Necrotizing enterocolitis – classification and two initial steps towards prevention

Sandra Meinich Juhl

This review has been accepted as a thesis together with three previously published papers by University of Copenhagen Nov 15 2016 and defended on Feb 2 2017

Tutors: Gorm Greisen, Karen Krogfelt and Elisabeth Iyore

Official opponents: Jørgen Thorup, Janet Berrington and Steffen Husby

Correspondence: Department of Neonatology, Rigshospitalet, Blegdamsvej 9, 2100 København Ø, Denmark

E-mail: sandrameinich@gmail.com

Dan Med J 2017;64(6):B5362

THE 3 ORIGINAL PAPERS ARE

1. Juhl SM, Hansen M, Fonnest G, Gormsen M, Lambaek I, Greisen G. Poor validity of the routine diagnosis of necrotising enterocolitis in preterm infants at discharge. Acta Paediatr 2017;106: 394-398
2. Petersen SM, Greisen G, Krogfelt KA. Nasogastric feeding tubes from a neonatal department yield high concentrations of potentially pathogenic bacteria— even 1 d after insertion. Pediatr Res 2016;80:395–400
3. Li Y[$], Juhl SM[$], Ye X, Shen RL, Iyore EO, Dai Y, Sangild PT, Greisen G. A step-wise, pilot study of bovine colostrum to supplement the first enteral feeding in preterm infants (Precolos): Study protocol and initial results Yanqi. Front Pediatr 2017;5:1–9.

[$]Co-first authors

BACKGROUND

Preterm birth, defined as a gestational age (GA) of less than 37 weeks at birth, is a global health issue which occurs in both high-income and low-income countries. The international preterm birthrate is 11.1 % which means that 14.9 million infants are born prematurely every year (1). Ten percent of these infants are born very preterm (28-32 weeks GA) and 5 % are born extremely preterm (before 28 weeks GA) (1). During the last decades, introduction of surfactant has led to increased survival of the extremely preterm infants (2), meaning that a higher proportion survive long enough to develop the complications associated to prematurity, such as intraventricular hemorrhage, bronchopulmonary dysplasia, retinopathy of prematurity and necrotizing enterocolitis (NEC) (3).

NECROTIZING ENTEROCOLITIS

NEC is an acute intestinal emergency which occurs in the neonatal period. It is primarily seen in preterm infants, but may also occur

ABBREVIATIONS

BC: Bovine colostrum
BW: Birth weight
CFU: Colony-forming units
CI: Confidence interval
CoNS: Coagulase negative *Staphylococci*
DM: Human donor milk
EGF: Epidermal growth factor
FWCH: Foshan Women's and Children's Hospital, China
GA: Gestational age
HC: Head circumference
HH: Hvidovre Hospital, Denmark
ICD-10: International classification of diseases version 10
IF: Infant formula
Ig: Immunoglobulin
IGF: Insulin-like growth factor
MALDI-TOF: Matrix-assisted laser desorption/ionization-time of flight
MM: Maternal milk
NEC: Necrotizing enterocolitis
NG-tube: Nasogastric feeding tube
NPV: Negative predictive value
PMA: Postmenstrual age
PPV: Positive predictive value
RH: Rigshospitalet, Denmark
SBMCH: Shenzhen Baoan Maternity and Child Healthcare Hospital, China
SD: Standard deviation
SP: Supplemental paper
SIP: Spontaneous intestinal perforation
TFF: Time to full enteral feeding
TGF-β: Transforming growth factor-beta
TTF120: Time to reach 120 ml·kg^{-1}·d^{-1} of enteral feeding

in hemodynamically compromised term infants (4,5). This thesis focuses primarily on the type of NEC that occurs in preterm infants. During the last 20 years, the proportion of deaths in preterm infants attributable to respiratory causes has decreased whereas the proportion of deaths attributable to NEC has increased (6).

The clinical findings of NEC are divided into abdominal and systemic signs. The abdominal signs are increased volumes and green discoloration of gastric residuals, emesis, gastrointestinal bleeding, increased abdominal circumference, abdominal tenderness and discoloration, and fewer or no abdominal sounds. The systemic signs vary from mild lethargy to rapidly developing clinical deterioration and shock, and the clinical presentation may imitate sepsis (7,8). When NEC is suspected, radiographic abdominal evaluation is conducted, but the pathognomonic signs of bacterial translocation to the intestinal wall, intestinal pneumatosis or portal venous gas, are not always present (7,9). Other radiographic findings such as a fixed bowel loop, gasless abdomen or pneumoperitoneum may be indicators of NEC but are not specific and may also be caused by other conditions (9,10).

Treatment is based on the clinical status of the infant and is defined as either conservative/medical or surgical. Conservative treatment includes withholding of feeds, bowel decompression, medical treatment with antibiotics and parenteral nutrition along with general supportive treatment, whereas surgical treatment consists of laparotomy or peritoneal drain (11). The indications for surgery are most often intestinal perforation or clinical deterioration despite maximum treatment. The extent of the disease can be determined at surgery and varies from local necrosis in a small part of the intestine to total necrosis of the entire intestine. Survival decreases with the extent of necrosis since no cure exists when the entire intestine has become necrotic (12) (**SP-II**). The most common histopathological findings are coagulation necrosis, inflammation, and bacterial overgrowth (13).

Mortality rates are as high as 15-40 % and increase with decreasing GA (14–20). The most fulminant form of NEC can lead to the death of the infant within less than 24 h from the onset of clinical symptoms despite maximal intensive care (16). Furthermore, follow-up studies showed that infants who survived NEC were more likely to suffer from neurodevelopmental impairment and bowel problems later in life than infants born at the same GA without developing NEC (21,22). Accordingly, prevention of NEC has been named one of the most important topics of interest to researchers, clinicians, parents - and infants (11).

DIAGNOSIS AND CLASSIFICATION

Unfortunately, the research community has not been able to make any substantial breakthroughs in the prevention of NEC despite immense research during the last forty years. It has been argued that until NEC is well-characterized and easily distinguished from other disease entities, it will be difficult to understand the pathogenesis of the disease and develop effective prevention strategies (23). At the moment, no specific test to determine if an infant suffers from NEC exists. Most neonatal units and researchers define NEC as stage II and III of Bell's staging system (**Table 1**). The staging system was developed in 1978 (7) and modified in 1987 (8). It was originally suggested as a diagnostic tool that in addition could be used bedside to evaluate the progression of the disease. Today, the staging system is also used as an epidemiologic tool to describe the severity of the disease in retrospect, e.g. at discharge. Despite widespread use, the system has never been validated. It has been criticized for not separating spontaneous intestinal perforation (SIP) from NEC which might pollute NEC databases and impair research with NEC as an outcome (24,25).

Table 1. The modified stages of Bell's staging system, adapted from (7,8,25)

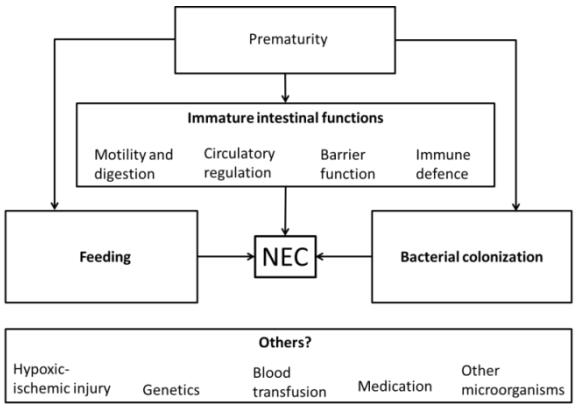
Stage	Abdominal findings	Systemic findings	Radiographic findings
I	Gastric residuals, emesis, occult blood in stool, mild abdominal distention.	Lethargy, apnea, bradycardia, temperature instability.	Normal gas pattern or mild ileus.
IIA	As stage I plus persistent or gross gastrointestinal bleeding or marked abdominal distention.	As stage I.	Ileus with one or more dilated loops, focal pneumatosis.
IIB	As stage IIA plus wall oedema with palpable loops and tenderness.	As stage I + mild metabolic acidosis and thrombocytopenia.	Widespread pneumatosis, portal-venous gas, ascites.
IIIA	As stage II plus worsening abdominal wall tenderness and oedema.	Mixed acidosis, oliguria, hypotension, coagulopathy.	Prominent bowel loops, worsening ascites, no free air.
IIIB	Perforated bowel.	Shock, deterioration in laboratory values and vital signs.	Pneumoperitoneum.

The incidence of NEC in very low birth weight infants admitted to the tertiary neonatal department of Rigshospitalet, Denmark (RH) was 6.0 % during the period 1996-2009 (**SP-I**), however, the incidence varies greatly around the world from 1.6 % in Japan to 10.0 % in the US (20,26–31). The large differences in incidence may not only be caused by differences in treatment strategies but also by discrepancies in diagnosing and classifying NEC. This speculation motivated the conduction of **study I**.

PATHOPHYSIOLOGY

The pathophysiology of NEC is still not completely understood, even though some contributing factors are relatively well-documented. The premature infant is prone to develop NEC because of immature intestinal functions; however being premature also indirectly affects the bacterial colonization and feeding (**Figure 1**). In this thesis, preventive strategies focused on feeding and bacterial colonization, since they were well established factors involved in the pathogenesis of NEC and easier to modify than the immature organ functions.

Figure 1. Pathophysiology of necrotizing enterocolitis (NEC) in the premature infant



Adapted from (32) and modified based on (33–35).

THE IMPACT OF FEEDING

When referring to feeding as a contributing factor to NEC development, both timing, amount and type of feeding may be considered (36). To avoid longer periods of parenteral nutrition and to stimulate the gastrointestinal tract, many preterm infants are given early trophic feeding, also called minimal enteral nutrition, however, the evidence that it improves feeding tolerance, growth outcomes or NEC-rates is lacking (37). Neither the timing of the introduction of progressive enteral feeds nor the advancement has an impact on NEC-rates according to the latest Cochrane reviews (38,39).

In contrast, there is solid evidence that the type of feeding given to preterm infants has a significant impact on NEC rates. Preterm infants fed maternal milk (MM) have significantly decreased NEC rates (40–42), but unfortunately MM is often not available in sufficient amounts to meet the requirements of the infant, especially in the first days of life (40). The current alternatives are preterm infant formula (IF) and pasteurized human donor milk (DM). The most recent Cochrane review concluded that DM was superior to IF in protecting against NEC (43), however DM is still considered inferior to MM (44,45). A double-blind, randomized controlled trial of DM versus preterm IF as a supplement to MM published in 2016 showed no difference in the pooled incidence of NEC, sepsis and death between the two groups, whereas across the groups, receiving more than 50 % MM was associated with a decreased risk of events (46). In studies of growth, preterm infants who received DM did not grow as well as those who received IF or MM (43,47,48), probably because DM is obtained from women late in lactation and therefore contains insufficient amounts of macronutrients for the preterm infant (49,50). Furthermore, the pasteurization of DM leads to a decreased quality of the milk which may lead to impaired uptake of nutrients, e.g. because of decreased fat-absorption due to decreased lipase activity (51,52).

