NECROTIZING ENTEROCOLITIS – GROWTH, BONE HEALTH AND INTESTINAL MICROBIOTA DURING CHILDHOOD

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"Give the children love, more love and still more love –

and the common sense will come by itself."

– **Astrid Lindgren**

To my family

Necrotizing enterocolitis –

growth, bone health and intestinal microbiota during childhood

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ABSTRACT

Necrotizing enterocolitis (NEC) is a dreaded gastrointestinal disease primarily affecting infants born preterm. Despite extensive research, NEC is still one of the most devastating diseases in neonatal intensive care. Improvements in neonatal care during the last decades have led to increased survival of more preterm infants, and thus, the group at risk to suffer from NEC has increased. Research is limited regarding if NEC in the neonatal period can affect growth, bone mass, body composition, fracture tendency and intestinal microbiota several years after the disease.

The aim of this thesis was to clarify if children born preterm with a history of NEC, had altered growth, body composition, bone mass, fracture tendency and intestinal microbiota during their childhood compared to preterm children without a history of NEC. Another aim was to investigate the occurrence of space-time clusters of NEC.

This thesis is based on the results of four different studies. Study I and II were retrospective cohort studies based on diagnostic codes from register data from children born between 1987 and 2009. Study I investigated the occurrence of space time-clustering of NEC, showing significant clustering on hospital level with a decreasing trend. Study II examined the risk for fractures and rickets in preterm children with and without a history of NEC, where a history of NEC appeared to increase the risk for rickets but not fractures. Study III and IV were

prospective cohort studies of growth, bone mass, body composition and intestinal microbiota in five-year-old children born preterm with and without a history of NEC. Study III showed that NEC-survivors were shorter, had lower weight, affected bone parameters and lower fat mass than matched controls. Study IV revealed significantly lower alpha diversity in the intestinal microbiota in NEC-survivors compared to controls. Significant differences between NEC-cases and controls were seen in relative abundance on both bacterial genus and species level. The differences in microbiota diversity were especially pronounced in the surgically treated NEC group.

In conclusion, these studies suggest that a history of NEC may have an impact on growth, bone mass and fat mass several years after the disease. However, a history of NEC does not seem to increase the risk for fractures. The results also indicate that intestinal dysbiosis after NEC is long-lasting, especially if NEC was surgically treated. A decrease of clustering of NEC on hospital level over time may reflect the improved routines in neonatal care to minimize the transmission of contaminants between patients.

Keywords: skeleton, preterm, NEC, microbiota

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SAMMANFATTNING PÅ SVENSKA

Nekrotiserande enterokolit (NEC) är en fruktad tarmsjukdom som framför allt drabbar underburna barn. Trots omfattande forskning om sjukdomen är det fortfarande en av de mest förödande sjukdomarna inom neonatal intensivvård och dödligheten är fortsatt hög. De senaste decennierna har betydande framsteg gjorts inom obstetrik och neonatalvård vilket har lett till att fler och mer underburna barn räddas och överlevnaden hos de allra minsta barnen har ökat kraftigt. Gruppen med risk att insjukna i NEC har därmed blivit större och som ett resultat av detta har incidensen av NEC ökat. Barn som insjuknar i NEC behandlas med tarmvila och antibiotika men vid svårare former av NEC kan kirurgisk åtgärd med tarmresektion behövas. Genomgången NEC, framför allt i de fall som behövt kirurgi, medför stor risk för långtidskomplikationer som tarmsvikt och kort-tarmsyndrom som kan ge nedsatt förmåga att ta upp näring vilket kan leda till tillväxthämning och påverkad benuppbyggnad. Barn med NEC behandlas dessutom ofta med långvariga antibiotikakurer vilket kan påverka koloniseringen av bakterier i tarmen och uppbyggnaden av en normal tarmflora.

Målet med denna avhandling var att studera om underburna barn som under sin neonatalperiod drabbats av NEC har påverkad tillväxt, bentäthet, frakturbenägenhet, kroppssammansättning och tarmflora flera år efter genomgången sjukdom. Detta område är ofullständigt utforskat och antalet långtidsuppföljningar är få. Ett annat mål var att undersöka förekomsten av anhopning av NEC, så kallad klustring, i tid och rum.

Denna avhandling baseras på resultaten från fyra olika studier. De två första studierna var retrospektiva registerbaserade kohortstudier på alla barn födda mellan 1987 och 2009, inklusive alla barn som fått diagnosen NEC. Studie I undersökte förekomsten av anhopning av NEC i tid och rum, där signifikant anhopning av NEC upptäcktes på sjukhusnivå i tidsperioden 1987–1997 men inte i tidsperioden 1998–2009. Studie II undersökte risken för rakit och frakturer under barndomen hos underburna barn med och utan genomgången NEC, där en genomgången NEC föreföll öka risken för rakit men inte risken för fraktur. Studie III och IV var prospektiva kohortstudier rörande tillväxt, kroppssammansättning, bentäthet och tarmflora vid fem års ålder hos underburna barn som har haft NEC och deras matchade kontroller. Studie III visade att vid fem års ålder var barn med genomgången NEC kortare och lättare än kontrollerna. De hade även mindre fettmassa och tendens till lägre bentäthet. Studie IV visade signifikant lägre alfa-diversitet i tarmfloran hos barn med genomgången NEC jämfört med kontrollerna. Avseende relativ förekomst av bakterier sågs signifikant skillnad mellan barn med genomgången NEC och

kontroller både på släktnivå och artnivå. Skillnaderna i diversiteten i tarmfloran sågs framför allt hos barn med kirurgiskt behandlad NEC.

Våra studier tyder på att genomgången NEC kan påverka tillväxt, kroppssammansättning och bentäthet flera år efter sjukdomen. Däremot verkar genomgången NEC inte öka risken för frakturer under barndomen. Resultaten antyder även att en bakteriell obalans i tarmen inte enbart har betydelse för uppkomsten av NEC utan kvarstår länge efter genomgången NEC, särskilt om sjukdomen behandlats kirurgiskt. Anhopningen av NEC-fall på sjukhusnivå har minskat de senaste decennierna, vilket kan återspegla neonatalvårdens förbättrade rutiner för minskad smitta mellan patienter.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Magnusson A**, Ahle M, Swolin-Eide D, Elfvin A, Andersson RE. Population-based study showed that necrotising enterocolitis occurred in space-time clusters with a decreasing secular trend in Sweden. Acta Paediatr. 2017;106(7):1097-102.

II. **Magnusson A**, Ahle M, Andersson RE, Swolin-Eide D, Elfvin A. Increased risk of rickets but not fractures during childhood and adolescence following necrotizing enterocolitis among children born preterm in Sweden. Pediatr Res. 2019;86(1):100-6.

III. **Magnusson A**, Swolin-Eide D, Elfvin A. Body composition and bone mass among five-year-old survivors of necrotizing enterocolitis. Pediatr Res. 2023;93(4):924-31.

IV. **Magnusson A**, Jabbari Shiadeh SM, Ardalan M, Swolin-Eide D, Elfvin A. Intestinal microbiota at five years of age among children born preterm with a history of necrotizing enterocolitis. In manuscript.

CONTENT

ABBREVIATIONS

ALP	Alkaline phosphatase
BMAD	Bone mineral apparent density
BMC	Bone mineral content
BMD	Bone mineral density
BPD	Bronchopulmonary dysplasia
CDR	National Cause of Death Register
DXA	Dual energy X-ray absorptiometry
DXL	Dual energy X-ray and laser
ELBW	Extremely low birth weight
GA	Gestational age
IF	Intestinal failure
IGF	Insulin like growth factor
LPS	Lipopolysaccharide
LS	Lumbar spine
MBD	Metabolic bone disease
MBR	Medical Birth Register
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care unit
NO	Nitric oxide
NPR	National Patient Register

OOP	Osteopenia of prematurity
PDA	Patent ductus arteriosus
PN	Parenteral nutrition
ROP	Retinopathy of prematurity
SBS	Short bowel syndrome
SDS	Standard deviation score
SIP	Spontaneous intestinal perforation
TBHE	Total body head excluded
TLR	Toll-like receptor
VEGF	Vascular endothelial Growth Factor
VLBW	Very low birth weight

THESIS AT A GLANCE

Paper	I	II	III	IV
Aims	To identify the presence of space-time clusters of NEC.	To determine if preterm NEC-survivors have increased risk of fractures, rickets and vitamin D deficiency compared to preterm children without a history of NEC.	To clarify if preterm NEC-survivors have an altered body composition and reduced growth and bone mass at 5 years of age compared to preterm children without a history of NEC.	To investigate if preterm NEC-survivors have an altered intestinal microbiota at 5 years of age compared to preterm children without a history of NEC.
Methods				
Study design	Retrospective cohort study	Retrospective cohort study	Prospective cohort study	Prospective cohort study
Population	All live births including all children with NEC born in Sweden between 1987 and 2009.	All children with NEC born <31+6 weeks in Sweden between 1987 and 2009 and matched controls without a history of NEC.	Children at 5 years of age with a history of NEC, born before 37 gestational weeks and matched controls without a history of NEC.	Children at 5 years of age with a history of NEC, born before 37 gestational weeks and matched controls without a history of NEC.
N of subjects	808 + 2 389 681	371 + 1839	25 + 25	15 + 24
Results	Clusters of NEC occurred on hospital level with a decreasing trend over the last decades. No sign of clustering on residential level.	NEC-survivors had an increased risk of rickets and malabsorption, but not more fractures or vitamin D deficiency during childhood and adolescence.	NEC-survivors were shorter and had lower weight, BMC, BMD Z-scores and fat mass compared to matched controls at 5 years of age.	NEC-survivors, especially the surgically treated, had different relative abundance and signs of lower species diversity in the intestinal microbiota at 5 years of age.
Conclusions	Clusters on hospital level indicate an infectious environmental effect after delivery. Decrease in clustering over time may reflect improved routines in neonatal care.	Despite an increased risk of rickets and malabsorption, a history of NEC was not associated with more fractures during childhood.	A reduced growth and hints of lower bone mass suggests that NEC-survivors need special attention regarding growth and bone health during childhood.	Surgical treatment of NEC appears to affect the microbiota composition for several years, indicating that treatment optimization may reduce dysbiosis.

1 INTRODUCTION

Necrotizing enterocolitis (NEC) is a devastating and life-threatening gastrointestinal disease, primarily affecting infants born preterm (1, 2). There's a wide spectrum of clinical presentations of NEC, ranging from mild forms, which can be misinterpreted as food intolerance, to a fulminant disease with necrosis and perforation of the bowel that requires surgery and removal of the affected part of the intestine (3, 4). Based on clinical and radiographic signs, the severity of NEC is classified by Bell's staging criteria from 1978, modified by Walsh and Kliegman in 1986, and the severity of the illness is playing a key role for the choice of treatment and the outcome (3, 5, 6). Despite decades of research, the exact pathogenesis is still not entirely understood, and NEC remains a major cause of death and long-term morbidity among neonates (2, 3, 7). The vast majority of NEC cases occur sporadically but reports of outbreaks or clusters of NEC indicate an infectious element as a possible contributing factor (8-10).

Conditions with close resemblance to NEC have been described since the early 19th century. However, it was only after the advent of neonatal intensive care units (NICUs) during the first half of the 20th century that NEC began to be described as a clinical entity (4, 11, 12). The term "necrotizing enterocolitis" to describe the condition with abdominal distension, shock, bleeding, and perforation in the gastrointestinal tract among preterm infants was first used in 1965 by Mizrahi (13).

The last decades there have been substantial improvements in the perinatal and neonatal care, especially with the introduction of surfactant treatment and antenatal steroids in the 1990s (14-16). These improvements have resulted in increased survival of extremely preterm infants with higher risk of developing NEC and, consequently, the population of patients with NEC has changed (17). Before the surfactant era, most patients with NEC were preterm infants with a gestational age over 30 weeks (4, 18). As a consequence of increased survival of patients more susceptible for NEC, the incidence of NEC has increased, and the mortality has remained high for the last decades (5, 15, 19). Along with the increased incidence of NEC, comes a larger population of survivors with possible long-term morbidities. These morbidities include, among other things, neurodevelopmental impairment, short bowel syndrome (SBS), intestinal failure (IF), malabsorption and poor growth (5). Many of the follow-up studies follow the children for up to 24 or 36 months, a few follow the children for a longer period of time (5).

The overall aim of this doctoral thesis was to clarify if children born preterm with a history of NEC, during their childhood had reduced growth, lower bone mass, increased fracture tendency and a different intestinal microbiota compared to children born preterm without a history of NEC. A further aim was to clarify if NEC occurs in space-time clusters.

2 BACKGROUND

2.1 GROWTH

The period with the fastest growth is before birth. In particular during pregnancy weeks 13-28 (the second trimester) the longitudinal growth is very fast. Several factors have an influence on intra-uterine growth, such as energy via the umbilical cord, maternal- and paternal size and hormones as insulin like growth factor -I (IGF-I) and IGF-II, growth hormone and androgens (20, 21). Growth during infancy is also regulated by genetic factors, hormones, vitamins and nutritional factors. Deficiency in any of the hormones may affect growth (22).

During the first year of extrauterine life, growth can initially be seen as an extension of the fetal growth. Growth rate is initially fast, with a declining rate over time. Special growth charts have been developed to describe the early extrauterine growth including the early extrauterine growth of extremely preterm infants (23). In recent years mathematical models have been developed as an aid to predict individual growth from fetal to adult life (24).

Regarding growth after preterm birth studies have shown that many children born very preterm have an initial period of poor growth, but then most of them reach normal height and weight before puberty, with parental height being the most important factor explaining variations (25). Another study showed that children born extremely preterm, with a gestational age at birth of 23-25 weeks in the 1990s showed an initial period of poor growth, followed by catch-up growth, but they remained significantly smaller than term born children at 11 years of age (26).

The initial period of poor growth among preterm infants may be affected by many things. Even if studies show that the majority of preterm infants will have a catch-up growth before puberty, the early postnatal growth failure may be of importance for other long term outcomes such as psychomotor development (27). Based on that, interventions to minimize the early period of poor growth in preterm infants, for example by optimizing nutrition, may be important, and is gaining more and more attention in modern neonatal care (28).

2.2 BONE MASS AND BODY COMPOSITION

2.2.1 BONE TISSUE

Bone is a metabolically active organ with numerous functions; it provides stability to the body and enables us to move, it serves as a protection of vital internal organs, it is a reservoir for phosphate and calcium, nurtures hematopoietic stem cells in the bone marrow and has metabolic functions (29, 30). The bone is under constant remodeling throughout life. The remodeling process is a close interaction between osteoblasts, responsible for bone formation, and osteoclasts, responsible for bone resorption (31-34). Bone consists of the compartments cortical bone, that is compact and solid, and trabecular bone that is more honeycomb-like. Both compartments provide strength to the bone but have different properties where the cortical bone has higher calcium content, but lower content of water compared to trabecular bone. Approximately 80% of the bone mass is cortical bone, which is located on the surface of the bones, and 20 % is trabecular bone, found in the vertebrae and in the metaphysis of the long bones (29). Approximately 20% of the trabecular volume is bone, the remaining 80% is fat and bone marrow.

