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Karolinska Institutet, Stockholm, Sweden

# NECROTIZING ENTEROCOLITIS: IMAGING AND RISK ASSESSMENT

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# **Necrotizing enterocolitis: Imaging and risk assessment**

THESIS FOR DOCTORAL DEGREE (PhD)

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## **ABSTRACT**

Despite decades of research on necrotizing enterocolitis (NEC), no major finding has improved the mortality and morbidity of the disease or changed the clinical management. The exact pathogenesis remains unclear, but several factors such as immature intestinal immunity, impaired bowel microcirculation, enteral nutrition and abnormal microbiota may play important roles. In the post-surfactant era, the NEC patient population has changed, with an increasing proportion of extremely preterm infants. Plain abdominal radiography is still considered the gold standard imaging technique for NEC. Unfortunately, abdominal radiography has low sensitivity and specificity, making the decision to intervene surgically very challenging. Recent studies have shown the increasing role of abdominal ultrasound and near-infrared spectroscopy in the diagnosis and monitoring of NEC. The overall aim of this project was to describe the preterm infants at risk of NEC and how those who develop severe NEC and need surgical treatment could be identified early, using new imaging techniques and monitoring.

The aim of Paper I was to describe the difference in the clinical and radiological presentation of NEC in extremely preterm infants compared with more mature ones. Extremely preterm infants show less typical signs of NEC, such as bloody stool or pneumatosis intestinalis, compared with more mature neonates.

The aim of Paper II was to assess if splanchnic oxygenation, as measured by near infrared spectroscopy (NIRS), in the first week of life is associated with the risk of developing necrotizing enterocolitis. Extremely preterm infants underwent NIRS monitoring during enteral nutrition. Low mean splanchnic oxygenation, below 30%, was associated with an increased risk of developing necrotizing enterocolitis during enteral nutrition in the first days of life.

The aim of Paper III was to determine whether a correlation exists between the sonographic findings and the clinical outcomes, defined as surgery or death in infants with NEC. Infants with a confirmed diagnosis of NEC, who underwent an abdominal ultrasound, were included in the study. The sonographic sign of complex fluid collections appeared to be strongly correlated with the need for surgery in infants with severe NEC.

The aim of Paper IV was to assess if hyponatremia, or worsening of already present hyponatremia, at the onset of necrotizing enterocolitis is associated with intestinal inflammation and ischaemia requiring surgery or death. In this cohort study, neonates with a confirmed diagnosis of NEC were included. Hyponatremia and a sudden decrease in plasma sodium concentration at the onset of NEC can be useful markers for severe intestinal inflammation/ischemia where an imminent need for surgery can be expected.



## LIST OF SCIENTIFIC PAPERS

This doctoral thesis is based on the following original papers, referred to in the text by their Roman numerals:

- I. **Palleri E**, Agham I, Bexelius TS, Bartocci M, Wester T. The effect of gestational age on clinical and radiological presentation of necrotizing enterocolitis. *J Pediatr Surg* 2018;53:1660-1664
- II. **Palleri E**, Wackernagl D, Wester T, Bartocci M. Low Splanchnic Oxygenation and Risk for Necrotizing Enterocolitis in Extremely Preterm Newborns. *J Pediatr Gastroenterol Nutr* 2020;71:401-406
- III. **Palleri E**, Kaiser S, Wester T, Arnell H, Bartocci M. Complex Fluid Collection on Abdominal Ultrasound Indicates Need for Surgery in Neonates with Necrotizing Enterocolitis. *Eur J Pediatr Surg* 2017;27:161-165
- IV. **Palleri E**, Frimmel V, Fläring U, Bartocci M, Wester T. Hyponatremia at onset of necrotizing enterocolitis is associated with severe intestinal inflammation and ischemia. Manuscript

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## LIST OF ABBREVIATIONS

NEC= necrotizing enterocolitis

PDA= patent ductus arteriosus

AUS= abdominal ultrasound

SGA= small for gestational age

SIP= spontaneous intestinal perforation

SBS= short bowel syndrome

IUGR= intrauterine growth restriction

HR= hazard ratio

CrSO<sub>2</sub> = cerebral regional oxygen saturation

SCOR= splanchnic cerebral oxygenation ratio

EPN= extremely preterm neonates

P-TN= preterm-term neonate

NIRS= near infrared spectroscopy

GA= gestational age

GI=gastrointestinal

CP=cerebral palsy

RR= risk ratio

OR= odds ratio

IVH= intraventricular hemorrhage

SrSO<sub>2</sub> = splanchnic regional oxygen saturation



# 1 INTRODUCTION

Necrotizing enterocolitis (NEC) is one of the most devastating gastrointestinal diseases among preterm neonates (1). The exact pathogenesis is still unclear. Despite decades of extensive research and experience in treating NEC patients, the disease remains a major cause of mortality and long-term morbidity among the most preterm infants (2-7).

The clinical presentation of NEC varies from a purely medical condition to a life-threatening event entailing an urgent surgical intervention, but the decision to operate is not easy.

Even though the first reports of what might have been NEC appeared in the early 20<sup>th</sup> century (8), necrotizing enterocolitis was not described as a clinical entity until the 1960s (7, 9, 10). At that time, most of the patients were full-term or near-term infants. In 1978, Bell et al published a severity staging system for NEC (11), which is, after a modification in 1986 (12), still in use today. The Bell staging system, based on clinical and radiological findings, is a support for therapeutic choices and indications for surgery. In the pre-surfactant era, NEC patients were mostly preterm neonates born after 30 weeks of gestation (13). Due to the improved survival of extremely preterm neonates, the incidence of NEC has increased over the last decades (14) and the patient population has now changed.

It has been difficult to define NEC over time. Different entities of neonatal gastrointestinal diseases, such as spontaneous intestinal perforation, have been included in NEC datasets, thus contaminating them. Over time, it has become increasingly clear that NEC is actually more than one single disease (15). Gordon described this in one of his lectures: “NEC is a common pathologic end point (10), but the disease arises from multiple triggers and etiologies, often requiring more than one such event to meet the threshold of disease” (16). This is one reason why there is still no specific biomarker for NEC, despite intensive research in the field. This thesis describes the clinical and radiological characteristics of NEC patients, assesses the risk of developing NEC by using near infrared spectroscopy in extremely preterm infants and, finally, analyzes the correlation between surgical NEC, ultrasound findings, and hyponatremia at NEC onset.

## **2 BACKGROUND**

### **2.1 EPIDEMIOLOGY**

NEC is a gastrointestinal inflammatory disease that mostly affects preterm neonates (2). Even though NEC is internationally widespread, the incidence rate varies remarkably across countries (17). For example, the incidence is between 7-10 in 10,000 live births in the USA (18, 19), while the overall incidence of NEC in Sweden was 3.4 in 10,000 live births during the period 1987-2009 (14). NEC occurs in 2-7% of infants born at less than 32 weeks of gestation in high income countries, with an incidence that varies from 5 to 22% among infants <1000g (17). The Swedish National Extremely Preterm Infants Study (EXPRESS) reported an increasing incidence from 6% in 2004-2007 to 10% in 2014-2016 among infants born before week 27 (20), probably due to improved early survival among the smallest infants. The same pattern has been seen in the Netherlands after the introduction of the new Dutch guidelines for the active treatment of extremely preterm infants (21). In a Canadian study, the reported incidence was 5.1% in infants below 33 weeks of gestation (22). It is known that NEC mainly affects preterm infants (8, 19), but the exact proportion also seems to vary across countries and over time (14, 19). In Sweden, the proportion of term infants decreased between 1987 and 2009 from 30% to 14% (14). Both incidence and case fatality rates increase with decreasing birth weight and gestational age (1, 23). The onset of disease often occurs between 27 and 34 weeks of gestation, with the highest incidence (13%) among extremely low birth weight infants (1, 8). The majority of NEC cases occurs sporadically, but “NEC outbreaks“ or “NEC clusters” have been reported, where a common etiology has been suggested (24, 25).

#### **2.1.1 Risk and protective factors**

Many risk factors have been shown to be related to NEC, but there is no general consensus among experts. Only three risk factors are universally accepted: gestational age, birth weight and enteral nutrition (formula) (26). Low gestational age and birth weight have been well studied as major risk factors for NEC (27, 28). Even though most cases of NEC occur among preterm infants, there is a small group of term (or late preterm) newborns who show a NEC-like clinical status, usually in association with other conditions (such as congenital heart disease, gastroschisis, etc.) (29).

The protective effect of breast milk, known for decades and confirmed by several randomized controlled trials, seems to be dose-related (30-33). Human milk includes a great diversity of beneficial bioactive factors and, among these, several have been indicated as protective factors, reducing both NEC incidence and progression (1). The intestinal immaturity and clinical instability of extremely preterm infants have always concerned clinicians, who often

delay the start of enteral feeding or advanced enteral nutrition at slow rates in order to protect the intestine. Neither early enteral fasting nor a slower advancement of enteral nutrition has been shown to reduce the risk of NEC, but most of these studies do not include the first days of life (34, 35). There are contradictory results concerning many other factors associated with NEC, most of which are selected from meta-analyses based solely on observational studies (36). The table below proposes a selection of factors associated with NEC, see Table 1.

<b>Suggested factors associated with NEC</b>	
Maternal or antenatal	<p>Pre-eclampsia (risk) (37)</p> <p>Antenatal steroids (protective) (38)</p> <p>Chorioamnionitis (risk) (39)</p> <p>Premature prolonged rupture of membranes (risk) (36)</p> <p>Maternal antibiotics (protective?) (40)</p> <p>Intrauterine growth restriction (risk) (41, 42)</p>
Infant baseline characteristics	<p>Low gestational age (risk) (27, 28, 43, 44)</p> <p>Low birth weight (risk) (27, 28, 43, 44)</p> <p>Small for gestational age (risk) (41, 43, 45)</p> <p>Congenital heart disease (risk) (43, 46, 47)</p> <p>Low APGAR/asphyxia (risk) (48)</p>
Postnatal	<p>Surfactant (risk) (45, 49, 50)</p> <p>Patent ductus arteriosus and treatment (risk) (43, 50, 51)</p> <p>Sepsis (risk) (50)</p> <p>Antibiotics (risk) (52)</p> <p>Severe anemia/red blood cell transfusion (risk) (53, 54)</p> <p>Fluid restriction (protective) (55)</p>
Feeding	<p>Breast milk (protective) (31, 32)</p> <p>Preterm formula (risk) (31, 32, 56)</p> <p>Donor human milk (protective) (57)</p> <p>Feeding protocol (protective) (58)</p> <p>Probiotics (protective) (59, 60)</p> <p>Acid suppression (risk) (61, 62)</p>

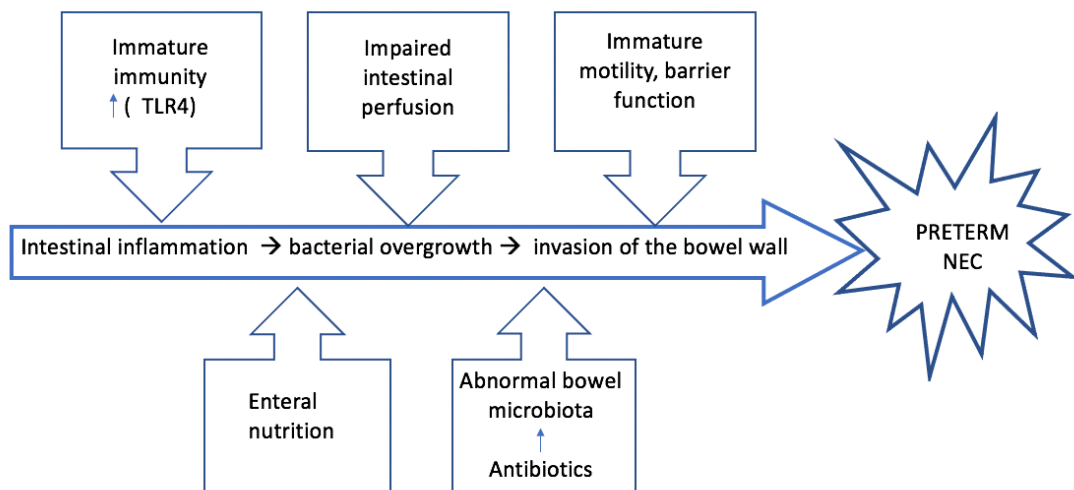
**Table 1:** *Factors associated with NEC*

## 2.2 PATHOPHYSIOLOGY

The pathogenesis of NEC has been extensively studied, but is still not clear. NEC is characterized by various degrees of mucosal or transmural inflammation and necrosis of the neonatal bowel, ultimately leading to bowel perforation, peritonitis and death (2, 63). The modern understanding is that necrosis of the intestinal mucosa is the ultimate endpoint of different triggers, which results in the diversity of clinical NEC presentations. There are some basic pre-conditions for developing NEC: 1) a recently colonized and naïve neonatal bowel; 2) a trigger that harms the intestinal mucosal integrity; and 3) the presence of food, substrate for bacteria, in the bowel (64). This chapter focuses mainly on the pathophysiology of the preterm NEC and only briefly on term/ischemic NEC that often develops in infants with underlying conditions (46).