Human and bovine colostrum

In theory, bovine colostrum (BC) may be the answer to the shortcomings of the supplemental diets used for preterm infants when MM is not available. The natural first nutrition for the newborn infant is colostrum which is produced in the mammary gland of the mother during the first 30-42 h after parturition (53). Colostrum contains a higher protein concentration than mature milk but a lower lipid, lactose and energy concentration (54). The protein fraction of milk consists of whey and casein. Colostrum contains mostly whey whereas mature milk contains more casein (**Table 2**). Casein is more slowly digested than whey as it clots at pH-values lower than 4.6 which affects digestion and gastric emptying (55,56). Additionally, colostrum contains a high concentration of bioactive factors involved in growth stimulation, immune modulation and microbial protection, which are only present in low concentrations in mature milk (**Table 2)** (57,58).

The number of factors detected in colostrum has increased with the development of new technologies (57,66). As such a large number of bioactive components are present in colostrum, the benefits are probably not caused by one or a few isolated factors but by the sum of them all; a concept known as food synergy (67). Therefore, only a small selection of the bioactive components will be mentioned here.

Table 2. Comparison of selected factors in the protein fraction of bovine colostrum, human colostrum, and mature human milk

	Bovine colostrum	Human colostrum	Mature human milk
Protein, g/l	51-160	11-32	09-12
Casein, g/l	26	3.0-5.6	3.5-4.4
Whey, g/l	57	4.3-11.1	5.3-6.6
Casein:whey	30:70	10:90	55:45
Whey proteins			
IgG1, g/l	90	0.087	0.010
IgG2, g/l	2.8	0.068	Not detected
IgA, g/l	1.6	8.98	0.68
Osteopontin, mg/l	N.D.	1493	138
Lactoferrin, g/l	1.0-2.0	5.0-7.0	1.0-2.0
Lactoperoxidase, mg/l	11-45	5.17	5.17
Lysozyme, mg/l	0.14-0.7	270-430	160-460
TGF-β, µg/l	150-1150	1366	953
IGF-I, µg/l	49-2000	29-49	3-6
IGF-II, µg/l	400-600	10.5	35
EGF, µg/l	4-324.2	35-438	20-111

Adapted from (59) with modifications based on (54,57,60–65). Casein and whey concentrations of bovine colostrum have been measured as described in Table 1 of **study II**. EGF: Epidermal growth factor. IGF: Insulin-like growth factor. Ig: Immunoglobulin. TGF-β: Transforming growth factor-beta.

Immunoglobulins have antigen-binding capacities and are stable enough to avoid complete digestion in the gastrointestinal tract (68,69). BC is rich in IgG, whereas the most abundant immunoglobulin in human colostrum is IgA **(Table 2)**. In the cow and several other species, IgG cannot pass the placental barrier and the offspring are born hypo-gammaglobulinemic. This leaves them completely reliant on receiving IgG from maternal colostrum which is then passed through the gut into the bloodstream (70). In humans, IgG is transferred via the placenta to the blood; a process which starts in the fetus at a GA of 16 weeks, accelerates from week 22, and reaches maternal levels in week 26 (71). If a newborn infant receives immunoglobulins orally, the beneficial effects may only occur locally in the gastrointestinal tract since immunoglobulins cannot pass from the intestines to the bloodstream in considerable amounts in newborn humans (68,70). Despite the potential antimicrobial properties, immunoglobulins given as oral supplementation to the enteral feeding has not

proven efficient in preventing NEC in the preterm infant population (72).

Lactoferrin has antimicrobial qualities and regulates immune functions (73). The most recent Cochrane review showed that oral supplementation with lactoferrin protected against NEC (74). Lactoperoxidase and lysozyme both have antimicrobial properties (58). Lactoperoxidase is available in higher concentrations in BC than in human colostrum and mature milk, whereas lysozyme is only available in small amounts in BC (**Table 2**). At last, several growth factors, which have the capabilities of stimulating cell growth, differentiation and proliferation (75), are present in higher concentrations in BC and human colostrum than in mature milk (**Table 2**).

Studies of bovine colostrum in preterms

The beneficial effects of whole BC have been intensively studied in an established piglet model of NEC in preterm infants (76). In this model, BC and DM protected equally against NEC compared to IF (77,78). Colostrum from the sow was not better than BC at protecting against NEC in the piglet model (79). Additionally, BC was superior to both DM and IF in stimulating growth, gut immunity and digestive functions (78). The piglet studies furthermore support that whole BC rather than isolated factors should be used, since BC was better at protecting against NEC than formulas enriched with sialic acids, gangliosides or osteopontin (80). Out of safety concerns, BC has to be processed which might lead to a loss of bioactive factors. In the piglet model, pasteurization and spray-drying did not affect the positive effects of BC, including NEC reduction, even though processing, especially pasteurization, reduced the concentration of bioactive proteins (81).

BC has not previously been given as the first nutrition to preterm infants, but it has been used for pediatric patients with short bowel syndrome. In a study of nine infants aged 17-169 months, no positive effect was seen on growth or intestinal function, however no adverse effects were detected either (82). In addition, the authors of the study conducted a randomized pilot study testing the efficacy of BC on intestinal adaptation in newborn infants after intestinal resection (unpublished). Infants older than one week and younger than two months, who required parenteral nutrition for more than one week after intestinal resection, were included. Five infants were allocated to the intervention group, which meant that 50 % of their feeds were replaced by BC for four weeks. One infant terminated the intervention before time because of a clinical suspicion of allergy, but the symptoms continued after cessation of the BC intervention. One infant dropped out due to parental request. The remaining three infants tolerated BC well. Thus, continuing with further studies of BC in this population was determined safe.

Based on the abovementioned literature, **study II** was planned as a pilot study of BC as supplementation to MM in the preterm population. The study would be the initial step towards conducting a large-scale study to determine if the NEC-reducing effects of BC seen in premature piglets were also present when used in premature infants.

THE IMPACT OF MICROBIOTA

Another modifiable factor involved in the pathogenesis of NEC is the bacterial colonization of the gut which has been investigated thoroughly in order to develop NEC-preventive strategies. When an infant is born, colonization with bacteria is inevitable and important. The microbiota of the gut plays a role not only in metabolizing the ingested feeds, but also in the intestinal barrier functions and epithelial growth (83). A heterogeneous population of commensals serves as competition against other bacteria thus protecting the host against colonization with potential pathogens (84). Animal studies have shown that bacteria and the products of bacterial fermentation regulate the production of the mucous layer of the intestine (85), enhance the function of the tight junctions of the epithelium (86), and contribute to the angiogenesis inside the villi (87). Furthermore, the microbiota plays an important role in immunological functions and intestinal homeostasis (88) and may even have an impact on behavior through the gut-brain axis (89,90).

The early colonization

The species found in the microbiota of newborn preterm infants differ markedly between individuals, but their microbiotas share the traits of low complexity and rapid changes (91). The early colonization of a preterm infant is affected by the degree of prematurity, meaning that the immaturity of the intestinal epithelium may independently affect the colonization (92,93). However, preterm infants also experience many external factors which modify the early colonization; delivery by cesarean section (94), admission to a neonatal department (95), treatment with antibiotics (96) and delayed oral feeding (97,98) are some examples.

NEC and microbiota

Bacteria are involved in the development of NEC. This is substantiated by the finding that germ-free mice and piglets treated with prophylactic, enteral antibiotics from the first day of life do not develop NEC (99,100). The immature intestine of mice and humans show dysregulated immunological and inflammatory responses to endotoxins which makes it plausible that bacteria can trigger the development of NEC in the preterm infant (101,102). Some studies have shown that infants who developed NEC had an altered fecal microbiota compared to infants who did not (103–108). The findings, however, did not point in one single direction, and other small studies found no differences between the microbiota of NEC and non-NEC infants (109,110). So despite some outbreaks of NEC being linked to a single species like Cronobacter Sakazakii (111), dysbiosis rather than a single causative pathogen is most likely to precede NEC in the majority of cases.

Since probiotics might prevent the development of dysbiosis, the NEC-reducing effects of prophylactic, orally administered probiotics to preterm infants has been investigated intensively (112–119). Even though the most recent Cochrane review concluded that probiotics had a positive impact on NEC incidence (120), the topic is still controversial. It has been argued that the studies included in the review were too heterogenic according to the probiotic strain, the amount and the timing, and that probiotics may even be detrimental to infants with a birth weight below 750 g due to the risk of sepsis (121). Meta-analyses of the individual strains have been inconclusive so far (122). In 2016, a well-powered, randomized controlled trial published in Lancet showed no effect of *Bifidobacterium breve* on NEC, sepsis or death (116). Hence, many questions regarding strain, dosage and timing remain. In order to answer these questions, knowledge about the bacterial load the preterm infant is subjected to in the first days of life is important.

Nasogastric feeding tubes

Feeding by a tube may contribute significantly to the bacterial load a preterm infant is subjected to in the neonatal department. Since the preterm infant is too immature to breastfeed, the infant is fed by a naso- or orogastric feeding tube from the very first meal until at least 34 weeks corrected GA. The feeding tube is inserted into the mouth or nose of the infant and kept there, thus exposed to body temperature, sometimes subjected to unpasteurized milk, and repeatedly handled by parents and personnel. In many neonatal units, the tube is only renewed once a week or if clotted or displaced. Previous studies found that a biofilm developed on the inside of used nasogastric feeding tubes (NG-tubes) from preterm infants (123–125), and that NG-tubes might be responsible for the exchange of bacteria between the infant and the environment, thereby affecting the early colonization (95,125).

One study of the contamination of NG-tubes showed that formula-fed infants with contaminated feeding tubes suffered from NEC more often than formula-fed infants with clean NG-tubes (124). In a quality improvement study from 2014, NEC incidence was significantly reduced from 16 % to 3 % after the implementation of several quality improvement interventions. Standardized weekly renewal of NG-tubes was one of them (126). The static biofilm inside used NG-tubes of preterm infants contained higher densities of bacteria when the tubes had been in use for more than 48 hours (123). None of the previous studies investigated how many of the viable bacteria from the biofilm were actually flushed into the upper gastrointestinal tract of the infant when a meal was given through the tube. This was addressed in **study III**.