Peak bone mass is the maximum amount of bone tissue accrued and is present at the end of bone maturation. Peak bone mass is genetically determined and occurs in early adulthood between 20 and 30 years of age. Thereafter, the bone mass slowly decreases throughout life (32, 35). Males have a prolonged bone maturation period than females, which is the main reason for the gender difference in in bone mass (36). Other factors that influence peak bone mass include genetic factors, physical activity, the intake of proteins and calcium and age at puberty. Hence, it is important to optimize physical activity and intake of calcium and proteins during growth for ideal peak bone mass acquisition (32, 37, 38). It is predicted that an increase of 10% in peak bone mass can delay the onset of osteoporosis with up to 13 years and the risk of fractures later in life decreases with 50% (39, 40).

2.2.2 CALCIUM, PHOSPHORUS AND MAGNESIUM ABSORPTION

The major minerals involved in bone mineralization are calcium, phosphorus and magnesium and most of the body content of these minerals are found in the skeleton (41, 42).

The most abundant mineral in the human body is calcium (35, 42). Calcium has importance in several functions in the body, for example cardiac function, muscle contraction, nerve transmission, intracellular metabolism and bone acquisition. Almost all, 99%, of the calcium in the body is contained in the bones and teeth and the remaining 1% is free and exchangeable. During the first 18 years of life, the bone calcium accretion is positive where the first year has the highest accumulation (35). The calcium concentration is maintained at an almost constant level through the hormones parathyroid hormone, $1,25(OH)_2$-vitamin D and calcitonin which regulate the intestinal calcium absorption, calcium excretion, renal reabsorption and the skeletal calcium stores (35, 43).

The main part, 80%, of the fetal calcium accumulation occurs during the third trimester, and this high mineral concentration is essential for bone mineralization. During this time, up to 150 mg calcium/kg/day and up to 90 mg phosphorus/kg/day is transferred across the placenta and the calcium content in the fetus increases fourfold (35, 44). Calcium is transported actively to the fetus across the placenta against a concentration gradient. To meet these higher calcium needs, the maternal calcium absorption increases with approximately one third during the last trimester (35, 41, 45). However, several animal studies have shown that the calcium level in the fetus is not affected but remains stable even though the maternal calcium levels are low (46, 47). This continuous, generous supply to the fetus of calcium, phosphorus and magnesium is terminated abruptly at birth. The newborn infant must then rely on enteral intake of minerals as the major source of minerals then shifts to the intestines while the fine balance of these minerals in serum and bone is maintained by the kidneys. After birth, the total and ionized calcium concentrations decrease to a reach a lowest level after 24-48 hours postnatally in term infants and thereafter gradually rise during the first week of life to adult levels comparable to the calcium levels found during childhood (35, 41). However, in preterm infants, the intestinal maturation, and therefore the rise in calcium levels, may be substantially delayed. This delayed rise in calcium levels along with the increased demands for mineral accretion, predispose preterm infants for osteopenia of prematurity (41).

Phosphorus is important for bone development since it has a central part in the formation of osteoid and the apoptosis of chondrocytes (41). The concentration of the minerals in serum in the fetus is primarily regulated by parathyroid hormone and parathyroid hormone related peptide (41, 48).

The bone mineral hydroxyapatite, which is where most of calcium and phosphorus are found, is formed only if the components calcium and phosphorus are available in ideal amounts at the same time. In addition to skeleton content, one fifth of phosphorus is found in tissue and is important for cell metabolism

(42). For optimal bone mineral accretion in the infant after birth, there needs to be a concurrent excess of calcium and phosphorus in the parenteral nutrition (PN). In deficiency of phosphorus, the available amount is mainly directed to the tissue since cellular metabolism has priority. Accordingly, in hypophosphatemia, the bone mineralization can be reduced or even bone demineralization can occur (49). Therefore, to avoid the mineral intake to be too low or too high in growing individuals, the intake should be adjusted individually regarding weight gain and growth (42). Since growth velocity changes over time, thus will also accretion rate change and can be different in boys and girls (42, 50).

Very low birth weight (VLBW) infants have a high need for phosphorus for their growth and is thus at risk for hypophosphatemia. Throughout hypophosphatemia, microcrystalline apatite cannot be formed which can lead to hypercalcemia, hypercalciuria and in the extension even bone demineralization and osteopenia (42, 51, 52).

In parenteral nutrition, calcium and phosphorus are directly available to bone mineralization. However, in enteral nutrition, the individual absorption of minerals must be taken in consideration and calcium absorption can vary substantially. Mineral losses through urine, feces and skin must also be considered (42, 49, 53).

2.2.3 OSTEOPENIA OF PREMATURITY

Infants born extremely preterm have an increased risk of reduced bone mineral content (BMC) and therefore to develop brittleness of the bones, a disease called osteopenia of prematurity (OOP) (54). OOP, also called metabolic bone disease (MBD) of prematurity, is characterized by a decreased mineralization of the bone and a reduction of BMC. The risk of OOP and severity of it is inversely proportional to gestational age and birth weight and it is most often observed in infants born before 28 weeks of gestation, occuring in up to 25% of VLBW infants and up to 55% of extremely low birth weight (ELBW) infants (55-58). Since the advances in neonatal care have led to increased survival of preterm infants, OOP remains a significant concern in the NICUs and the incidence is rising (58, 59).

The minerals calcium and phosphate, essential for bone mineralization, are transported actively across the placenta from the mother to the fetus. During the third trimester, 80% of the mineral accretion and bone mineralization occurs (56, 60). Hence, preterm infants born before or during the third trimester miss out of the accretion and mineralization in the uterus in varying extent and have

inadequate reserves of these essential minerals (54, 61, 62). In addition to inadequate reserves, preterm infants also have an increased loss of minerals. Preterm infants are, for example, frequently treated with diuretics and steroids, which increase the losses of calcium and phosphate through the kidneys (58, 59, 63, 64). Furthermore, since the placental transfer of the essential minerals stops at birth, the preterm infant is reliable of enteral and parenteral intake and there are difficulties in maintaining a sufficient supply of minerals this way (35). OOP is caused by a combination of an increased loss of these important minerals and difficulties to maintain sufficient intake to replace the losses (45, 54, 59, 63).

Alkaline phosphatase (ALP) is a marker of bone formation and osteoblast activity. ALP is a biomarker that increases in bone turnover. High levels of ALP have been associated with OOP or MBD (56, 64, 65). The consequences of OOP range from mild osteopenia to severe forms that can cause rickets and fractures (42, 64, 66-68). OOP with high ALP can affect postnatal growth, with slower growth rate during the neonatal period, and may even have a long-term effect with stunted height during childhood (64, 65, 69, 70).

2.2.4 MEASUREMENT OF BONE MASS AND BODY COMPOSITION

DUAL-ENERGY X-RAY ABSORPTIOMETRY (DXA)

The method with DXA, which uses X-rays instead of gamma rays, was first described in 1987 by Cullum et al (71). It is a two-dimensional X-ray method and compared to previous methods for measuring bone mass, DXA has both improved precision and accuracy and a reduced radiation dose. DXA is now the golden standard for bone density measurements and the International Society for Clinical Densitometry, including their pediatric section, have recommended DXA as the ideal method to measure BMC and bone mineral density (BMD) (38, 72-74). When introduced in 1987 it the DXA measured bone density in the spine and hips, but now measurements of the whole body can be performed (71). However, in children, the recommended skeletal sites for measurement of BMC and BMD are the posterior-anterior spine and total body head excluded (TBHE), since the skull represents a considerable percentage of the skeletal mass (38, 72).

DXA uses X-rays at two different energy levels, which makes it possible to measure two different types of tissues; bone tissue and soft tissue, and measures body composition with fat mass and lean mass, figure 1 (74, 75).

BMC is reported in gram and BMD is reported in gram/cm^2. BMD is often also reported in Z-scores, defined as the number of standard deviations above or below the gender- and age-matched mean. A BMD Z-score ≤ -2 is referred to as "low bone mineral density for age" (38, 72, 76). The bone mass results can be confounded due to small bone size and should be adjusted in children with delayed growth or short stature (38). Furthermore, BMD measured with DXA is areal, or two-dimensional, and it is not possible to get a three-dimensional or volumetric bone density in g/cm^3. This affects the evaluation of bones of different sizes and growing skeletons (77). Adjustment can be done using bone mineral apparent density (BMAD) (see section with DXL) (38).

There are numerous advantages with measurements with DXA. One strength is the rapid scanning time, which means that the child doesn't have to hold still for a long period of time minimizing the risk for motion artifacts. Another strength is the small radiation dose which is similar to that of a single day's background radiation. It is also relatively easy to position the child in the scanner and the configuration of the scanner is open and therefore rather reassuring for the child (38, 74) .

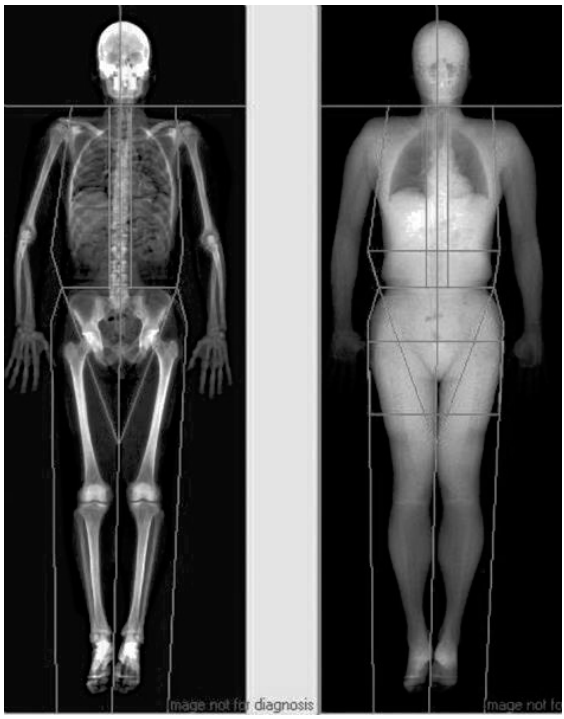

Figure 1. *De-identified DXA-scans of total body of an adult. Picture provided by GE Healthcare.*

DUAL-ENERGY X-RAY ABSORPTIOMETRY AND LASER (DXL)

The DXL Calscan technique measures the bone mass in the calcaneus in the heel of the foot with a combination of DXA and laser, where the laser measures the thickness of the calcaneus. This technique allows the calcaneal BMC and BMD to be measured with greater accuracy (78, 79). The pediatric version of the DXL Calscan enables manual measurement of the calcaneal height. This height, together with the BMD, enables calculation of the volumetric BMAD (g/cm^3), which makes adjustment for different bone sizes possible (80). Osteopenia is well predicted with the DXL method and BMD values acquired from DXA and DXL correlates well in children and adolescents (81, 82).

2.3 INTESTINAL MICROBIOTA

2.3.1 INTESTINAL MICROBIOTA IN THE INFANT

Intestinal microbiota refers to the microorganisms in the gut and is estimated to include trillions of microbial cells, which is more than all other cells in the human body. The microbiota serves several important functions; it helps with digestion, provides with essential vitamins and nutrients, protects against pathogens and stimulates angiogenesis to name a few of the functions (83, 84). It is influenced by several factors including delivery mode, diet, age, medication, geography, travel, and stress and can differ substantially between individuals. Imbalances in the microbiota are known to correlate with several disorders, including NEC (83, 85-88). *Enterobacter* species, *Klebsiella* species, *E. Coli* and gram-positive organisms like coagulase-negative *Staphylococcus* are the most common organisms from bacterial cultures from NEC-cases (2, 89).

Previously, it was assumed that the intrauterine environment was sterile and hence, the infant in utero had a sterile gastrointestinal tract, and it was during birth that the colonization of microbes begun. Later studies have, however, shown that the placenta, umbilical cord, and amniotic fluid contain microbes and for example by swallowing amniotic fluid the fetus might begin to colonize the gastrointestinal tract (85, 90). It has also been shown that microbes are present in the meconium (91). However, even if there is some intrauterine exposure, the majority of the intestinal microbiota in the newborn infant is obtained after birth. Since the vaginal microbiota differs from the microbiota on the skin, the mode of delivery, vaginal or caesarian section, influences the composition of the intestinal microbiota in the infant and can affect the progression of the microbiota for several months after birth (83, 85-87, 92). The altered microbial colonization may be of importance to the susceptibility to inflammatory processes in the intestines (93).

It is shown that the intestinal microbiota in hospitalized preterm infants is different from the microbiota in healthy infants born at term (84). Abnormal colonization of the intestines in a preterm infant may affect the maturation of the immune system. It may also influence the intestinal microbiota for a long time (94). The number of follow-up studies regarding the evolution of intestinal microbiota of children born preterm are very sparse (94). Intestinal dysbiosis appears to have increased in the last century in developed countries, partially because of the use of antibiotics (95). The type of feeding also plays an important role in determination of the intestinal microbiota (92).

The literature regarding the composition and the factors that shape the infant intestinal microbiota is contradictory (83). Some reports describe that *Bifidobacteria* is predominated over potentially harmful bacteria in the microbiota of breast-fed infants, while others describe that *Bifidobacteria* are much less abundant. The microbiota of formula-fed infants has, however, a less abundance of *Bifidobacteria* and a higher abundance of aerobic bacteria like *Enterococci* (83, 84, 96).

In contrast to the intestinal microbiota in adults, that remains stable for several months, the composition of the infant intestinal microbiota is more variable and less stable (83). Anaerobes like *Bacteroides, Clostridium, Eubacterium, Faecalibacterium and Ruminococcus* constitutes a large majority of the adult intestinal microbiota (83).

Bacteria are classified as shown in Table 1.

Table 1. *The taxonomic classification of bacteria using Klebsiella Pneumoniae as an example.*

Kingdom	Prokaryote
Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Klebsiella*
Species	*Klebsiella pneumoniae*

Early in the colonization process of the newborn infant there are primarily aerobic, potentially pathogenic, organisms that colonize the gut, for example *Staphylococci, Streptococci* and *Enterobacteria* (85, 92). There is strong evidence supporting the administration of prophylactic probiotics to prevent sepsis, necrotizing enterocolitis and death in preterm infants (95). A Cochrane review from 2014 showed that probiotic supplementation, to imitate the natural colonization in the gut, significantly reduced the incidence of severe NEC (stage II and higher), mortality, and NEC related mortality in preterm infants (97). Probiotics have also been shown to reduce the length of hospital stay and feeding intolerance and improve growth velocity and weight gain (98).

Early in the 1900s, it was seen that drinking fermented milk products could be associated with a prolonged life, hypothetically with healthy bacteria from the fermented milk competing with harmful bacteria in the gut. It was indicated that the gut microbiome is modifiable (85).

The variation in diversity in intestinal microbiota is greater between infants than between adults and this variation may be due to differences in the immune response during colonization, diet, lifestyle, or other random events during colonization, for example antibiotic treatment. At birth, the diversity is at its lowest and then increases over time (99). Antibiotic treatment can be followed by lower diversity and changes in diet can increase the diversity. However, when exposed for the same antibiotic more than once, the diversity may not be as affected as when exposed for the first time. This may indicate that the microbiota has an adaptive power when exposed multiple times for the same antibiotic (99).