### 2.2.1 Preterm NEC

The preterm bowel is more prone to develop NEC than the full-term bowel. This is explained by differences in bacterial colonization, microcirculatory perfusion, and maturity of the enteric immune system (1, 65). Each of these factors cannot explain the pathogenesis of NEC by itself, hence it is probably multifactorial. (Figure 1)



**Figure 1:** Pathogenesis of preterm NEC

#### 2.2.1.1 Immunity

Until enteral nutrition is effectively established, the neonatal intestine depends mainly on the innate system for its immunity. In this developing phase, toll-like receptors survey the lumen for macromolecules specific to pathogens (64). Nino et al (1) recently suggested a unified

hypothesis on the pathogenesis of preterm NEC, where the preterm bowel exists in a “hyper-reactive state” compared with the full-term bowel. This benefits the development of the disease after colonization with an appropriate microbial flora. The fetal bowel matures in an environment with reduced exposure to bacteria, but preterm infants are later exposed to a great quantity and diversity of microbes, especially after enteral feeding is initiated (30). Evidence suggests that an increased baseline reactivity of the preterm intestine to microbial ligands, predisposes the preterm infant to an increased risk of gastrointestinal damage. In fact, toll-like receptor 4 (TLR4) has been shown to be more expressed in the premature bowel than in the full-term bowel in both animals and humans (66). TLR4 activation by gram-negative bacteria leads to inflammatory cytokine production, increased enterocyte apoptosis, reduction of enterocyte proliferation, impaired mucosal healing and reduction in intestinal blood flow (1, 64, 67). The deleterious consequences of TLR4 activation can be the development of NEC. This explanation is called “the cross-switching hypothesis“ and partially explains why NEC occurs upon bacterial colonization in the premature infant (1).

#### *2.2.1.2 The gut microbiota*

Although the exact role of the intestinal microbiota in the development of NEC is uncertain, numerous factors implicate its involvement. The bowel colonization in the early neonatal period occurs in two waves: the first depending mainly on the delivery mode, the second on feeding type (68). In premature infants, the second wave is less influenced by feeding type and seems to follow a specific rate and pattern, related mostly to the degree of prematurity. In more premature infants, the advancement of bacterial colonization is slower but the same pattern of colonization is still followed (1). Many authors have shown an association between dysbiosis in preterm newborns and NEC development beyond three weeks of postnatal age (69, 70). Microbial colonization of the bowel is essential for the development of NEC, as NEC occurs just after this event and not in utero (69-71). However, diverse organisms are grown from isolated outbreaks so it cannot be claimed that a single and specific organism is responsible for the development of the disease, even though gram-negative bacteria are still the key suspects (30, 72). The use of prophylactic antibiotics in premature infants suppresses the intestinal flora and is associated with lower proportions of potentially protective anaerobes, which is a risk factor for NEC (70). Several studies suggest a loss of microbial diversity to occur directly before the onset of NEC (73, 74).

#### *2.2.1.3 Microcirculation*

Ischemia and the impairment of splanchnic microcirculation definitely play a role in the pathogenesis of NEC. Experimental and animal studies have suggested that an impairment in

intestinal microcirculation could be present in NEC patients (75). Immature intestinal perfusion, with a less pronounced vasodilatory response and an increased oxygen extraction to systemic stress, has been shown in fetuses and newborn animals compared with older ones (76, 77). In healthy subjects, digestion and absorption of food cause an increased intestinal perfusion response to the increase in oxygen demand, which is called postprandial hyperemia (78, 79). As a result of intestinal immaturity, splanchnic microcirculation is not fully capable of increasing intestinal perfusion in response to an increased metabolic demand, such as during feeding. In fact, during the first days of life, as opposed to an intestine older than two weeks, the neonatal intestine compensates for different stress factors mainly with increased oxygen extraction rather than with vasodilation (76, 77). Studies in neonatal pups have shown that feeding-induced bowel hypoxia is associated with poor postprandial intestinal hyperemia in the first days of life (56).

The intestinal blood flow is mainly regulated by the vasodilator nitric oxide (NO) and the vasoconstrictor endothelin-1 (ET-1), both produced by endothelium (75). As previously discussed, the preterm bowel expresses high levels of TLR4. Studies have shown that deletion of TLR4 in the endothelium of the intestinal cells increases NO-dependent microcirculation and decreases both the incidence and severity of NEC (67). Accordingly, studies on intestinal perfusion measured by near infrared spectroscopy have reported that splanchnic oxygenation is somehow impaired before the onset of NEC in preterm infants (80, 81).

#### *2.2.1.4 Other factors*

Other factors that predispose the preterm bowel to NEC are immature motility, digestion, absorption and barrier function (30). Gastric acid secretion is reduced in the preterm neonate and this limitation has been related to an increased risk of NEC. Furthermore, the decreased number of mucus-producing goblet cells in the preterm bowel and the reduced clearance of intraluminal contents result in an impaired mucosal protection that leads to an augmented exposure to pathogenic bacteria (1, 64, 66).

### **2.2.2 Enteral feeding**

As already mentioned, enteral nutrition is implicated in the pathogenesis of NEC. In fact, almost all premature infants who develop NEC have already been fed (64, 82-84). The start of enteral nutrition results in colonization of the bowel and increased food amounts lead to an increased oxygen demand in the splanchnic perfusion (68, 78, 79). In the past, an early introduction of enteral feeding to a preterm intestine, as well as increasing enteral nutrition too fast, has been thought to increase the risk of NEC. A recent randomized control trial

comparing a slow and fast rate of feeding advancement showed no evidence of an increased risk of NEC, but the first days of life were not included in the trial (35). Neither an early nor late introduction of enteral nutrition has been shown to decrease the incidence of NEC (85). The type of feeding seems to be important in the pathogenesis of NEC as breast milk has been shown to decrease its incidence (32, 33). There are many beneficial bioactive factors in breast milk, such as lactoferrin and secretory IgA, with different positive effects in the neonatal bowel (86). One example is the vasodilatory effect. Breast milk is much richer in nitrate anions, needed for the production of NO, than formula (56, 67). It has also been shown *ex vivo* that breast milk, known to have a protective effect against NEC (87), is a potent inhibitor of TLR4 signaling (88).

### **2.2.3 Ischemic NEC (Term NEC)**

Term NEC occurs often during the first week of life and seems to follow hypoxic/ischemic events that lead to mesenteric bowel ischemia (64). Ischemia-reperfusion injury due to relative splanchnic hypoperfusion is believed to be an important factor in the pathogenesis of term NEC. Infants with congenital cardiac disease, with low peripheral perfusion and a compromised superior mesenteric artery perfusion are known to have a higher risk of NEC (89-91). Infants who were growth restricted in utero and those with perinatal-ischemic events also belong to this group (13). Intrauterine growth restriction is a well-known risk factor for NEC, possibly caused by hypoxic-ischemic injury of the gut due to circulatory redistribution and alterations in intestinal microcirculation (75, 92). Hypoxic events are thought to induce TLR4 expression *de novo*, as in infant rodent models where ischemic events/asphyxia are triggers for NEC (64).

## **2.3 CLINICAL PRESENTATION AND DIAGNOSIS**

The clinical presentation of NEC can vary, from subtle signs of feeding intolerance that progress over many hours or days to a life-threatening condition requiring urgent surgical intervention. The early classical signs and symptoms of NEC are usually gastrointestinal and include abdominal pain and distention, feeding intolerance, increased gastric residuals or vomiting and bloody stools (65). Many of these signs are not specific for NEC and can occur in other diseases, such as neonatal sepsis or simply feeding intolerance (93, 94). The disease can develop quickly resulting in abdominal wall erythema, bowel necrosis, perforation, and peritonitis (65). Abdominal wall erythema is highly predictive of NEC but is rarely present (95). Ultimately, patients may require increased ventilator and vasopressor support. The distinction between infants with real NEC and those with bacterial sepsis without NEC is important since the clinical management of these two conditions is quite different regarding



type of antibiotics, length of fasting, parenteral nutrition and, eventually, the need for surgery (96). Full-term infants who develop NEC postnatally are usually systemically ill and have a history of other predisposing conditions, such as birth asphyxia or congenital heart disease. Preterm babies are at risk of developing NEC for several weeks after birth and their disease can be more difficult to diagnose (97). More specifically, the gestational age at birth determines the window of vulnerability within which a neonate typically develops the disease (98). The postnatal age at which infants develop NEC is inversely correlated to gestational age (19, 93). Over the past few decades, the NEC patient population has changed, with an increasing proportion of the smallest preterm infants, and some studies have shown an effect of the gestational age in the clinical presentation of the disease (93, 99).

### **2.3.1 Staging and diagnosis**

The clinical severity of NEC is still classified according to the modified Bell's staging system from 1986 (12, 100). Bell's staging system is based on three wide stages that include systemic signs, gastrointestinal symptoms or signs, and radiographic findings. In this staging system, the presence of intramural gas is the hallmark to confirm the diagnosis. A modification of Bell's criteria is shown in Table 2. At the present, there is a lack of a comprehensive definition and staging of NEC (101), probably because it has become increasingly clear that NEC, rather than being one single disease (15), is actually a common pathologic final stage of different etiologies and triggers (10). Currently, the most commonly used definitions of NEC include that of the Vermont Oxford Network (102) and the modification of Bell's criteria (12, 100). Another score system for NEC, recently proposed by Battersby et al, is gestational age-specific and based on a combination of clinical and radiological signs (97). They assigned 1-3 points to different signs, or a combination of signs, of NEC and defined a gestational age-specific cut-off score for the diagnosis of NEC. An important topic that has been raised over the past few years is the need of keeping other acquired intestinal diseases (such as spontaneous intestinal perforation or cow's milk protein allergy) out of NEC datasets in order to improve data quality. This way of thinking is called NEC reductionism (13, 101). Both the Vermont Oxford Network staging system and the one suggested by Battersby et al include the presence of pneumoperitoneum in the diagnosis of NEC and do not efficiently exclude spontaneous intestinal perforation. The Bells system is still the most commonly used one but, due to a change in patient population and understanding of the disease, its adequacy has been questioned (13, 103). Lately, Gordon et al proposed the "two out of three rule" for the bedside diagnosis of preterm NEC, see Table 3 (101, 103).

<b>Stage</b>	<b>Systemic symptoms</b>	<b>Intestinal symptoms</b>	<b>Radiological signs</b>	<b>Treatment</b>
IA- suspected	Temperature instability, apnea, bradycardia	Increased gastric residuals, mild abdominal distension, emesis, occult blood in stool	Normal or intestinal dilation, mild ileus	NPO, antibiotics x 3 days
IB- suspected	Same as above	Bright red blood from rectum	Same as above	Same as IA
IIA- definite	Same as above	Same as above, plus absent bowel sounds with or without abdominal tenderness	Intestinal dilation, ileus, pneumatosis intestinalis	NPO, antibiotics x 7 to 10 days
IIB- definite	Same as above, plus mild metabolic acidosis, mild thrombocytopenia	Same as above, plus absent bowel sounds, definite tenderness, with or without abdominal cellulitis or right lower quadrant mass	Same as IIA, plus portal vein gas, with or without ascites	NPO, antibiotics x 14 days
IIIA- advanced	Same as IIB, plus hypotension, bradycardia, severe apnea, combined respiratory and metabolic acidosis, DIC, neutropenia	Same as above, plus signs of generalized peritonitis, marked tenderness, and abdominal distention	Same as IIB, plus ascites	NPO, antibiotics x 14 days, fluid resuscitation, inotropic support, ventilator therapy, paracentesis
IIIB- advanced	Same as stage IIIA	Same as IIIA	Same as above, plus pneumoperitoneum	Same as IIIA, plus surgery

**Table 2:** Bell's Criteria, modified by Walsh and Kliegman (12, 100). NPO= nil per os, DIC= disseminated intravascular coagulation.

Patients may be given a diagnosis of preterm NEC if they have abdominal distension, ileus and/or bloody stools and meet at least two of the criteria below:

- 1- Pneumatosis intestinalis and/or portal gas by ultrasound or abdominal radiography at presentation.
- 2- Persistent platelet consumption (<150,000 for 3 days after diagnosis).
- 3- Post-menstrual age at disease onset more consistent with NEC than SIP

Differential diagnosis:

Exclusion: full-term infants ( $\geq 36$  weeks of gestation), infants with known SIP and complex congenital abnormalities and infants with less than 80 ml/kg/day of enteral nutrition.

**Table 3:** *Two out of three rule(101, 103)*

### **2.3.2 Imaging**

Without validated and specific biomarkers for NEC, imaging has always played a central role in the diagnosis and management of the disease. Plain abdominal radiography is still considered the standard method and first choice to detect and monitor NEC, despite the overall low sensitivity (104-106). The presence of pneumatosis intestinalis (intramural gas) or portal venous gas on plain abdominal radiography usually confirms the diagnosis of NEC (1, 103, 104, 107, 108). Pneumatosis intestinalis is caused by bacterial fermentation within the bowel wall and is a sign of bacterial translocation (109, 110). This sign, even though transient, is almost always considered pathognomonic of NEC, although neonates with other intestinal diseases may also present with pneumatosis intestinalis (107, 111). The early diagnosis of NEC is indeed very difficult because of the low specificity and sensitivity of these radiological findings and the high inter-observer variability in the interpretation of abdominal radiographs (105, 109, 112). Recently, a new standardized evaluation of abdominal plain radiography has been proposed in order to improve diagnostic accuracy: The Duke Abdominal Assessment Scale (DAAS). DAAS is a scoring system based on radiological findings at the abdominal radiographs, where the score increases with the severity of the disease and the likelihood of surgical treatment, a score of 0 corresponds to a normal gas pattern and a score of 10 to pneumoperitoneum (113, 114). Pneumoperitoneum is a sign of intestinal perforation and, so far, the only universally accepted indication for laparotomy (95, 115). Unfortunately, pneumoperitoneum is not pathognomonic of NEC and not always present in babies with bowel perforation or bowel necrosis (95, 116). Lately, ultrasonography (US) has been widely

recognized to provide information that is not depicted on radiographs and it is now used to assess NEC (109, 117, 118). Routine US is used to detect pneumatosis intestinalis, bowel wall thickening/thinning, bowel wall perfusion, portal venous gas and other signs which are difficult to detected on abdominal radiographs (104, 109, 119). The main advantages of US are that it offers, at bedside, real-time images of abdominal organs, of the bowel and, eventually, of peritoneal fluids (104, 109, 116, 117, 119), all avoiding radiation exposure. Abdominal US seems to be more sensitive than plain abdominal radiographs in the detection of pneumatosis intestinalis at earlier stages, portal venous gas and, sometimes, even pneumoperitoneum (109). Faingold et al (119) showed that color Doppler US was extremely useful in assessing the perfusion of the intestinal wall. Unfortunately, Doppler sonography at low velocities is difficult to assess. Various studies have now proved that abdominal ultrasound outperforms plain abdominal radiology for the diagnosis and monitoring of NEC and should be part of the standard protocol for NEC diagnosis (103, 109, 120, 121). On the other hand, the technique is highly operator-dependent and has thus been difficult to implement in clinical practice (122). Faingold recently published a paper on the technical aspects of abdominal ultrasound that can help in the implementation of this technique (123). Especially in cases with complicated NEC where the patient's clinical deterioration does not correspond with the radiographic picture, US gives more information about the viability of the bowel wall, suggesting that it is an accurate screening tool to determinate the need for surgical intervention (116, 119, 124-126). Research with contrast-enhanced US, which has the ability to evaluate the microvascular perfusion, might also be valuable in certain subsets of infants with NEC (127).

