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The pathophysiology of necrotizing enterocolitis in preterm infants

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INTRODUCTION- TION AND AIMS OF THE THESIS

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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.⁴ 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.^{3,4,15} Common laboratory findings are leukocytosis, thrombocytopenia, metabolic acidosis, and increased C-reactive protein levels.^{4,15} 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.^{4,16} 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.³

Several staging systems have been developed to help and guide clinicians in diagnosing and treating NEC.¹⁷⁻¹⁹ In our NICU, the modified Bell's staging system is used,¹⁸ 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.³ These patients may only have abdominal distention, without intraluminal bowel gas, on presentation.²⁰ Therefore, the ominous progression of the disease may be missed, with a failure to intervene early enough.³ 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.

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

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

Modified from Walsh MC, Kliegman RN. Necrotizing enterocolitis: treatment based on staging criteria. Pediatr Clin North Am 1986; 33:179–201.¹⁸

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.²¹ Symptomatic treatment of the infant with NEC begins with prompt recognition of the symptoms and medical stabilization.²² Medical interventions typically include abdominal decompression, bowel rest, and broad-spectrum intravenous antibiotics.³ 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.^{3,23} The assessment of intestinal necrosis and the timing of surgery, especially in the absence of perforation, remains a key problem in NEC.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.^{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.⁴ Mortality is inversely related to GA.^{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.^{25,26} Subacute complications include intestinal strictures, recurrent NEC, dysmotility of the bowel, malabsorption, short bowel syndrome, and cholestatic liver disease.²⁷⁻³⁰ Severe NEC is associated with growth delay that can persist beyond infancy into childhood and with poor neurodevelopmental outcomes.^{25,31,32}

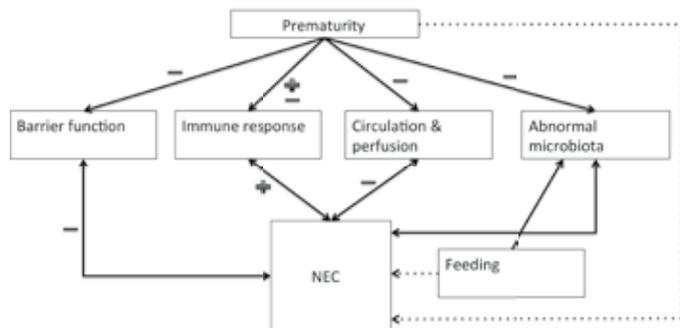
Pathophysiology

Current evidence supports a complex, multifactorial model of NEC (Figure 1).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.^{3,33} 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.^{4,33} Therefore, these events may predispose preterm infants to intestinal mucosal injury.^{4,33} 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.³³ 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.

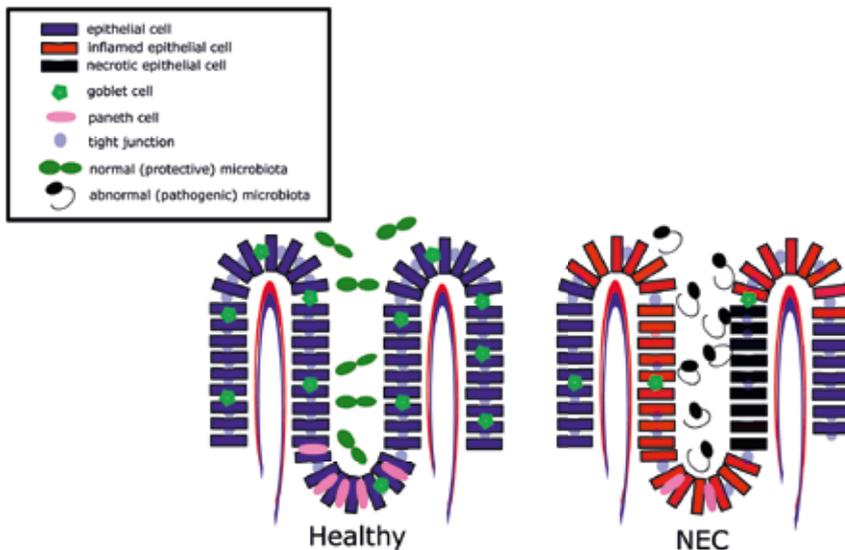


FIGURE 2:

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.³⁴

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.³⁵ Preterm infants have an increased intestinal permeability, perhaps to allow passage of important macromolecules from amniotic fluid or breast milk.³⁶ However, this same increased permeability can lead to an increased bacterial translocation.³⁴ Maintenance of an intact intestinal epithelium is crucial to maintain intestinal barrier integrity.³⁷ 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.^{34,37,38} The inflammatory response of the intestinal barrier can be triggered by either commensal, opportunistic, or pathogenic bacteria.³⁹

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).³⁵ 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.^{35,40} 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.⁴¹ 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.⁴¹ The upstream mechanisms that initiate intestinal differentiation remain largely unknown, but TLR expression is discovered to be important.^{34,35} The expression of TLR4 in the intestinal lining increases during embryonic development and decreases significantly after (term) birth.³⁵ 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).^{33,42} IL-8 can increase the production of acute-phase proteins in the intestine as seen in NEC and finally induce ischemia and necrosis.⁴³⁻⁴⁵