When measuring species diversity, both relative abundance, evenness and richness are considered. Abundance, or absolute abundance, is the number of organisms found in a sample or defined area. In other words, every organism or individual in an area is identified and counted. Relative abundance describes the rarity or commonness of a species or organism in relation to other species or organisms in a defined community or area. Richness refers to the number of species in an area or community. Evenness refers to how equal species are in proportion to each other where a higher equality of species provides a higher evenness. Low evenness indicates that a low number of species are dominating a community and a low evenness gives a lower alpha diversity (100, 101).

Example A and B in figure 2 both have ten organisms and four different species, hence they have the same abundance and richness, but they differ in evenness and relative abundance. In example A in figure 2, the red organism has high relative abundance compared to the other organisms and the evenness and diversity is low. However, in example B the relative abundance is more evenly distributed, the evenness is higher, and the diversity is higher.

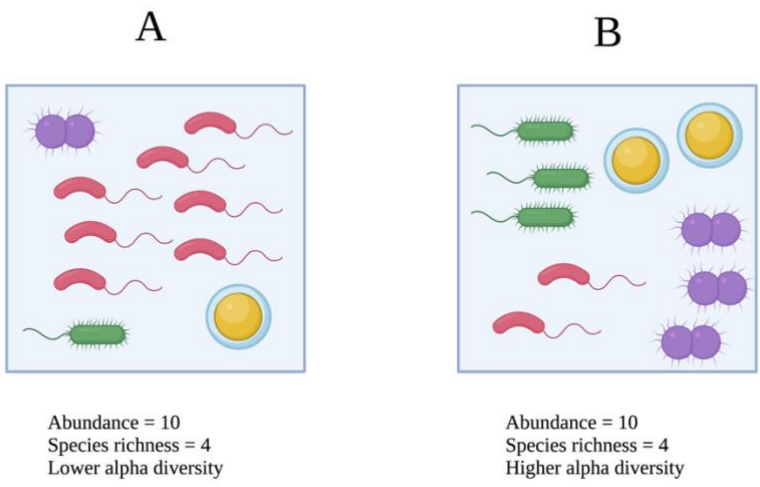

Figure 2. *Illustration showing bacterial abundance, richness and alpha diversity. Created with BioRender.com with inspiration from dr Seyedeh Marziyeh Jabbari Shiadeh.*

Alpha diversity is a measurement of the diversity within a defined area or community where a higher number of different species and a higher evenness results in a higher alpha diversity. Beta diversity, on the other hand, is a measurement of the diversity, or commonness, of species between two areas or communities. If two communities have many unique species and share only a low number of species, the beta diversity is high. On the contrary, if two communities have several species in common, the beta diversity is low, figure 3.

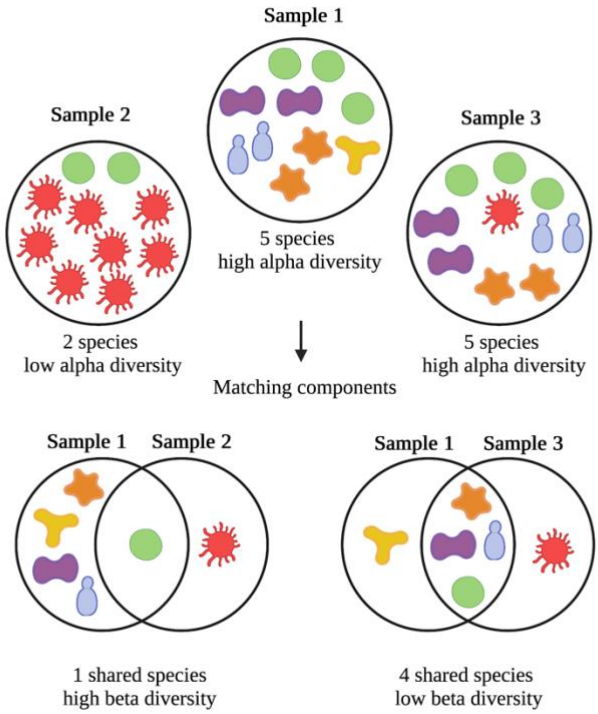

Figure 3. *Alpha- and beta diversity. Sample 1 and 3 have high alpha diversity with a high number of different species, while sample 2 only have two species and a low alpha diversity. Sample 1 and 2 only have one species in common and hence, the beta diversity is high. Sample 1 and 3 have four different species in common and only one unique species in each sample and the beta diversity is therefore low. Created with BioRender.com with inspiration from dr Seyedeh Marziyeh Jabbari Shiadeh.*

2.4 NECROTIZING ENTEROCOLITIS

2.4.1 HISTORICAL BACKGROUND

NEC is not a new disease and already in the 19th century there were reports of cases with symptoms as inflammation and necrosis in the gastrointestinal tract and where perforations in the intestinal wall were found at the postmortem examinations (4, 12). However, it was only after the advent of NICUs during the first half of the 20th century that NEC began to be described as a clinical entity (4, 11, 12). The term "necrotizing enterocolitis" to describe the condition with abdominal distension, shock, bleeding, and perforation in the gastrointestinal tract among preterm infants was first used in 1965 by Mizrahi (13).

2.4.2 EPIDEMIOLOGY

NEC is the most common gastrointestinal emergency and a leading cause of morbidity among preterm infants (6, 11, 102, 103). It affects primarily preterm infants with VLBW (<1500 grams), where the incidence is approximately 7% (11). However, there is a considerable variation in incidence between studies with reported incidences from below 3% to above 16% among VLBW infants (2, 3, 14, 19, 104, 105). The overall incidence of NEC among all newborns in Sweden is 0,34 per 1000 live births, but this also differs between studies and countries (15). Due to the improvements in neonatal care, preterm infants that would not survive decades ago are now surviving and are susceptible for NEC and the incidence is therefore rising (5, 19, 106-108). Although NEC is widespread worldwide, there is a variation in incidence between different populations and geographical locations (6, 17). For example, Japan has a lower reported incidence of NEC among extremely preterm and VLBW infants than other high-income countries like Australia, Canada and USA (6). In low and middle-income countries, the incidence of NEC seems to be lower in countries where preterm infants are uncommon (2, 14). Of those affected by NEC, approximately 30-40% require surgery and the incidence of surgical NEC is higher among those who have an earlier onset of NEC (109, 110).

The mortality rates vary between studies and range from 10% to 50% with the highest rates among the most preterm infants with ELBW (<1000 g) and among those requiring surgery (1, 6, 11, 107). The mortality in NEC has increased over the last decades, both in absolute numbers and the relative contribution to deaths. This may be a result of the improvements in survival of preterm infants that previously would have died before they suffered from NEC (111, 112). In summary, both the incidence and the mortality are inversely proportional to gestational age and birth weight with higher rates among smaller infants (2, 5, 113). Infants with NEC are shown to be hospitalized longer than unaffected

preterm infants, 20 days longer if medically treated and 60 days longer if surgically treated (11, 114).

NEC is rare among infants born at or close to term, below 10% of the infants that develop NEC are born at term (1). NEC among those infants is often associated with other anomalies or predisposing conditions such as congenital heart disease, intestinal anomalies, polycythemia, perinatal asphyxia, chorioamnionitis, low Apgar scores, and sepsis. These conditions might reduce the mesenteric blood flow and therefore predispose to NEC (113, 115, 116). Hence, there is some discrepancy of the term NEC between preterm and term infants (116).

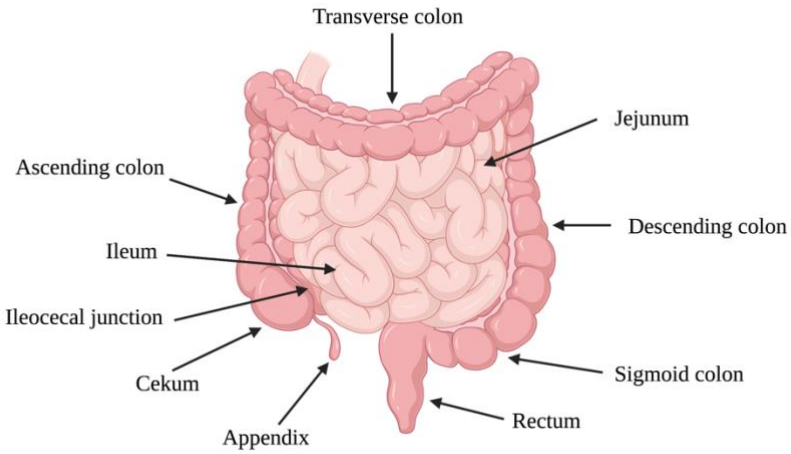

Figure 4. *Schematic illustration of the small and large intestine. NEC in preterm infants most often affect the ileum. Created with BioRender.com.*

NEC is most often seen in the ileum, but can involve any section of the intestines with variable length of the affected part, figure 4 (109, 117)

Spontaneous intestinal perforation (SIP), which is a sudden onset of pneumoperitoneum due to a focal or isolated perforation of the intestine, but usually without inflammation or necrosis, is a NEC-like condition and has at times been categorized as NEC but is a different disease with a different pathogenesis (79).

2.4.3 PATHOPHYSIOLOGY OF NEC

The pathophysiology of NEC is not entirely clear but is most certainly multifactorial and may differ depending on the maturity level of the infant. Prematurity and low birth weight are predisposing factors, and the intestine of a preterm infant is more prone to develop NEC than the intestines of a term infant (2, 11, 17, 118). The disease is characterized by various degrees of inflammation in the intestines, mucosal or transmural, where the end point is necrosis of the intestinal mucosa. The necrosis can lead to bowel perforation and peritonitis and, in the most severe cases, death. Sometimes the necrosis can involve the entire intestine which is referred to as "NEC totalis" (1, 2, 109, 119).

Although the pathophysiology is multifactorial, there are certain prerequisites for the disease to develop including abnormal bacterial colonization, presence of food in the intestines as a substrate for the bacteria, a change in the vascular tone, intestinal ischemia, intestinal injury and intestinal immaturity with an exaggerated inflammatory response, figure 5 (117, 120, 121).

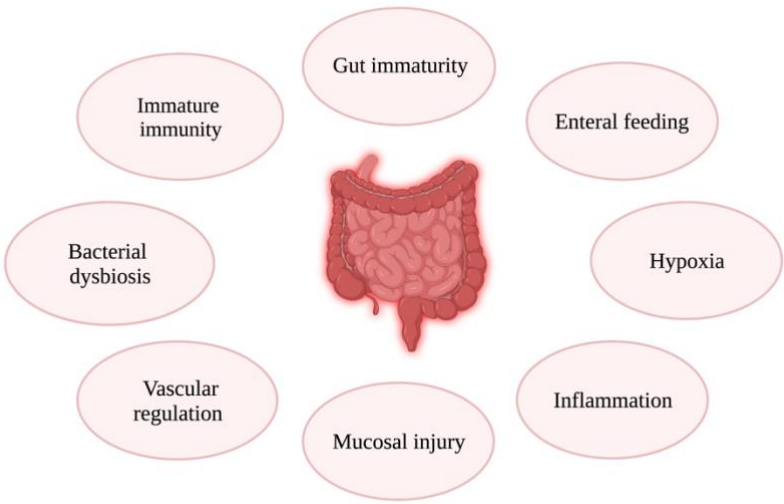

Figure 5. *Risk factors involved in the development of NEC in preterm infants. Created with BioRender.com.*

NEC in term infants is rare, and these infants often have underlying conditions like congenital heart disease or sepsis. There is a difference in pathophysiology and clinical presentation in term NEC compared to preterm NEC, and it is suggested that term NEC may be a different disease (113, 122, 123). Term or late

preterm NEC most often occurs earlier than preterm NEC, where the median age is under one week in term or late preterm infants and approximately two weeks or more in preterm infants (110, 122-124). Also, term NEC is more often localized to colon than preterm NEC that more often affect the small intestine and the ileocecal valve (125).

Where preterm NEC primarily is an inflammatory disease with necrosis as a secondary event, it is suggested that term infants with congenital heart disease often develop NEC due to reduced organ perfusion with inflammation as a secondary event (125).

The following text will mainly focus on the pathogenesis of preterm NEC.

GUT IMMATURITY

The motility of the intestines develops during the third trimester of pregnancy. Hence, the bowel in a preterm infant has an immature motility, which makes the intestinal epithelium more exposed to potential harmful substances (2).

The onset of NEC is believed to be partly due to an increased reactivity to microbial ligands in the preterm intestinal mucosa, compared to the intestinal mucosa in term infants. The preterm intestine also has an increased expression of Toll-like receptors (TLRs) compared to the intestine in a term infant. TLRs are bacterial receptors and an essential component of the innate immune defense in the preterm intestine (118). The TLRs are activated by different microbes in the intestine. Toll-like receptor 4 (TLR4) is activated by Lipopolysaccharide (LPS) on the wall of gram-negative bacteria which leads to an inflammatory cascade with subsequent disruption of the mucosa, vasoconstriction and eventually necrosis (109). Excessive Toll-like receptor 4 (TLR-4) signaling in response to LPS in the outer membrane of Gram-negative bacteria as well as an excessive immune inflammatory response is associated with a higher risk of NEC-onset. These TLRs are activated by different microbes in the gut, which results in an inflammatory cascade that induces a transcription factor, nuclear factor kappaβ, which in turn results in transcription of inflammatory mediators, such as cytokines and chemokines (118).

ENTERAL FEEDING

Typically, NEC occurs after enteral feeding is initiated. However, a delay in the initiation of enteral feed may also be associated with an increased risk for NEC, possibly due to development of mucosal atrophy when food in the intestines is absent (109). However, it has also been shown that there is no difference in prevalence of NEC between delaying the introduction of enteral feeding for over three days from birth or not (126).

Breast milk, however, has a well-known protective effect and this effect seems to be dose-related (127, 128). Cow´s milk-based formulas have shown to increase the risk of NEC in preterm infants. The use of human milk for preterm infants is therefore recommended (129, 130). A resent Cochrane report showed that a delay in the introduction of progressive enteral feeding to very preterm or VLBW infants might not reduce the risk for necrotizing enterocolitis (131).

BACTERIAL DYSBIOSIS

The microbial environment, or more specific dysbiosis, in the intestines are considered to be an essential factor in the development of NEC (109). Treatment with antibiotics as a standard for preterm infants, even in the absence of sepsis, can be related to an increased risk for NEC (118).

The intestinal epithelium is formed by tight junctions and a layer of glycoprotein mucin. It acts as a barrier and is crucial in protecting the intestines against possible pathogens. In preterm infants, the tight junctions and mucin layer are immature which can lead to an increased permeability in the intestines and a rise in bacterial adherence (2) Immune dysregulation linked to microbial dysbiosis has been proposed in the pathogenesis of NEC. Proteobacteria phylum includes many of the Gram-negative species known to be involved in NEC development, table 2.