### **2.3.3 Laboratory**

Many biomarkers to detect NEC have been assessed so far, but none of them is “ideal”. Current biomarkers for the clinical assessment of NEC are usually divided into non-specific and specific biomarkers.

#### *2.3.3.1 Non-specific biomarkers*

The most common non-specific biomarkers for NEC are mediators of the inflammatory cascade. Acute phase reactants, such as C-reactive protein (CRP) and serum amyloid A (SAA), are often used in the diagnosis of NEC. CRP is a “late-warning” biomarker, therefore it is not as useful for early diagnosis of inflammatory conditions as for monitoring the disease progress (128, 129). SAA is another acute phase reactant that is synthesized in response by activated macrophages to cytokine release after acute-phase stimuli, such as infection and tissue injury (130). SAA is quite similar to CRP, but has a faster upregulation, it is not specific for NEC and its levels are increased in both infants with NEC and sepsis (131). Platelet-activating factor

(PAF), an endogenous inflammatory phospholipid, has also been studied in both plasma and feces for NEC diagnosis/monitoring with promising results (129, 132). Other acute phase proteins, such as procalcitonin, have been suggested as valuable indicators for diagnosing or predicting surgical NEC (96). Interleukin (IL)-6 and tumor necrosis factor (TNF)- $\alpha$ , regulated upon activation of normal T-cell expression, are known to be early biomarkers for diagnosing both neonatal sepsis and NEC (128, 133). Some authors have reported significantly higher IL-6 and IL-8 levels in surgical NEC than in matched controls (134). Other non-specific immunoregulatory mediators of the inflammatory pathway, such as neutrophil CD64, IL-6, and apolipoprotein CII, are early warning biomarkers, but they cannot discriminate NEC from sepsis and other inflammatory diseases (96, 128). Markers for metabolic derangement, such as acidosis, hyponatremia and thrombocytopenia have also been studied in combination as predictors of surgical NEC (135, 136). Thrombocytopenia is often present in NEC and has recently been included by Gordon as a criterion for NEC (103, 137). CRP and platelet count are still the most widely used biochemical markers for diagnosing and monitoring NEC (138).

#### 2.3.3.2 *Specific biomarkers*

Organ-specific biomarkers indicate gastrointestinal mucosal injury and/or intestinal barrier impairment. They include intestinal fatty acid-binding protein (i-FABP), liver fatty acid-binding protein (l-FABP), and fecal calprotectin (1). When the bowel mucosa is severely injured, damaged enterocytes and epithelial cells release large amounts of these intestinal barrier proteins into the blood. Theoretically, measuring their concentrations in serum or urine can allow an early diagnosis of NEC and quantify the severity of intestinal damage (139). The most encouraging is i-FABP, a cytoplasmatic protein released in the blood after intestinal damage and secreted in the urine that seems to correlate with the extent of intestinal necrosis (131, 140-142). Levels of l-FABP in combination with i-FABP were found to be higher in infants who later developed NEC compared with those who did not, suggesting that intestinal mucosal damage is detectable before NEC symptoms develop (131). Unfortunately, despite its high specificity, i-FABP has a medium-low specificity for NEC. One explanation could be that an elevated level of i-FABP is suggestive of mucosal damage rather than damage extending into the muscle layers (141, 143). Calprotectin is an antibacterial protein expressed in granulocytes and monocytes upon inflammatory activation that is widely used in the screening and monitoring of inflammatory bowel disease (129, 144). Fecal calprotectin has been evaluated for the diagnosis of NEC as well, and a recent metanalysis defines it as a “promising biomarker with high diagnostic value”, even though it remains unclear which cut off should be used (145). None of these bowel-specific proteins can be used as an early screening or warning

tool for detecting minor forms of intestinal injury (139). The latest systematic review of the available evidence concludes that serum markers for necrotizing enterocolitis generally have a low diagnostic accuracy, thence the use of many of them in clinical practice is so far unjustified (146).

### **2.3.4 Near Infrared Spectroscopy**

Near infrared spectroscopy (NIRS) is a bedside non-invasive monitoring technique that measures the difference between oxyhemoglobin and deoxyhemoglobin. It measures the balance of oxygen that is delivered minus what is extracted at tissue level (147, 148). Biological tissue is quite transparent to near-infrared light (700-1000nm), which is absorbed by different chromophores in the bloodstream. Hemoglobin, which is the main chromophore in the bloodstream, absorbs NIR-light depending on its oxygenation level (148-150). NIRS technology generally uses wave-lengths of 700-850 nm. At these lengths oxygenated and deoxygenated hemoglobin have characteristic spectra (149, 151). Using a modified version of the Beer-Lambert equation, NIRS devices are able to measure changes in the concentration of oxyhemoglobin (150, 151). This noninvasive measurement is reported as the regional oxygen saturation ( $rSO_2$ ) by INVOS<sup>®</sup> devices, as well as in this thesis (147, 149). The  $rSO_2$  is a mixed saturation value. Since the greater proportion of blood is venous, veins account for 80-85% of the  $rSO_2$ , while arteries provide 15%-20%, and capillaries 5% (149, 150). A low  $rSO_2$  measurement can either reflect increased oxygen extraction at tissue level or reduced blood flow altering the oxygen supply in the tissue monitored.

#### *2.3.4.1 NIRS and NEC*

NIRS is widely used for the assessment of cerebral oxygenation (150, 152) and could potentially allow continuous monitoring of intestinal perfusion as well. Since bowel ischemia, or at least impaired intestinal perfusion, seems to be associated with the development of NEC (75), measuring splanchnic oxygenation and extraction might detect changes compatible with the initial stages of NEC at an earlier point. Splanchnic  $rSO_2$  has been validated against gastric pH measurements, systemic mixed venous oxygenation, venous saturation from umbilical catheters, and serum lactate in neonates (153, 154). Splanchnic oxygenation ( $rSO_2$ ) seems to change with both gestational and postnatal age in preterm infants during the first weeks of life (155) and it is known to have a greater variability than cerebral  $rSO_2$ . Previous studies have shown that splanchnic NIRS measurements differ between preterm infants with and without intestinal ischemia (156, 157). One study reported that by measuring both cerebral and splanchnic oxygenation it was possible to differentiate complicated from uncomplicated NEC (158). The ratio between splanchnic and cerebral

oxygenation (SCOR or CSOR depending on the authors) has also been studied and described as a marker for abdominal pathology or anemia in infants (156, 159). Whether splanchnic oxygenation can be used in prediction models for NEC is still under debate. Corvaglia et al used splanchnic NIRS monitoring at the first feed to predict feeding intolerance in preterm infants (160). Actually, animal models have shown that low splanchnic rSO<sub>2</sub> within the first days of life might be a risk factor for NEC (161) and impaired intestinal oxygenation before the onset of clinical NEC has been reported in preterm infants (80, 81). It remains, however, that NIRS monitoring has so far not proven to be helpful to distinguish between definite NEC and no NEC in preterm infants with clinical signs suspicious of the disease. It could possibly be a useful tool in identifying infants who will develop NEC and those who will have a more complicated course. All the studies mentioned are quite small and further studies are needed to understand the potential role of NIRS in intestinal perfusion and NEC (162).

## **2.4 MANAGEMENT AND INDICATIONS FOR SURGERY**

The management of a suspected case of NEC consists of providing supportive care with fasting and administration of broad-spectrum intravenous antibiotics, after having secured blood cultures (2, 71). Patients are closely monitored for progression of the disease and the abdomen is regularly assessed using both clinical examinations and imaging (71, 138). Medical treatment (such as fasting and antibiotics) is continued for 7 to 14 days as long as the patient is stable and improving. There is no evidence of any specific antibiotic regime being superior to others (163) and empiric antibiotic therapy should be tailor-made according to the hospital's antibiogram. Surgical treatment is needed in approximately 50% of the NEC patients in large population-based studies (22, 164). Frank intestinal perforation, diagnosed by pneumoperitoneum on plain abdominal radiography or, rarely, by paracentesis positive for stool, is the only absolute indication for surgical treatment (115, 119, 165, 166). However, pneumoperitoneum is not always present in neonates with bowel perforation or necrosis, especially when the infant is very premature (93, 95, 116). The diagnosis of surgical NEC is not easy and the decision to operate is difficult considering the lack of consensus on how to manage surgical NEC (138). A deteriorating clinical condition (acidosis, severe persistent thrombocytopenia, hypotension) despite optimal medical management, or the presence of widespread pneumatosis intestinalis, portal venous gas, or fixed bowel loops on serial radiographs are relative indications for surgery (95, 136, 165, 167). Patients who need surgical intervention usually undergo explorative laparotomy with the goals of resecting only clearly gangrenous non-viable bowel, reducing the systemic sepsis and subsequent risk of multiorgan failure, and preserving any bowel that has a chance of survival (165). This approach focuses on bowel preservation with the intention of

reducing the number of patients who will later develop short bowel syndrome (137). Peritoneal drainage is an alternative for the most unstable patients, being used as both a temporary and a definite solution (138). Several studies have shown that patients undergoing peritoneal drainage have comparable outcomes to those undergoing laparotomy (164, 168-171). Given that most infants treated with peritoneal drainage require a laparotomy later on (164, 171), peritoneal drainage is reserved for small (<750g), unstable patients with elevated abdominal pressure that impairs ventilation.

## **2.5 OUTCOMES**

NEC remains one of the leading causes of death in neonatal intensive care units (172). The overall mortality reported is around 15-30% and decreases substantially with increasing birth weight (3, 18, 19, 21, 30, 137). The majority of NEC cases can be managed medically, although approximately 20-50% of affected infants require surgery (21, 45, 173). Many authors report unchanged mortality rates over the last decades (21, 45). It is important to make a distinction between medical and surgical NEC, since surgical NEC has a much higher mortality rate, up to 50% in extremely low birth weight infants, and is strongly associated with many long-term complications (5, 137, 173, 174).

### **2.5.1 Long-term outcomes**

NEC survivors are at a higher risk of developing long-term complications, some related to the gastrointestinal (GI) tract, others affecting general growth and neurodevelopment.

#### *2.5.1.1 Gastrointestinal complications*

Bowel strictures are the most common GI complications. They occur in 20% of NEC patients, usually after surgical NEC, but they may also develop after medical NEC (175, 176). The formation of strictures is caused by fibrosis of the bowel loop affected by NEC, as an attempt to heal after severe ischemic damage, and does not seem to depend on the surgical procedure as such (115, 176).

NEC remains the most common cause of pediatric short bowel syndrome (SBS) (137, 177, 178). Intestinal failure is defined as “the reduction of gut function below the minimum necessary for the absorption of macronutrients and/or water and electrolytes, such that intravenous supplementation is required to maintain health and/or growth” (179, 180). The inability to wean off parenteral nutrition results in increased mortality and additional complications such as risk of intestinal failure-associated liver disease and central line-associated blood stream infections in these infants (176). A recent meta-analysis reported that intestinal failure rates vary from 15% in all NEC to 35% in surgical NEC (174). On the bright



side, however, studies have shown that NEC children have a better chance of achieving enteral autonomy when compared with other causes of short bowel syndrome (181). This could be explained by considering that NEC is a disease that mainly affects very premature infants, and one can assume that the intestines of these neonates have better abilities to adapt over time than in other patients with SBS (3). Long-term growth and development may be compromised in NEC survivors, especially in infants with short bowel syndrome (178). Even NEC survivors without intestinal failure are likely to fall below the 50th percentile for weight and height several years later (182).

#### *2.5.1.2 Neurodevelopmental outcomes*

NEC survivors can develop neurodevelopmental dysfunction, including speech and motor impairment, intellectual delays, and problems with social skills. This occurs in up to 40-50% of cases in very low birth weight infants (176, 183). Several studies have reported long-term neurodevelopmental impairment in neonates who survived NEC (174, 184, 185). A systematic review from 2007 indicates that a definite diagnosis of NEC (Bell's stage II or higher) is associated with an increased risk of long-term neurodevelopmental impairment in very low birth weight infants (183). The risk seems to be even higher in the presence of surgical NEC (6, 183). No significant differences have been reported in neurodevelopmental outcomes between very low birth weight infants with spontaneous intestinal perforation and those without surgical diseases, when the influences of severe IVH (grades 3 and 4) and sepsis were excluded (186).

Neurodevelopmental impairment includes, in most cases, at least one of the following: scores less than two standard deviations of normal on the Bayley or Griffiths scale of assessment, cerebral palsy, severe visual impairment and severe hearing impairment (183). In this review, there are two important issues that need to be discussed; the differences in definition of neurodevelopmental impairment and the short follow-up time in some studies. Differences in definition of the severity of cerebral palsy and different cut offs for psychomotor and cognitive impairment can over- or underestimate the impairment rates. Simon et al (187) reported a higher incidence of motor delays at 8 and 15 months of corrected age in very low birth weight neonates requiring surgery for NEC. However, in most of the cases, the motor delays had resolved by 24 months, underlining the importance of a longer follow-up (187). Since the diagnosis of definite NEC is associated with long-term neurodevelopmental impairment, prevention, early diagnosis, optimal management, and long-term follow-up after the disease are extremely important in this group of patients.