AIMS

Three aims were set in order to study the validity of the NEC diagnosis and to perform the initial studies of two possible means of prevention:

STUDY I

Aim: To determine the validity of the NEC diagnosis given at discharge.

STUDY II

Aim: To design a pilot study of bovine colostrum used for preterm infants in the first days of life as a supplementation to maternal milk and to determine the feasibility and initial safety of such a study.

STUDY III

Aim: To determine the bacterial contamination of feeds given through used nasogastric feeding tubes from a neonatal department and to relate the contamination to the duration of use.

STUDY I

METHODS

In a previous study, two cohorts of infants born at less than 30 weeks GA and admitted to RH were given a NEC classification code not only at discharge but also at a round table conference by an expert panel (114). We used the data from this process to determine the validity of the NEC diagnosis.

At discharge, all infants were evaluated and given a NEC code by the attending clinician, both according to the International classification of diseases (ICD-10) and according to a simplified version of Bell's stages. There was no option to give a separate code for SIP. The expert panel consisted of a neonatologist, a paediatric surgeon and a paediatric radiologist who were blinded to the NEC codes given to the infant at discharge. They evaluated clinical summaries, radiographs, surgical descriptions and any abdominal ultrasounds of all infants in the cohorts who received metronidazole during the admission. The infants were then classified into one of six groups; no NEC, NEC stage I-III according to Bell's stages, SIP, or a group called 'other gastrointestinal diseases'. Infants who did not receive metronidazole during the admission were classified as not having NEC. Since the definitions of Bell's stages II and III varied slightly between the discharge classification and the expert panel classification, all infants classified with stage II and III were pooled as the NEC-group. The classification given by the expert panel was defined as the golden standard.

RESULTS

A total of 714 infants were included in the study. At discharge, the ICD-10 code DP77.9 (necrotizing enterocolitis) was given to 84 infants, and according to the simplified Bell's stages 34 and 44 infants were diagnosed with NEC II and III, respectively. The expert panel, in contrast, diagnosed 26 infants with NEC II, 31 with NEC III, and 7 with SIP. This resulted in poor positive predictive values of the NEC diagnoses given at discharge, both according to the ICD-10 system and according to Bell's staging system (**Table 3**).

Table 3. Validity of the NEC diagnoses given at discharge.

	Sensitivity (95 % CI)	**Specificity (95 % CI)**	**PPV (95 % CI)**	**NPV (95 % CI)**
SIP considered as NEC				
ICD-10 code DP77.9	0.75 (0.62-0.85)	0.94 (0.92-0.96)	0.57 (0.46-0.68)	0.97 (0.96-0.98)
Bell's stages II-III	0.75 (0.62-0.85)	0.95 (0.93-0.97)	0.61 (0.49-0.71)	0.97 (0.96-0.99)
SIP not considered as NEC				
ICD-10 code DP77.9	0.72 (0.58-0.83)	0.93 (0.91-0.95)	0.49 (0.38-0.60)	0.97 (0.96-0.98)
Bell's stages II-III	0.72 (0.58-0.83)	0.94 (0.92-0.96)	0.52 (0.40-0.63)	0.97 (0.96-0.99)

CI: Confidence interval. ICD-10: International classification of diseases version 10. NEC: Necrotizing enterocolitis. NPV: Negative predictive value. PPV: Positive predictive value. SIP: Spontaneous intestinal perforation.

The incidence of NEC was significantly higher when using the discharge diagnosis rather than the diagnosis given by the expert panel (11.1 and 8.0 % respectively, p =0.003), even if SIP was included as NEC (11.1 and 9.0 %, p= 0.03). Nine infants, who had been diagnosed with NEC III at discharge, were classified as suffering from another gastrointestinal diseases by the expert panel. Three were diagnosed with ileus without signs of NEC, three with stenosis or volvulus with necrotic lesions, two with intra-abdominal perforation because of a misplaced feeding tube and one with an intra-abdominal abscess.

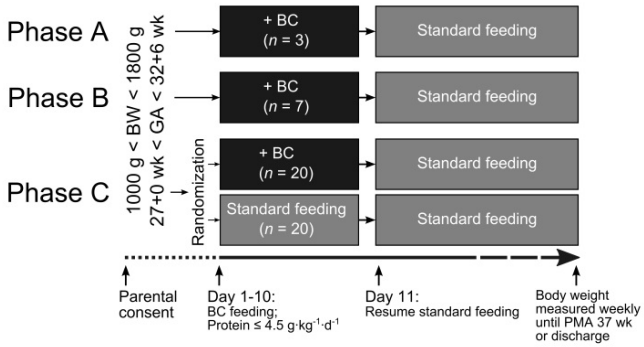
STUDY II

PROTOCOL

The Precolos study, a pilot study of BC as the initial nutrition for preterm infants, was designed with three-phases to proceed with care (**Figure 2**). In phase A, an infant was included and followed

until reaching full enteral feeding and thriving, before the next could be included. After phase A was completed with no safety concerns, seven infants were included and studied in parallel in phase B. After completion of phase B without safety concerns, phase C was initiated as an open-label, randomized controlled trial. The study was approved by ethical committees in both Denmark and China.

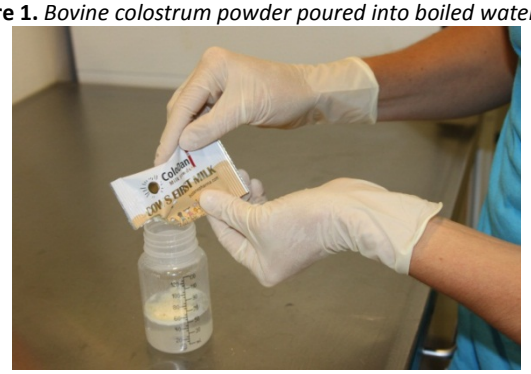
Figure 2. Design of the Precolos study



BC: Bovine colostrum. BW: Birth weight. GA: Gestational age. PMA: Postmenstrual age.

It was decided to conduct the study in two dissimilar clinical settings in two different parts of the world. In this way, we could study the feasibility of using BC both in a site using DM and early enteral feeding and in a site using IF and delayed enteral feeding. RH, Denmark and Foshan Women's and Children's Hospital, China (FWCH) were the two study sites of phase A and B, whereas Hvidovre Hospital, Denmark (HH) and Shenzhen Baoan Maternity and Child Healthcare Hospital, China (SBMCH) entered the study after the initiation of phase C. The BC used in the study was prepared from BC powder made of unmodified, whole colostrum collected from healthy Danish dairy cows at the second milking after parturition. The BC was gently pasteurized (62.5 °C for 30 min) and spray-dried. Reconstituted BC was made by mixing BC powder with cooled boiled water at room temperature (10 g powder in 50 ml water).

Picture 1. *Bovine colostrum powder poured into boiled water.*



In phases A and B, BC was given to all infants in the first ten days of life as a supplement when MM was insufficient. It was given in adherence with local guidelines and replaced DM feeding at RH and IF feeding at FWCH. Since BC has a high protein concentration compared to human colostrum and term milk (**Table 2**), the intervention could, however, not be given as an unlimited supplement to MM. The study was designed so that the daily available volume of MM together with the BC supplementation would provide a maximum of 4.5 $g \cdot kg^{-1} \cdot d^{-1}$ protein, which is the upper limit of protein intake for preterm infants recommended by European Society of Pediatric Gastroenterology, Hepatology and Nutrition (127). If MM and BC could not meet the required enteral feeding volume due to protein limitations, DM at RH or IF at FWCH was supplemented to fulfill the needs.

To determine the safety and tolerability of BC feeding, several clinical outcomes were recorded daily from the medical file. Furthermore, routine blood samples were evaluated, and intact bovine IgG levels in plasma (only RH) along with plasma amino acids (both sites) were measured on day 7 and 14. Safety and tolerability were evaluated by a safety monitoring board after completion of phase A and B who determined that initiation of phase C was safe.

In the randomized phase C, the infants were allocated into two groups, an intervention group and a control group (**Figure 2**). Infants in the intervention group received BC as described for phases A and B. Infants in the control group received the respective standard diets at each site which were DM at RH and HH and IF at FWCH and SBMCH. The last patient has recently been included in phase C and the same outcomes as those of phase A and B will be evaluated. In addition, a simplified three-sugar test was performed on study day seven to assess intestinal functions as described by others previously (128,129). In short, after two hours of fasting the infants received 2 $ml \cdot kg^{-1} \cdot d^{-1}$ of a sugar solution containing 5% lactose, 5% lactulose, and 2% mannitol. A blood sample was taken 40 min later, and urine was collected continuously for 6 hours. Lactose digestion and galactose uptake capacity will be assessed by measuring the level of galactose in the blood. Lactose digestion will also be assessed by measuring the ratio of lactulose/lactose in the urine. Intestinal permeability will be assessed by measuring the lactulose/mannitol ratio in the urine.

RESULTS OF PHASE A AND B

In the first two phases, twelve infants were included in the study, seven at RH and five at FWCH (**Table 4**). BC was given as the first diet; on day one at RH and on day two or three at FWCH. BC was provided for an average of seven days, and the infants received a total average of 257 ml BC during the entire intervention period (**Table 5**). During the first week of life, infants at RH received 97 $ml \cdot kg^{-1} \cdot d^{-1}$ fluid, 3.4 $g \cdot kg^{-1} \cdot d^{-1}$ protein and 69 $kcal \cdot kg^{-1} \cdot d^{-1}$. Correspondingly, FWCH infants received 91 $ml \cdot kg^{-1} \cdot d^{-1}$ fluid, 2.7 $g \cdot kg^{-1} \cdot d^{-1}$ protein and 67 $kcal \cdot kg^{-1} \cdot d^{-1}$. BC provided 54 % and 46 % of the total amount of protein given to the infants in the first week of life at RH and FWCH, respectively.

Table 4. Neonatal characteristics of the infants recruited in phase A and B[a]

	RH	FWCH
n	7	5
Gestational age (weeks)	29.6 ± 1.6	31.8 ± 1.6

Birth weight (g)	1346 ± 344	1526 ± 222
Birth weight Z–score	-0.15 ± 0.31	-0.72 ± 0.42
Birth length (cm)	38.9 ± 3.3	42.8 ± 2.4
Birth HC (cm)	27.4 ± 3.0	27.4 ± 2.4
Male gender, *n*	3	2
Antenatal steroids, *n*	5[b]	2
Caesarian section, *n*	6	1

HC: Head circumference. [a]Unless otherwise indicated, values are given as means ± standard deviation [b]Missing data for two infants

No adverse clinical reactions to BC feeding were observed. Feeding intolerance was observed in seven infants during the first week and in one infant during the second week (**Table 5**). Plasma amino acids were within the normal range, except for tyrosine. Hypertyrosinemia was observed in five of the twelve infants after the first week of life (**Table 5**) but returned to normal in all cases after the second week. Plasma levels of bovine IgG were below the assay detection limit in all samples.