Table 2. *The most frequently found bacterial phyla in the intestinal microbiota and examples of bacteria in each phylum.*

Phylum	Examples of genus of bacteria	Gram-positive/-negative aerobic/anaerobic
Actinobacteria	*Actinomyces, Atopobium, Bifidobacterium, Cornyebacterium, Cutibacterium, Dermabacter, Lawsonella, Rhodococcus, Rothia, Streptomyces* and more	Gram-positive, aerobic and anaerobic
Bacteriodetes	*Alistipes, Bacteroides, Flavobacterium, Parabacteroides, Prevotella, Tanerella, Sphingobacterium* and more	Gram-negative, aerobic and anaerobic
Firmicutes	*Clostridium, Enterococcus, Faecalibacterium, Lactobacillus, Ruminococcus, Staphylococcus, Streptococcus* and more	Mostly Gram-positive, aerobic and anaerobic
Fusobacteria	*Cetobacterium, Fusobacterium, Leptotrichia, Streptobacillus* and more	Gram-negative, obligate anaerobic
Proteobacteria	*Acinetobacter, Bilophila, Enterobacter, Escherichia, Shigella, Haemophilus, Helicobacter, Klebsiella, Neisseria, Serratia* and more	Gram-negative, facultative anaerobic

A dominance of Proteobacteria phylum in the early intestinal colonization among preterm infants who develop NEC has been described in several studies. A relative increase in the Proteobacteria phylum compared to other bacteria phylum such as Bacteroidetes and Firmicutes has been linked to an increased risk of developing NEC (103, 132-136). As Lipid A of the LPS in the cell wall is hexacylated in Proteobacteria, it is a strong activator of TLR4 and thus a more powerful trigger of the immune system. In Bacteroidetes phylum, the LPS is pentacylated, making it less potent in activating TLR4. As Proteobacteria activates the TLR4 this might lead to intestinal inflammation, damage of the enterocytes, and decreased proliferation of the epithelium, as well as induction of apoptosis, which all promote the development of NEC (132, 135, 137).

The use of antacid drugs for the stomach has been described to increase the growth of Proteobacteria over Firmicutes in the intestine. This could be one explanation to why these drugs increase the risk of NEC development (138).

Among the species of the Proteobacteria phylum, *Klebsiella* species, and more specific *Klebsiella pneumonia* are the bacteria that most often are linked to onset of NEC. According to Chen et al., *Klebsiella* species and their fimbriae-encoding genes were significantly increased in feces that was collected immediately prior to the NEC-onset. They suggest that these fimbriae may lead to the overexpression of TLR4-receptors seen in preterm infants (139). In addition to this, Torrazza et

al. detected *Klebsiella* spp. more frequently close to the diagnoses of NEC, and more specific *Klebsiella pneumoniae* that was strongly associated with the development of NEC (103).

Colonization with bacteria of the genus *Bifidobacterium* from the phylum Actinobacteria has been described as protective of intestinal pathology among preterm infants. Given the crucial role that *Bifidobacteria* play in maintaining a normal gut microbiota and epithelial resistance against pathogens, this is a highly reasonable finding (140). The importance of early colonization with *Bifidobacteria* as NEC protection needs to be further studied.

INFECTION

NEC is often related to a septic episode in preterm infants. The disease may start as a sepsis with bacteremia, followed by a necrotic inflammation in the intestine, or it may start with a bacterial translocation from the intestinal lumen into the intestinal wall, and further into the blood (141). Early treatment with antibiotics is often used in preterm infants due to concerns about early onset sepsis. However such exposure of early antibiotics might contribute to intestinal microbial dysbiosis that could lead to an increased risk of NEC (142).

BLOOD PERFUSION

Arterial circulation and NEC

Poor arterial circulation is a risk factor for NEC development. In term infants, development of NEC is often associated with an underlying condition affecting the circulation or oxygenation of the intestine, such as asphyxia or congenital heart disease (143, 144). In preterm infants affected arterial circulation due to a patent ductus arteriosus (PDA) may be related to increased risk of NEC development (145). Both the presence of a PDA and the medical or surgical treatment to close the PDA may affect the arterial circulation and be related to NEC development (146). Despite a large number of studies addressing if and when to treat PDA in preterm infants there still remains uncertainty regarding treatment of PDA in relation to the risk of NEC development (147).

Prostaglandins and nitric oxide (NO) are part of the regulation of the intestinal epithelium. Prostaglandins assist in the regulation of the assembly of tight junctions. The role of NO on the intestinal epithelium is rather contradictory. Low levels of NO are essential to sustain the intestinal homeostasis. On the other

hand high levels of NO, that are seen during inflammation, can have a harmful effect on the intestinal epithelium that can lead to disruption in the essential barrier function and, hence, make the infant susceptible for NEC (2). NO stimulates vasodilatation in the intestinal circulation in the newborn and endothelin-1 stimulates vasoconstriction. Normally, there is a balance between these two stimuli with an excess of vasodilatation, but with dysfunction in the epithelium the vasoconstriction is favored, leading to a reduce in the blood flow with intestinal ischemia as a possible consequence (2).

Venous circulation and NEC

On top of a poor arterial circulation causing hypoxia in the intestine there have also been studies suggesting the venous circulation as an important factor in NEC development. Disturbed venous blood flow could cause edema in the intestine that could contribute to the development of NEC. Closure of ductus venosus followed by a transient portal hypertension has in animal studies been described to cause cellular damage in the intestine (148). Umbilical venous catheter is commonly used in preterm infants. A study suggests a relationship between malposition of umbilical venous catheter and increased risk of developing NEC (149).

Microcirculation and NEC

Immature or pathologic development of the microvasculature of the intestinal mucosa might play an important role in NEC pathogenesis (150). Vascular endothelial growth factor A (VEGF-A) is involved in intestinal angiogenesis after birth among preterm infants. It has been described that the early intestinal microbiota can influence VEGF-A, and thereby on the angiogenesis of the gut. Sabnis et al showed that VEGF-A is decreased in infants with NEC (151).

It has been described in a mouse model that during fetal life there are high levels of VEGF-A and VEGF-receptor 2 proteins in the developing gut and after birth the expression of the proteins is downregulated. Furthermore, the activity of the VEGF-receptor 2 is of importance for the microvasculature of the gut mucosa during the immediate neonatal period, and deficiency of VEGF-receptor 2 function increases the risk of NEC development in newborn mice (152).

2.4.4 CLUSTERING OF NEC

In neonatal units, outbreaks of bacteria causing sepsis is not uncommon, for example outbreaks of *Serratia marcescens* and *Enterobacter cloacae* in overcrowded

NICUs (153, 154). An outbreak of *Staphylococcus haemolyticus* has also been described from a Swedish NICU (155). Cases of NEC usually occur sporadically. However, there are several reports of clusters of outbreaks (10, 156-159). Clustering of NEC due to seasonal variation and overcrowding in the NICU was described already in 1999 by Hentschel et al and in relation to a Klebsiella outbreak in 1974 by Hill et al (160, 161). Several organisms have been proposed to cause NEC, for example coagulase-negative *Staphylococcus species, Eschericia coli*, *Klebsiella pneumoniae* and *Clostridium species* (10, 162-164). In some outbreaks viral agents have been isolated, for example rotavirus, coronavirus, norovirus and enterovirus (158, 162, 165-167). However, there are also outbreaks where no etiologic microorganisms were found (10, 159, 168).

Even though there are reports of clustering of NEC, there are also reports that have not been able to see variation in incidence of NEC between NICUs (169).

2.4.5 CLINICAL PRESENTATION AND DIAGNOSIS OF NEC

The clinical presentation of NEC ranges from mild forms with subtle signs to fulminant with intestinal perforation and systemic hypotension. The progress from mild to fulminant can be very rapid, often within hours (11).

The NEC-diagnosis is based on radiographic and clinical findings. The typical infant with NEC presents with increased feeding intolerance, bloody stools, abdominal distension, and tenderness, typically when the infant is 8 to 10 days old (11, 116). Other non-specific signs are temperature instability, bradycardia, apnea, and lethargy. The initial subtle symptoms may then progress quickly to a discolored abdomen, intestinal perforation, and peritonitis (2, 116).

In the most extremely preterm infants the clinical signs and radiological findings are described as less specific (170).

Laboratory tests can show nonspecific signs of inflammation, it is common with either leukocytosis or neutropenia. When NEC progresses to more advanced stages, laboratory tests often show thrombocytopenia and acidosis, and a poor prognostic sign is when the platelets fall rapidly (2). However, there are no specific diagnostic biomarkers (109). In a recent study, sudden development of hyponatremia is associated with a worse outcome (171).

Abdominal radiograph early in the disease may show no abnormalities or just mild distension or ileus. When the disease advances, radiology can show dilated bowel loops and fixated gas-filled loops that look the same on repeated radiographs. Free air on radiographs, as a mark of perforation of the intestines, is a sign of even more advanced NEC. Radiographic signs of NEC often associated with NEC are portal venous gas and pneumatosis intestinalis (11). However, pneumatosis intestinalis may in rare cases be caused by allergic colitis, such as cow's milk protein allergy in infants (172). This uncommon presentation of cow's milk protein allergy may easily be misinterpreted as NEC (173).

NEC is classified based on Bell's staging criteria, first described in 1978 and modified in 1986 by Walsh and Kliegmann and is after more than four decades still in use and is a support for therapeutic decisions, table 3 (174-176). The staging system is based on radiological and clinical findings and classifies the severity of NEC into suspected (stage IA and IB), definite (stage IIA and IIB) and advanced (stage IIIA and IIIB), where stage II-III most often is the definition of NEC in international papers (177). The staging system can, unfortunately, not accurately differentiate NEC from other diseases with different etiology but with similar radiological or clinical findings and there has been complains to its poor validity (18, 109, 177). It has been shown that Bell's staging criteria might not be adequate for diagnosis or staging of NEC in preterm infants born at <28 weeks of gestational age since these extremely preterm neonates show less specific radiological and clinical signs (170).

Table 3. *Bell's criteria modified by Walsh and Kliegman. Table inspired by the tables in the original papers* (174, 175, 178).

	Stage	Clinical signs	Gastrointestinal signs	Radiographic signs
IA	**Suspected**	Apnea, bradycardia, temperature instability	Mild abdominal distension, increased gastric residuals, emesis, occult blood in stool	Normal or intestinal dilation, mild ileus
IB	**Suspected**	Same as IA	Bright red blood from rectum	Same as IA
IIA	**Definite**, mildly ill	Same as IA	IB + absent bowel sounds with/without abdominal tenderness	Intestinal dilation, ileus, pneumatosis intestinalis
IIB	**Definite**, moderately ill	IIA + metabolic acidosis and mild trombocytopenia	IIA + absent bowel sounds, definite abdominal tenderness with/without abdominal cellulitis or mass in right lower quandrant	IIA + portal venous gas, with/without ascites
IIIA	**Advanced**, severely ill, bowel intact	IIB + bradycardia, severe apnea, hypotension, respiratory and metabolic acidosis, neutropenia, DIC	IIB + signs of peritonitis, marked tenderness, and abdominal distension	IIB + definite ascites
IIIB	**Advanced**, severely ill, bowel perforated	Same as IIIA	Same as IIIA	IIB + pneumoperitoneum

DIC = disseminated intravascular coagulation

The modified Bell's staging criteria are based on radiological and clinical findings and do not include novel diagnostic tools, such as ultrasound. Over the last decades, abdominal ultrasound has evolved as a useful tool in diagnosing NEC. It is often used in combination with abdominal radiograph (179, 180). As point-of-care ultrasound is rapidly developing in neonatal units, bowel ultrasound of suspected NEC is likely to be more available in the future (181, 182).

2.4.6 TREATMENT

Based on the clinical presentation or the severity of the disease, NEC may require medical or surgical management. If treatment is initiated as soon as NEC is suspected, many cases of NEC can be managed medically. Medical treatment typically includes bowel rest and abdominal decompression with a gastric tube, broad-spectrum antibiotics, and intravenous fluids (2, 11). Several abdominal

radiographs and examinations are often done during treatment to follow the progress of the disease. If the clinical status or radiographs are deteriorating, a surgical intervention is generally required. An absolute indication for surgery is the presence of free intraperitoneal air on the abdominal radiograph, as an evidence of intestinal perforation (2). Although most of the cases can be managed medically, about one third of the infants with NEC will need surgery, either after progression of the disease when medically treated or immediately when the progress of the disease is rapid (2). Laparotomy, with resection of the affected part of the intestine and creation of a stoma, or peritoneal lavage are the two most common methods when treating NEC surgically (11, 183, 184) However, in Sweden, laparotomy is the preferred surgical method and peritoneal lavage is very uncommon. Remote ischemic conditioning is a novel treatment option currently evaluated for NEC (185).

2.4.7 MORTALITY AND SHORT-TERM OUTCOMES OF NEC

NEC-survivors are at high risk for SBS, up to 25% of the infants that require surgery for their NEC will develop SBS and NEC is the most common cause to SBS. SBS is a malabsorptive condition where the intestines cannot absorb adequate nutrients needed for growth most often because of extensive resection of the small intestine (2, 186, 187). SBS is affected by the length and health of the residual intestine after surgery, what part of the intestines that were removed and the presence of the ileocecal valve. There is a correlation between length of dependence of parenteral nutrition and the length of the residual intestine, where the remaining intestine must increase the absorptive capacity (186-188). The location of the resection is important for the subsequent gastrointestinal function as there is a diversity in the ability to adapt to demands on absorptivity (2, 187). Additionally, infants with NEC managed medically or infants with only a small resection can also develop intestinal failure, since the intestines may have suffered severe mucosal damage that affects the absorptive ability (2)

Approximately one third of the infants with NEC, both medically and surgically treated, develop intestinal strictures (2). Bowel strictures are common both after surgically treated and medically treated NEC (189). After medical treatment of suspected NEC, it is of outmost importance that the treating clinician is aware of the possibility of bowel stricture development. Bowel strictures as a complication of NEC have been described in several publications already between 1970 to 1980, and it still today is an important complication of medical and surgical NEC (190, 191). Resection of the strictures may impair the gastrointestinal function among these infants, and even further put them at risk for SBS (2).

2.4.8 LONG-TERM COMPLICATIONS OF NEC

SHORT BOWEL SYNDROME AND INTESTINAL FAILURE FOLLOWING NEC

Intestinal failure (IF) following NEC may occur as a result of resection of a large part of the small bowel, resulting in SBS, but IF may also occur among medically treated NEC infants, suggesting that factors independent of bowel resection, such as inflammatory changes may be involved in IF development. In many cases, especially after medically treated NEC, the IF is resolving over a period of time (192). Even in cases of surgical NEC with SBS the chances of reaching full enteral autonomy are good compared to other causes of SBS. This is likely because NEC mainly affects preterm infants and the ability of the remaining bowel to adapt over time is good (193).

In some cases, with SBS however the IF may be long lasting, and in need for long term follow up, and interventions. The most commonly used definition of IF today is "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" (194). Poor enteral function following surgical NEC may affect long term growth and development (195, 196).

NEC AND GROWTH

NEC is a serious inflammatory condition that is described as having an impact on long term growth. Compared to preterm infants surgically treated for SIP, infants treated surgically for NEC have been shown to have a delay in achieving enteral autonomy, and a poor catch up growth over the first years of life (197). Some studies have suggested that infants born preterm with medically treated NEC have growth similar to infants without any history of NEC (198, 199). Others claim that adolescents with a history of medical or surgical NEC have poorer growth compared to controls with no history of NEC (200).