## 2.6 PREVENTION

The protective effect of breast milk has been confirmed by several randomized controlled trials (30, 188). A variety of beneficial bioactive factors contained in human breast milk have been shown to reduce NEC incidence and progression (1). The use of probiotics to prevent NEC has been extensively studied. The World Health Organization define probiotics as “live micro-organisms, which, when administered in adequate amounts, confer a health benefit on the host” (189). Even if several randomized controlled trials have shown conflicting results, different meta-analyses have demonstrated a statistically significant positive effect of probiotics in both preventing NEC and reducing all-cause mortality (190-192). Among the many different probiotic strains that have been tested, the European Society for Paediatric Gastroenterology Hepatology and Nutrition Working Group for Probiotics and Prebiotics has now recommended: *Lactobacillus rhamnosus* GG ATCC53103 or the combination of *Bifidobacterium infantis* Bb-02, *Bifidobacterium lactis* Bb-12, and *Streptococcus thermophilus* TH-4 (59). At the same time, there is still not enough evidence for the use of probiotics in infants with a birthweight of less than 1000 g (193). The use of lactoferrin supplementation has also been studied for the prevention of NEC. Lactoferrin seems to decrease late-onset sepsis but not NEC stage II or III in preterm infants (194) and so, at this point, it cannot be recommended for NEC prevention.

### **3 HYPOTHESIS AND AIMS**

The overall aim of this project was to describe the clinical and radiological characteristics of NEC patients, to assess the risk of developing NEC using NIRS in extremely preterm infants and, finally, to study how infants who develop severe NEC or need surgical treatment can be identified early, using new imaging techniques or biochemical status.

#### **3.1 SPECIFIC AIMS:**

- 1- To examine the effect of gestational age on the clinical and radiological presentation of NEC (Paper I).
  
- 2- To study the role of NIRS as a tool to identify patients with a high risk of NEC (Paper II).
  
- 3- To study the correlation between sonographic findings and outcomes in NEC patients (Paper III).
  
- 4- To assess if hyponatremia, or a decrease in plasma sodium levels, at the onset of NEC, is a marker for intestinal inflammation/ischemia requiring surgery (Paper IV).

## 4 STUDY DESIGN AND METHODS

### 4.1 STUDY DESIGN AND POPULATION

The study design and population used in the included studies are described below and summarized in Table 4.

Paper	Study	Study design	Population and setting	N of subjects
I	NEC and gestational age study	Retrospective observational study	NEC patients treated in Stockholm from 1 <sup>st</sup> January 2009 to 31 <sup>st</sup> December 2014	89
II	NIRS study	Prospective cohort study	Extremely preterm infants born at the Karolinska University Hospital from 1 <sup>st</sup> September 2014 to 31 <sup>st</sup> December 2016	45
III	Abdominal ultrasound study	Retrospective observational study	NEC patients who underwent abdominal ultrasound from September 2010 to August 2013 at the Karolinska University Hospital	25
IV	Hyponatremia study	Retrospective cohort study	NEC patients treated in Stockholm from 1 <sup>st</sup> January 2009 to 31 <sup>st</sup> December 2014	88

**Table 4:** Study design and population for each study

#### **4.1.1 Papers I and IV: Population and setting**

In *Papers I* and *IV* the same study population was used. All neonates treated in Stockholm County Council (SLL) from 1<sup>st</sup> January 2009 to 31<sup>st</sup> December 2014 with a NEC diagnosis (P.779, according to ICD-10) were identified in the Swedish National Quality Register (SNQ). Data were recorded manually and prospectively from the medical records to validate the diagnosis. All infants included in the study were admitted to a NICU level II or higher, in community or academic settings. Infants were included in the study if they had a verified NEC diagnosis ( $\geq$  Bell's stage II). If the diagnosis was uncertain, one fellow in neonatology and one experienced neonatologist reviewed the patients' charts. If radiological findings were uncertain, the radiographs were reviewed by an experienced pediatric radiologist. Infants with NEC Bell's stage  $<$  II were not considered to have NEC and were hence not included in the study. Patients with major abdominal malformations or with spontaneous intestinal perforations were also excluded (101, 170). All patients who required surgery were treated in the same center, the Karolinska University Hospital in Stockholm. In Paper IV, only patients with verified NEC combined with available data on plasma sodium at NEC onset were included.

#### **4.1.2 Paper II: Population and setting**

This was a prospective cohort study. Extremely preterm infants, born before 28 weeks of gestational age and admitted to the neonatal intensive care unit of the Karolinska University Hospital, Stockholm, from September 2014 to December 2016, were enrolled. Exclusion criteria were infants born before 23 weeks of gestation, major abdominal wall defects, major known intra-abdominal malformations and critically ill neonates who did not tolerate abdominal ultrasound or NIRS monitoring. The infants were included during the first six days of life.

#### **4.1.3 Paper III: Population and setting**

In Paper III, all neonates with a verified NEC (Bell's stage  $\geq$  II), admitted to the Department of Neonatology at the Karolinska University Hospital, Stockholm, Sweden, from September 2010 to August 2013, were identified. Only patients who underwent at least one AUS and plain radiography the same day were included in the study. Patients with pneumoperitoneum (free gas) were excluded since pneumoperitoneum, in itself, is an indication for surgical treatment.

## **4.2 CLINICAL VARIABLES**

The demographic, clinical and radiological data, laboratory findings and histology reports were manually collected from medical charts. The demographic and clinical data collected were: gestational age, birth weight, maternal and delivery-related information, IUGR, intraventricular hemorrhage, patent ductus arteriosus, respiratory distress syndrome and clinical findings at NEC onset (increased and/or bilious aspirates, feeding intolerance, blood in stool, abdominal erythema, abdominal distension). The radiological data (such as gasless abdomen, portal venous gas, fixed loop, pneumatosis intestinalis, ileus, and pneumoperitoneum) were collected from reports and, when needed, images were reviewed as well. Histological reports were mainly from surgical specimens and autopsy. Laboratory findings included: CRP, platelets count, plasma lactate, plasma glucose, plasma sodium and creatinine. The clinical variables of main interest are listed in Table 5.

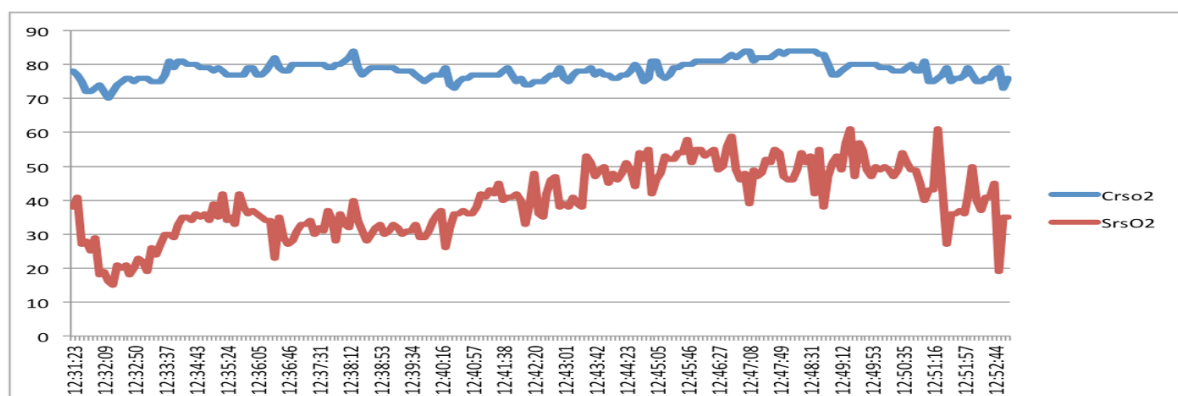
In Paper II, the risk of NEC was the primary outcome and age at full enteral nutrition was our secondary outcome. In Paper III, the need for surgery and death were listed as outcomes. In Paper IV, the risk of severe NEC was the primary outcome and hyponatremia was the exposure.

Clinical variables	Definition	Paper
<b>NEC diagnosis/verified NEC</b>	Bell's stage $\geq$ II, based on the presence of pneumatosis intestinalis on abdominal X-ray, visual inspection at laparotomy, and/or histological evidence of NEC. Infants with spontaneous intestinal perforation were also excluded.	I-IV
<b>Surgical NEC/need for surgery</b>	When the neonate underwent surgical treatment, and/or peritoneal drainage. Used as a proxy parameter for bowel gangrene or perforation.	I and III
<b>Age to full enteral nutrition</b>	The first day when the patient tolerated full enteral nutrition, without any parenteral nutrition.	II
<b>Pneumoperitoneum</b>	Free gas at the abdominal radiography.	I,III,IV
<b>Pneumatosis intestinalis</b>	Intramural gas at the abdominal radiography or at the abdominal ultrasound in Study III.	I, III
<b>Gasless abdominal radiography</b>	Absence of intraluminal gas at the abdominal radiography.	I
<b>Bloody stools</b>	The presence of bloody stools reported in the medical chart.	I
<b>Bilious gastric aspirates</b>	The presence of bilious gastric aspirates causing the withdrawal of enteral nutrition reported in the medical chart.	I
<b>Hyponatremia</b>	A plasma sodium (P-Na) $<$ 135 mmol/L at the onset of NEC.	IV
<b>Difference in P-Na (<math>\Delta</math>Na)</b>	The difference in P-Na obtained before and at NEC onset.	IV
<b>Severe NEC</b>	The need for intestinal resection and/or NEC-related death within 2 weeks of the onset of NEC.	IV

**Table 5:** Clinical variables and definitions.

### 4.3 NEAR INFRARED SPECTROSCOPY AND NIRS DATA, PAPER II

In Paper II, NIRS technology was used for monitoring splanchnic oxygenation ( $SrSO_2$ ) during the first week of life. The hypothesis was that low splanchnic oxygenation was associated with a higher risk of NEC. A single NIRS monitoring was performed at postnatal age 2 to 6 days, within 96 hours after the first enteral feeding. The infants included underwent an abdominal ultrasound to help position the sensors for splanchnic NIRS monitoring. The study protocol details are described in Paper II. We used an INVOS-5100c (Medtronic™, USA) monitor with neonatal INVOS sensors to measure cerebral and splanchnic  $rSO_2$  (Figure 2). The measurements were performed for one hour. High-quality recordings for at least 20 consecutive minutes after the baseline recording were required to be included in the analyses. NIRS data were analysed for periods of artefacts. Artefacts were defined as changes in  $rSO_2$  that could not be physiologically explained (e.g., a 30% change between 2 subsequent data points) or missing  $rSO_2$  values because of measurement failure. Values of 15% and 95% (the minimum and maximum values for INVOS) from our analyses were not excluded. To determine whether low mean  $SrSO_2$  values predicted the onset of NEC, mean values from the NIRS measurements, which were obtained between 48 and 144 hours after birth, were used. The mean  $rSO_2$  (cerebral and splanchnic) with standard deviation was calculated for each measurement. The splanchnic cerebral oxygenation ratio (SCOR) was calculated as splanchnic oxygenation divided by cerebral oxygenation ( $SrSO_2/CrSO_2$ ). Unless a documented misplacement of the sensor occurred or the measurement was shorter than 20 minutes, all these NIRS data were included in the analysis. Cut off for mean  $SrSO_2$  was based on the receiver operating characteristic curve analysis. Infants who developed verified NEC and those who did not develop NEC were compared for different explanatory variables.



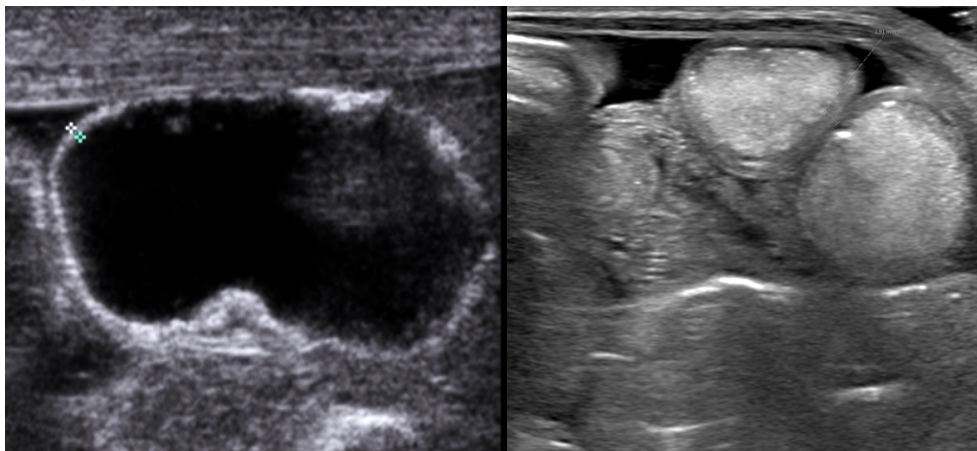
**Figure 2:** Example of cerebral (blue) and splanchnic (red) oxygen saturation in a stable extremely preterm newborn during enteral nutrition



#### 4.4 ABDOMINAL ULTRASOUND, PAPER III

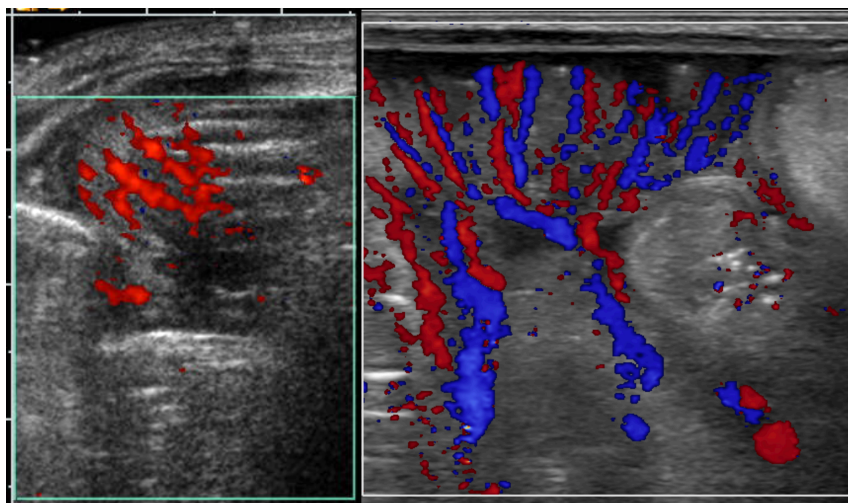
In Paper III, the association between sonographic signs and outcomes (need of surgical intervention or death in NEC patients) was studied. All US examinations were performed according to our local protocol, which is based on the technique discussed by Faingold et al (119). All four abdominal quadrants were evaluated using vector and linear array transducers functioning between 8 and 15 MHz. An experienced pediatric radiologist reviewed all the patients' sonograms. The US features were categorized into eight findings and are shown in the images below:

- Bowel wall thickness, measured by calipers, normal values between 1.1 and 2.6 mm. (Figure 3)



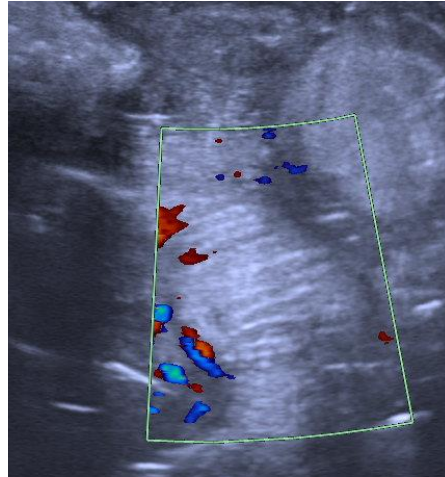
**Figure 3:** US images in grey scale. On the left, a bowel loop with thin bowel wall. On the right, two bowel loops with bowel wall measured with calipers, 1.9 mm.