Table 5. Selected clinical and paraclinical outcomes of the infants included in phase A and B[a]

	RH	FWCH
n	4-7	4-5
Days on parenteral nutrition (d)	3 ± 6	22 ± 9
Bovine colostrum feeding (d)	7 ± 3	7 ± 1
Total BC intake (ml)	273 ± 199	234 ± 92
TFF (d)	22 ± 11	21 ± 8
TTF120 (d)	8 ± 3	21 ± 8
Feeding intolerance in week 1, *n*	6	1
Feeding intolerance in week 2, *n*	1	0
Gastric residuals in week 1 (ml)	80 ± 41	5 ± 5
Z–score change at 37 weeks/discharge	-1.2 ± 0.6	-0.5 ± 0.5
Growth velocity ($g \cdot kg^{-1} \cdot d^{-1}$)	11.8 ± 0.9	12.9 ± 2.7
Regain birth weight (d)	12 ± 2	6 ± 2
Hypertyrosinemia on day 7, *n*	3	2
Blood urea nitrogen on day 7 (mmol/l)	5.8 ± 3.3	3.3 ± 1.1
Blood urea nitrogen on day 14 (mmol/l)	2.0 ± 0.3	1.7 ± 0.6

FWCH: Foshan Women's and Children's Hospital. HC: Head circumference. RH: Rigshospitalet. TFF: Time to full enteral feeding. TTF120: Time to 120 $ml \cdot kg^{-1} \cdot d^{-1}$ enteral feeding. [a]Unless otherwise indicated, values are given as means ± standard deviation

STUDY III

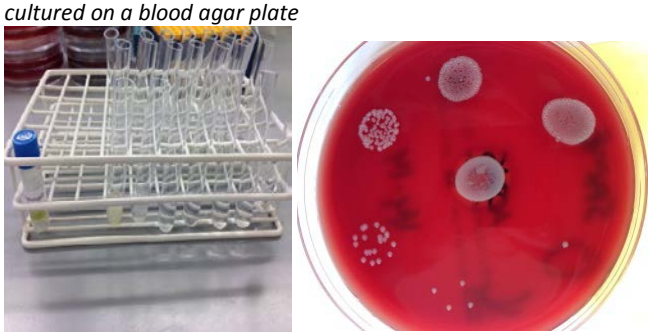
METHODS

A prospective, observational study at the tertiary neonatal department at RH in Denmark was performed. The study was approved by the Danish ethical committee. All admitted infants who had a NG-tube could be included if written parental consent was given. During a two-month period, used NG-tubes of included infants were collected for the study. According to the guidelines of the department, the NG-tubes should be renewed weekly, but more often if they clotted or got displaced. The infants were fed every two or three hours. Infants born with a GA below 30 weeks were given probiotics (two capsules of 10^9 CFU *Lactobacillus rhamnosus* GG (LGG) + 10^8 CFU *Bifidobacterium animalis ssp. Lactis* (BB12)) through the NG-tube from day three of life as standard treatment.

Picture 2. *Left) Preterm infant with c-pap system and nasogastric feeding tube. Right) Collected nasogastric feeding tubes.*

In order to imitate a meal, the collected NG-tubes were flushed with one ml saline each (the "flush") which was then subjected to culture methods. Both aerobic, 5% CO_2, and anaerobic culturing was performed in order to detect as many different species as possible. Direct and ten-fold-dilution culturing was performed to determine the concentration of the bacteria in the flush expressed as colony-forming-units (CFU) per ml. A NG-tube was defined as contaminated if the flush contained more than 1000 CFU of at least one isolate, except for the probiotics supplemented to the infants. Gram-negative rods and *Staphylococcus aureus* were defined as potentially pathogenic bacteria.

Picture 3. *Left) Ten fold dilutions performed on the saline flushed through the nasogastric feeding tube Right) Ten fold dilutions cultured on a blood agar plate*



The VITEK-2 system was used as the first attempt to identify the isolates. If the VITEK-2 system failed to provide a valid identification of the isolate, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) or standard laboratory methods were used. The VITEK-2 system relies on automated biochemical testing of the isolate which is suspended in saline and inoculated into identification cards. The pattern of reactions is used to determine the identity of the microorganism (130). MALDI-TOF relies on mass spectroscopy in which the microorganism is determined based on the pattern of observed mass spectral peaks, i.e. how long it takes ionized proteins from the organism to travel from the matrix they are dissolved in to an analyzer (131). Both meth-

ods provided one or more suggestions of the identity of the microorganism along with a probability that the suggestion was true. Standard laboratory methods were used if both systems failed to provide a plausible suggestion with a probability above the pre-defined cut-off level.

RESULTS

A total of 94 NG-tubes from 34 infants were collected during the study period. The infants included in the study were born at a median GA of 30.1 weeks with a birth weight of 1083 g. Their postnatal age at the time of NG-tube collection ranged from 1 to 119 days with a median of 37 days. The NG-tubes had been in use for a median of 3.25 days, but the duration of use ranged from 8 hours to 14.2 days. The flushes of 89 % of the NG-tubes were contaminated, and the flushes of 55 % of the NG-tubes were contaminated with the possible pathogens *Enterobacteriaceae* or *Staphylococcus aureus* (**Table 6**). The mean concentration of bacteria in the flush was 5.3 log_{10}CFU/ml bacteria, and the maximum concentration was 9.4 log_{10}CFU/ml.

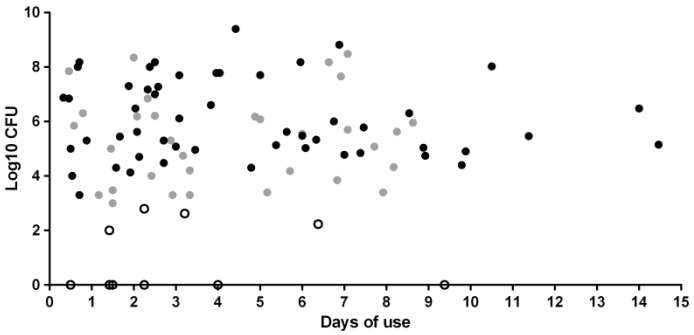
Table 6. Bacterial isolates cultured from one-ml saline 'meals' flushed through 94 used NG-tubes from a neonatal department

Isolate	NG-tubes, n (%) (n = 94)	Infants, n (%) (n = 34)	Concentration mean ± SD Log10CFU/ml
CoNS	44 (46.8)	23 (67.6)	4.6 ± 1.3
S. epidermidis	33 (35.1)	21 (61.8)	4.6 ±1.3
S. haemolyticus	14 (14.9)	10 (29.4)	4.9 ± 1.5
S. hominis	5 (5.3)	4 (11.8)	4.5 ± 0.95
Unspecified	7 (7.4)	6 (17.6)	4.1 ± 0.86
Enterococcus sp.	25 (26.6)	13 (38.2)	5.5 ± 1.4
E. faecium	15 (16.0)	5 (14.7)	6.0 ± 1.5
E. faecalis	10 (10.6)	8 (23.5)	4.8 ± 1.2
Klebsiella sp.	20 (21.3)	7 (20.6)	5.9 ± 1.8
K. oxytoca	15 (16.0)	5 (14.7)	6.0 ± 1.8
K. pneumoniae	7 (7.4)	4 (11.8)	5.5 ± 1.9
Enterobacter cloacae complex	19 (20.2)	5 (14.7)	6.0 ± 1.7
Lactobacillus rhamnosus	14 (14.9)	10 (29.4)	6.4 ± 1.8
Staphylococcus aureus	13 (13.8)	10 (29.4)	4.8 ± 1.0
Alpha-hemolytic *Streptococcus*	10 (10.6)	7 (20.6)	4.3 ± 1.2
Micrococcus lutea	5 (5.3)	5 (14.7)	4.0 ± 0.9
Escherichia coli	3 (3.2)	3 (8.8)	6.0 ± 2.9
Serratia marcescens	2 (2.1)	2 (5.9)	4.7 ± 1.9
Yeast	2 (2.1)	2 (5.9)	3.4 ± 0.12
Bifidobacterium animalis	1 (1.1)	1 (2.9)	3.6
Neisseria mucosa	1 (1.1)	1 (2.9)	4.3
Rothia mucigalinosa	1 (1.1)	1 (2.9)	5.2
Bacillus cereus	1 (1.1)	1 (2.9)	3.0

CoNS: Coagulase negative *Staphylococci*. CFU: Colony forming units. NG-tubes: Nasogastric feeding tubes. SD: Standard deviation.

There was no correlation between the number of days the NG-tubes had been in use and the concentration of bacteria in the flush (Spearman correlation $r_s = 0.065$, $p = 0.53$) or the concentration of potentially pathogenic bacteria in the flush (Spearman correlation $r_s = -0.11$, $p = 0.43$) (**Figure 3**). Probiotics given through the NG-tubes did not protect against contamination (88 % versus 90 % of probiotic and non-probiotic NG-tubes, respectively) or against contamination with potential pathogens (56 % versus 52 % of probiotic and non-probiotic NG-tubes, respectively).

Figure 3. The number of days the nasogastric feeding tube had been in use plotted against the concentration of bacteria in the saline flushed through the used tube



White circles: Not identified/no bacteria/only probiotic bacteria. Gray circles: No potential pathogens. Black circles: Potential pathogens, defined as Gram negative rods or *Staphylococcus aureus.* CFU: Colony forming units.

DISCUSSION AND PERSPECTIVES

In **study I**, we found a poor validity of the NEC diagnosis given at discharge when compared to the diagnosis given by the expert panel, which was considered the golden-standard. It must be kept in mind that the physician giving the diagnosis at discharge might have had clinical knowledge not stated in the medical records, whereas the expert panel only saw an abstract of the clinical data. Nevertheless, the study showed that there might be disagreements when diagnosing NEC. This may explain some of the differences in the reported incidence of NEC around the world. Furthermore, it may affect the conclusions in clinical trials with NEC as an outcome.