Postnatal growth failure has historically been common among preterm infants and may be influenced by many things such as gestational age, birth weight, suboptimal nutrition, parallel diseases, and medical treatment. With an optimized early nutrition growth failure may be prevented in many of these infants (28). Among children and adolescents with a history of NEC poor growth is still a problem that needs special attention. It has been proposed that not just the postsurgical bowel length but also variations in bowel physiology among NEC survivors affect growth (200).

NEC AND BONE HEALTH

As a large part of fetal bone mineralization occurs during the third trimester extreme preterm infants miss a major part of the fetal bone development. Osteopenia of prematurity is a condition of reduced bone mineral content among preterm infants (201). NEC with inflammation and often need for resection of bowel may have an impact on bone development during childhood, but there is a lack of studies of bone mass during childhood and adolescents among individuals with a history of NEC. NEC is well described as one of the risk factors for development of osteopenia of prematurity (202). The effect of NEC on development of osteopenia may be related to inflammation and nutrition, and it has also been described that NEC increases bone resorption in preterm infants (203). Cakir et al conclude that a single episode of NEC increases bone resorption in preterm infants, and that longitudinal studies are needed to evaluate the long-term effect of this on BMC and bone mass (203).

NEC AND NEURODEVELOPMENTAL OUTCOME

It is a well-known fact that several diseases related to preterm birth tend to commonly occur together in one individual, for example severe NEC tend to occur more often in individuals with retinopathy of prematurity (ROP) and bronchopulmonary dysplasia (BPD) (204).

As NEC is a serious inflammatory condition it is not surprising that NEC in preterm infants results in a poor neurodevelopmental outcome. Several studies have shown a relationship between NEC in the neonatal period and neurodevelopmental impairment (198, 205). The intestine is very immunoreactive in NEC and the inflammatory response that follows NEC is extreme and dysregulated. The inflammatory response can spread and damage other organs, for example the retina or the brain, and hence increase the risk of ROP and neurodevelopmental delay (11, 116). Compared to SIP, NEC is a more severe inflammatory condition, and studies show more prevalent neurodevelopmental problems after NEC compared to SIP in preterm infants (206). The exact mechanisms in how NEC affects neurodevelopment are not fully understood. It is accepted that the initial inflammation may have a negative effect on neurodevelopment during childhood. There are also several recent publications discussing the possibility of a dysbiosis of the intestinal microbiota following NEC can cause disturbances in the Gut-brain axis resulting in an impaired neurological outcome (207, 208). Further understanding of how NEC affects neurodevelopment and if interventions not only during the NEC episode, but also possible interventions regarding microbiota during childhood can be of importance to improve outcome of these individuals in the future.

3 AIMS

The overall aim of this doctoral thesis was to clarify if children born preterm with a history of NEC, during their childhood had reduced growth, lower bone mass, increased fracture tendency, altered body composition and a different intestinal microbiota compared to children born preterm without a history of NEC. A further aim was to clarify if NEC occurs in space-time clusters.

SPECIFIC AIMS FOR EACH PAPER:

I. To identify the presence of space-time clusters of NEC during the years 1997 to 2009, both on hospital level and on residential level.

II. To determine if children born preterm with a history of NEC had an increased risk of rickets, fractures and vitamin D deficiency compared to children born preterm without a history of NEC.

III. To clarify if children born preterm with a history of NEC had reduced growth, reduced bone mass and altered body composition at 5 years of age compared to children born preterm without a history of NEC.

IV. To investigate if children born preterm with a history of medically or surgically treated NEC had altered intestinal microbiota at 5 years of age compared to children born preterm with no history of NEC.

4 METHODS

4.1 OVERVIEW OF THE METHODS

Paper	**I**	**II**	**III**	**IV**
Study design	Retrospective cohort study	Retrospective cohort study	Prospective cohort study	Prospective cohort study
Population	All children with NEC + all live births born in Sweden between 1987 and 2009.	All children with NEC born in Sweden between 1987 and 2009 with gestational age <31+6 weeks + matched controls without NEC.	5-year-old children with a history of NEC, admitted to the neonatal unit in Gothenburg Sweden + matched controls without a history of NEC.	5-year-old children with a history of NEC, admitted to the neonatal unit in Gothenburg Sweden + matched controls without a history of NEC.
Inclusion criteria	Born between 1987 and 2009 and a diagnosis of NEC	Born between 1987 and 2009, a diagnosis of NEC and gestational age <31+6 weeks	Gestational age <37 weeks, admitted to the neonatal unit in Gothenburg, Sweden and 5 years of age at measurements.	Gestational age <37 weeks, admitted to the neonatal unit in Gothenburg, Sweden, 5 years of age at measurements and provided a stool sample.
N of subjects	808 + 2 389 681	371 + 1839	25 + 25	15 + 24
Study period	1987–2009	1987–2009	2013–2017 (One child measured 2020)	2013–2017 (One child measured 2020)
Main research question	Does NEC occur in space-time clusters?	Do NEC-survivors have more fractures, rickets and vitamin D deficiency?	Is the growth, bone mass and body composition affected in 5-year-old NEC-survivors?	Is the intestinal microbiota altered in 5-year-old NEC-survivors?

4.2 METHODOLOGICAL CONSIDERATIONS

4.2.1 REGISTER-BASED STUDIES - PAPER I – II

In paper I and II a cohort of infants was collected from three national registers. Studies on data collected from registers have the advantage of giving the possibility to include large numbers of study subjects, but the disadvantage of being retrospective with limited possibilities to control laboratory data or clinical data in patient records.

On the website of the Swedish National Data Service (www.snd.se) register-based research is described as research based on data from records kept by government agencies or other organizations, where the data can be traced back to individuals. In Sweden we have a long tradition on national registers that can be used for research. Sweden is one of few countries with unique personal identity numbers making it possible to link information from different registers to each other regarding a specific individual. The Swedish Research Council has created a website called Registerforskning.se (www.registerforskning.se) with information and tools to enable efficient and high-quality register-based research.

In a publication from 2015 Ludvigsson et al discussed the ethical aspects of registry-based research in the Nordic countries (209). They discussed the role of informed consent in registry-based research and the integrity of study participants, children in particular.

Olsen argued in a publication from 2011 that register-based research has many advantages compared to collecting data for each specific study (210). One argument is that data collection is very time consuming, and by using registers large quantities of data are already collected and ready to be used. Register-based data also have the advantage that selection bias due to non-responders is not a problem in these registers, as it can be if data is collected from the study subjects for each study.

There are a number of methodological challenges that needs to be delt with when register-based research is becoming more and more common. The data in the registers need to be as correct as possible. The definitions of conditions in the registers need to be clear, and well known to the people doing the administrative work with the registry. The ethical integrity of the persons registered needs to be clear.

4.2.2 BODY COMPOSITION AND BONE MASS MEASUREMENTS – PAPER III

Multiple tools are available for measuring body composition during childhood, DXA and Air-Displacement Plethysmography becoming the most used methods in clinic and in research. Air-Displacement Plethysmography has the advantage of being relatively easy to use, but the disadvantage of providing information only regarding body composition, but not on bone mass or BMD. DXA is considered the golden standard for body composition and bone mass measurements and reference values are available. As Air-Displacement Plethysmography is a relatively new method there have not been any good reference values, especially not for children in preschool age and for children born preterm (211).

As we aimed to measure both bone mass and body composition, we used DXA in paper III.

4.2.3 ANALYZING MICROBIOTA - PAPER IV

There are different techniques for analyzing the intestinal microbiome. Most recent studies use non-culture-based techniques such as 16s rRNA sequencing, or shotgun metagenomic sequencing (212).

The most traditional method is the culture based method (213). An advantage with culture-based methods is that you will identify only live bacteria, whereas with non-culture-based methods you will risk identifying also dead bacteria. When studying for example the microbiome in the newborn infant, identifying the live bacteria that will thrive in the environment of the newborn infant is a desire. Many of the maternal bacteria transferred during delivery will rapidly die in the oxygen rich environment of for example infant skin and mucosa. With the non-culture-based techniques there is a risk to identify these dead bacteria as belonging to the infant flora.

A technical problem with culture-based methods is the need to keep the bacteria alive until the analysis. The culture will have to take place in direct relationship to the collection of the sample.

The new non-culture-based methods, often called next generation sequencing have the advantage that samples can be stored in -80° C for long periods of time. Bacteria that will not grow on culture are possible to analyze. These methods are developing fast and will be more common in future research and in every day clinical work.

The cheapest option of these new methods, the 16s rRNA sequencing is well suited for analysis of large number of samples for example from multiple patients or for a limited number of patients in longitudinal studies. A problem with 16s rRNA sequencing is that it offers limited taxonomical and functional resolution. The first developed methods of 16s could only identify bacteria on genus level, and not on species level (214). Moreover, by using primers for different regions of the 16S rRNA gene the results may be discordant. This is explained by resolution of each variable region across taxa and the distinct binding affinities for the conserved regions (215). Metagenomics on the other hand is becoming the more frequently used method as it, even though it is more expensive, it offers better resolution, giving a more specific taxonomic and functional profile as well as the discovery of new bacterial genes and genomes (216).

In paper IV the metagenomics method was used for bacterial analysis.

4.3 STUDY DESIGN AND POPULATION

PAPER I-II

Paper I and II are register-based studies; a retrospective cohort study (study I) and a longitudinal retrospective cohort study (study II).
The studies are based on anonymized data of all children born between 1987-2009 obtained from the following registers held by the Swedish National Board of Health and Welfare: the Medical Birth Register (MBR), the National patient register (NPR), and the National Cause of Death Register (CDR). MBR is abbreviated as SMB (Swedish Medical Birth register) in paper II. The data were retrieved from the registers by Margareta Ahle, MD, PhD, in Linköping for her PhD-project.

The NPR contains discharge diagnoses according to the International Classification of Disease (ICD) coding system for all hospital admissions in Sweden, both specialist and outpatient care. In addition to discharge diagnoses and perinatal data, the registers also contain information about demographic data, i.e delivery hospital and the mother's municipality.
For paper II the last linkage with the NPR dates from December 31, 2012.

PAPER III-IV

Paper III and IV are both prospective cohort studies based on preterm children born between 2008 and 2011 with a discharge diagnosis of NEC, who were treated for their NEC at the Queen Silvia's Children's Hospital in Gothenburg, Sweden. All children with a minimum of NEC stage IIA according to Bell's

staging criteria were eligible for inclusion. The children were included at five years of age.
For every NEC case, a preterm control matched for gestational age, sex, and age at the time for measurement with DXA but without a history of NEC, was included.

4.4 DATA COLLECTION PAPER I-II

PAPER I

All children in the registers born between 1987-2009 with a discharge diagnosis of NEC, according to the 9th and 10th revision of the World Health Organization ICD9 and ICD10, i.e 777F and P77 respectively, were identified. The date of birth of the child was used as the date of NEC diagnosis, since the exact date for the diagnosis was not possible to extract from the registers.

In total, 808 children with a discharge diagnosis of NEC were identified, of which 720 had a full personal identification number and 88 children were manually identified by unique information (date of birth, date of discharge, sex and others) even if the personal identification number were missing in the NPR.

For the cluster analysis, each twin pair with NEC were counted for as one child. After correcting for twin pairs and missing data of delivery hospital and the mother's residential municipality there were 774 and 769 children respectively available for analyses on delivery hospital and residential municipality.
The background population is a complete extract from MBR covering all children born in Sweden during 1987-2009, a cohort of 2 389 681 individuals.

To study variations in clustering of NEC over time, the cohort was divided in two according to the children's birth year, 1987-1997 and 1998-2009.

Two NEC-cases were considered as close if their birth dates were close and if they were geographically close at the time for birth. Three time windows defined closeness in time of birth: seven, 14 and 21 days apart. Closeness in space were defined at two geographical levels: the delivery hospital and the mother's residential municipality.

PAPER II

All children born in Sweden 1987-2009 with a gestational age <31+6 weeks, and with a discharge diagnosis of NEC, were included. For every preterm child with a history of NEC, up to six randomly selected controls found, matched for

gestational age and birth year. Due to a limited number of eligible controls in the subgroups with the most preterm children, some NEC cases had fewer than six controls. Infants surviving <28 days were not eligible for analyses of morbidities connected to bone health and were thus excluded. The study population thus consisted of 371 children with a gestational age <31+6 weeks and a history of NEC surviving >28 days and their 1839 controls.

Diagnoses connected to bone, growth and nutrition were retrieved from the NPR and included all categories of fractures, rickets, vitamin D deficiency, malnutrition, malabsorption, disorders of bone development and growth and more.

Every diagnosis, except for fractures, was counted once per individual, even if it occurred multiple times in the NPR. Regarding fractures, the diagnosis was counted as one episode if it reoccurred within one year, otherwise it was counted as a new fracture.

4.5 DATA COLLECTION PAPER III-IV

4.5.1 MEDICAL RECORDS

Medical records were used for collecting information regarding birth data, infant characteristics, and surgical events. Growth data were collected from growth charts from the child health care centers and from the neonatal unit.

4.5.2 QUESTIONNAIRES

Questionnaires regarding the child's medical conditions and current lifestyle were answered by caregivers after written informed consent.

4.5.3 COLLECTION OF DATA AT 5 YEARS OF AGE

During an appointment at Queen Silvia's Children's Hospital, Gothenburg, Sweden, when the children were five years of age, the children's body height and body weight were measured, all by the same trained nurse and with the same calibrated scale. Calculations of body mass index (BMI) were based on these data. The values were then compared to reference values (217, 218).

During the same appointment, areal BMD (aBMD), BMC and body composition were measured for total body head excluded (TBHE), hip, lumbar spine (LS), trunk, left arm and left leg by the same trained nurse. Z-scores for BMD were

provided from Lunar international reference database for TBHE, LS and the hips. Measurements were performed with Lunar Prodigy DXA (GE Lunar Corp., Madison, WI) until March 2016 when the DXA equipment was replaced with Lunar iDXA (GE Lunar Corp., Madison, WI), which was used subsequently, figure 6. A reliability study for comparison of the results from Lunar Prodigy DXA with the results from Lunar iDXA has been described (219). Based on high intraclass correlation and low coefficient of variation, the reliability between Lunar Prodigy DXA and Lunar iDXA was evaluated as acceptable.

The head represents a large portion of the total bone mass in children and could therefore disguise bone mass deficits in other body sites. Hence, the head was excluded from the scans in this study and the scans of TBHE were used.

Figure 6. *Lunar iDXA. Picture provided by GE Healthcare.*

To get reliable data from the DXA measurements, the person must lie completely still, otherwise movement artifacts make the data unreliable. Since this can be hard for a five-year-old, the number of accounted DXA-scans in some calculations were fewer than the number of included children, due to movement artifacts.

A DXL Calscan technique (Scanflex/Demetec AB, Täby, Sweden) was used to assess calcaneal BMD and BMC. This is a combination of DXA and laser and the technique has been modified for pediatric use (80). The pediatric version of the DXL Calscan enables manual measurement of the height of the calcaneus. The height can then be calculated together with the areal calcaneal BMD to give the volumetric BMAD (g/cm^3).

4.5.4 STORAGE OF SAMPLES

The fecal specimens were obtained at home using a specific kit with a spatula and a tube. The collection was done at five years of age before the appointment to the hospital for DXA measurement. The families transported the samples on ice to the hospital, and the sample was then frozen to -80°C.