- Bowel perfusion, with a velocity setting of 0.09 m/s, was considered increased when there was an increased color flow signal (in a loop signal). Increased perfusion patterns: ring or Y or zebra patterns of flow. Evaluation was generally subjective. (Figure 4)



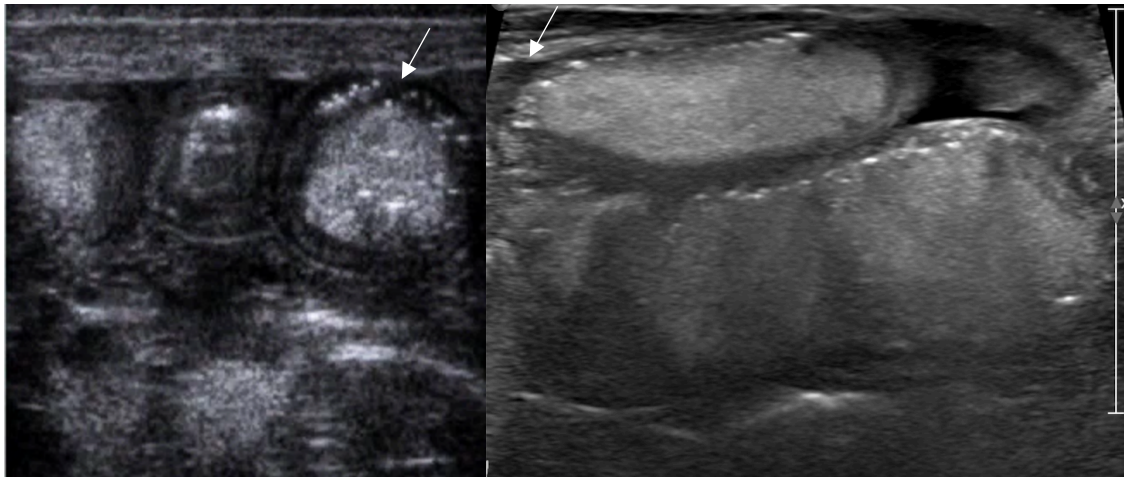
**Figure 4:** US images, both demonstrating hyperemic bowel perfusion with zebra patterns.

- Bowel perfusion was considered absent when no flow was detectable within the bowel wall on Doppler sonography after decreasing the velocity setting. Evaluation was subjective, as above. (Figure 5)



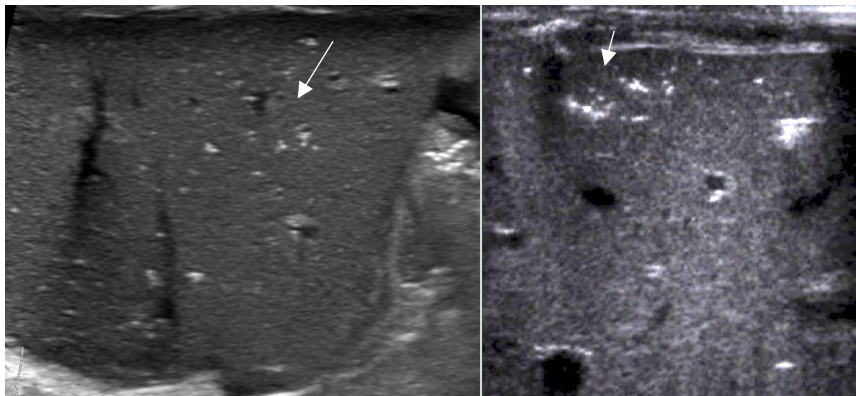
**Figure 5:** *US image, bowel loop with non-detectable Doppler flow.*

- Pneumatosis intestinalis was considered to be present when there were punctuate or granular increased echogenic foci within the bowel wall (195). (Figure 6)



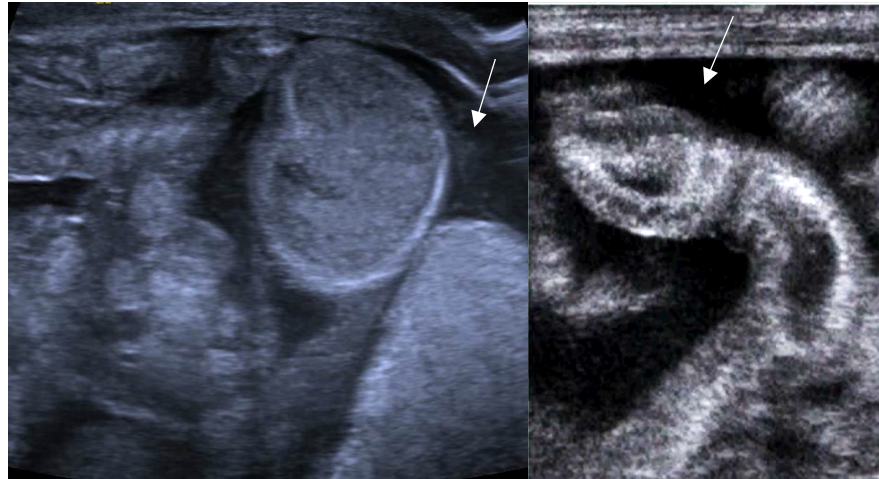
**Figure 6:** *US images in grey scale showing pneumatosis intestinalis (arrow) in the bowel wall.*

- Portal venous gas was considered present when punctuate echogenic foci were noted within the main portal vein or its intrahepatic branches.(Figure 7)



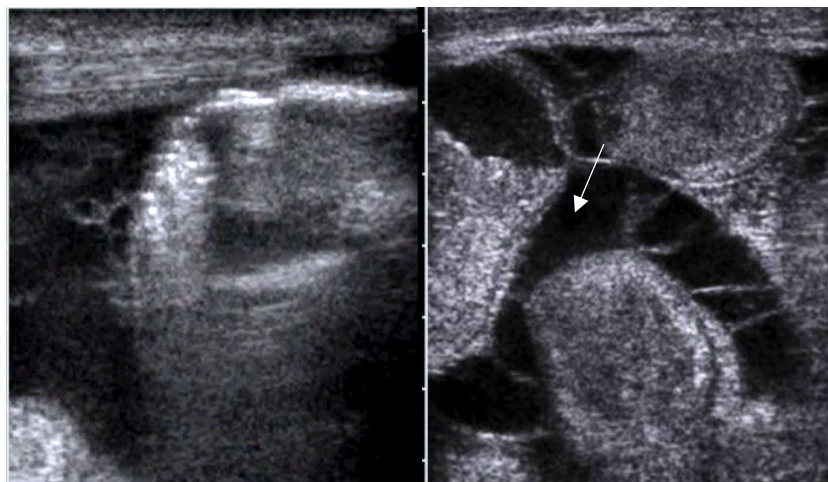
**Figure 7:** *US images of liver parenchyma in grey scale with portal venous gas (arrow).*

- Anechoic free peritoneal fluid. (Figure 8)



**Figure 8:** *US images in grey scale showing bowel loops and surrounding anechoic fluid (arrow).*

- Focal fluid collections were considered complex when echogenic material or septa were noted. (Figure 9)



**Figure 9:** *US images in grey scale. Complex fluid collections with septa (arrow).*

- Peristalsis was assessed according to the presence or absence of bowel contractions on a cine sequence.

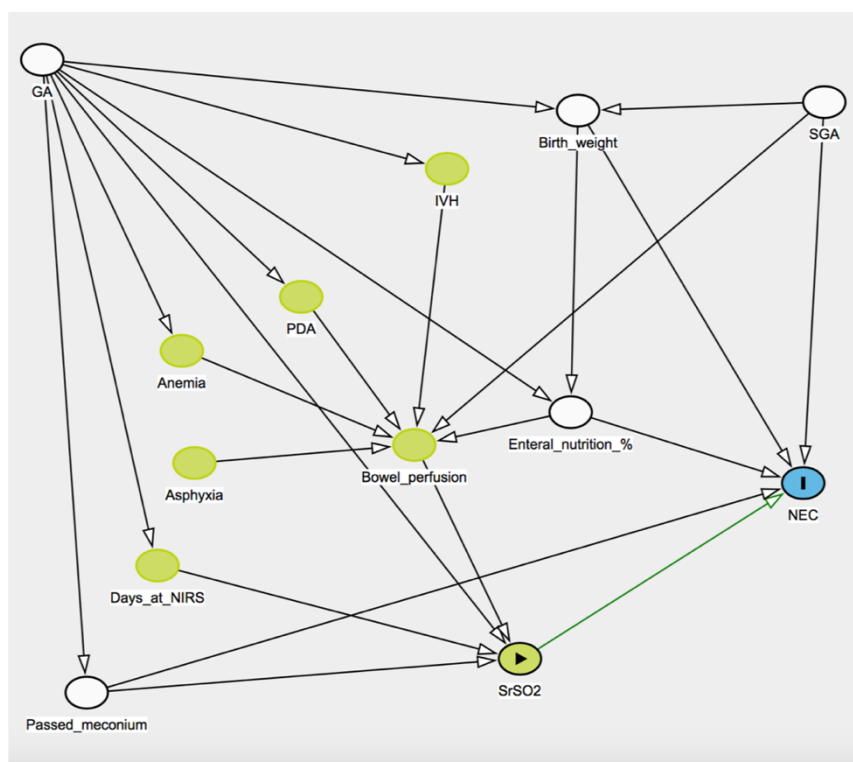
## 4.5 STATISTICAL ANALYSIS

### 4.5.1 Directed Acyclic Graph

Directed Acyclic Graphs (DAGs) are graphs that simplify the interpretation and communication in causal inference and are used for covariate selection in observational studies (196). DAGs were used to identify possible confounders that could induce a non-causal association between our variable of interest and outcome in our cohort studies. Each arrow represents a causal influence.

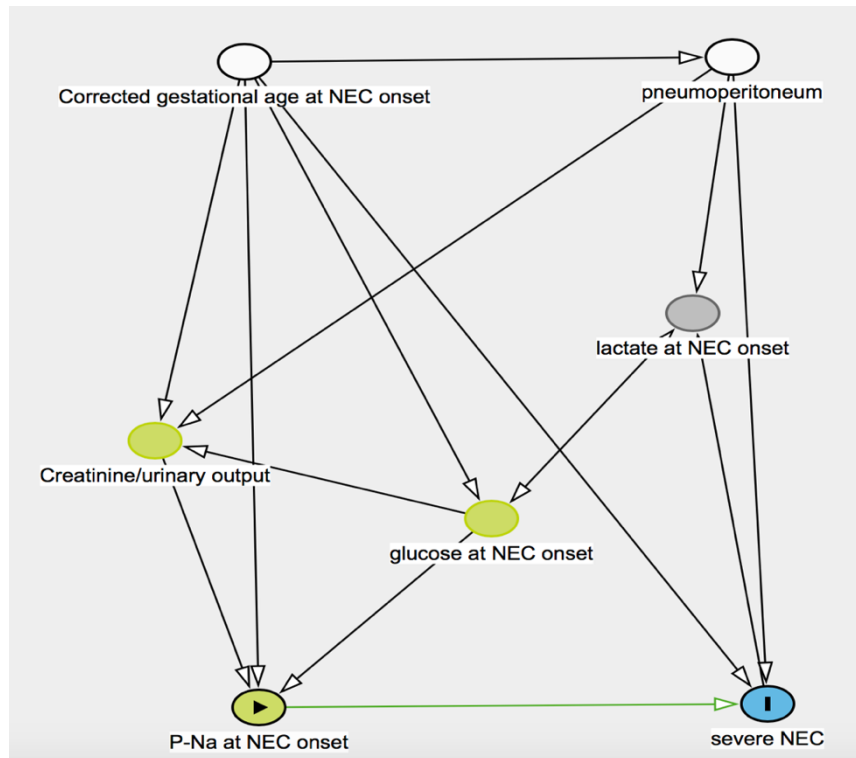


In Paper II, we wanted to identify possible confounders between mean splanchnic oxygenation (SrSO<sub>2</sub>) and risk of NEC (Figure 10). In white are the confounders that could induce a non-causal association between mean SrSO<sub>2</sub> and risk of NEC according to the DAG.



**Figure 10:** The DAG for Paper II. In white are the confounders that could induce a non-causal association between exposure and outcome (blue) according to the DAG, (GA, gestational age; PDA, patent ductus arteriosus; SGA, small for gestation age; IVH, Intraventricular hemorrhages).