The intestinal barrier includes two components: the intrinsic barrier and the extrinsic barrier.⁴⁶ 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).^{38,46} The extrinsic barrier includes mucus that coats the epithelial lining.⁴⁶ 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.³⁸ The primary function of enterocytes is to absorb nutrients apically and export them basally.³⁸ 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.⁴⁷ As current evidence supports, enterocyte damage is not primarily causing NEC, but is part of a vicious cycle of inflammation-inflicted epithelial damage.⁴⁸ Pathogenic bacteria can interact with specific apical surface receptors on the enterocytes.⁴⁹ 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.⁴⁹

Intestinal fatty acid-binding protein (I-FABP), a marker for enterocyte damage, might be a possible biomarker for NEC.^{24,50-52} When enterocytes are damaged, I-FABPs are released from the enterocytes.⁵⁰⁻⁵² I-FABP is a small cytoplasmic protein with high organ sensitivity found in the enterocytes located at the tip of the villi.⁵⁰ 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.⁵⁰⁻⁵² 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.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.⁵⁶ TJs thereby contribute significantly to the defense against microbes.⁵³⁻⁵⁵

TJs develop early in the second trimester of pregnancy.^{56,57} They mature during the remainder of pregnancy. During this maturation the TJ proteins get more embedded in the epithelium.^{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).^{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.^{50,53} Yang et al.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.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.⁶¹ Thuijls et al.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.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.^{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.^{56,60,62,63}

Immune cells contributing to the intestinal barrier

The extrinsic barrier includes post-mitotic 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^{38,64} PCs and goblet cells both are related to the pathogenesis of NEC.^{34,37,38} 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.⁶⁵ PCs secrete (among others) human α -defensins (HD5/HD6).¹⁶ These defensins show protective activity against bacterial agents, and are thought to be associated with initiating and adapting immunity.¹⁵ As such PCs can be considered the “guardians of the crypt.”⁶⁵ 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.^{1,16,66,67} 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.^{8,65,68,69} 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.⁶⁸⁻⁷⁰ In the first hypothesis, it is suggested that there is a relative deficiency of (immune competent) PCs in the immature intestine.^{65,71} This deficiency could lead to a limited protection against opportunistic bacteria involved in NEC development.^{65,71} 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.⁷²⁻⁷⁴ 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.⁷²⁻⁷⁴ 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.^{72,75,76} 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.⁶⁵ 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.⁶⁵ 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.⁶⁵ 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.⁶⁵ 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.⁶⁵ Mucins coat the epithelial lining and provide the extrinsic barrier function and protect, on this manner, against bacterial invasion.⁶⁵ 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.² Mucus produced in the immature intestinal tracts has a different viscosity, buoyancy, and carbohydrate composition than mucus produced by adults.⁷⁷ 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.^{2,51}

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.⁷⁸ 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.²

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).²²

Preterm infants are also more vulnerable for intestinal ischemia because their system for regulating vascular resistance is immature.²² 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.²² NO causes vasodilatation, while ET1 causes vasoconstriction.²²

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.^{4,79} Increased levels of NO in the preterm infant causes a defective splanchnic autoregulation in response to hypotension when the infant suffers from hemodynamic instability, causing hypoxia of the intestine.²² 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.²² Also secondary factors, such as inflammatory mediators, can cause hypoxic-ischemic insults.^{4,80} 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.⁸¹ 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.^{81,82}

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.⁸³⁻⁸⁹ NIRS uses light in the near-infrared range (wavelength between 700 and 1000nm), which can be effectively transmitted through biological tissue over longed

distances.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.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.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 (rSO₂), which represents oxygen uptake in tissue. When at the same moment transcutaneous arterial oxygen saturation (SpO₂) is measured, fractional tissue oxygen extraction (FTOE) can be calculated, with the following formula: $FTOE = (SpO_2 - rSO_2) / SpO_2$.⁹⁰ FTOE reflects the balance between tissue oxygen supply and consumption.⁹⁰ FTOE can be used to gain information on tissue perfusion and possible hypoxic-ischemic events.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.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 tissue, 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 rSO₂ 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

