information about surface tissue oxygen saturation and the deep detector information about the oxygen saturation of deeper tissues. The rSO\textsubscript{2} is calculated by subtracting the oxygen saturation of the surface path from the deeper path and represents the venous weighted oxygen saturation of tissues at a depth of approximately 20 mm.

**Bacterial colonization**

The intestine is colonized with around $10^{14}$ bacteria and characterized by a genomic content (microbiota), which represents more than 100 times the human genome.\textsuperscript{96} The intestinal microbiota can consists out of commensal, opportunistic and pathogenic bacteria. Commensal bacteria are bacteria which are beneficial for maintaining the intestinal barrier and do not trigger an immune response. Opportunistic bacteria include bacteria that require a systemic immunosuppression in order to establish an infection. Pathogenic bacteria require no systemic immunosuppression to establish an infection.
The intestinal tract is colonized with a variety of ingested environmental and maternal microbiota rapidly after birth. The microbiota plays a crucial role in protecting the infant from disease by acting as a barrier against pathogens, exerting metabolic functions and stimulating the development of the immune system. The immune system’s ability to coevolve with the microbiota during the perinatal period allows the host and the microbiota to coexist in a relationship of mutual benefit. In the preterm infant this relationship is still developing, and thus very vulnerable to many environmental disturbances, as presented in Figure 3. In the current thesis we focus on the relation between the intestinal microbiota and NEC.

**FIGURE 3:**
Most important environmental factors influencing the infants’ intestinal microbiota

The bacterial colonization process might start already in utero. Afterwards the intestinal microbiota undergoes rapid maturation during the first year after birth and is securely established in an adult form by three years of age. Within the first weeks after birth the bacterial colonization of the intestines is most important, because it effects the composition of the individuals’ future intestinal microbiota via a variety of factors. The microbiota is established via a complex interplay between a variety of environmental factors, the initial colonizing microbiota, genes, intestinal development, and diet. Importantly, for this thesis, an abnormal colonization of bacteria in the intestine during the first weeks after birth is linked with inflammatory intestinal diseases, such as NEC.

**Preterm- versus term microbiota**

In the term infant, *Enterobacteriaceae* (in particular *Eschierichia coli* and *Klebsiella* species) are the initial colonizers of the gut. These bacteria reduce the high redox
potential in the intestinal and allow other bacteria to colonize the gut.\textsuperscript{98} By introducing feeding to the infant bacteria, such as bifidobacteria and lactobacilli, will dominate colonization within the intestine. Preterm infants, especially very-low-birth-weight infants, have a different intestinal microbiota which is less favorable to maintain health compared to term infants.\textsuperscript{98} This altered microbiota might be associated with the development of NEC. Multiple factors contribute to the development of the intestinal microbiota besides intestinal immaturity, and include among others: preterm prolonged rupture of membranes, maternal infection, increased incidence of Cesarean delivery, perinatal and postnatal broad-spectrum antibiotic exposure, exposure to other intestinal-modifying medications such as H2-blockers, altered intestinal motility, periods of fasting, intensive care infection control standards and selection for resistant microbes, and decreased exposure to human milk (Figure 3).\textsuperscript{98–100} Given these factors the preterm infant’s intestinal microbiota has a reduced microbial diversity with an increase in colonization with opportunistic and/or pathogenic organisms compared to term infants.\textsuperscript{98,101} Arboleya et al.\textsuperscript{102} demonstrated that when compared with full-term infants, preterm infants showed increased populations of facultative anaerobes such as enterococci and \textit{Enterobacteriaceae}, increased numbers of staphylococci, and decreased numbers of anaerobes like bifidobacteria, \textit{Bacteroides}, and \textit{Atopobium}. Butel et al.\textsuperscript{103} suggest that there might be GA threshold for colonization with certain microbes. For example, 33 weeks of gestation appears to be the milestone for appearance of bifidobacteria species, the organism most commonly implicated in the development and maintenance of a healthy intestinal microbiota.\textsuperscript{103} Why these thresholds appear, the mechanisms of these thresholds, and the possible relation with NEC, still need to be revealed.