The DAG for Paper IV is shown below (Figure 11). We wanted to identify possible confounders between hyponatremia at NEC onset and risk of severe NEC. In this paper we used a DAG to see the relationship between the variables. Hyponatremia was investigated at NEC onset as an intermediate step in the causal way, or biologically speaking in the pathophysiology, of severe NEC.



**Figure 11:** The DAG for Paper IV. Severe NEC is the outcome (in blue). In white are the confounders that could induce a non-causal association between hyponatremia and risk of severe NEC, (P-Na, plasma sodium concentration).

#### 4.5.2 Statistical analysis

Papers II and IV are designed as cohort studies where a “population at risk” is followed over time. The risk is, respectively, to develop NEC or severe NEC. Therefore, a measure of effect was chosen to present the results. Generalized linear models were applied with respectively log and logit link to analyse the primary outcome and presented as, respectively, the risk ratio of NEC and the odds ratio of severe NEC. For the secondary outcome in Paper II, age at full enteral nutrition, a Cox regression analysis was performed and presented as a hazard ratio (HR). Time had an important role in the secondary outcome of Paper II. Therefore an instantaneous effect measure such as HR was chosen instead of a cumulative effect measure such as risk ratio (RR) or odds ratio (OR). In Paper IV, a measure of effect (OR) instead of the accuracy of a test was presented for two reasons; firstly, the retrospective design of the study could easily induce selection bias, secondly it is not possible to study a continuous variable as such (for example plasma sodium) when presenting sensitivity and specificity. In all the papers, the Mann-Whitney U-test, Student's t-test and chi-square or Fisher's exact test were used, whenever appropriate, to compare clinical characteristics between patients who developed NEC and those who did not, or between infants who needed surgery or not.

A p-value <0.05 was considered statistically significant. The statistical program STATA version 14.2 (StataCorp, College Station, TX, USA) was used to perform the analyses.

### 4.5.3 Sensitivity analysis

In all four papers, the patient sample size was quite small. Because a small sample size can easily induce significant associations or results that are based, for instance, on a single patient multiple sensitivity analyses were performed, especially in Papers II and IV to test the robustness of our results.

## 4.6 ETHICAL APPROVAL

The ethical approval numbers are shown in Table 6.

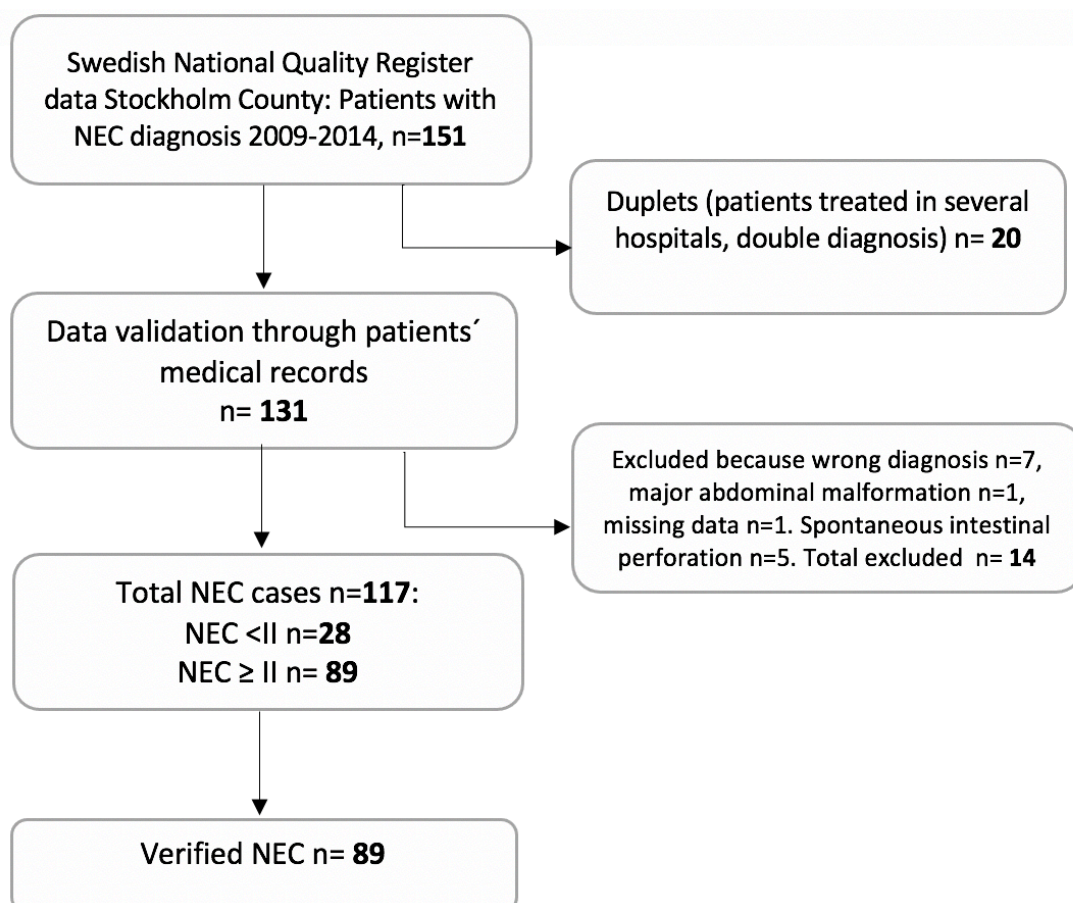
Paper	Ethical approval number
I	2015/2144-31/1
II-III	2014/500-31
IV	2017/1237-31, 2019/06289

**Table 6:** *Ethical approval numbers.*

## 5 RESULTS

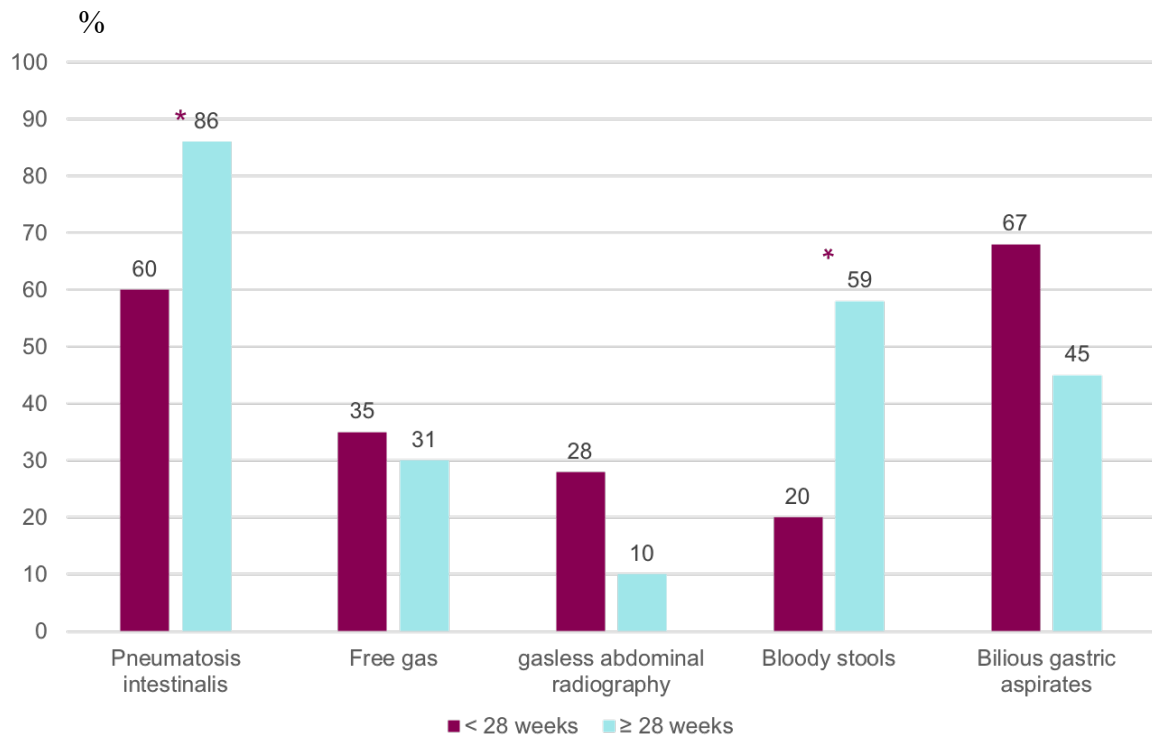
### 5.1 THE EFFECT OF GESTATIONAL AGE IN NECROTIZING ENTEROCOLITIS (PAPER I)

For Paper I, 131 medical charts and radiographic images were reviewed of infants with a NEC diagnosis. (Figure 12)



**Figure 12:** Flow chart

Eighty-nine patients (median gestational age 26.3 weeks, IQR 24.9-29) were considered to have verified NEC and were thus included in the study. Most of them were males (67.4%) and delivered by cesarean section (65%). Sixty infants were born at < 28 weeks GA and 29 infants were born at  $\geq 28$  weeks GA. Fifty-seven patients underwent surgical treatment and more than 70% of them were extremely preterm. As expected, extremely preterm infants developed more complications related to prematurity compared with the more mature infants, IUGR/SGA was, on the other hand, more common in NEC patients born at  $\geq 28$  weeks of gestation. Extremely preterm neonates develop NEC symptoms later in life compared with more mature infants. Clinical and radiological data are shown in a graph (Figure 13) and with more details in tables in Paper I.

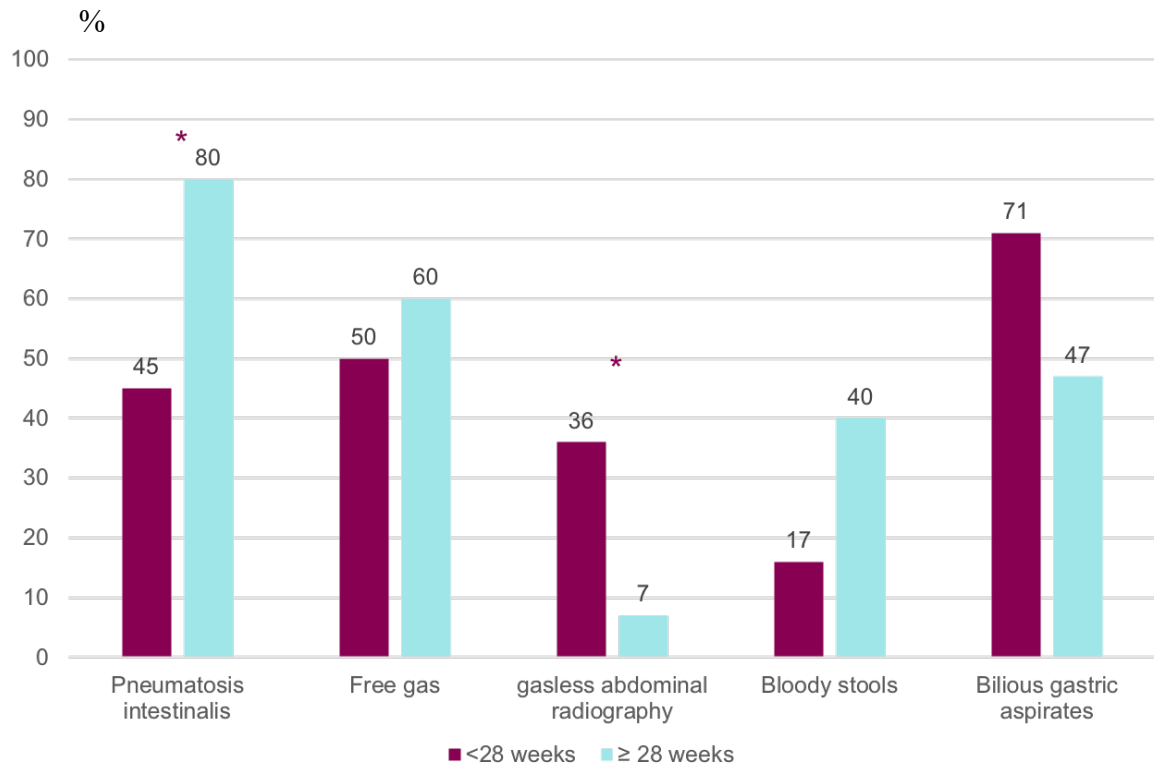


**Figure 13:** Clinical and radiological differences between neonates with NEC, born at < 28 weeks in purple and at ≥ 28 weeks gestational age in blue, \*=  $p < 0.05$ .

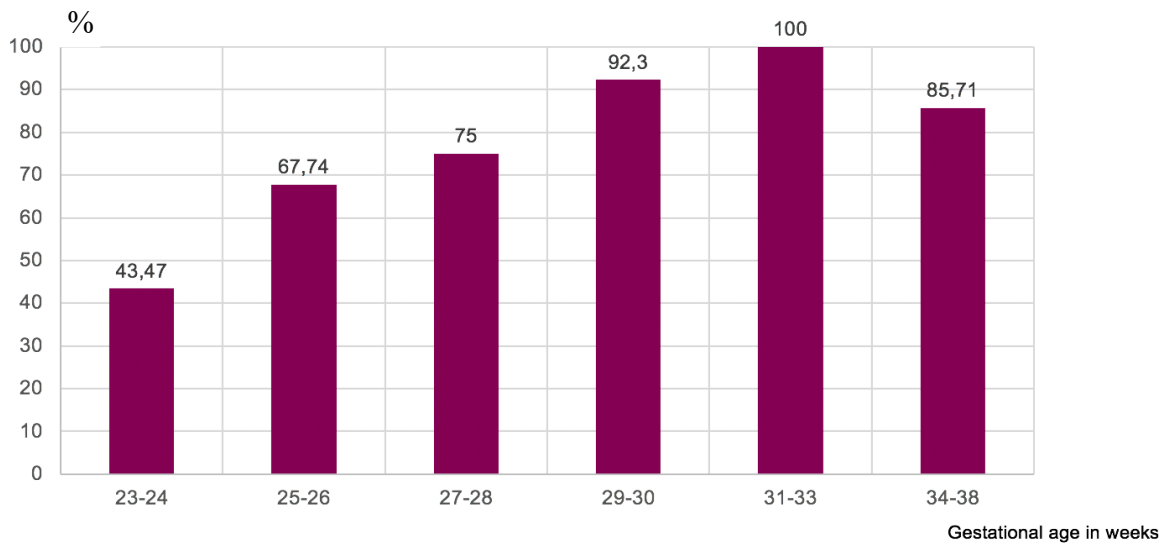
Bloody stools, which are considered quite a typical indication for NEC, were present in only 20.0% of the extremely preterm infants compared with 58.6% of the more mature ones. Pneumatosis intestinalis was detected in only 60.0% of infants born before 28 weeks of gestation vs 86.2% of infants born at ≥ 28 weeks of gestation ( $p=0.013$ ), while this finding was still significant in the subgroup of surgical NEC infants (Figure 14). The relative frequencies of pneumatosis intestinalis per gestational age are shown in Figure 15. Almost 48% of the infants (50% born at <28 weeks vs 40% born at ≥28 weeks) who required surgical intervention did not present pneumoperitoneum at the abdominal radiography. Extremely preterm neonates who required surgery more often presented with a gasless abdomen on abdominal radiographs compared with the more mature neonates.