4.5.5 FAECES – MICROBIOME ANALYSIS

All fecal samples were analyzed using the shotgun metagenomics sequencing method (220). The DNA extraction and metagenomics sequencing was performed at Clinical Microbiomics in Copenhagen, Denmark. All analysis of the microbiome data was performed in collaboration with dr Seyedeh Marziyeh Jabbari Shiadeh, and dr Maryam Ardalan, Sahlgrenska Academy, University of Gothenburg.

From all fecal samples DNA was extracted using the NucleoSpin 96 Soil (Macherey-Nagel) kit. As a positive control a standardized microbiomal community was used (ZymoBIOMICS, Zymo Research).

The Clinical Microbiomics Human Gut HG04 gene catalog was used as a reference gene catalog, and for taxonomic abundance profiling, the Clinical Microbiomics HGMGS version HG4.D.2 set of 2095 metagenomic species (MGS) was used. Each metagenomic species was processed with CheckM (221), and updated the annotation with the CheckM result if this resulted in a lower taxonomic rank. For sequencing read pairs in which both reads passed filtering with a length of at least 100 bp were retained. These were classified as high-quality non-host reads. High-quality non-host reads were then mapped to a gene catalog, and a gene count table was created with the number of uniquely mapped read pairs for each gene.

To examine alpha diversity of the fecal microbiota three indices were used. The Shannon diversity index was primarily used to analyze differences between groups. The Simpson index and the Berger Parker Dominance index were then used to check and confirm the findings. For beta diversity the Bray-Curtis dissimilarity matrix was calculated.

4.6 STATISTICS

4.6.1 PAPER I

To identify significant space-time clustering of NEC, two different statistical methods were used: the Knox space-time cluster analysis and Kulldorff's scan

statistic (222, 223). The statistical analyses were performed in collaboration with Roland E. Andersson, Department of Surgery, County Hospital Ryhov, Jönköping, Sweden.

Kulldorff's scan statistic scans for clusters in space that depend on time. The method identifies observed events within all births that occurred in a circle with a varying radius in space in one dimension and with a varying duration of time in the other dimension, like a cylinder changing in width and height, figure 7. To look for possible clusters, the statistic is centered at all geographical locations. Consequently, the cylindrical window is flexible in not only size and time, but also location. Closeness in space were defined at two geographical levels: the delivery hospital and the mother's residential municipality. The number of observed cases in a cluster was compared to the expected number of cases, if all cases were independent to each other in terms of time and space.

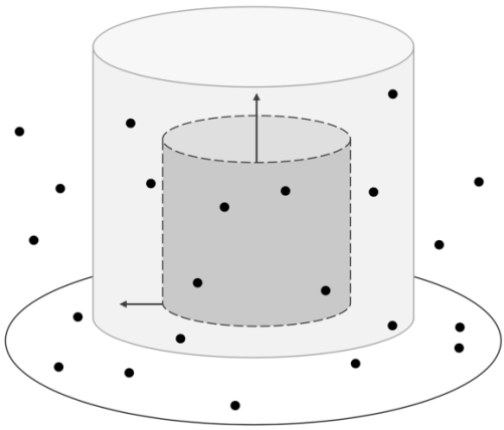

Figure 7. *Kulldorff's scan statistic can be described as a cylinder with space in one dimension and time in the other dimension. The cylinder is flexible and increases in size to include further geographic space and time intervals until it reaches an upper limit. The cylinder is moved through the study's geographic and temporal space to find observed cases.*

The Knox space-time cluster analysis is a test for the proximity of pairs of cases within a defined space and time-period. The test observes all possible distinct pairs of cases, n(n-1)/2. The number of observed pairs are then compared to the expected number of pairs within the same defined space and time-period and if the observed number of cases exceed the expected number of cases it is

considered as evidence of space-time clustering. According to if the two parts in the pairs are close or not close to each other considering geographical distance and time, every distinct pair is organized into one of four cells in a 2x2 table with space (close/not close) and time (close/not close) on the two axes. If the two parts in a pair were close to each other in date of birth and if their geographical distance at time of birth were close, the pair was considered as being in close proximity. Three time windows were used to define closeness in date of birth in a pair: seven, 14 and 21 days apart. As for the Kulldorff's scan statistic, closeness in space were defined at two geographical levels: the delivery hospital and the mother's residential municipality.

The Knox space-time cluster analysis was also used to study changes in NEC clustering over time. The population was thus divided into two cohorts, 1987-1997 and 1998-2009, according to the year of birth of the individuals. To compare the change in NEC incidence in the two time periods, the binomial test was used.

4.6.2 PAPER II

Mann-Whitney U test was used for quantitative data and Chi-square test and Fischer's exact test was used for categorical data. Since the latest linkage with NPR dates to December 31, 2012, there was a difference in the length of follow-up, and risk comparisons for fractures were made by Kaplan-Meier curves and univariate and multivariate Cox proportional hazard regressions.

4.6.3 PAPER III

Mann-Whitney U test was used for quantitative data and Chi-square test and Fischer's exact test was used for categorical data. To correct for height as a confounding factor on BMC, a multivariable linear regression was performed.

4.6.4 PAPER IV

In paper IV statistical analyses were performed in collaboration with Dr Seyedeh Marziyeh Jabbari Shiadeh and Dr Maryam Ardalan, Sahlgrenska Academy, University of Gothenburg. The R-based iNEXT software was used to compute sequencing data. To show any differences in the distribution of fecal microbiota (alpha diversity) a Lorenz curve was generated.

The Mann–Whitney U test and Kruskal–Wallis test were used for comparison of data between control and NEC subjects and between control, NEC-medical

treatment and NEC-surgical treatment subjects Spearman correlation test was used to analyze correlations between the parameters.

Significance values have been adjusted by the Bonferroni correction for multiple tests.

A p-value <0.05 was considered significant in all papers.

4.6.5 SAMPLE SIZE CALCULATION FOR PAPER III-IV

In collaboration with Statistiska Konsultgruppen in Gothenburg a power calculation was made, where the desired power was set to 0,8. The power calculation was based on the NEC cases and their controls matched for gestational age, sex and age at the time for the DXA measurement. A relatively good similarity between the groups can therefore be assumed. BMC (TBHE) was used as primary outcome for the power calculation. To detect significant differences between the groups 25 children in each group were needed, 50 children in total, according to the power calculation.

For statistical analysis in paper III and IV SPSS (IBM Corp. Version 28.0. Armonk, NY, USA). Graphs were created using Prism 8 (GraphPad Software Inc., USA) and Excel (Microsoft, Excel, 2019).

4.7 ETHICAL CONSIDERATIONS

PAPER I-II

Study I and II were retrospective register-based cohort studies with data from over 2.3 million individuals. The studies were approved by the Regional Ethical Review Board of Linköping, approval number M213-07, with supplement 2010/405-32. Informed consent was not obtained from the individuals, according to standard research practice in register-based research. Approval from each individual would not have been reasonable or possible to obtain. The data were obtained retrospect from the registers.

PAPER III-IV

Study III and IV were prospective cohort studies with 5-year-old children born preterm with and without a history of NEC. The studies were approved by the Regional Ethical Review Board of Gothenburg, approval numbers 720-13 and 319-12, and the radiation protection committee of Region Västra Götaland. An

amendment was approved by the Swedish Ethical Review Authority with Dnr 2022-03402-02. Since the participants were under 18 years of age, written informed consent was obtained from the caregivers from all participants. The caregivers were sent a letter with written information about the study and were then called a few days later to receive verbal information. They could thereafter decide whether they approved their child to participate or not. Before the appointment with the DXA-measurement, the children were primarily informed by the caregivers. Subsequently, when they arrived at the appointment, the child could at any time refuse participation and it was important that the child did not feel forced to do the measurements. Even though the children were well under 18 years of age and, hence, the caregivers signed the written informed consent, it was of great importance that the 5-year-old children's own will was taken into account. The families could at any time withdraw their participation.

The DXA-measurements in study III did not cause physical pain for the children. However, it was important that the study subjects stayed completely still during the DXA-measurement to get reliable data. This can be very difficult for a 5-year-old and sometimes it was necessary with a hand holding, for example, their legs to keep still. However, since participation was optional it was also important that they did not feel forced or restrained. If a child had major difficulties holding still, it was better to cancel the measurement. The total radiation dose in the studies was very low, less than 20 μSv. For comparison, the natural background radiation dose is approximately 2400 μSv per year and a chest X-ray approximately 100 μSv.

In study IV, fecal samples from the children were collected at home by the caregivers, a moment that would not cause physical pain but could eventually cause discomfort for the child. Also here, the families could withdraw their participation at any time. The research persons were not present at the collection, they did only handle the collected samples.

From an integrity perspective, no identifiable data can be linked to a specific study subject in any of the studies. The importance of the knowledge from these studies outweighed the risk for the subjects.

5 RESULTS

5.1 CLUSTERING OF NEC - PAPER I

Paper I investigated the occurrence of space-time clustering of NEC with two different statistical methods; the Knox space-time cluster analysis and Kulldorff's scan statistics.

All children born in Sweden between 1987 and 2009 with a discharge diagnosis of NEC were identified in the previously described registers. The background population were all children born in Sweden during the same time period. Three time-windows were used to define closeness in date of birth, seven, 14 and 21 days, and two different geographical levels were used to define closeness in space, the delivery hospital end the mother's residential municipality. To study changes of clustering and incidence of NEC over time, the population was split into two cohorts based on year of birth, 1987-1997 and 1998-2009. The number of NEC cases identified were 808, where 289 were born in the first time period and 519 were born in the second time period. The background population consisted of 2 389 681 children, where 1 113 946 were born in the first time period and 1 275 735 were born in the second time period. The incidence of NEC was significantly higher during 1998-2009 compared to 1987-1997 ($p<0.001$).

THE KNOX SPACE-TIME CLUSTER ANALYSIS

Significant clustering of NEC was found at hospital level, both when looking at the entire time period and the first time period. A decrease in clustering at hospital level over time was seen, since there was no clustering at hospital level during the second time period. There was no sign of clustering of NEC at residential level.

KULLDORFF'S SCAN STATISTIC

At hospital level, seven significant clusters of NEC at seven different hospitals were found. The time intervals for the clusters ranged from one to 24 days. Four of the clusters consisted of only two cases, yet they occurred at hospitals with very low expected number of cases. Most of the clusters occurred during November to April. One single cluster was found at residential level, consisting of four cases of NEC diagnosed within 17 days, where the mothers came from four different municipalities within a radius of 34 kilometers.

5.2 RICKETS AND FRACTURES FOLLOWING NEC - PAPER II

Paper II was based on the same cohort as paper I. For every NEC case, up to six controls were found, matched for gestational age and birth year. Since paper II focused on children born preterm only children with a gestational age <31+6 weeks were included. We identified 465 NEC cases and 2127 controls, of which 371 and 1839 survived >28 days and were eligible for morbidity analyses.

FRACTURES

Fractures were identified and counted in total and in subgroups of fractures for both NEC cases and controls. No difference in fracture incidence was found between the groups, either in total or in any of the subgroups. In total, 39 (10.5%) fractures were identified in the 371 NEC cases and 204 (11.1%) fractures were identified in the 1839 controls. Furthermore, no difference was seen in fracture incidence between the groups when merging all fractures in the upper limb and lower limb respectively, figure 8. NEC cases were significantly older at the time for fracture compared to controls, with mean age 11.2 years and 9.2 years respectively (p=0.037)

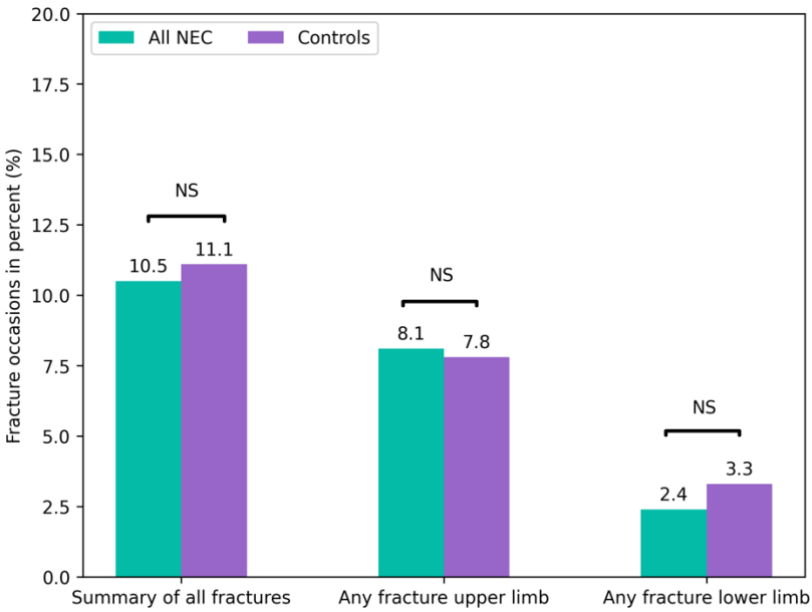

Figure 8. *Prevalence of fractures among the 371 NEC cases and 1839 controls. No significant differences were found. NS= Not significant*

RICKETS

It was significantly more common with rickets during the first year of life among NEC cases, 3%, compared to controls, 1.1% (OR 2.65, p=0.007), figure 9. Two of the children with rickets, both controls, also had a fracture diagnosis.

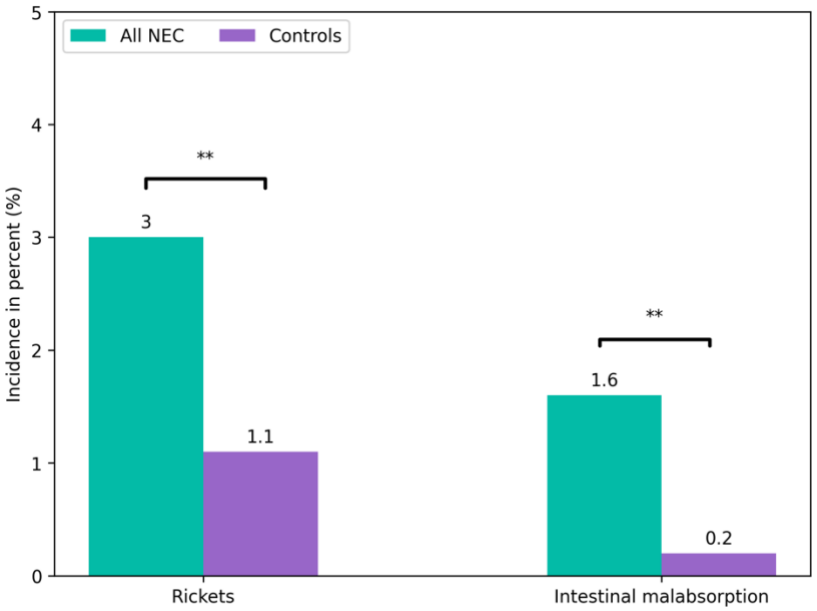

Figure 9. *Diagnosis of morbidities related to intestinal function and metabolic bone disease among the 371 NEC cases and 1839 controls. Significant differences were found regarding the diagnoses rickets and intestinal malabsorption. ** = p<0.01*

OTHER MORBIDITIES

None of the children, neither NEC cases nor controls, were diagnosed with vitamin D deficiency. It was significantly more common with malabsorption and postprocedural disorders in the digestive system among NEC cases compared to controls, figure 9. However, no significant differences were seen between the groups regarding short stature, failure to thrive, obesity, malnutrition or retarded development or sequelae following malnutrition.