The validity of the NEC diagnosis according to both ICD-10 and Bell's classification system was in the lower end compared to other discharge diagnoses (132–134). This might be because NEC is a difficult disease to define, as it is probably not one disease but a final common pathway for many conditions. It may be difficult to draw the line between other pathologies and NEC, considering that nine of the infants, who were diagnosed with NEC at discharge, were determined to suffer from other gastrointestinal diseases by the expert panel. In other cases, there was not enough information to determine whether or not an infant suffered from NEC or SIP; e.g. in the case of a preterm infant with radiographic signs of pneumoperitoneum but no pneumatosis who did not undergo surgery or autopsy. Taken to the extreme, there will always be an uncertainty of the NEC diagnosis when the infant does not undergo surgery or autopsy. The presence or absence of intestinal pneumatosis is used to guide the decision, however it can occur in various degrees and radiologists do not always agree on its presence (135).

The poor validity of the NEC diagnosis could, nevertheless, also be caused by an inadequate classification system. The findings of the present study may inspire the neonatal community to discuss if classification according to Bell's stages is the best option. The staging system was developed almost 40 years ago when the preterm population was different from today and it did not take SIP into account. The stages are vaguely described e.g. with regards to separating other disease entities from NEC. On the other hand,

the system is so widely used that creating another system to be taken up by the neonatal community will be almost impossible, and it will cause problems when comparing the results of different NEC studies. NEC research may, however, still benefit from a revision of Bell's staging system with clear guidelines describing how to separate NEC from other disease entities and how to classify infants when certain information is missing.
In **study II**, whole BC was given as the initial enteral nutrition to the first twelve infants in the world. The study challenged the present trend in neonatal nutrition which is to use as much human milk as possible (136). At RH, several parents objected to giving their infant a bovine based product because DM was available, and even though they wanted to support research, they did not want to let their infant participate in the Precolos study. Thus, the acceptability of the intervention by the parents is an aspect of the Precolos study and translational research in general to consider in the planning of future studies (**SP-III**).

The human milk movement in the preterm community is partly based on the reduced NEC-incidence seen in infants fed MM. There are two main hypotheses of why this reduced incidence is seen. The first is that IF is NEC-inducing. Bovine protein has been suggested as the factor in IF than induces NEC. The theory has gained ground because of two studies of a human-milk-based fortifier in which a sole human milk diet protected significantly against NEC (137,138). The studies were small, and the NEC-rate in the control group of both studies was 20 % which is higher than expected even in an IF-fed population. Another theory of why IF could be NEC inducing is that the processing of IF and addition of artificial elements cause intestinal inflammation as shown in piglet studies (139–141). However, the recent large randomized controlled trial from the Netherlands of DM versus IF could not confirm that avoiding IF and bovine based fortifiers in the first ten days of life reduced the risk of NEC, sepsis and death as a composite outcome (46).

The second hypothesis of why infants fed MM have a decreased risk of NEC is that MM contains NEC-protective factors and that the depletion of these factors will leave the infant vulnerable to developing NEC. This would explain the findings in the Dutch study where the group allocation to receive IF or DM as a supplement to MM had no influence on the outcome, whereas the proportion of MM fed to the infants was significantly associated to the combined outcome of NEC-sepsis-death (46). If this hypothesis holds true, the bioactive ingredients in BC might make up for the depletion of NEC-protective factors in the first days of life until the production of MM is adequate.

When writing this thesis, recruitment of infants for phase C has just finished and the discharge of the last infant is imminent. However, the studies of BC as the first enteral nutrition for preterm infants have probably just begun. Not much can be concluded from a pilot study of 52 infants in total, except for feasibility, initial tolerability and safety along with an evaluation of possible primary outcomes for the next study. If the results from phase C show that BC was well tolerated and that there were no adverse clinical or paraclinical effects, a larger study will be planned to determine if feeding BC as a supplement to MM can protect against NEC in preterm infants.

NEC is however, as shown in s**tudy I**, a difficult disease to study. If NEC is used as the primary outcome, the NEC diagnosis should be given according to pre-defined guidelines based on Bell's staging system, preferably with central rather than local evaluation by experts who should be blinded to the allocation of the infants. The relatively infrequent occurrence of NEC also challenges the research. The incidence of NEC is highest in the extremely premature infants, but our pilot study only included infants born after 27 weeks GA. Hence, there is no initial safety data for more immature infants. BC may be more beneficial in this group; however it could also be more harmful. The preterm piglets, in which the initial BC studies have been performed, model infants born at approximately 30-32 weeks GA (76), so the data from these studies cannot be used to substantiate the argument that BC is beneficial for extremely preterm subjects. Special safety monitoring should be conducted for infants below 27 weeks, if they are included in a large-scale trial.

Given that the aim is to show a reduction in NEC-incidence from 10 % to 5 % with a power of 0.8 at a significance level of 0.05 in a randomized controlled trial, 435 infants are needed in each arm. Hence, even if we expect an unrealistically large effect of the intervention, we still need to include almost 900 infants to show it. Thus, a combined outcome of NEC-sepsis-death is intriguing and even meaningful due to the immune-modulatory potential of BC and the fact that oral lactoferrin prophylaxis was shown to reduce the incidence of sepsis in preterm infants (74). A reduction of the composite incidence from 30 % to 20 % will require 294 infants in each group which is more realistic, although still a great task. Furthermore, a proper sample size calculation will have to take into account that a small proportion of infants will not need supplementation due to adequate supply of MM. This proportion can be approximated based on the results from the Precolos study and used in the final sample size calculation.

The design of a large scale study must be as simple as possible for several reasons. First, it will be easier to convince other centers to participate, if the task is not too overwhelming. Second, the simpler the protocol, the higher the possibility that the study is conducted according to the protocol at all sites in a multi-center trial. Third, keeping the number of tests at a minimum will increase the recruitment rate. The individual sites can still choose to expand the study with extra tests if they have the capacity and motivation. Despite the intention to keep the study as simple as possible, long-term follow up investigation of growth, neurodevelopmental outcome and the development of cow's milk protein allergy at approximately two years of age should be aimed for.

The pilot study tested feasibility and was therefore conducted in two very different settings, however the lesson learnt from this was that a large-scale study should be conducted in centers with more homogenous settings. The results of a trial where BC is tested against both DM and IF will be difficult to interpret and comparisons between the sites instead of between groups will disturb the message. A study located in China comparing BC to IF as the supplement to MM is the most logical first step considering the human milk trend and parent acceptability. If BC turns out to be superior to IF, a randomized trial comparing BC and DM may be planned.

In **study III**, we showed that high concentrations of potentially pathogenic bacteria were flushed into the stomach of infants fed by NG-tubes. In the light of the hygienic precautions taken in neonatal departments to avoid infections in general, the finding was disturbing and underscored the need for hygienic handling of NG-tubes. However, it is still uncertain if and how the contamination

of meals affects the infants. Probiotics are given through the NG-tubes and the probiotic bacteria may be weakened by the competition from the bacteria of the NG-tubes. Considering the general notion that a heterogeneous bacterial population is healthier than a population with a few dominating species, contamination with low concentrations of several species may be unimportant while contamination with a high concentration of one single strain could be problematic.

To study the impact of NG-tubes further, we have commenced a small, randomized controlled trial to determine if exchanging the NG-tube once a day rather than once a week affects the early gut colonization of preterm infants (clinicaltrials.gov: H-15021673). The primary aim is to determine if the bacterial concentration in gastric aspirates collected after one week of life is lower if the NG-tube is renewed daily. In order to get a realistic sample size, the primary outcome is microbiological, so the study will not be powered to show if there are any clinical benefits or disadvantages from daily exchange of the feeding tube. Ultimately, a large randomized controlled trial of the frequency of renewal is needed in order to understand the clinical impact of contaminated NG-tubes. A design could include time to full enteral feeding as primary outcome, and colonization of the gut, contamination of meals by the NG-tube, duration of parenteral nutrition, length of admission, growth and the incidence of NEC, sepsis and death as secondary outcomes.

The origin of the bacteria inside the NG-tubes may also be investigated in order to understand the hygienic implications. Bacteria may enter the NG-tube when gastric residuals are aspirated into the tube before each meal to evaluate feeding tolerance. A randomized controlled trial found that infants with no routine aspiration reached full enteral feeding earlier and had fewer days with central lines compared to infants undergoing routine aspiration (142). This may be due to reduced concerns about the appearance of the residuals. However, it may also be due to a reduced development of bacterial biofilms inside the NG-tubes in the group with no routine aspirations. Reduced contamination of feeds may then have led to fewer incidents of feeding intolerance and a faster attainment of full enteral feeding. To substantiate the claim that the bacteria of the NG-tubes derived from the gastric aspirates of the infants rather than from external sources, full-genome sequencing will be performed on 47 isolates collected in **study III** from 8 infants. The aim is to determine if the NG-tubes collected from the same infant contained the same bacterial clone every time.

CONCLUSION

In this thesis, the aims were to investigate if the NEC diagnosis was valid and to study two possible NEC-preventive approaches. It has been highlighted that there were disagreements when diagnosing NEC which should be kept in mind when conducting epidemiological NEC studies and especially when conducting trials with NEC as an outcome. A revision of Bell's staging system may improve studies on NEC in the future. A pilot study of bovine colostrum for preterm infants has been undertaken and if the findings of the randomized phase of the study indicate a positive effect of bovine colostrum and do not give rise to concerns regarding feasibility, safety or tolerability, a large-scale randomized controlled trial with NEC as the primary outcome will be planned. The results and lessons learned from the three phases of the pilot study will be used to guide the design of such a study. Hopefully, the results will show whether the use of BC as a supplement to MM can prevent NEC and sepsis in the preterm population. At last, meals given through used NG-tubes were contaminated with high concentrations of bacteria with no association to the duration of use. This finding might seem disturbing, but the clinical impact on the infants still needs to be determined. A randomized controlled trial of the frequency of feeding tube exchange in preterm infants has been commenced to study the impact on the early colonization. In all, the studies of this thesis serve as a base for future studies on the prevention of NEC which will continue to be an area of high priority in neonatology.

SUMMARY

The premature infant suffers from immaturity of all organ systems, one of them being the gastrointestinal tract. When the infant is born, the immature gastrointestinal tract is exposed to milk and simultaneously colonized by high densities of bacteria. The combination of milk, microbiota and an immature gut, leaves the infant vulnerable to developing the dreaded intestinal emergency necrotizing enterocolitis (NEC). NEC is often very aggressive and no cure exists, which means that prevention is an utmost important topic to researchers, physicians, parents - and infants.

Despite immense research during the last decades, no specific test to determine if an infant suffers from NEC exists. Most neonatal units use Bell's staging criteria, which are based on clinical and radiographic findings, as a diagnostic tool; however the diagnosis given according to Bell's stages has not been validated. In **study I**, we aimed to determine the validity of the NEC diagnosis given at discharge. An expert panel consisting of a neonatologist, a paediatric surgeon and a paediatric radiologist served as the golden standard. We found that the diagnosis given at discharge had a poor validity which significantly affected the reported incidence of NEC in the neonatal department at Rigshospitalet, Denmark. The validity of the NEC diagnosis was worse than the validity of most other paediatric diagnoses that had been investigated.