B



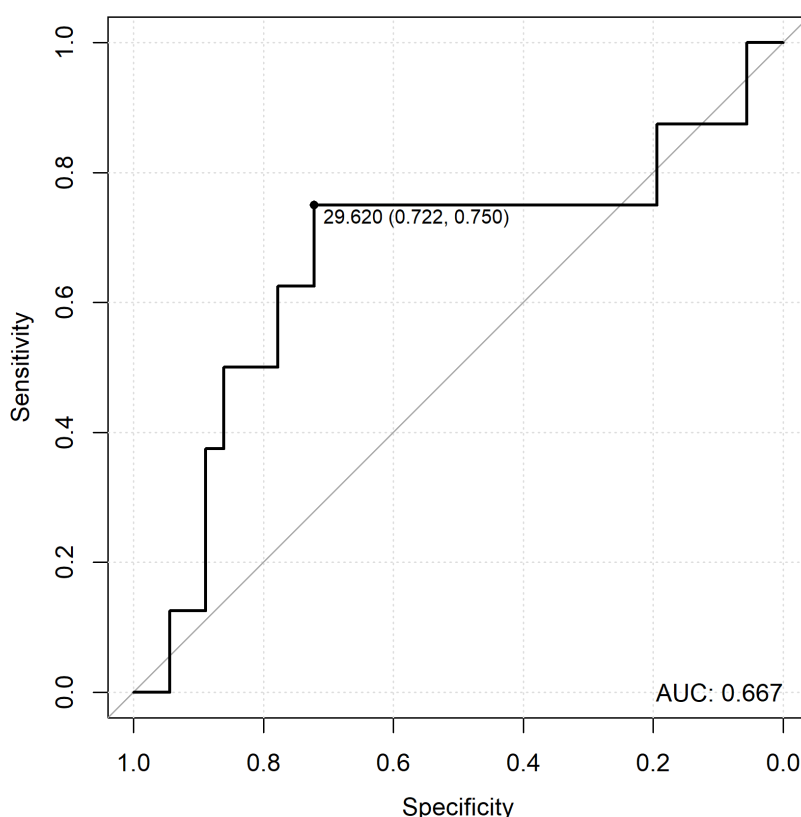
**Figure 14:** Clinical and radiological differences between neonates with surgical NEC, born at < 28 weeks in purple and born at ≥ 28 weeks gestational age in blue.



**Figure 15:** Relative frequencies of pneumatosis intestinal per gestational age.

## 5.2 LOW SPLANCHNIC OXYGENATION AND RISK OF NEC (PAPER II)

Forty-nine infants completed the study and underwent NIRS monitoring. Forty-five patients had complete NIRS data. One infant developed NEC within one day of NIRS monitoring and was therefore excluded from the study. Forty-four patients were included in the final analysis, 61% of the infants were male. Median gestational age was 25.6 weeks (range 23.0-27.9) and median birth weight 698 grams (range 485-1353), 18% of the infants were SGA. Eight infants developed NEC during the neonatal period at a median postnatal age of 15 days (range 6-35 days) and six of them required surgery. The mean age at full enteral nutrition was  $11.7 \pm 4.6$  days. We defined a cut off of 30% for SrSO<sub>2</sub> which was supported by the receiver operating characteristic curve analysis, shown in Figure 16.



**Figure 16:** Receiver operating characteristic curve for splanchnic oxygenation (SrSO<sub>2</sub>) and risk of NEC. AUC= area under the ROC curve

Sixteen infants had a mean SrSO<sub>2</sub> < 30% in total: 75.0% of the infants who developed NEC vs 27.8% of the infants who did not (p=0.019). All infants who developed surgical NEC had a mean SrSO<sub>2</sub> <30%. There was no difference in median SrSO<sub>2</sub>, CrSO<sub>2</sub> or SCOR between the two groups. The risk ratio to develop NEC was assessed using a generalized linear model analysis, with NEC as the dependent variable. Infants with a mean SrSO<sub>2</sub> < 30% had a higher risk of developing NEC compared with those with a mean SrSO<sub>2</sub> >30% (Crude RR 5.25, 95% CI 1.19-23.01). Adjusted risk ratios for gestational age, birth weight, SGA, days at NIRS

monitoring, passed stool and CrSO<sub>2</sub>, still showed a significant positive association between SrSO<sub>2</sub> < 30% and NEC.

Since all infants who developed NEC had obtained more than 60 ml/kg/d of enteral feeds, a sub-analysis in this subgroup was performed showing that infants with a mean SrSO<sub>2</sub> < 30% had a still higher risk of developing NEC compared with those with SrSO<sub>2</sub> > 30% (Crude RR 6.00, 95% CI 1.61-22.42).

No statistically significant association was seen between age at full enteral nutrition and SrSO<sub>2</sub> (HR 1.05, 95% CI 0.57-1.96), even when adjusted for gestational age or presence of a patent ductus. A sub-analysis excluding infants born at a gestational age of 26 and 27 weeks was performed showing that the risk of NEC was still significantly higher in infants with a mean SrSO<sub>2</sub> < 30%, but no correlation was found between age at full enteral nutrition and SrSO<sub>2</sub>.

### **5.3 ABDOMINAL ULTRASOUND AND NEED FOR SURGERY (PAPER III)**

Thirty-six out of 58 screened patients (62%) with a verified diagnosis of NEC underwent AUS and plain abdominal radiography on the same day. Eleven patients were excluded; five because of missing data and six because they developed free gas, thus presenting an absolute indication for surgery. Twenty-five patients, with a median gestational age of 25.6 (range 23-35) weeks were included in the study, 72% of them were male. Eleven patients underwent surgery and five patients died (only one of which did not undergo NEC surgery). We did not find any association between surgery and bowel wall thickness, absent peristalsis, free fluids, increased or absent bowel flow and intramural or portal gas. Absent flow at Doppler sonography and portal gas were actually only present in infants who required surgery. Only the sonographic finding of complex fluid collections was more frequently present in infants who underwent surgery compared with those who did not (7.14% vs 63.6%, p=0.007). There was no significant difference between NEC survivors and those who died in any of the sonographic findings studied.

### **5.4 HYPONATREMIA AND RISK OF SEVERE NEC (PAPER IV)**

Eighty-nine patients with verified NEC were identified as described in Paper I. Since no data on plasma sodium were found for one patient, only eighty-eight infants were included in the study. Median gestational age was 26.2 (range 23-38.4) weeks, 60 out of 88 patients were extremely preterm infants and 57 out of 88 male. Fifty-four infants had severe NEC and 34 had non-severe NEC. Infants who developed severe NEC had a younger postnatal age at NEC onset than those who had non-severe NEC, but this difference was attenuated when infants with pneumoperitoneum were excluded. For infants with severe NEC, median time to surgery or

death from NEC onset was 1 day (IQR 0-2). Hyponatremia was very common in both severe and non-severe NEC, but P-Na at NEC onset was significantly lower in the severe NEC group (130 vs 134.5 mmol/L,  $p=0.0016$ ). Platelet counts were lower in the severe NEC group and plasma lactate concentration was slightly higher. The lactate difference was even higher in the sub-analysis excluding infants with pneumoperitoneum. P-Na before NEC onset did not differ between the two groups. We assessed the odds ratio of developing severe NEC with a generalized linear model analysis. Infants with hyponatremia had a higher odds ratio of developing NEC compared with those with normal P-Na (crude OR 3.91, 95% CI 1.52-10.04,  $p=0.005$ ). Our results also showed that the more the plasma sodium had dropped at NEC onset, the higher the odds ratio of developing severe NEC (crude OR 1.19, 95% CI 1.07-1.33,  $p=0.002$ ). Adjusted odds ratios for corrected gestational age and creatinine value were still significant. The sub-analysis excluding infants with pneumoperitoneum showed that infants with hyponatremia had an even higher odds ratio of developing NEC compared with those with normal P-Na (crude OR 23.0, 95% CI 2.78-190.08,  $p=0.004$ , adjusted OR 21.8, 95% CI 2.52-188.03,  $p=0.005$ ). This positive association was still consistent for the sodium difference ( $\Delta$ Na crude odds ratio 1.24, 95% CI 1.07-1.43,  $p=0.004$ , adjusted OR 1.24, 95% CI 1.06-1.43,  $p=0.005$ ) in this subgroup. Because extremely preterm newborns were more likely to have pre-existing hyponatremia (40% vs 21%,  $p=0.087$ ), we controlled if our findings were still consistent in this subgroup and found that the hyponatremia crude OR was 4.27 (95% CI 1.34-13.55,  $p=0.014$ ), and  $\Delta$ Na crude OR was 1.22 (95% CI 1.05-1.41,  $p=0.010$ ).

## 6 DISCUSSION

### 6.1 THE EFFECT OF GESTATIONAL AGE (PAPER I)

Extremely preterm infants show a different clinical and radiological presentation of NEC compared with more mature infants, often failing to present features known to be typical for NEC, such as pneumatosis intestinalis and bloody stools. Our findings show that the neonatal intestine's response to NEC seems to be affected by the degree of intestinal maturity. In this cohort, extremely preterm neonates represent two thirds of NEC cases and 74% of the surgical NEC cases. As expected, extremely preterm infants developed NEC symptoms at an older postnatal age compared with the more mature neonates (19, 93). Many factors can explain this; the differences in immunity maturity, the small volume of trophic feeding, the delayed microbial colonization of the intestine, and the use of broad spectrum antibiotics (1). IUGR/SGA was more common in the late preterm-term group. As has been previously discussed, term NEC more often occurs in infants with pre-existing risk factors. Bloody stools, a sign of colitis, is three times more frequent in the late preterm-term group than in the extremely preterm group, as already described by other authors (93, 97). The delayed gastric emptying, longer transit of nutrients, and reduced bowel motility are possible reasons for the fact that NEC-colitis is rarely seen in the most preterm infants (94, 197). Extremely preterm infants more often present with signs of ileus or sub-ileus, such as bilious gastric aspirates, feeding intolerance and a gasless abdomen. Gasless abdominal radiographs are actually quite common in the smallest infants with surgical NEC. Studies concerning neonatal intestinal motility patterns have shown that preterm infants react to feeding with decreased motor activity (called an immature response), resulting in a less efficient propulsion of nutrients from stomach to colon (94). At the radiographs, the extremely preterm group had a lower rate of pneumatosis intestinalis compared with the late preterm-term group, even considering that pneumatosis intestinalis was actually a requirement for medical NEC in order to be included in this cohort. This difference still remains significant in the surgical NEC subgroup. Pneumatosis intestinalis is caused by bacterial fermentation within the bowel wall and is a transient sign of bacterial translocation (109, 110). Extremely preterm infants have thin immature bowel walls, immature tight junctions and tend to receive small volumes of trophic feeds for several days (93, 198, 199). Considering the low sensitivity of plain abdominal radiographs it comes as no surprise that this radiological sign is more difficult to detect in this particular group (93, 200). On the other hand, the presence of large amounts of nutrients in the bowel leading to bacterial fermentation in the more mature infants likely accounts for the presence of pneumatosis intestinalis as a radiological sign of NEC in this group (93). Half of the infants who required

surgical intervention did not present with pneumoperitoneum at the radiographs. The fact that failing medical treatment was the relative indication for surgery in these cases highlights how challenging the decision to operate on NEC patients can be. Considering that more than 40 years have passed since Bell et al described the clinical stages of NEC (11) it is hardly surprising that the NEC patient population has changed. The lack of specific signs of NEC in extremely preterm infants makes early diagnosis, especially in this group which currently represents the majority of NEC patients, clearly problematic. Acknowledging this fact evokes two thoughts; first, so far, prevention is probably the best strategy to reduce NEC consequences and, second, we need to be better equipped to identify infants who require surgical treatment.