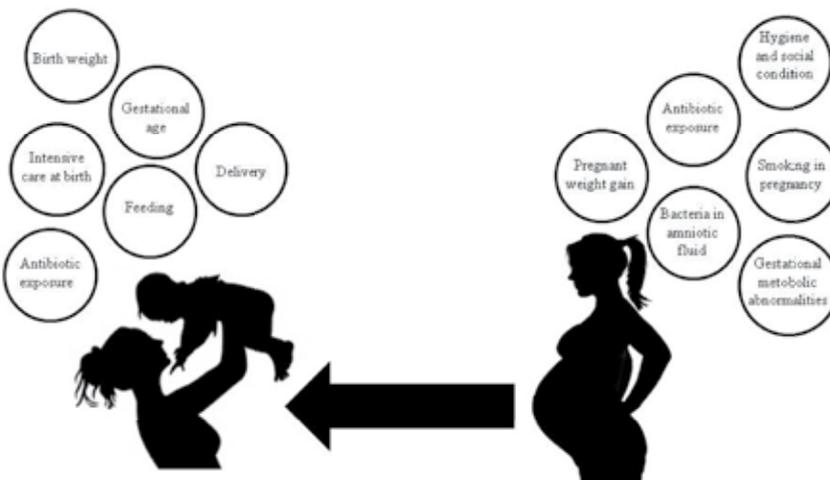
The intestine is colonized with around 10¹⁴ bacteria and characterized by a genomic content (microbiota), which represents more than 100 times the human genome.⁹⁶ The intestinal microbiota can consist out of commensal, opportunistic and pathogenic bacteria. Commensal bacteria are bacteria which are beneficial 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.^{80,96,97} The microbiota plays an 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.^{80,97} 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.^{80,97} In the preterm infant this relationship is still developing, and thus very vulnerable to many environmental disturbances, as presented in Figure 3.⁹⁶ 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 *Escherichia coli* and *Klebsiella* species) are the initial colonizers of the gut.⁹⁸ These bacteria reduce the high redox

potential in the intestinal and allow other bacteria to colonize the gut.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.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).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.^{98,101} Arboleya et al.¹⁰² demonstrated that when compared with full-term infants, preterm infants showed increased populations of facultative anaerobes such as enterococci and *Enterobacteriaceae*, increased numbers of staphylococci, and decreased numbers of anaerobes like bifidobacteria, *Bacteroides*, and *Atopobium*. Butel et al.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.¹⁰³ Why these thresholds appear, the mechanisms of these thresholds, and the possible relation with NEC, still need to be revealed.

Mother-child symbiosis

The hypothesis supposes that during the uterine life, the fetus develops in a sterile environment.⁹⁶ The presence of bacteria in the amniotic fluid, when revealed, causes amnionitis, funisitis, and chorioamnionitis. Such infections are associated with a preterm delivery.^{96,104} Within days after birth the intestine is colonized by bacteria mainly maternal derived maternally, but also from the external environment.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 *Prevotella* species.⁹⁸ Infants born via Cesarean delivery are colonized by epidermal rather than vaginal species, such as *Clostridium spp*, staphylococci, *Propionibacterium*, and *Corynebacterium* and they have a deficiency of anaerobes with lower numbers of *Bacteroides* and bifidobacteria when compared to vaginally born infants.⁹⁸ Multiple studies stated a possible relationship between an altered bacterial colonization by mode of delivery and the development of NEC.^{96,98,105} While we did not investigate the

mother's intestinal microbiota in this thesis, we did investigate if there are relations between maternal factors (such as (chorio)amnionitis, preterm premature rupture of membrane and mode of delivery) and the intestinal microbiota of the preterm infant.

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.⁹⁸ 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.^{96,98,106} Bacteria are also observed in breast milk, including staphylococcus, streptococcus, bifidobacteria and lactobacilli.¹⁰⁷ 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).⁹⁸ Conversely, formula-fed infants are exposed to a different array of carbohydrates, bacteria and nutrients, which results in different colonization patterns.⁹⁸ The intestinal microbiota in formula-fed infants is colonized with bacteria, including *Escherichia coli*, *Clostridium difficile*, *Bacteroides*, *Prevotella* and lactobacilli.¹⁰⁸⁻¹¹¹ 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.¹¹²

Intestinal microbiota associated with NEC

The linkage of NEC to bacterial colonization was recognized by Santulli et al.¹¹³ over three decades ago. Additional observations showing case clustering, outbreaks in institutions, the finding of pneumatosis intestinalis, and the common finding of bacteremia and endotoxemia in affected infants, all support a microbial role in the pathogenesis of NEC.⁹⁷ 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.^{96,114} Studies have suggested that a decreased bacterial diversity and the presence of microorganisms, such as clostridia, *Klebsiella pneumoniae*, and *E. coli*, increases the risk for NEC development.¹¹⁴⁻¹¹⁸ 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.¹¹⁷ NEC will develop when additional insults or vulnerabilities will occur, including later exposures of pathogens or oxidative stress.¹¹⁷ 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.^{98,119} 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.^{98,119} 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.¹²⁰⁻¹²² Prospective randomized trials during the past decade have evaluated the effects of various probiotics to prevent NEC.^{123,124} 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.³ The Cochrane review by Alfaleh, et al.¹¹⁹ 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.¹¹⁹ 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.¹²⁵ 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.^{57,65,126} Raveh-Sadka et al.¹²⁷ 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.¹²⁵ 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.¹¹⁴⁻¹¹⁸ 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