\textbf{Mother-child symbiosis}

The hypothesis supposes that during the uterine life, the fetus develops in a sterile environment.\textsuperscript{96} The presence of bacteria in the amniotic fluid, when revealed, causes amnionitis, funisitis, and chorioamnionitis. Such infections are associated with a preterm delivery.\textsuperscript{96,104} Within days after birth the intestine is colonized by bacteria mainly maternal derived maternally, but also from the external environment.\textsuperscript{96} Mode of delivery is an important event that influences the intestinal microbiota of the newborn. Infants born via vaginal delivery are colonized with bacteria derived from the maternal vaginal flora, such as lactobacillus and \textit{Prevotella} species.\textsuperscript{98} Infants born via Cesarean delivery are colonized by epidermal rather than vaginal species, such as \textit{Clostridium spp}, staphylococci, \textit{Propionibacterium}, and \textit{Cornynebacterium} and they have a deficiency of anaerobes with lower numbers of \textit{Bacteroides} and bifidobacteria when compared to vaginally born infants.\textsuperscript{98} Multiple studies stated a possible relationship between an altered bacterial colonization by mode of delivery and the development of NEC.\textsuperscript{96,98,105} While we did not investigate the
Influence of nutrition on the intestinal microbiota
Nutrition, via breast milk and/or formula feeding, during the early life of the newborns influences the composition of the intestinal microbiota. Nutrition can alter the composition of the intestinal microbiota, and could therefore be related to NEC development. Oligosaccharides, glycoconjugates, and natural components of breast milk stimulate the growth of bifidobacteria. Bacteria are also observed in breast milk, including staphylococcus, streptococcus, bifidobacteria and lactobacilli. In infants who are breast-fed, transmission of sIgA from the mother is reflective of her own microbiota providing protection against pathogens that could lead to dysbiosis (the disruption of a healthy, functional microbiota). Conversely, formula-fed infants are exposed to a different array of carbohydrates, bacteria and nutrients, which results in different colonization patterns. The intestinal microbiota in formula-fed infants is colonized with bacteria, including Escherichia coli, Clostridium difficile, Bacteroides, Prevotella and lactobacilli. Interestingly, even relatively small amounts of formula supplementation of breast-fed infants will result in a shift from a breast-fed to a formula-fed pattern.

Intestinal microbiota associated with NEC
The linkage of NEC to bacterial colonization was recognized by Santulli et al. over three decades ago. Additional observations showing case clustering, outbreaks in institutions, the finding of pneumatosis intestinalis, and the common finding of bacteremia and endotoxinemia in affected infants, all support a microbial role in the pathogenesis of NEC. While we know that prematurity, mode of delivery, and nutrition are associated to an altered microbiota, the details of the relationship between an altered microbiota and NEC pathogenesis remain poorly understood. Studies have suggested that a decreased bacterial diversity and the presence of microorganisms, such as clostridia, Klebsiella pneumonia, and E. coli, increases the risk for NEC development. The results of these studies vary and only few analyzed the microbiota during the whole interval between birth and NEC development. An abnormal colonization after birth is hypothesized to be associated with an increased risk of the development of NEC. NEC will develop when additional insults or vulnerabilities will occur, including later exposures of pathogens or oxidative stress. As we did in the current thesis, further exploration of the alterations in the intestinal microbiota of infants at risk for NEC should be considered as an important research priority in order to gain insight in the role of microbiota within the pathophysiology of NEC and to develop new diagnostic and preventive tools.
Probiotics, live microorganisms supplements, possibly promotes the acquisition of a ‘healthy’ intestinal microbiota in the preterm neonatal population. Probiotics might be valuable in the prevention of NEC and its associated morbidity by prevention of (pathogenic) bacterial invasion, prevention against dysbiotic conditions, and by enhancing the immune responses of the host. Probiotics result in an enhanced epithelial barrier function, direct antagonism against pathogens, enhanced mucosal IgA responses, prevention of apoptosis of cells, production of anti-inflammatory cytokines, and down-regulation of pro-inflammatory pathways. Prospective randomized trials during the past decade have evaluated the effects of various probiotics to prevent NEC. The most recently reported multicenter trial of probiotics suggested that the probiotic approach decreased the incidence of NEC, but did not decrease mortality from NEC. The Cochrane review by Alfaleh, et al. concluded that supplementation with probiotics reduced the risk of severe NEC and lowered the mortality in infants with a birth weight >1000 grams. This Cochrane review concluded that there were no available studies concerning on the use of probiotics in the population infants born with a birth weight <1000 grams, and therefore, a reliable estimation of the safety and effectiveness of the use of probiotics cannot be made in this population. To investigate the use of probiotics and its (beneficial- and side) effects a large multicenter randomized controlled trial is needed.