## **6.2 LOW SPLANCHNIC OXYGENATION AND RISK OF NEC (PAPER II)**

In Paper II we tested if NIRS could be helpful in the identification of extremely preterm infants with impaired splanchnic perfusion and therefore at high risk of developing NEC. We found that low mean splanchnic oxygenation ( $SrSO_2 < 30\%$ ) in the first week of life is associated with an increased risk of developing NEC in extremely preterm newborns receiving enteral nutrition. The regulation of bowel perfusion in neonates changes over time and initially depends mainly on oxygen demands (76, 201). Nowadays, splanchnic microcirculation impairment and enteral nutrition are acknowledged to be important factors in the pathogenesis of preterm NEC (64, 75, 83). Several studies have assessed NIRS as a non-invasive tool for the continuous monitoring of splanchnic oxygenation as a measure of intestinal perfusion in preterm infants (158, 202, 203). Kuik et al reported that preterm infants with both low gestational and postmenstrual age were not able to increase their splanchnic oxygenation after bolus feeding, which was postulated to be essential in order to meet metabolic demands (204). Our data show that the risk ratio of developing NEC is significantly higher in infants with one-hour mean  $SrSO_2 < 30\%$  during enteral feeding in the first week of life. In accordance with our results, other authors have reported lower  $SrSO_2$  in infants who develop NEC or feeding intolerance compared with those who did not (80, 160). This positive association was even stronger when adjusted for gestational age. We had fewer infants born in weeks 26 and 27 of gestation in our cohort than expected, and a selection bias could not be ruled out. In order to see if our results were still consistent, we performed a sub-analysis excluding infants born at GA 26 and 27 weeks and we found a significant correlation between mean  $SrSO_2 < 30\%$  and risk of NEC. In our cohort, all infants who developed NEC had been fed more than 60 ml/kg/d, making enteral feeding an important confounder. In a sub-analysis of this subgroup, we did not find any other explanatory variable that could increase the risk of NEC, except mean  $SrSO_2 < 30\%$  and mean  $SrSO_2$  as a continuous variable. Extremely preterm infants are constantly

under stress and feeding causes an increased metabolic demand. It has also been shown that continuous enteral nutrition does not increase  $SrSO_2$ , as bolus feeding does (205, 206). We postulated that low mean  $SrSO_2$  values in the NEC group could be the expression of the maximal oxygen extraction in a very immature bowel. This could explain why only those infants fed more than 60 ml/kg/d with mean  $SrSO_2 < 30\%$  during the first days of life developed NEC while those fed with smaller amounts did not. The local autoregulatory control of intestinal blood flow does not mature until the fifth week after birth (204, 207). An alternative explanation could be that those infants who are already fed more than 60 ml/kg/d at day 4 receive fortifiers earlier which contain cow's milk proteins and increase milk osmolarity, which could be an extra trigger in the development of NEC (208, 209). Advancing enteral feed volumes at lower daily increments does not reduce the risk of NEC or death in extremely preterm newborns, but most studies do not include the very first days of life (35, 210). Monitoring splanchnic oxygenation with NIRS might help the clinician to individualize enteral feeding advancement in the very first days and identify those who are at the highest risk of NEC early.

### **6.3 INDICATION FOR SURGERY IN SEVERE NEC (PAPERS III AND IV)**

The decision to operate on a critically ill extremely preterm infant can be frightening, but early intervention on the necrotic bowel does give the infant a better chance of survival without major complications. Early diagnosis of bowel necrosis is probably the key to improving the long-term outcomes of NEC patients by promoting an earlier transfer to surgical centers and surgery, possibly resulting in salvage of viable bowel (166). In the best case scenario, surgical intervention would occur in infants with irreversible intestinal ischemia, prior to the occurrence of bowel perforation (166, 211). Predicting the progression of bowel inflammation to necrosis and the identification of NEC infants who ultimately will require surgical intervention is very difficult, especially in extremely preterm infants (211, 212). When there is radiographic evidence of pneumoperitoneum it may already be too late and actually this sign is not always present in the case of bowel ischemia (95, 99, 213). In Paper I, we showed that almost 50% of the NEC infants who required surgery did not present with pneumoperitoneum on the plain abdominal radiography. Thus, clinical deterioration despite optimal medical care often becomes a relative indication for surgical treatment, but the timing is still a challenge (165, 166). Many factors can be helpful in the decision-making for surgical intervention.

In Paper III, we found that the sonographic finding of complex fluid collections was associated with the need for surgery. Complex fluid collections are a sign of intraperitoneal fluid with debris levels and septa, which may represent a sign of peritonitis, intestinal necrosis and sometimes occult bowel perforation (104, 124, 200). This finding is in accordance with the current literature (120, 200, 214) and complex fluid collections have been suggested as an indication for surgery in the latest review (121). No significant association was found between absent bowel flow and need of surgery or adverse outcome in our population. However, only infants who required surgical treatment presented with absent bowel perfusion at the abdominal sonography. Faingold et al (119) were the first to report the correlation between bowel necrosis and absence of bowel perfusion at the Doppler sonography. In our study, the small number of patients studied and the fact that patients in our unit are often mechanically ventilated with high frequency oscillatory ventilation (HFOV) can have masked this correlation; the HFOV oscillations generate Doppler-artefacts at the slowest Doppler velocity making the assessment of the intestinal perfusion very difficult. It is possible that we failed to detect absent bowel perfusion in some infants. Absence of peristalsis, free fluid and bowel wall thinning at the AUS were not specific signs of bowel necrosis and were not related to our outcomes, even though these signs were more often present in the population who required surgery. The important role of abdominal ultrasound in the diagnosis and management of NEC is now well acknowledged (120, 121, 126), especially in situations where abdominal radiology is not conclusive.

In Paper IV our main finding was that hyponatremia and/or a sudden decrease in plasma sodium at the onset of NEC is associated with an increased odds ratio for severe intestinal inflammation or ischemia, especially in infants without pneumoperitoneum. We found that for each mmol in plasma sodium decrease at the onset of NEC, the odds ratio for severe NEC increases by 1.2. We wanted to draw attention to infants who are going to require surgery, because they carry the highest mortality and long-term morbidity. Severe hyponatremia or a sudden decrease in plasma sodium could be an early predictor for the failure of conservative treatment in infants with NEC. These findings are in accordance with Tepas et al (135) who described hyponatremia as one of the metabolic derangements to consider when timing surgery in NEC patients. Since hyponatremia is a common condition in preterm infants from the second week of life, we also focused on the decrease in plasma sodium from the level when the infants were in a clinically stable condition. Hyponatremia has been studied as a predictor of tissue ischemia in different surgical conditions (215-219). The exact etiology of hyponatremia in surgical conditions is not well understood. Both hypovolemia and severe inflammation could result in the activation of arginine-vasopressin (AVP) production resulting in water retention and hyponatremia (215, 217). Recent studies show that proinflammatory cytokines may regulate



AVP secretion and the development of hyponatremia (220). P-Na is easily obtained and may often serve as a bedside analysis, available around the clock in all kinds of neonatal units. To know that a greater decrease in P-Na corresponds to an increased risk of severe NEC at NEC onset can be helpful in the clinical management of NEC infants. This is true even for extremely preterm infants where hyponatremia is very common. We decided to exclude infants with pneumoperitoneum in Paper III and perform a sub-analysis in Paper IV for two reasons; first, to exclude cases with misdiagnosed spontaneous intestinal perforation, since SIP has another etiology and, second, because pneumoperitoneum is the universally accepted indication for surgery (95, 221).

## **6.4 LIMITATIONS**

The major limitation throughout this thesis is the small number of patients. This is of course an issue with power, increasing the probability of failing to reject our null hypothesis when false (a type II error). For instance, in Paper III, absent Doppler flow at the abdominal ultrasound was not associated with a need for surgery as reported by other authors, probably because of the small number of patients. In Papers I, III and IV we decided to use validated cases of NEC and not just register data, which would easily have increased the sample size, because of the issues related to the definition and classification of NEC (222, 223). Reviewing medical case records is time consuming, but we wanted to keep the dataset as “clean” as possible from spontaneous intestinal perforation and other acquired intestinal diseases (98). In order to avoid including patients without NEC, we excluded all those with suspected medical NEC who did not have a radiological validation of the disease. We might have therefore excluded medical NEC that did not show radiological signs and, therefore, were considered uncertain. This can explain the numerical imbalance between surgical and medical NEC in this dataset. Bell’s staging system was used to validate the NEC cases because at the time (and still today), it was the most commonly used in the literature. One might argue that Bell’s staging system was actually not meant for case definition (11), that it is not really appropriate with a changing NEC population and that it increases the risk of misdiagnosing SIP for NEC (13, 223). It remains, however, the most used system. The retrospective design is of course a limitation in Papers I, III, and IV. The retrospective design is the reason why we decided not to study the accuracy (specificity and sensitivity) of abdominal ultrasound in NEC patients. To avoid bias (especially selection or verification bias) the study of accuracy of a diagnostic test should be prospective, and where a standard protocol is strictly followed. On the other hand, patients were consecutive and we collected the data prospectively and when this was not possible (as in Paper IV, where NEC cases were

already defined), an independent, second researcher collected the data, for instance on plasma sodium. In Paper II we were not able to invite and include consecutively all the extremely preterm infants born during the study period because, at the time, there were several ongoing clinical studies in our institution addressing the same study population. Missing data on specific variables, such as plasma glucose in Paper IV is, of course, a limitation. Because of missing data, plasma glucose was excluded from our model. In Papers II and IV all the analyses performed are complete case analyses.

There are many limitations related to NIRS measurements and technology. Short and not-repeated measurements as well as the presence of artefacts are the two most important ones. The presence of artefacts due to movements, air and stools may have had an impact on the interpretation of the data. We opted for short measurement durations because it was easier to ensure the quality of the SrSO<sub>2</sub> in the smallest infants and to better estimate when artefacts were caused by movements. An attempt to compensate for artefacts was to adjust for the first passage of stool, because meconium contains substances with a chromophoric effect (224). Further limitations are discussed in detail in Paper II.

## 7 CONCLUSION

The population of NEC patients has changed over time and our findings suggest that the clinical and radiological presentation of NEC depends on gestational age. We believe that the findings in this thesis might help to identify infants with bowel necrosis, especially when the radiological sign of pneumoperitoneum is not present.

- Extremely preterm neonates with NEC do not show the specific clinical and radiological signs of NEC, which can result in doctor's delay for both medical and surgical treatment.
- In order to identify extremely preterm infants with a high risk of NEC early, we tested monitoring splanchnic oxygenation during the first week of life. A mean low splanchnic oxygenation during enteral nutrition seems to be associated with a higher risk of developing NEC in extremely preterm infants. Validation in a larger cohort is therefore needed.
- To help in the decision to intervene surgically, abdominal ultrasound might be a useful tool. Our study found that the sonographic finding of complex fluid collections was associated with the need for surgery.
- Hyponatremia and/or a decrease in plasma sodium at the onset of NEC is associated with the risk of surgical NEC or death.

## 8 FUTURE PROSPECTIVES

NEC has been far from being completely understood and the clinical management probably needs an update. The future NEC staging system should consider the different clinical presentations in infants with different gestational ages and should include abdominal ultrasound in the standard care, as Gordon already suggested in the “two out of three rule” (101, 103, 104, 109, 119, 225). The validation of our findings in the NIRS study in a larger cohort is our next step. We began a collaboration with University Medical Center Groningen, the Netherlands, for a validation of low splanchnic oxygenation as an early marker of impaired microcirculation in order to identify infants who will develop NEC. We think the microcirculation has an important role in the progression of NEC as well.

The emergent technique of Remote Ischemic Conditioning has proven to be helpful in containing the progression of intestinal inflammation and ischemia in animal models (226, 227). In collaboration with Sick Kids in Toronto, where these experimental studies have been performed, we are going to start a pilot study on preterm infants with NEC to assess if remote ischemic conditioning under NIRS monitoring can stop the progression to surgical NEC.

## 9 SUMMARY IN SWEDISH

Nekrotiserande enterokolit (NEC) är en allvarlig tarmsjukdom som framför allt drabbar för tidigt födda barn. Tillståndet kan ge allt från ytliga slemhinneskador med inflammation och svullnad till perforation av tarmen. Trots årtionden av forskning saknas fortfarande avgörande landvinningar som påtaglig förbättrar överlevnaden och minskar de långsiktiga konsekvenserna av NEC. Orsaken till sjukdomen är ännu inte klarlagd. Omogen tarmimmunitet, nedsatt tarm-cirkulation, enteral näringstillförsel och onormal bakterieflora i tarmen är sannolikt viktiga bidragande orsaker. Genom åren har NEC-populationen förändrats med en ökande andel extremt prematura barn. Vanlig slätröntgen av buken anses vara det bästa metoden för bilddiagnostik vid NEC. Dessvärre är slätröntgen inte en tillräckligt specifik metod och flera barn riskerar att behandlas för sent med allvarliga konsekvenser för tarmfunktionen som följd. Det gör att beslutet om och när ett kirurgiskt ingrepp är nödvändigt fortfarande är mycket utmanande. Preliminära studier har visat att ultraljud på tarmen kan upptäcka NEC tidigare än enbart slätröntgen.

Det övergripande syftet med detta projekt var att beskriva de för tidigt födda barn som riskerar att utveckla NEC och hur de som utvecklar svår NEC och behöver kirurgisk behandling kan identifieras tidigt med hjälp av ny bilddiagnostik.

Syftet med arbete I var att beskriva skillnaden i den kliniska och radiologiska bilden vid NEC hos extremt prematura barn jämfört med mer mogna barn. Extremt prematura barn visar inte typiska tecken på NEC, såsom blodig avföring eller intramural gas på röntgen.

Syftet med arbete II var att bedöma abdominell syresättning under enteral näringstillförsel hos extremt tidigt födda barn. Abdominell syresättning mäts tack vare infraröd spektroskopi ("Near Infrared Spectroscopy: NIRS). Med hjälp av en liten sensor kan NIRS detektera blodflödesförändringar i djupare vävnader och beskriver på så sett tarmcirkulationen. Låg genomsnittlig abdominell syresättning, mindre än 30%, under den första levnadsveckan var förknippad med en ökad risk att utveckla NEC.

Syftet med arbete III var att avgöra om det finns en korrelation mellan ultraljudsfynd och sämre kliniska utfall, definierade som kirurgi eller död. Nyfödda med radiologiskt och / eller histopatologiskt bekräftad nekrotiserande enterokolit, genomgick ultraljud av buken. Komplexa vätskesamlingar i buken korrelerade starkt med behovet av kirurgi hos spädbarn med svår NEC.

Syftet med arbete IV var att bedöma om hyponatremi, eller försämring av redan befintlig hyponatremi, i samband med debut av nekrotiserande enterokolit var associerat med operationskrävande tarminflammation och ischemi alternativt ledde till död. Hyponatremi och en plötslig minskning av plasmanatriumkoncentrationen vid insjuknandet i NEC kan vara användbara markörer för svår tarminflammation/ischemi där ett överhängande behov av operation kan förväntas.

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