In studies II and III, we aimed to explore possible means of NEC prevention. The role of nutrition in NEC development is well established with mother's milk as the best option to avoid NEC in the preterm infant. Maternal milk is, however, most often not available in sufficient amounts during the first days of life, and preterm infant formula or human donor milk is used in its absence. Studies in preterm piglets showed that bovine colostrum equally to human donor milk protected against NEC compared to infant formula. Furthermore, bovine colostrum was superior to human donor milk in stimulating gut immunity and digestive functions.

Hence, in s**tudy II** we aimed to design a pilot study of bovine colostrum used as a supplement to maternal milk in the first days of life and to determine if the study was feasible. In the paper, we present the protocol and the results of the first two phases of the Precolos study in which 12 infants were included and received pasteurized, spray-dried and reconstituted bovine colostrum during the first days of life as the first infants in the world. We found that the infants tolerated bovine colostrum without clinical adverse effects, but we also observed a transient hypertyrosinemia on day seven of life in five infants. The results were evaluated by a safety management board which encouraged us to continue the pilot study with the last phase, which was a randomized con-

trolled trial of 20+20 infants comparing supplementation with bovine colostrum to supplementation with standard nutrition. The randomized trial has just finished recruitment.

At last, we wanted to shed light on a possible microbiological angle of NEC prevention. Dysbiosis and bacterial translocation are believed to play a crucial role in the development of NEC as intestinal pneumatosis, which occurs when bacteria produce gas inside the intestinal wall, is a pathognomonic radiographic finding. In a quality improvement study from the US published in 2014, NEC incidence was significantly reduced after the implementation of several quality improvement interventions. Standardized weekly exchange of nasogastric feeding tubes was suggested as one of the potential NEC-reducing interventions.

In the neonatal unit at Rigshospitalet, Denmark, preterm infants are fed 8-12 times daily through a resident nasogastric feeding tube which is exposed to body temperature, contains milk residuals from the last meal and is handled by both parents and personnel. Since bacterial pollution of milk given through the nasogastric feeding tube might be NEC-inducing, we aimed in **study III** to determine the bacterial load given to the infants when feeding them through a tube. We collected 92 used nasogastric feeding tubes and flushed them with one ml saline each to imitate a meal given through them. Eighty-nine percent of the tubes contaminated the meals with more than 1000 colony-forming units of bacteria and fifty-five percent contaminated the meals with the possible pathogens *Enterobacteriaceae* or *Staphylococcus aureus*. The concentration of bacteria in the saline flushed through the tubes was as high as 10^9 colony-forming units per ml; however, neither the risk of contamination nor the concentration of bacteria in the flush was associated with the duration of use. Implementation of standardized weekly exchange of feeding tubes would therefore not prevent the contamination of meals.

In conclusion, the studies included in this thesis serve as a base for future studies investigating the prevention of NEC. We found a poor validity of the NEC diagnosis given at discharge. This should be kept in mind when conducting epidemiological studies of NEC and especially when conducting interventional trials with NEC as an outcome. If the findings of the randomized part of the Precolos study indicate a positive effect of bovine colostrum and do not give rise to concerns regarding feasibility, safety or tolerability, a large-scale randomized controlled study with NEC as the primary outcome will be planned. Based on the high concentrations of bacteria found in the nasogastric feeding tubes, a randomized controlled trial investigating whether the frequency of feeding tube exchange affects the early colonization has been commenced in the neonatal department at Rigshospitalet. Hopefully, the results of these studies will bring us closer to preventing NEC in the future.

REFERENCES

1. Blencowe H, Cousens S, Oestergaard MZ, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: A systematic analysis and implications. Lancet 2012;379:2162–72.
2. Stoll BJ, Hansen NI, Bell EF, et al. Trends in Care Practices, Morbidity, and Mortality of Extremely Preterm Neonates, 1993-2012. Jama 2015;314:1039–51.
3. Stoelhorst GMSJ, Rijken M, Martens SE, et al. Changes in neonatology: comparison of two cohorts of very preterm infants (gestational age <32 weeks): the Project On Preterm and Small for Gestational Age Infants 1983 and the Leiden Follow-Up Project on Prematurity 1996-1997. Pediatrics 2005;115:396–405.
4. Ahle M, Drott P, Andersson RE. Epidemiology and trends of necrotizing enterocolitis in Sweden: 1987-2009. Pediatrics 2013;132:e443-51.
5. Lambert DK, Christensen RD, Henry E, et al. Necrotizing enterocolitis in term neonates: data from a multihospital health-care system. J Perinatol 2007;27:437–43.
6. Berrington JE, Hearn RI, Bythell M, Wright C, Embleton ND. Deaths in Preterm Infants: Changing Pathology Over 2 Decades. J Pediatr 2012;160:49–53.e1.
7. Bell M, Ternberg J, Feigin R. Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging. Ann Surg 1978;187:1–7.
8. Kliegman RM, Walsh MC. Neonatal necrotizing enterocolitis: pathogenesis, classification, and spectrum of illness. Curr Probl Pediatr 1987;17:213–88.
9. Tam AL, Camberos A, Applebaum H. Surgical decision making in necrotizing enterocolitis and focal intestinal perforation: Predictive value of radiologic findings. J Pediatr Surg 2002;37:1688–91.
10. Muller A, Schurink M, Bos AF, et al. Clinical importance of a fixed bowel loop in the treatment of necrotizing enterocolitis. Neonatology 2014;105:33–8.
11. Neu J, Walker WA. Necrotizing enterocolitis. N Engl J Med 2011;364:255–64.
12. Fasoli L, Turi RA, Spitz L, Kiely EM, Drake D, Pierro A. Necrotizing enterocolitis: Extent of disease and surgical treatment. J Pediatr Surg 1999;34:1096–9.
13. Ballance WA, Dahms BB, Shenker N, Kliegman RM. Pathology of neonatal necrotizing enterocolitis: A ten-year experience. J Pediatr 1990;117.
14. Fitzgibbons SC, Ching Y, Yu D, et al. Mortality of necrotizing enterocolitis expressed by birth weight categories. J Pediatr Surg 2009;44:1072–6.
15. Fullerton BS, Sparks EA, Morrow KA, et al. Hospital transfers and patterns of mortality in very low birth weight neonates with surgical necrotizing enterocolitis. J Pediatr Surg 2016;51:932–5.
16. Clark RH, Gordon P, Walker WM, Laughon M, Smith PB, Spitzer AR. Characteristics of patients who die of necrotizing enterocolitis. J Perinatol 2012;32:199–204.
17. Kastenberg ZJ, Lee HC, Profit J, Gould JB, Sylvester KG. Effect of deregionalized care on mortality in very low-birth-weight infants with necrotizing enterocolitis. JAMA Pediatr 2015;169:26–32.
18. Sharma R, Hudak ML, Tepas JJ, et al. Impact of gestational age on the clinical presentation and surgical outcome of necrotizing enterocolitis. J Perinatol 2006;26:342–7.
19. Elfvin A, Dinsdale E, Wales PW, Moore AM. Low birthweight, gestational age, need for surgical intervention and gram-negative bacteraemia predict

intestinal failure following necrotising enterocolitis. Acta Paediatr 2015;104:771–6.
20. Guillet R, Stoll BJ, Cotten CM, et al. Association of H2-blocker therapy and higher incidence of necrotizing enterocolitis in very low birth weight infants. Pediatrics 2006;117:e137-42.
21. Rees CM, Pierro A, Eaton S. Neurodevelopmental outcomes of neonates with medically and surgically treated necrotizing enterocolitis. Arch Dis Child Fetal Neonatal Ed 2007;92:F193–8.
22. Pike K, Brocklehurst P, Jones D, et al. Outcomes at 7 years for babies who developed neonatal necrotising enterocolitis: the ORACLE Children Study. Arch Dis Child - Fetal Neonatal Ed 2012;97:F318–22.
23. Obladen M. Necrotizing enterocolitis - 150 years of fruitless search for the cause. Neonatology 2009;96:203–10.
24. Gordon P, Young ML, Marshall DD. Focal small bowel perforation: an adverse effect of early postnatal dexamethasone therapy in extremely low birth weight infants. J Perinatol 2001;21:156–60.
25. Gordon P, Swanson JR, Attridge JT, Clark R. Emerging trends in acquired neonatal intestinal disease: is it time to abandon Bell's criteria? J Perinatol 2007;27:661–71.
26. Uauy RD, Fanaroff AA, Korones SB, Elizabeth A, Philips JB, Wright LL. Necrotizing enterocolitis in very low birth weight infants : Biodemographic and clinical correlates. J Pediatr 1991;119:630–938.
27. Hack M, Wright L, Shankaran S. Very-low-birth-weight outcomes of the National Institute of Child Health and Human Development Neonatal Network, November 1989 to October 1990. Am J Obstet Gynecol 1995;172:457–64.
28. Sankaran K, Puckett B, Lee DSC, et al. Variations in incidence of necrotizing enterocolitis in Canadian neonatal intensive care units. J Pediatr Gastroenterol Nutr 2004;39:366–72.
29. Gagliardi L, Bellu R, Cardilli V, Curtis MD. Necrotising Enterocolitis in Very Low Birth Weight Infants in Italy : Incidence and Non-nutritional Risk Factors. J Pediatr Gastroenterol Nutr 2008;47:206–10.
30. Luoto R, Matomäki J, Isolauri E, Lehtonen L. Incidence of necrotizing enterocolitis in very-low-birth-weight infants related to the use of Lactobacillus GG. Acta Paediatr 2010;99:1135–8.
31. Isayama T, Lee SK, Mori R, et al. Comparison of mortality and morbidity of very low birth weight infants between Canada and Japan. Pediatrics 2012;130:e957-65.
32. Lin PW, Stoll BJ. Necrotising enterocolitis. Lancet 2006;368:1271–83.
33. Gordon P, Christensen R, Weitkamp JH, Maheshwari A. Mapping the New World of Necrotizing Enterocolitis (NEC): Review and Opinion. EJ Neonatol Res 2013;2:145–72.
34. Ohlsson A, Walia R, Shah SS. Ibuprofen for the treatment of patent ductus arteriosus in preterm or low birth weight (or both) infants. Cochrane database Syst Rev 2015;CD003481.
35. Coggins SA, Wynn JL, Weitkamp JH. Infectious causes of necrotizing enterocolitis. Clin Perinatol 2015;42:133–54.
36. Ramani M, Ambalavanan N. Feeding practices and necrotizing enterocolitis. Clin Perinatol 2013;40:1–10.
37. Morgan J, Bombell S, Mcguire W. Early trophic feeding versus enteral fasting for very preterm or very low birth weight infants (Review). Cochrane database Syst Rev 2013;3:CD000504.
38. Morgan J, Young L, McGuire W. Delayed introduction of progressive enteral feeds to prevent necrotising enterocolitis in very low birth weight infants. Cochrane database Syst Rev 2014;12.
39. Morgan J, Young L, McGuire W. Slow advancement of enteral feed volumes to prevent necrotising enterocolitis in very low birth weight infants. Cochrane database Syst Rev 2015;10:CD001241.
40. Corpeleijn WE, Kouwenhoven SMP, Paap MC, et al. Intake of own mother's milk during the first days of life is associated with decreased morbidity and mortality in very low birth weight infants during the first 60 days of life. Neonatology 2012;102:276–81.
41. Meinzen-Derr J, Poindexter B, Wrage L, Morrow AL, Stoll B, Donovan EF. Role of human milk in extremely low birth weight infants' risk of necrotizing enterocolitis or death. J Perinatol 2008;29:57–62.
42. Sisk PM, Lovelady CA, Dillard RG, Gruber KJ, O'Shea TM. Early human milk feeding is associated with a lower risk of necrotizing enterocolitis in very low birth weight infants. J Perinatol 2007;27:428–33.
43. Quigley M, McGuire W. Formula versus donor breast milk for feeding preterm or low birth weight infants. Cochrane database Syst Rev 2014;CD002971.
44. Arslanoglu S, Corpeleijn W, Moro G, et al. Donor Human Milk for Preterm Infants. J Pediatr Gastroenterol Nutr 2013;57:535–42.
45. Section on Breastfeeding. Breastfeeding and the use of human milk. Pediatrics 2012;129:e827-41.
46. Corpeleijn WE, de Waard M, Christmann V, et al. Effect of Donor Milk on Severe Infections and Mortality in Very Low-Birth-Weight Infants. JAMA Pediatr 2016;170:1–8.
47. Dritsakou K, Liosis G, Valsami G, Polychronopoulos E, Skouroliakou M. Improved outcomes of feeding low birth weight infants with predominantly raw human milk versus donor banked milk and formula. J Matern Fetal Neonatal Med 2015;7058:1–8.
48. Montjaux-Régis N, Cristini C, Arnaud C, Glorieux I, Vanpee M, Casper C. Improved growth of preterm infants receiving mother's own raw milk compared with pasteurized donor milk. Acta Paediatr 2011;100:1548–54.
49. Wojcik KY, Rechtman DJ, Lee ML, Montoya A, Medo ET. Macronutrient Analysis of a Nationwide Sample of Donor Breast Milk. J Am Diet Assoc 2009;109:137–40.
50. Michaelsen KF, Skafte L, Badsberg JH, Jørgensen M. Variation in macronutrients in human bank milk: influencing factors and implications for human milk