1. Underwood MA. Paneth cells and necrotizing enterocolitis. *Gut Microbes* **3**, 562–565 (2012).
2. McElroy SJ, Prince LS, Weitkamp JH, Reese J, Slaughter JC, Polk DB. Tumor necrosis factor receptor 1-dependent depletion of mucus in immature small intestine: a potential role in neonatal necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol* **301**, 656–666 (2011).
3. Neu J & Walker WA. Necrotizing enterocolitis. *N Eng J Med* **364**, 255–264 (2011).
4. Sharma R & Hudak MLA. Clinical Perspective of Necrotizing Enterocolitis: Past, Present, and Future. *Clin Perinatol* **40**, 27–51 (2013).
5. Obladen M. Necrotizing enterocolitis - 150 years of fruitless search for the cause. *Neonatology* **96**, 203–210 (2009).
6. Gordon P, Christensen R, Weitkamp JH, Maheshwari A. Mapping the new world of necrotizing enterocolitis (NEC): review and opinion. *EJ Neonatal Res* **2**, 145–172 (2012).
7. Kim JH. Necrotizing enterocolitis: The road to zero. *Sem Fetal Neonatal Med* **19**, 39–44 (2014).
8. Yee WH, Soraisham AS, Shah VS, *et al.* Incidence and timing of presentation of necrotizing enterocolitis in preterm Infants. *Pediatrics* **129**, e298–e304 (2012).
9. Meinen-Derr J, Morrow AL, Hornung RW, Donovan EF, Dietrich KN, Succop PA. Epidemiology of necrotizing enterocolitis temporal clustering in two neonatology practices. *J Pediatr* **154**, 656–661 (2009).
10. Neu J. Necrotizing enterocolitis: the mystery goes on. *Neonatology* **106**, 289–295 (2014).
11. Guillet R, Stoll BJ, Cotten CM, *et al.* Association of H2-blocker therapy and higher incidence of necrotizing enterocolitis in very low birth weight infants. *Pediatrics* **117**, e137–e142 (2006).
12. Fitzgibbons SC, Ching Y, Yu D, *et al.* Mortality of necrotizing enterocolitis expressed by birth weight categories. *J Pediatr Surg* **44**, 1072–1076 (2009).
13. Stoll BJ, Hansen NI, Bell EF, *et al.* Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics* **126**, 443–456 (2010).
14. Gephart SM, McGrath JM, Effken JA, Halpern MD. Necrotizing enterocolitis risk: state of the science. *Adv Neonatal Care* **12**, 77–87 (2012).
15. Dominguez KM & Moss RL. Necrotizing enterocolitis. *Clin Perinatol* **39**, 387–401 (2012).
16. Salzman NH, Underwood MA, Bevins CL. Paneth cells, defensins, and the commensal microbiota: A hypothesis on intimate interplay at the intestinal mucosa. *Sem Immunol* **19**, 70–83 (2007).

17. Bell MJ, Ternberg JL, Feigin RD, *et al.* Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging. *Ann Surg* **187**, 1–7 (1978).
18. Walsh MC & Kliegman RM. Necrotizing enterocolitis: treatment based on staging criteria. *Pediatr Clin North Am* **33**, 179–201 (1986).
19. Gordon PV, Swanson JR, Attridge JT, Clark R. Emerging trends in acquired neonatal intestinal disease: is it time to abandon Bell's criteria? *J Perinatol* **27**, 661–671 (2007).
20. Epelman M, Daneman A, Navarro OM, *et al.* Necrotizing enterocolitis: review of state-of-the-art imaging findings with pathologic correlation. *Radiographics* **27**, 285–305 (2007).
21. Lin PW, Nasr TR, Stoll BJ. Necrotizing enterocolitis: recent scientific advances in pathophysiology and prevention. *Sem Perinatol* **32**, 70–82 (2008).
22. Schnabl KL, Van Aerde JE, Thomson AB, Clandinin MT. Necrotizing enterocolitis: a multifactorial disease with no cure. *World J Gastroenterol* **14**, 2142–2161 (2008).
23. Reisinger KW, Kramer BW, van der Zee DC, *et al.* Non-invasive serum amyloid A (SAA) measurement and plasma platelets for accurate prediction of surgical intervention in severe necrotizing enterocolitis (NEC). *PLoS One* **9**, (2014).
24. Heida FH, Hulscher JB, Schurink M, *et al.* Intestinal fatty acid-binding protein levels in Necrotizing enterocolitis correlate with extent of necrotic bowel: results from a multicenter study. *J Pediatr Surg* **50**, 1115–1118 (2015).
25. Kasivajjula H & Maheshwari A. Pathophysiology and current management of necrotizing enterocolitis. *Indian J Pediatr* **81**, 489–497 (2014).
26. Henry MCW & Moss RL. Necrotizing enterocolitis. *Annu Rev Med* **60**, 111–124 (2009).
27. Amin SC, Pappas C, Iyengar H, Maheshwari A. Short Bowel Syndrome in the NICU. *Clin Perinatol* **40**, 53–68 (2013).
28. Kastenbergh, ZJ. & Sylvester, KG. The surgical management of necrotizing enterocolitis. *Clin Perinatol* **40**, 135–148 (2013).
29. Heida FH, Loos MHJ, Stolwijk L, *et al.* Risk factors associated with post-necrotizing enterocolitis strictures in infants. *J Pediatr Surg*. (2015) doi:10.1016/j.jpedsurg.2015.09.015
30. Lin PW & Stoll BJ. Necrotising enterocolitis. *Lancet* **368**, 1271–1283 (2006).
31. Hintz SR, Kendrick DE, Stoll BJ, *et al.* Neurodevelopmental and growth outcomes of extremely low birth weight infants after necrotizing enterocolitis. *Pediatrics* **115**, 696–703 (2005).
32. Pike K, Brocklehurst P, Jones D, *et al.* Outcomes at 7y for babies who developed neonatal necrotizing enterocolitis: the ORACLE children study. *Arch Dis Child Fetal Neonatal Ed* **97**, 318–322 (2012).