5.3 BONE MASS, BODY COMPOSITION AND GROWTH FOLLOWING NEC - PAPER III

Paper III investigated body composition, bone mass and growth at five years of age in children born preterm, with and without a history of NEC. In total, 50 children born preterm were included; 25 NEC cases, of which eleven were surgically treated for their NEC, and 25 controls. Both NEC cases and controls had a median age of 5.1 years at the time for measurements, no difference between the groups (p=0.648). Birth- and neonatal characteristics did not differ between the groups. Neither did, apart from NEC, the most common health issues affecting preterm infants differ between the groups. The initiation of feeding was the same in the two groups. However, the median number of days on parenteral nutrition was significantly higher in the NEC group compared to controls, 30 vs 7 days (p<0.001). The total number of days on parenteral nutrition ranged from 9-216 days in the NEC group and from 0-21 days in the control group.

GROWTH

NEC cases had lower median weight, 16.6 kg vs 18.3 kg (p=0.006), and lower median height, 106.5 cm vs 108.6 cm (p=0.041), compared to controls, figure 10. Weight standard deviation score (SDS) was significantly lower in the NEC group, -1.3 SDS vs -0.7 SDS (p=0.014). Height SDS was significantly lower when comparing surgical NEC cases to controls, -1.3 vs -0.5 (p=0.013), but not when comparing all NEC cases to controls. BMI did not differ between the groups.

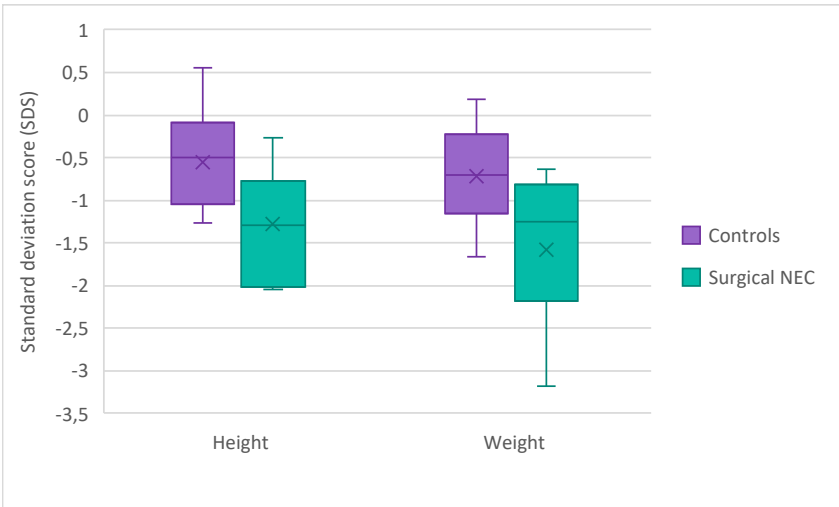

Figure 10. *Boxplot of height and weight SDS at five years of age. All differences are significant, p<0.05. SDS = standard deviation score.*

BONE MASS - DXA

Values from TBHE, LS, hip, trunk, left leg and left arm were analyzed for bone mass.

BMC was significantly lower in the NEC cases in TBHE, LS, trunk, left leg and left arm, compared to controls, figure 11. Since BMC is size-dependent, correction for height at measurement was made. The difference in BMC remained significant in TBHE, trunk, left leg and left arm even after height correction.

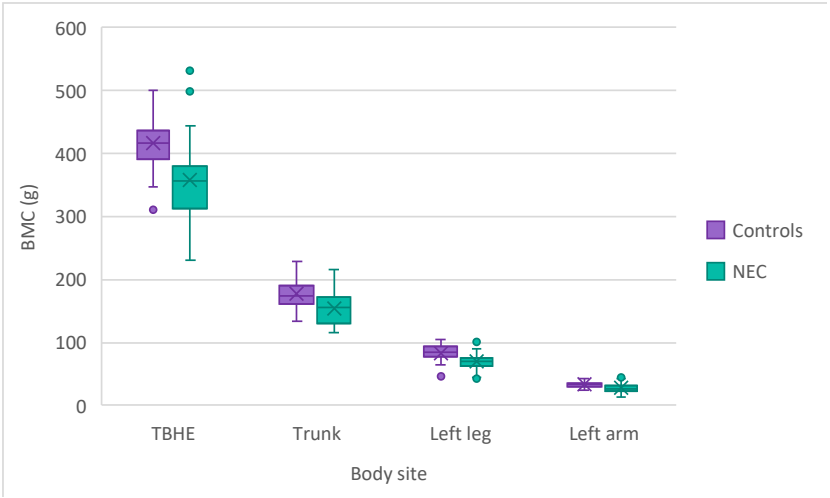

Figure 11. *Boxplot of BMC at five years of age.. All differences are significant, p<0.05. TBHE = total body head excluded.*

The extracted group of eleven surgical NEC cases had lower BMC in TBHE, LS, trunk, left leg and left arm compared to their matched controls. However, this difference disappeared when correcting for height.

BMD did not differ between the groups at any site. However, the NEC cases had significantly lower BMD Z-scores in TBHE and LS, figure 12. The surgical NEC cases had lower BMD Z-scores in TBHE and LS, but in addition they also had lower BMD in LS compared to their matched controls.

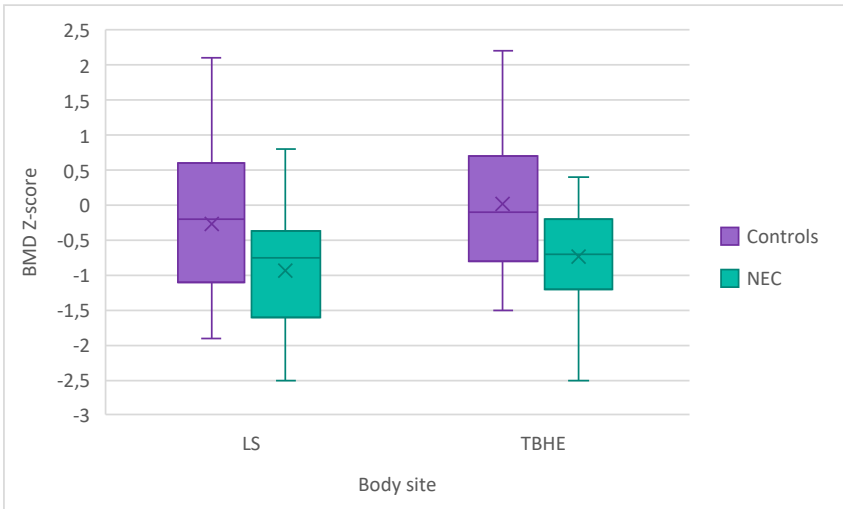

Figure 12. *Boxplot of BMD Z-score at five years of age. All differences are significant, p<0.05. LS = lumbar spine, TBHE = total body head excluded.*

BONE MASS - CALSCAN DXL

No differences were found between the groups BMC, BMD or BMAD in the left foot.

BODY COMPOSITION - DXA

For body composition, values from TBHE, trunk, left leg and left arm were analyzed.

Fat mass was significantly lower in the NEC cases in TBHE, trunk, left leg and left arm, figure 13. Also, fat percentage was significantly lower in the NEC cases in TBHE, trunk and left leg, but not in the left arm. The results were similar when comparing individuals with surgical NEC cases and controls.

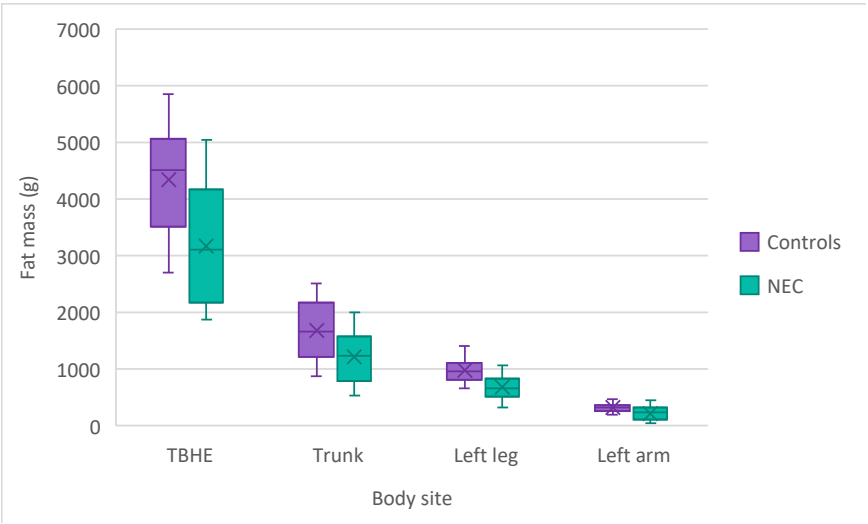

Figure 13. *Boxplot of fat mass at five years of age. All differences are significant, p<0.05.*

5.4 INTESTINAL MICROBIOTA FOLLOWING NEC - PAPER IV

The cohort of children in paper IV was based on the same 50 preterm children as in paper III, but only the children who provided fecal samples were included. The fecal samples were analyzed with shotgun metagenomics sequencing method.

In total 39 children were included; 15 children with a history of NEC, of which eight were surgically treated, and 24 controls without a history of NEC. Both groups had a median age of 5.1 years at inclusion in the study and sample collection. Preterm children with a history of NEC had antibiotic treatment and

parenteral nutrition for a significantly longer time during their neonatal period compared to controls.

Relative abundance of microbiota was tested at different phylogenetic levels. On phylum level there were no significant differences between the groups. As shown in table 4, the surgical NEC group showed significantly lower levels of numerous bacteria at genus level compared to the control group. Both the surgical and medical NEC groups showed a higher relative abundance compared to controls concerning two and three bacteria on genus level respectively, see table 4.

Table 4. *Relative abundance of microbial composition at genus level.*

	Lower relative abundance compared to controls – **genus** level	**Higher relative abundance** compared to controls – **genus** level
Medical NEC		- *Ellagibacter* - *Holdemanella*
Surgical NEC	- *Anaerotignum* - *Hydrogeniiclostidium* - *Subdoligranulum* - *Haemophilus* - *Intestinibacter* - *Parasutterella*	- *Megaspheaera* - *Lacticaseibacillus* - *Sutterella*

Significant differences ($p<0.05$) between medical NEC group compared to controls and surgical NEC group compared to controls are shown.

At species level, there were both decreased and increased relative abundance in the NEC group compared to controls table 5. There was a stronger difference between the surgical NEC cases, than the medical NEC cases, compared to controls. This is in conformity with what was seen on genus level.

Table 5. *Relative abundance of microbial composition at species level.*

	Lower relative abundance compared to controls - **species** level	**Higher relative abundance** compared to controls – **species** level
Medical NEC	- *[Ruminococcus] lactaris*	- *Clostridium sp. AF15-31* - *Ellagibacter isourolithinifaciens* - *Staphylococcus aureus* - *Ruminococcus sp. AF21-42*
Surgical NEC	- *[Clostridium] saccharogumia* - *Anaerotignum faecicola* - *[Ruminococcus] lactaris* - *Clostridium sp. AF37-5* - *Intestinibacter bartlettii* - *Hydrogeniiclostidium mannosilyticum* - *Pseudoflavonifractor sp. NSJ-25* - *Haemophilus parainfluenzae* - *Subdoligranulum sp. APC924/74*	- *Bifidobacterium animalis* - *Lacticaseibacillus paracasei* - *Coprococcus sp. AM27-12LB* - *Megasphaera micronuciformis* - *Sutterella wadsworthensis* - *Limosilactobacillus vaginalis*

Significant differences ($p<0.05$) between medical NEC group compared to controls and surgical NEC group compared to controls are shown.

When evaluating alpha diversity between the groups Shannon index showed significantly lower index in the NEC group compared to control ($p<0.05$). When the NEC cases were divided into medically and surgically treated there was a significantly lower Shannon index in the surgical NEC group compared to controls ($p<0.01$), figure 14. The medical NEC cases showed no significant difference compared to controls. The Simpson index of alpha diversity was significantly higher in the surgical NEC group confirming the findings of lower alpha diversity among the surgically treated NEC cases.

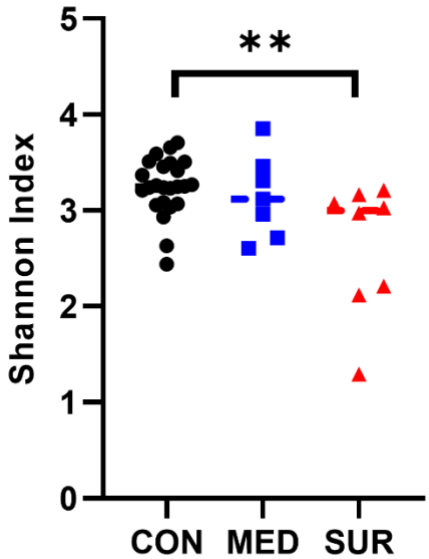

Fig 14. *Shannon index showing lower alpha diversity of the fecal microbiome of the surgical NEC cases compared to controls. CON= controls. MED= medically treated NEC cases, SUR= surgically treated NEC cases. **p<0.01*

Regarding beta diversity, using Bray-Curtis dissimilarity matrix, and regarding species richness no significant differences could be identified between the three groups.

6 DISCUSSION

6.1 GENERAL DISCUSSION

The results presented in this thesis have contributed to increased knowledge of space time clustering of NEC and of long-term outcomes following NEC regarding growth, bone health and microbiota.

Using the Knox space-time cluster analysis and Kulldorff's scan statistics paper I showed that NEC occurred in clusters on hospital level. The study was not able to link any specific contagious agent to clustering. Previous studies by others have shown that clustering of NEC have been associated with specific bacteria in the NICU such as *Klebsiella pneumoniae* and *Clostridium* species (161, 163, 224).

Early fecal dysbiosis with a relative increase in the Proteobacteria phylum compared to other phyla such as Bacteroidetes and Firmicutes has been linked to an increased risk of developing NEC (103, 132-136).

Paper II and III aimed to see if NEC during the neonatal period had an impact on bone health and risk of fractures during childhood and adolescence. The results of paper II concluded that children born preterm with a history of NEC had an increased risk of rickets and diagnosis of malabsorption, but not fractures compared to children born preterm without a history of NEC. Paper III did not show any differences of diagnoses related to vitamin D deficiency. However, the study had only access to diagnostic codes and not laboratory results.

Paper III revealed that in the study population of 25 NEC cases and their matched controls, a history of NEC was associated with lower BMC, altered body composition and lower weight at five years of age, indicating that a history of neonatal NEC has a direct or indirect effect on bone health.

In the same group of children paper IV showed that at five years of age a history of surgical NEC was associated with dysbiosis of the fecal microbiota with a lower alpha diversity compared to controls.