banking. J Pediatr Gastroenterol Nutr 1990;11:229–39.
51. Henderson TR, Fay TN, Hamosh M. Effect of pasteurization on long chain polyunsaturated fatty acid levels and enzyme activities of human milk. J Pediatr 1998;132:876–8.
52. Andersson Y, Sävman K, Bläckberg L, Hernell O. Pasteurization of mother's own milk reduces fat absorption and growth in preterm infants. Acta Paediatr 2007;96:1445–9.
53. Kulski JK, Hartmann PE. Changes in human milk composition during the initiation of lactation. Aust J Exp Biol Med Sci 1981;59:101–14.
54. Gidrewicz DA, Fenton TR, Kramer M, et al. A systematic review and meta-analysis of the nutrient content of preterm and term breast milk. BMC Pediatr 2014;14:216.
55. Kunz C, Lönnerdal B. Human-milk proteins: Analysis of casein and casein subunits by anion-exchange chromatography, gel electrophoresis, and specific staining methods. Am J Clin Nutr 1990;51:37–46.
56. Fried MD, Khoshoo V, Secker DJ, Gilday DL, Ash JM, Pencharz PB. Decrease in gastric emptying time and episodes of regurgitation in children with spastic quadriplegia fed a whey-based formula. J Pediatr 1992;120:569–72.
57. Moles L, Manzano S, Fernández L, et al. Bacteriological, Biochemical, and Immunological Properties of Colostrum and Mature Milk From Mothers of Extremely Preterm Infants. J Pediatr Gastroenterol Nutr 2015;60:120–6.
58. Tripathi V, Vashishtha B. Bioactive compounds of colostrum and its application. Food Rev Int 2006;22:225–44.
59. Chatterton DEW, Nguyen DN, Bering SB, Sangild PT. Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. Int J Biochem Cell Biol 2013;45:1730–47.
60. Kehoe SI, Jayarao BM, Heinrichs AJ. A Survey of Bovine Colostrum Composition and Colostrum Management Practices on Pennsylvania Dairy Farms. J Dairy Sci 2007;90:4108–16.
61. Ballard O, Morrow AL. Human milk composition: nutrients and bioactive factors. Pediatr Clin North Am 2013;60:49–74.
62. Morrill KM, Conrad E, Lago A, Campbell J, Quigley J, Tyler H. Nationwide evaluation of quality and composition of colostrum on dairy farms in the United States. J Dairy Sci 2012;95:3997–4005.
63. Tsioulpas A, Grandison AS, Lewis MJ. Changes in physical properties of bovine milk from the colostrum period to early lactation. J Dairy Sci 2007;90:5012–7.
64. Godden S. Colostrum Management for Dairy Calves. Vet Clin North Am - Food Anim Pract 2008;24:19–39.
65. Kunz C, Lönnerdal B. Re-evaluation of the whey protein/casein ratio of human milk. Acta Paediatr 1992;81:107–12.
66. Nissen A, Bendixen E, Ingvartsen KL, Røntved CM. In-depth analysis of low abundant proteins in bovine colostrum using different fractionation techniques. Proteomics 2012;12:2866–78.
67. Jacobs DR, Tapsell LC. Food synergy: the key to a healthy diet. Proc Nutr Soc 2013;72:200–6.
68. Hurley WL, Theil PK. Perspectives on immunoglobulins in colostrum and milk. Nutrients 2011;3:442–74.
69. Jasion VS, Burnett BP. Survival and digestibility of orally-administered immunoglobulin preparations containing IgG through the gastrointestinal tract in humans. Nutr J 2015;14:22.
70. Van De Perre P. Transfer of antibody via mother's milk. Vaccine 2003;21:3374–6.
71. Saji F, Samejima Y, Kamiura S, Koyama M. Dynamics of immunoglobulins at the feto-maternal interface. Rev Reprod 1999;4:81–9.
72. Foster JP, Seth R, Cole MJ. Oral immunoglobulin for preventing necrotizing enterocolitis in preterm and low birth weight neonates. Cochrane database Syst Rev 2016;4:CD001816.
73. Sherman MP. Lactoferrin and necrotizing enterocolitis. Clin Perinatol 2013;40:79–91.
74. Pammi M, Abrams SA. Oral lactoferrin for the prevention of sepsis and necrotizing enterocolitis in preterm infants. Cochrane database Syst Rev 2015;CD007137.
75. Gauthier SF, Pouliot Y, Maubois JL. Growth factors from bovine milk and colostrum: composition, extraction and biological activities. Lait 2006;86:99–125.
76. Sangild PT, Thymann T, Schmidt M, Stoll B, Burring DG, Buddington RK. Invited review: The preterm pig as a model in pediatric gastroenterology. J. Anim. Sci. 2013;91:4713–29.
77. Jensen ML, Sangild PT, Lykke M, et al. Similar efficacy of human banked milk and bovine colostrum to decrease incidence of necrotizing enterocolitis in preterm piglets. Am J Physiol Regul Integr Comp Physiol 2013;305:R4–12.
78. Rasmussen SO, Martin L, Østergaard MV, et al. Bovine colostrum improves neonatal growth, digestive function, and gut immunity relative to donor human milk and infant formula in preterm pigs. Am J Physiol - Gastrointest Liver Physiol 2016;311:G480–91.
79. Bjornvad CR, Thymann T, Deutz NE, et al. Enteral feeding induces diet-dependent mucosal dysfunction, bacterial proliferation, and necrotizing enterocolitis in preterm pigs on parenteral nutrition. Am J Physiol Gastrointest Liver Physiol 2008;295:G1092-103.
80. Møller HK, Thymann T, Fink LN, Frokiaer H, Kvistgaard AS, Sangild PT. Bovine colostrum is superior to enriched formulas in stimulating intestinal function and necrotising enterocolitis resistance in preterm pigs. Br J Nutr 2011;105:44–53.
81. Støy ACF, Sangild PT, Skovgaard K, et al. Spray Dried, Pasteurised Bovine Colostrum Protects Against Gut Dysfunction and Inflammation in Preterm Pigs. J Pediatr Gastroenterol Nutr 2016;63:280–7.
82. Aunsholt L, Jeppesen PB, Lund P, et al. Bovine colostrum to children with short bowel syndrome: a randomized, double-blind, crossover pilot study. J Parenter Enter Nutr 2012;38:99–106.