33. Lim JC, Golden JM, Ford HR. Pathogenesis of neonatal necrotizing enterocolitis. *Pediatr Surg Int* **31**, 509–518 (2015).
34. Claud EC. Neonatal necrotizing enterocolitis - inflammation and intestinal immaturity. *Antiinflamm Antiallergy Agents Med Chem* **8**, 248–259 (2009).
35. Sodhi CP, Neal MD, Siggers R, *et al.* Intestinal epithelial Toll-like receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in mice. *Gastroenterology* **143**, 708–18.e1–5 (2012).
36. Rouwet EV, Heineman E, Buurman WA, *et al.* Intestinal permeability and carrier-mediated monosaccharide absorption in preterm neonates during the early postnatal period. *Pediatr Res* **51**, 64–70 (2002).
37. Huang XZ, Zhu LB, Li ZR, Lin J. Bacterial colonization and intestinal mucosal barrier development. *World J Clin Pediatr* **2**, 46–53 (2013).
38. Noah TK, Donahue B, Shroyer NF. Intestinal development and differentiation. *Exp Cell Res* **317**, 2702–2710 (2011).
39. Claud EC, Lu L, Anton PM, Savidge T, Walker WA, Cherayil BJ. Developmentally regulated IkappaB expression in intestinal epithelium and susceptibility to flagellin-induced inflammation. *Proc Natl Acad Sci USA* **101**, 7404–7408 (2004).
40. Sodhi CP, Shi XH, Richardson WM, *et al.* Toll-like receptor-4 inhibits enterocyte proliferation via impaired beta-catenin signaling in necrotizing enterocolitis. *Gastroenterology* **138**, 185–196 (2010).
41. Kandasamy J, Huda S, Ambalavanan N, Jilling T. Inflammatory signals that regulate the intestinal epithelial renewal, differentiation, migration and cell death: implications for necrotizing enterocolitis. *Pathophysiology* **21**, 67–80 (2014).
42. Akira S & Sato S. Toll-like receptors and their signaling mechanisms. *Scand J Infect Dis* **35**, 555–562 (2003).
43. Benkoe T, Baumann S, Weninger M, *et al.* Comprehensive evaluation of 11 cytokines in premature Infants with surgical necrotizing enterocolitis. *PLoS One* **8**, (2013).
44. Benkoe T, Mechtler TP, Weninger M, *et al.* Serum levels of interleukin-8 and gut-associated biomarkers in diagnosing necrotizing enterocolitis in preterm infants. *J Pediatr Surg* **49**, 1446–1451 (2014).
45. Mukaida N. Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation. *Int J Hematol* **72**, 391–398 (2000).
46. Brandtzaeg P. Gate-keeper function of the intestinal epithelium. *Benef Microbes* **4**, 67–82 (2013).
47. Sellin ME, Müller AA, Felmy B, *et al.* Epithelium-intrinsic NAIP/NLRC4 inflammasome drives infected enterocyte expulsion to restrict Salmonella replication in the intestinal mucosa. *Cell Host Microbe* **16**, 237–248 (2014).

48. Grishin A, Bowling J, Bell B, Wang J, Ford HR. Roles of nitric oxide and intestinal microbiota in the pathogenesis of necrotizing enterocolitis. *J Pediatr Surg* **51**. (2015). doi: 10.1016/j.jpedsurg.2015.10.006.
49. Neu J, Mihatsch WA, Zegarra J, Supapannachart S, Ding ZY, Murguia-Peniche T. Intestinal mucosal defense system, Part 1. Consensus recommendations for immunonutrients. *J Pediatr* **162**, S56–63 (2013).
50. Thuijls, G, Derikx JP, de Haan JJ, *et al*. Urine-based detection of intestinal tight junction loss. *J Clin Gastroenterol* **44**, e14–e19 (2010).
51. Schurink M, Scholen IG, Kooi EM, *et al*. Intestinal fatty acid-binding protein in neonates with imminent necrotizing enterocolitis. *Neonatology* **106**, 49–54 (2014).
52. Schurink M, Kooi EM, Hulzebos CV, *et al*. Intestinal fatty acid-binding protein as a diagnostic marker for complicated and uncomplicated necrotizing enterocolitis: a prospective cohort study. *PLoS One* **10**, e0121336 (2015).
53. Caricilli AM, Castoldi A, Câmara NOS. Intestinal barrier: A gentlemen's agreement between microbiota and immunity. *World J Gastrointest Pathophysiol* **5**, 18–32 (2014).
54. Högberg N, Stenbäck A, Carlsson PO, Wanders A, Lilja HE. Genes regulating tight junctions and cell adhesion are altered in early experimental necrotizing enterocolitis. *J Pediatr Surg* **48**, 2308–2312 (2013).
55. Schneeberger EE & Lynch RD. The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol* **286**, C1213–C1228 (2004).
56. Jakaitis BM & Denning PW. Commensal and probiotic bacteria may prevent NEC by maturing intestinal host defenses. *Pathophysiology* **21**, 47–54 (2014).
57. van Elburg RM, Fetter WPF, Bunkers CM, Heymans HSA. Intestinal permeability in relation to birth weight and gestational and postnatal age. *Arch Dis Child Fetal Neonatal Ed* **88**, F52–F55 (2003).
58. Anderson JM & Van Itallie CM. Physiology and function of the tight junction. *Cold Spring Harb Perspect Biol* **1**, 1–16 (2009).
59. Nusrat A, Turner JR, Madara JL. Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. *Am J Physiol Gastrointest Liver Physiol* **279**, G851–G857 (2000).
60. Yang F, Wang A, Zeng X, Hou C, Liu H, Qiao S. *Lactobacillus reuteri* I5007 modulates tight junction protein expression in IPEC-J2 cells with LPS stimulation and in newborn piglets under normal conditions. *BMC Microbiol* **15**, (2015).
61. Halpern MD, Denning PW. The role of intestinal epithelial barrier function in the development of NEC. *Tissue Barriers* **3**, e1000707 (2015).

62. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* **118**, 229–241 (2004).
63. Hooper LV, Wong MH, Theijn A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. *Science* **291**, 881–884 (2001).
64. Kazanjian A, Noah T, Brown D, Burkart J, Shroyer NF. Atonal homolog 1 is required for growth and differentiation effects of notch/gamma-secretase inhibitors on normal and cancerous intestinal epithelial cells. *Gastroenterology* **139**, 918–928 (2010).
65. McElroy SJ, Underwood MA, Sherman MP. Paneth cells and necrotizing enterocolitis: A novel hypothesis for disease pathogenesis. *Neonatology* **103**, 10–20 (2012).
66. Huff DS. in *Color atlas of fetal and neonatal histology* (ed. Ernst LM, Ruchelli ED, H. D.) 39–63 (Springer, London, UK, 2011).
67. Mallow EB, Harris A, Salzman N, *et al.* Human enteric defensins: Gene structure and developmental expression. *J Biol Chem* **271**, 4038–4045 (1996).
68. González-Rivera R, Culverhouse RC, Hamvas A, Tarr PI, Warner BB. The age of necrotizing enterocolitis onset: an application of Sartwell’s incubation period model. *J Perinatol* **31**, 519–523 (2011).
69. Clevers HC & Bevins CL. Paneth cells: maestros of the small intestinal crypts. *Annu Rev Physiol* **75**, 289–311 (2013).
70. Sherman MP, Bennett SH, Hwang FFY, Sherman J, Bevins CL. Paneth cells and antibacterial host defense in neonatal small intestine. *Infect Immun* **73**, 6143–6146 (2005).
71. Arciero J, Ermentrout GB, Siggers R, *et al.* Modeling the interactions of bacteria and Toll-like receptor-mediated inflammation in necrotizing enterocolitis. *J Theor Biol* **321**, 83–99 (2012).
72. Gregory KE, Deforge CE, Natale KM, Phillips M, van Marter LJ. Necrotizing enterocolitis in the premature infant: neonatal nursing assessment, disease pathogenesis, and clinical presentation. *Adv Neonatal Care* **11**, 155–164 (2011).
73. Hackam DJ, Good M, Sodhi CP. Mechanisms of gut barrier failure in the pathogenesis of necrotizing enterocolitis: Toll-like receptors throw the switch. *Semin Pediatr Surg.* **22**, 76–82 (2013).
74. Bevins CL & Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. *Nat Rev Microbiol* **9**, 356–368 (2011).