**Microbiota analysis via 16S rRNA sequencing**

Studies describing microbial composition in infants with NEC have rapidly expanded in the last decade. Advances in 16S rRNA based sequencing technologies nowadays allow for a rapid and detailed analysis of the bacterial composition of feces, the so-called microbiota. This includes the accurate measurement of unculturable bacterial species. Raveh-Sadka et al. clearly demonstrated the opportunities of the rapidly evolving sequencing technologies as a tool for research on the bacterial involvement in NEC. The majority of the studies on intestinal microbiota composition reported disease-specific abnormalities as compared with controls. 16S rRNA sequencing can be considered as a potential future predictive/diagnostic tool.

**Aims of the thesis**

Both preventive and treatment options in NEC are limited due to the incomplete understanding of the underlying multifactorial pathophysiology of NEC. Therefore, the overall aim of this thesis is to enlarge the present state of knowledge on the pathophysiology of NEC by focusing on three major contributing components: the intestinal barrier function, intestinal perfusion, and the intestinal microbiota.
In chapter 2 we retrospectively analyzed the changes in incidence, clinical presentation and mortality of NEC during the last nine years in three academic referral centers in the Netherlands. We hypothesized that the incidence and mortality had not changed during the last decade, making NEC an important research priority.

In section 2 and 3 of this thesis we zoomed in on the pathophysiology of NEC. In section 2 we investigated intestinal barrier integrity and the circulation. In chapter 3 we focused on the relation between PCs and NEC. Because knowledge is limited about the development of PCs in the human gut we retrospectively determined when PCs arise and when they become immune competent in the developing human gut. In chapter 4 and 5 we studied epithelial damage and the role of an impaired perfusion contributing to the pathophysiology of NEC. In chapter 4, we prospectively determined whether cerebral and splanchnic FTOE values are related to mucosal damage in NEC. We also investigated whether cerebral and splanchnic FTOE values together with I-FABP levels give insight in the pathological cascade of uncomplicated and complicated NEC cases. In chapter 5 we prospectively studied whether the extent of mucosal damage, measured via I-FABP levels, correlated with the extent of necrotic bowel.

Section 3 describes studies concerning bacterial colonization and NEC. In chapter 6, 7 and 8 we focus on bacterial involvement in NEC. In chapter 6 we retrospectively investigated whether bloodstream infections predisposed to NEC development (e.g. by activating the pro-inflammatory response) or resulted from the loss of intestinal barrier integrity during NEC development.

In chapter 7 we concentrate on the intestinal microbiota in infants with a high risk for NEC development. Previous studies have suggested that an abnormal intestinal microbiota increases the risk for NEC development. Therefore, we determined in chapter 7 the diversity and the composition of the intestinal microbiota in preterm infants at risk for NEC and its relation to NEC development in a prospective study. In the same chapter we investigated possible associations between maternal- and/or neonatal factors and the intestinal microbiota, which could lead to NEC development.

In chapter 8 we focused on the intestinal microbiota during NEC. There is limited information on the identity and abundance of bacteria in the intestine during complicated NEC. Therefore, we investigated in chapter 8 retrospectively the bacterial invasion of the intestinal wall in surgical (complicated) NEC cases.

We discuss the main findings and the hypotheses of the pathophysiology of NEC in a general discussion presented in chapter 9. In the same chapter we offer implications for clinical practice and directions for future research. A summary of the main findings and conclusions of this thesis are given in chapter 10 and 11.
References