83. Guarner F, Malagelada JR. Gut flora in health and disease. Lancet 2003;360:512–9.
84. Van der Waaij D, Berghuis-de Vries JM, Lekkerkerk-van der Wees JEC. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J Hyg (Lond) 1971;69:405–11.
85. Hill RR, Cowley HM, Andremont A. Influence of colonizing micro-flora on the mucin histochemistry of the neonatal mouse colon. Histochem J 1990;22:102–5.
86. Peng L, Li Z, Green RS, Holzman IR, Lin J. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. J Nutr 2009;139:1619–25.
87. Stappenbeck TS, Hooper L V, Gordon JI. Nonlinear partial differential equations and applications: Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc Natl Acad Sci USA 2002;99:15451–5.
88. Hooper L, Littman DR, Macpherson AJ. Interactions Between the Microbiota and the Immune System. Science 2012;336:1268–73.
89. Clarke G, O'Mahony SM, Dinan TG, Cryan JF. Priming for health: gut microbiota acquired in early life regulates physiology, brain and behaviour. Acta Paediatr 2014;103:812–9.
90. Sherman MP, Zaghouani H, Niklas V. Gut microbiota, the immune system, and diet influence the neonatal gut–brain axis. Pediatr Res 2015;77:127–35.
91. Grishin A, Papillon S, Bell B, Wang J, Ford HR. The role of the intestinal microbiota in the pathogenesis of necrotizing enterocolitis. Semin Pediatr Surg 2013;22:69–75.
92. La Rosa PS, Warner BB, Zhou Y, et al. Patterned progression of bacterial populations in the premature infant gut. Proc Natl Acad Sci 2014;111:12522–7.
93. Butel MJ, Suau A, Campeotto F, et al. Conditions of bifidobacterial colonization in preterm infants: a prospective analysis. J Pediatr Gastroenterol Nutr 2007;44:577–82.
94. Gregory KE, LaPlante RD, Shan G, Kumar DV, Gregas M. Mode of Birth Influences Preterm Infant Intestinal Colonization With Bacteroides Over the Early Neonatal Period. Adv Neonatal Care 2015;15:386–93.
95. Brooks B, Firek BA, Miller CS, et al. Microbes in the neonatal intensive care unit resemble those found in the gut of premature infants. Microbiome 2014;2:1.
96. Greenwood C, Morrow AL, Lagomarcino AJ, et al. Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of enterobacter. J Pediatr 2014;165:23–9.
97. Patel K, Konduru K, Patra AK, Chandel DS, Panigrahi P. Trends and determinants of gastric bacterial colonization of preterm neonates in a NICU setting. PLoS One 2015;10:e0114664.
98. Parm Ü, Metsvaht T, Ilmoja ML, Lutsar I. Gut colonization by aerobic microorganisms is associated with route and type of nutrition in premature neonates. Nutr Res 2014;35:496–503.
99. Musemeche CA, Kosloske AM, Bartow SA, Umland ET. Comparative efects of ischemia, bacteria, and substrate on the pathogenesis of intestinal necrosis. J Pediatr Surg 1986;21:536–8.
100. Birck MM, Nguyen DN, Cilieborg MS, et al. Enteral but not parenteral antibiotics enhance gut function and prevent necrotizing enterocolitis in formula-fed newborn preterm pigs. Am J Physiol Gastrointest Liver Physiol 2016;310:G323-33.
101. Nanthakumar NN, Fusunyan RD, Sanderson I, Walker WA. Inflammation in the developing human intestine: A possible pathophysiologic contribution to necrotizing enterocolitis. Proc Natl Acad Sci USA 2000;97:6043–8.
102. Gribar SC, Sodhi CP, Richardson WM, et al. Reciprocal expression and signaling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enterocolitis. J Immunol 2009;182:636–46.
103. Warner BB, Deych E, Zhou Y, et al. Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: A prospective case-control study. Lancet 2016;387:1928–36.
104. Wang Y, Hoenig JD, Malin KJ, et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. ISME J 2009;3:944–54.
105. Mai V, Young CM, Ukhanova M, et al. Fecal microbiota in premature infants prior to necrotizing enterocolitis. PLoS One 2011;6:e20647.
106. Stewart CJ, Marrs ECL, Nelson A, et al. Development of the Preterm Gut Microbiome in Twins at Risk of Necrotising Enterocolitis and Sepsis. PLoS One 2013;8:1–9.
107. Stewart CJ, Marrs ECL, Magorrian S, et al. The preterm gut microbiota: changes associated with necrotizing enterocolitis and infection. Acta Paediatr 2012;101:1121–7.
108. Smith B, Bodé S, Skov TH, Mirsepasi H, Greisen G, Krogfelt K a. Investigation of the early intestinal microflora in premature infants with/without necrotizing enterocolitis using two different methods. Pediatr Res 2012;71:115–20.
109. Normann E, Fahlén A, Engstrand L, Lilja HE. Intestinal microbial profiles in extremely preterm infants with and without necrotizing enterocolitis. Acta Paediatr 2013;102:129–36.
110. Millar MR, Linton CJ, Cade A, Glancy D, Hall M, Jalal H. Application of 16S rRNA gene PCR to study bowel flora of preterm infants with and without necrotizing enterocolitis. J Clin Microbiol 1996;34:2506–10.
111. van Acker J, de Smet F, Muyldermans G, Bougatef A, Naessens A, Lauwers S. Outbreak of necrotizing enterocolitis associated with Enterobacter sakazakii in powdered milk formula. J Clin Microbiol 2001;39:293–7.
112. Denkel LA, Schwab F, Garten L, Geffers C, Gastmeier P, Piening B. Protective Effect of Dual-Strain Probiotics in

Preterm Infants: A Multi-Center Time Series Analysis. PLoS One 2016;11:e0158136.
113. Demirel G, Erdeve O, Celik IH, Dilmen U. Saccharomyces boulardii for prevention of necrotizing enterocolitis in preterm infants: A randomized, controlled study. Acta Paediatr 2013;
114. Lambæk ID, Fonnest G, Gormsen M, Brok J, Greisen G. Probiotics to prevent necrotising enterocolitis in very preterm infants. Dan Med J 2016;63:1–2.
115. Dilli D, Aydin B, Fettah ND, et al. The ProPre-Save Study: Effects of Probiotics and Prebiotics Alone or Combined on Necrotizing Enterocolitis in Very Low Birth Weight Infants. J Pediatr 2015;166:545–551.e1.
116. Costeloe K, Hardy P, Juszczak E, Wilks M, Millar MR. Bifidobacterium breve BBG-001 in very preterm infants: A randomised controlled phase 3 trial. Lancet 2015;387:649–60.
117. Härtel C, Pagel J, Rupp J, et al. Prophylactic Use of Lactobacillus acidophilus/Bifidobacterium infantis Probiotics and Outcome in Very Low Birth Weight Infants. J Pediatr 2014;165:285–289.e1.
118. Janvier A, Malo J, Barrington KJ. Cohort Study of Probiotics in a North American Neonatal Intensive Care Unit. J Pediatr 2014;164:980–5.
119. Oncel MY, Sari FN, Arayici S, et al. Lactobacillus Reuteri for the prevention of necrotising enterocolitis in very low birthweight infants: a randomised controlled trial. Arch Dis Child Fetal Neonatal Ed 2013;99:F110-5.
120. AlFaleh K, Anabrees J. Probiotics for prevention of necrotizing enterocolitis in preterm infants. Evid Based Child Health 2014;9:584–671.
121. Neu J. The Developing Intestinal Microbiome: Probiotics and Prebiotics. 2014;110:167–76.
122. Mihatsch WA, Braegger CP, Decsi T, et al. Critical systematic review of the level of evidence for routine use of probiotics for reduction of mortality and prevention of necrotizing enterocolitis and sepsis in preterm infants. Clin Nutr 2012;31:6–15.
123. Hurrell E, Kucerova E, Loughlin M, et al. Neonatal enteral feeding tubes as loci for colonisation by members of the Enterobacteriaceae. BMC Infect Dis 2009;9:146.
124. Mehall J, Kite C, Saltzman D. Prospective study of the incidence and complications of bacterial contamination of enteral feeding in neonates. J Pediatr Surg 2002;37:1177–82.
125. Gómez M, Moles L, Melgar A, et al. Early Gut Colonization of Preterm Infants: Effect of Enteral Feeding Tubes. J Pediatr Gastroenterol Nutr 2016;62:893–900.
126. Patel AL, Trivedi S, Bhandari NP, et al. Reducing necrotizing enterocolitis in very low birth weight infants using quality-improvement methods. J Perinatol 2014;34:850–7.
127. Agostoni C, Buonocore G, Carnielli VP, et al. Enteral nutrient supply for preterm infants: commentary from the European Society of Paediatric Gastroenterology, Hepatology and Nutrition Committee on Nutrition. J Pediatr Gastroenterol Nutr 2010;50:85–91.
128. Corpeleijn WE, Elburg RM Van, Kema IP, Goudoever JB Van. Assessment of Intestinal Permeability in (Premature) Neonates by Sugar Absorption Tests. Methods Mol Biol 2011;763:95–104.
129. Corpeleijn WE, van Vliet I, de Gast-Bakker DA, et al. Effect of enteral IGF-1 supplementation on feeding tolerance, growth, and gut permeability in enterally fed premature neonates. J Pediatr Gastroenterol Nutr 2008;46:184–90.
130. Shetty N, Hill G, Ridgway GL. The Vitek analyser for routine bacterial identification and susceptibility testing: protocols, problems, and pitfalls. J Clin Pathol 1998;51:316–23.
131. Demirev PA, Ho YP, Ryzhov V, Fenselau C. Microorganism identification by mass spectrometry and protein database searches. Anal Chem 1999;71:2732–8.
132. Agergaard P, Hebert A, Bjerre J, Sørensen KM, Olesen C, Østergaard JR. Children diagnosed with congenital cardiac malformations at the national university departments of pediatric cardiology: Positive predictive values of data in the Danish National Patient Registry. Clin Epidemiol 2011;3:61–6.
133. Moth G, Vedsted P, Schiøtz P. National registry diagnoses agree with medical records on hospitalized asthmatic children. Acta Paediatr 2007;96:1470–3.
134. Pedersen M, Klarlund M, Jacobsen S, Svendsen AJ, Frisch M. Validity of rheumatoid arthritis diagnoses in the Danish National Patient Registry. Eur J Epidemiol 2004;19:1097–103.
135. Di Napoli A, Di Lallo D, Perucci CA, et al. Inter-observer reliability of radiological signs of necrotising enterocolitis in a population of high-risk newborns. Paediatr Perinat Epidemiol 2004;18:80–7.
136. Menon G, Williams TC. Human milk for preterm infants: why, what, when and how? Arch Dis Child Fetal Neonatal Ed 2013;98:F559-62.
137. Cristofalo EA, Schanler RJ, Blanco CL, et al. Randomized trial of exclusive human milk versus preterm formula diets in extremely premature infants. J Pediatr 2013;163:1592–5.
138. Murthy K, Yanowitz TD, DiGeronimo R, et al. Short-term outcomes for preterm infants with surgical necrotizing enterocolitis. J Perinatol 2014;34:736–40.
139. Thymann T, Møller HK, Stoll B, et al. Carbohydrate maldigestion induces necrotizing enterocolitis in preterm pigs. Am J Physiol Gastrointest Liver Physiol 2009;297:G1115–25.
140. Li Y, Jensen ML, Chatterton DEW, et al. Raw bovine milk improves gut responses to feeding relative to infant formula in preterm piglets. Am J Physiol Gastrointest Liver Physiol 2014;306:G81-90.
141. Li Y, Østergaard MV, Jiang P, et al. Whey protein processing influences formula-induced gut maturation in preterm pigs. J Nutr 2013;143:1934–42.
142. Torrazza RM, Parker LA, Li Y, Talaga E, Shuster J, Neu J. The value of routine evaluation of gastric residuals in very low birth weight infants. J Perinatol 2014;35:57–60.