75. Heneghan AF, Pierre JF, Tandee K, *et al.* Parenteral nutrition decreases paneth cell function and intestinal bactericidal activity while increasing susceptibility to bacterial enteroinvasion. *JPEN J Parenter Enteral Nutr* **Epub**, 1–9 (2013).
76. Shen B, Porter EM, Reynoso E, *et al.* Human defensin 5 expression in intestinal metaplasia of the upper gastrointestinal tract. *J Clin Pathol* **58**, 687–694 (2005).
77. Shub MD, Pang KY, Swann DA, Walker WA. Age-related changes in chemical composition and physical properties of mucus glycoproteins from rat small intestine. *Biochem J* **215**, 405–411 (1983).
78. Clark J, Doelle SM, Halpern MD, *et al.* Intestinal barrier failure during experimental necrotizing enterocolitis: protective effect of EGF treatment. *Am J Physiol Gastrointest Liver Physiol* **291**, G938–G949 (2006).
79. Nowicki PT, Caniano DA, Hammond S, *et al.* Endothelial nitric oxide synthase in human intestine resected for necrotizing enterocolitis. *J Pediatr* **150**, 40–45 (2007).
80. Stanghellini V, Barbara G, Cremon C, *et al.* Gut microbiota and related diseases: Clinical features. *Intern Emerg Med* **5**, 57–63 (2010).
81. Barlow BST. Importance of multiple episodes of hypoxia or cold stress on the development of enterocolitis in an animal model. *Surgery* **77**, 687–690 (1975).
82. Caplan MS, Hedlund E, Adler L, Hsueh W. Role of asphyxia and feeding in a neonatal rat model of necrotizing enterocolitis. *Pediatr Pathol* **14**, 1017–1028 (1994).
83. Watkins DJ & Besner GE. The role of the intestinal microcirculation in necrotizing enterocolitis. *Semin Pediatr Surg* **22**, 83–87 (2013).
84. Cortez J, Gupta M, Amaram A, Pizzino J, Sawhney M, Sood BG. Noninvasive evaluation of splanchnic tissue oxygenation using near-infrared spectroscopy in preterm neonates. *J Matern Fetal Neonatal Med* **24**, 574–582 (2011).
85. Gay AN, Lazar DA, Stoll B, *et al.* Near-infrared spectroscopy measurements are lower in preterm infants at risk for necrotizing enterocolitis. *Pediatr Crit Care Med* **15**, 735–741 (2014).
86. Gillam-Krakauer M, Cochran CM, Slaughter JC, *et al.* Correlation of abdominal rSO₂ with superior mesenteric artery velocities in preterm infants. *J Perinatol* **33**, 609–612 (2013).
87. McNeill S, Gatenby JC, McElroy S, Engelhardt B. Normal cerebral, renal and abdominal regional oxygen saturations using near-infrared spectroscopy in preterm infants. *J Perinatol* **31**, 51–57 (2011).
88. Pellicer A, Greisen G, Benders M, *et al.* The SafeBoosC phase II randomised clinical trial: A treatment guideline for targeted near-infrared-derived cerebral tissue oxygenation versus standard treatment in extremely preterm infants. *Neonatology* **104**, 171–178 (2013).

89. Patel AK, Lazar DA, Burrin DG, *et al.* Abdominal near-infrared spectroscopy measurements are lower in preterm infants at risk for necrotizing Enterocolitis. *Pediatr Crit Care Med* **15**, 735-741 (2014).
90. Naulaers G, Meyns B, Miserez M, *et al.* Use of tissue oxygenation index and fractional tissue oxygen extraction as non-invasive parameters for cerebral oxygenation: A validation study in piglets. *Neonatology* **92**, 120–126 (2007).
91. Wyatt JS, Cope M, Delpy DT, Wray S, Reynolds EO. Quantification of cerebral oxygenation and haemodynamics in sick newborn infants by near infrared spectrophotometry. *Lancet* **2**, 1063–1066 (1986).
92. Jöbsis FF. Noninvasive, infrared monitoring of cerebral and myocardial oxygen sufficiency and circulatory parameters. *Science* **198**, 1264–1267 (1977).
93. Edwards AD, Wyatt JS, Richardson C, Delpy DT, Cope M, Reynold EO. Cotside measurement of cerebral blood flow in ill newborn infants by near infrared spectroscopy. *Lancet* **2**, 770–771 (1988).
94. McCormick PW, Stewart M, Geotting MG, Dujovny M, Lewis G, Ausmann JI. Noninvasive cerebral optical spectroscopy for monitoring cerebral oxygen delivery and hemodynamics. *Crit Care Med* **19**, 89–97 (1991).
95. Kooi EMW, van der Laan ME, Hulscher JBF, Schurink M, Bos AF. Measuring splanchnic tissue oxygenation in preterm neonates with necrotizing enterocolitis using multi-site near infrared spectroscopy: a pilot study comprising two abdominal locations. *Pediatr Res* **70**, 810–810 (2011).
96. Putignani L, del Chierico F, Petrucca A, Vernocchi P, Dallapiccola B. The human gut microbiota: a dynamic interplay with the host from birth to senescence settled during childhood. *Pediatr Res* **76**, 2–10 (2014).
97. Torrazza RM & Neu J. The Altered Gut Microbiome and Necrotizing Enterocolitis. *Clin Perinatol* **40**, 93–108 (2013).
98. Gritz EC & Bhandari V. The human neonatal gut microbiome: a brief review. *Front Pediatr* **3**, (2015).
99. Groer MW, Luciano AA, Dishaw LI, Ashmeade TL, Miller E, Gilbert JA. Development of the preterm infant gut microbiome: a research priority. *Microbiome* **2**, 38 (2014).
100. Berrington JE, Stewart CJ, Embleton ND, Cummings SP. Gut microbiota in preterm infants: assessment and relevance to health and disease. *Arch Dis Child Fetal Neonatal Ed* **98**, 286–291 (2012).
101. Stewart CJ, Marss EC, Nelson A, *et al.* Development of the preterm gut microbiome in twins at risk of necrotising enterocolitis and sepsis. *PLoS One* **8**, e73465 (2013).

102. Arboleya S, Sanchez B, Milani C, *et al.* Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics. *J Pediatr* **166**, 538–544 (2015).
103. Butel MJ, Suau A, Campeotto F, *et al.* Conditions of bifidobacterial colonization in preterm infants: a prospective analysis. *J Pediatr Gastroenterol Nutr* **44**, 577–582 (2007).
104. DiGiulio DB. Diversity of microbes in amniotic fluid. *Semin Fetal Neonatal Med* **17**, 2–11 (2012).
105. Collado MC, Cernada M, Neu J, Perez-Martinez G, Gormaz M, Vento M. Factors influencing gastrointestinal tract and microbiota immune interaction in preterm infants. *Pediatr Res* **77**, 726–731 (2015).
106. Dai D & Walker WA. Protective nutrients and bacterial colonization in the immature human gut. *Adv Pediatr* **46**, 353–382 (1999).
107. Solís G, de los Reyes-Gavilan CG, Fernández N, Margolles A, Gueimonde M. Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. *Anaerobe* **16**, 307–310 (2010).
108. Jain N & Walker WA. Diet and host-microbial crosstalk in postnatal intestinal immune homeostasis. *Nat Rev Gastroenterol Hepatol* **12**, 14–25 (2015).
109. Power SE, O’Toole PW, Stanton C, Ross RP, Fitzgerald GF. Intestinal microbiota, diet and health. *Br J Nutr* **111**, 387–402 (2014).
110. Gomez-Llorente C, Plaza-Diaz J, Aquillera M, *et al.* Three main factors define changes in fecal microbiota associated with feeding modality in infants. *J Pediatr Gastroenterol Nutr* **57**, 461–466 (2013).
111. Jost T, Lacroix C, Braegger CP, Chassard C. New insights in gut microbiota establishment in healthy breast fed neonates. *PLoS One* **7**, e44595 (2012).
112. Guaraldi F & Salvatori G. Effect of breast and formula feeding on gut microbiota shaping in newborns. *Cell Infect Microbiol* **2**, (2012). doi: 10.3389/fcimb.2012.00094
113. Sántulli TV, Schullinger JN, Heird WC, *et al.* Acute necrotizing enterocolitis in infancy: a review of 64 cases. *Pediatrics* **55**, 376–387 (1975).
114. Morowitz MJ, Poroyko V, Caplan M, *et al.* Redefining the role of intestinal microbes in the pathogenesis of necrotizing enterocolitis. *Pediatrics* **125**, 777–785 (2010).
115. McMurtry VE, Gupta RW, Tran L, *et al.* Bacterial diversity and Clostridia abundance decrease with increasing severity of necrotizing enterocolitis. *Microbiome* **3** (2015) doi: 10.1186/s40168-015-0075-8.
116. La Rosa PS, Warner BB, Zhou Y, *et al.* Patterned progression of bacterial populations in the premature infant gut. *Proc Natl Acad Sci* **111**, e 1409497111 (2014).

117. Morrow AL, Lagomarcino AJ, Schibler KR, *et al.* Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. *Microbiome* **1**, (2013). doi: 10.1186/2049-2618-1-13 (2013).
118. Sim K, Shaw AG, Randell P, *et al.* Dysbiosis anticipating necrotizing enterocolitis in very premature infants. *Clin Infect Dis* **60**, 389–397 (2015).
119. Alfaleh K, Anabrees J, Bassler D, Al-Kharfi T. Probiotics for prevention of necrotizing enterocolitis in preterm infants. *Cochrane Database Syst Rev* **4**, (2011). doi:10.1097/AOG.0b013e3181726f2f.
120. Panigrahi P. Probiotics and prebiotics in neonatal necrotizing enterocolitis: new opportunities for translational research. *Pathophysiology* **21**, 35–46 (2014).
121. Patel RM & Denning PW. Therapeutic use of prebiotics, probiotics, and postbiotics to prevent necrotizing enterocolitis: what is the current evidence? *Clin Perinatol* **40**, 11–25 (2013).
122. Ofek Shlomai N, Deshpande G, Raob S, Patole S. Probiotics for preterm neonates: what will it take to change clinical practice? *Neonatology* **105**, 64–70 (2013).
123. Lin HC, Su BH, Chen AC, *et al.* Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants. *Pediatrics* **115**, 1–4 (2005).
124. Bin-Nun A, Bromiker R, Wilschanski M, *et al.* Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. *J Pediatr* **147**, 192–196 (2005).
125. Niemarkt HJ, de Meij TG, van de Velde ME, *et al.* Necrotizing enterocolitis: a clinical review on diagnostic biomarkers and the role of the intestinal microbiota. *Inflamm Bowel Dis* **21**, 436–444 (2015).
126. Stewart CJ, Marss EC, Magorrian S, *et al.* The preterm gut microbiota: Changes associated with necrotizing enterocolitis and infection. *Acta Paediatr Int J Paediatr* **101**, 1121–1127 (2012).
127. Raveh-Sadka T, Thomas BC, Singh A, *et al.* Gut bacteria are rarely shared by co-hospitalized premature infants, regardless of necrotizing enterocolitis development. *Elife* **4**, (2015). doi: 10.7554/eLife.05477.