Is the finding of fecal dysbiosis at five years of age among children with a history of surgical NEC associated to the finding of lower BMC in the same individuals? Does resection of the bowel cause both the dysbiosis and lower BMC, or does surgical NEC cause microbial dysbiosis that then cause effects on bone mineralization? Do other factors such as long antibiotic treatment during the neonatal period or long periods of parenteral nutrition among NEC patients

cause both dysbiosis and effects on bone mineralization? These questions remain to be answered in future studies.

This thesis has contributed to increased knowledge that neonatal NEC may occur in clusters and that, in particular surgically treated NEC, could have an impact on bone mass and fecal microbiota at five years of age. Many questions on the exact mechanisms remain to be answered.

6.2 CLUSTERING OF NEC (PAPER I)

It is clinical knowledge among NICU staff that cases of NEC sometimes seem to occur in clusters.

Most cases of NEC occur sporadically, but there are numerous reports of clusters or outbreaks of NEC (10, 156, 159). Some of these reports were published as early as in the 1970s (159, 161). There are also a number of recent publications describing clusters of NEC (225, 226).

In the present study data of 808 children diagnosed with NEC in a national cohort of 2 389 681 children born between 1987 and 2009 in Sweden was used. Using the Knox space–time cluster analysis and Kulldorff's space–time permutation scan statistic paper I showed space–time clustering of NEC on hospital level in Sweden, but not at the level of the mother's residential municipality, suggesting a contagious environmental effect after delivery (222, 223).

Several publications have described different explanations of why NEC cluster on hospital level. A few groups have described associations with NEC and viruses. One group found an association with NEC and the norovirus (158). Another group showed already in 1982 a strong association between NEC and coronavirus infections (166).

Other studies have suggested transmission of bacteria from healthcare workers during periods of overcrowding and understaffing as the cause of outbreaks (154). *Serratia marcescens* is a Gram-negative bacterium often described with a high incidence of horizontal transmission in NICUs, and it can cause outbreaks. *Serratia marcescens* may cause NEC, but it is not one of the most common causes of the disease (227)

6.3 NEC AND BONE HEALTH (PAPER II-III)

Paper II concluded that children born preterm with a history of NEC had increased risk of being diagnosed with rickets, but no increased risk of fractures

during childhood and adolescence compared to controls. Paper III concluded that preterm survivors of NEC at five years of age had indications of lower bone mass, altered body composition and lower weight compared to matched controls.

It is described by others that preterm birth may have a negative impact on bone development both on short term with a risk of osteopenia of prematurity and in the long-term outcome (228, 229). It has been speculated if this could lead to an increased risk for osteoporosis later in life among children born preterm (230). Regarding bone development during childhood there are studies showing lower BMC and BMD among children born preterm compared to those born at term (66). Other studies could however not show any such differences in bone mass, but found different body composition, during childhood among children born preterm compared to those born at term (231).

A study by Wagner et al from 2019 could not find any increased risk of fractures at five years of life related to being born preterm (57). Their approach and conclusions were similar to our study on NEC and risk of fractures (paper II), but their sample size was smaller, and their follow up time was much shorter than in our study. NEC as being a risk factor for development of OOP during the neonatal period is described in several studies (202, 232). The inflammation related to NEC in combination with post NEC malabsorption and long-term parenteral nutrition have been described as risk factors of OOP (202). So, the effects of NEC on short term bone health are well described.

However, whether NEC is a risk factor for bone health during childhood and adolescence is not as well described. In one study by Davies et al from 1999 they performed total body and lumbar spine DXA measurements on 21 children at a median age of 7,4 years. The children had undergone limited ileal resection during the neonatal period, 17 due to NEC, and the rest for other reasons such as intussusception. The NEC infants were born preterm with a median GA at birth of 30 w. DXA at 7 years of age did show low BMD only in one child, with a history of NEC, compared to an age matched control group of term infants. The rest of the infants in the ileal resection group were within normal range for age (233).

In an older study by Abbasi et al from 1984 they showed that recovery of the gastrointestinal function after NEC can be expected if not a major part of the small intestine is resected (234). They performed measurements of growth, nutritional status and hydrogen breath test at one year of age among NEC patients. However, they did not do any DXA measurements, and their group of NEC patients was different from the patients we would expect to see today.

In a review on NEC and long term complications by Bazacliu and Neu from 2019 the authors mention the common complications of NEC including neurodevelopmental delay, failure to thrive and short bowel syndrome with or without intestinal failure (5). They do not specifically mention bone mass or bone health, but indeed intestinal failure may be related to effects on bone development and bone accrual.

Han et al described long term outcomes of severe surgical NEC in a publication from 2020 (235). They concluded that in children who survive the initial course of NEC the long-term survival is good, but there are several long-term complications. They describe that many of these complications are directly related to the length of the remaining bowel and suggest that more focus in follow up should be related to remaining small bowel length.

In summary there are several previous publications regarding preterm birth and bone development and a few publications on NEC and short-term effects on OOP. There has been a need for studies regarding NEC and long-term effects on bone health. Papers II and III add new knowledge regarding bone health during childhood in children born preterm with a history of NEC.

6.4 NEC AND MICROBIOTA (PAPER IV)

There are several recent studies on the alteration of fecal microbiota prior to onset of NEC in preterm infants (133, 236). There seems to be a microbial dysbiosis or at least a different microbiome with more Proteobacteria before onset of NEC (237). A few studies have reported that the dysbiosis in NEC was characterized by increased relative abundances of Proteobacteria and lower relative abundances of Firmicutes and Bacteroidetes (137, 226). There have been reports of specific gut bacteria linked to the onset of NEC such as *Klebsiella* or some *Clostridium* species (136, 226, 238). Others however claim that there is no specific microbial signature among preterm infants that can be linked to development of a specific disease, rather is the microbiome in preterm infants highly personalized, but can be affected by for example antibiotics and then change in relation to development of for example NEC.

In this thesis, paper I studied clustering of NEC and discussed the possible connection between clustering of NEC and the spreading of certain bacteria within a neonatal unit. In paper IV however, the focus was on the long-term perspective. One question that is debated is whether children born preterm with a history of NEC have alterations in their microbiota at five years of age compared

to controls born preterm without NEC. In 2021, Marti et al published a follow up on two-year-old children born preterm that had been supplemented with probiotics during the neonatal period. They did not find any differences in diversity of the microbiota at two years between cases and controls (239). In the limited number of subjects studied in paper IV, children with a history of NEC had a decreased Shannon index for alpha diversity and exhibited a different relative abundance for several bacteria on genus and species level compared to controls. These differences in alpha diversity and in relative abundance were mainly driven by the differences among the surgical NEC cases compared to controls. The groups studied in paper IV were small, but the findings were clearly significant. If the results from paper IV can be repeated in larger studies, this implies that surgical intestinal resection following NEC in the neonatal period does have an impact on intestinal microbiota several years later. The clinical impact of this is not fully understood yet, but the more knowledge we gain on microbiota the more we will understand how important it is for growth, health and disease. The exact mechanisms behind the findings remain to be studied. Is the resection of bowel, including in some cases the ileocecal valve what causes the dysbiosis, or are other factors such as prolonged antibiotic treatment or prolonged use of parenteral nutrition more important?

What other studies are there to support the findings of long-term changes in fecal microbiota following surgical NEC?

In one study from 2023 Lin et al performed fecal sampling at onset of NEC, and then again when the infant had reached full enteral feeds (240). They found that infants with surgically treated NEC had a fecal microbiota with lower alpha diversity even after reaching full enteral feeds. They concluded that it might take more time to reestablish the normal intestinal flora of NEC infants after surgery. In their study the median age for last fecal sample collection in the NEC group was 54 days. In paper IV the NEC cases had lower alpha diversity of the fecal microbiota even after five years.

In a review from 2023 Wang et al discussed that the neurodevelopmental impairment following NEC could be related to the Gut-brain axis (207). They proposed that the dysbiosis related to NEC could be an important factor in the damaged connections in the gut-brain axis, and thus contributing to impaired neurodevelopment. Their review contains a lot of references on fecal microbiota before onset, or just after onset of NEC, and also to other reviews on that topic (241, 242). There are no references on long term microbial dysbiosis following NEC and how that may affect the gut-brain axis during childhood.

To the best of our knowledge there are no previous studies on fecal microbiota from five-year-old, or older, children born preterm with a history of medically or surgically treated NEC, and their controls. In that case our study, even if small, is the first to show this important outcome with lower alpha diversity and differences in relative abundance of the fecal bacteria in children with a history of surgically treated NEC.

6.5 MICROBIOTA AND BONE HEALTH (PAPER III-IV)

The original hypothesis for this project was that NEC in the neonatal period would result in long-term changes of the microbiota, and that this dysbiosis could influence bone development. Hence, a history of NEC would result in poor bone development. The results presented in paper III showed that NEC might have an impact on BMC, and possibly on BMD at five years of age. Furthermore, paper IV showed that surgically treated NEC has an impact on fecal microbiota at five years of age. However, paper III and paper IV have not been able to conclude that the alterations in microbiota following NEC had a direct impact on bone development.

There are several studies that link changes in microbiota to poor bone development. One Swedish animal study by Sjögren et al in 2012, showed that germ-free mice exhibit increased bone mass associated with reduced number of osteoclasts, and that colonization of germ-free mice with normal gut microbiota did normalize the bone mass. In conclusion they could show a direct relationship between intestinal microbiota and bone mass (243).

Since then, there have been a number of publications confirming this relationship. Ohlsson and Sjögren have in 2015 published a review on effects of the gut microbiota on bone mass (244).

D´Amelio and Sassi have published a study where they reported that gut microbiota can improve bone health by improving the absorption of calcium and modulating the production of gut serotonin (245).

Furthermore, Yan et al suggests in 2016 that gut microbiota could promote bone formation and growth by inducing IGF-I (246). As IGF-I is an important growth factor for the fetus and preterm infant this could be of importance for preterm infants following NEC. They also suggest that the effect could be mediated via

short-chain-fatty-acids, a factor that is gaining increasing interest in the development of preterm infants.

The results presented in this thesis have been able to show that NEC has a long-term impact on gut microbiota (paper IV) and on bone mass (paper III). Even though we have not been able to show the exact connections between changes in microbiota and bone mass, our studies support the idea of a link between gut microbiota and bone mass.

6.6 LIMITATIONS

Paper I and II consisted of a large study population collected from different registers. This has the advantage of making it possible to identify differences between groups that would have not been visible in a smaller material. However, data collected from registers have a number of limitations. One limitation is the retrospective design, which makes it impossible to follow-up clinical and laboratory data with a medical record review to confirm the diagnosis. All diagnostic data in paper I and II are based on the diagnostic codes as they are reported by the treating clinician. For example, in study I we were able to conclude that NEC does occur in space-time clusters, but we could not identify any specific reason for the clustering such as spreading of a specific gut bacterium etc. As the use of patient registers is becoming increasingly common in medical research it is of outmost importance that data in the registers are as correct and accurate as possible.

In paper III and IV the problems were inverse compared to paper I and II. In paper III and IV we had direct contact with every study subject and their families, I was personally present at all the DXA examinations, and personally received all the collected stool samples from the families. The main problem in these studies was the small sample size. In preparation for paper III we performed a power calculation based on the primary question in paper III. We decided to use controls matched for gestational age at birth, sex and age at DXA-measurements. The samples were then analyzed as groups, not as pairs. The power calculation in paper III suggested that 25 individuals in each group would be sufficient for the primary outcome. In NEC research, being able to study 25 NEC patients at five years of age is considered a large number. As the power calculation was based on the primary research question, there are limitations in any conclusion not based on the primary research question.

For paper IV stool samples were collected from the individuals included in paper III. They were supposed to collect the sample in a specific container at home, and bring it frozen to the hospital. One problem with collecting stool from five-year-old children was that they did not all bring samples. We tried to organize for them to bring the samples at a second occasion, and in a few cases we went to their homes to pick up the samples from their freezer. Unfortunately, we were not able to collect samples from all 25+25 individuals. As we decided to use all the samples collected, the children in study IV were no longer matched as perfectly as in study III. As the NEC group consisted of medically and surgically treated cases the number of individuals in each group was even smaller. This was an important limitation as the difference in alfa diversity and in relative abundance was mainly in the surgically treated NEC group. It would be of great interest to perform larger follow up studies on microbiota following NEC to confirm our findings.

6.7 CLINICAL RELEVANCE

The papers presented in this thesis do contribute with confirmatory data and new results that are important in the clinical everyday work for hospital staff working with these patients.

We have been able to confirm clustering of NEC on hospital level. All staff working in any NICU are aware of the importance of following the hygiene instructions to avoid spreading of bacteria between patients. Further knowledge of clustering of NEC, in periods of overcrowding or understaffing in the NICU, and the risk of spreading specific contagious agents between patients can help us avoiding this in the future.

Paper I showed a decreasing trend in clustering of NEC on a hospital level when comparing two time periods. This may indicate that improved routines are effective in minimizing the transfer of contagious agents between patients in the NICU. However, continued awareness is still warranted to further minimize the risk of environmental factors for NEC being transferred from one patient to another.

The findings in papers II, III and IV showed that NEC has a long-term impact on bone health and microbiota. We could identify lower bone mass and lower microbial diversity in the stool at five years of age among children with a history of NEC, even though we could not identify any increased risk of fractures during childhood and adolescence related to NEC.

Being able to identify changes in bone mass, body composition, growth and microbiota at five years of age among children with NEC during infancy, in particular surgically treated, provides important knowledge that NEC in infancy is not a condition limited to the neonatal period. NEC in infancy is a condition that you must live with for the rest of your life. We do need to pay special attention to this group of individuals in our follow up programs. We have been able to identify important changes in bone mass and microbiota at five years of age in these individuals, but we still do not know how their life will be as adults or elderly. Do they have an increased risk of osteoporosis? Will the dysbiosis of fecal microbiota at five years of age be of importance for health and disease later in life? These questions remain to be answered.

The need for an increased understanding of the long-term complications of NEC is addressed in a recent publication by Canvasser et al (247). Their study focuses on the mental and physical health of NEC survivors and their families beyond two years after discharge from the NICU. They conclude that NEC affects many aspects of life of NEC survivors for many years or even decades after the actual NEC episode. They highlight the need for more knowledge on many aspects of the long-term consequences of NEC, and they refer to a previous study from our group regarding intestinal failure following NEC (247, 248).

7 CONCLUSIONS

Paper I: NEC occurred in clusters at hospital level in Sweden, as found with both the Knox space-time cluster analysis and Kulldorff's scan statistic. Clustering did not occur at the level of the mother's residential municipality. The study could identify a decrease in clustering on a hospital level over the last few decades.

Several possible explanations for clustering on a hospital level have been described. One explanation is that NEC is associated with a contagious agent spread from one child to another in a NICU. Paper I could not make any conclusions regarding the specific causes of clustering.

Paper II: Children born preterm with a history of NEC had an increased risk of rickets and diagnosis of malabsorption compared to those without NEC. The diagnosis of rickets was usually limited to the first year of life. A history of NEC was not related to an increased risk of fractures or vitamin D deficiency during childhood and adolescence.

Paper III: Measurements with DXA showed that at five years of age children born preterm and diagnosed with NEC during infancy had an altered body composition, with lower amount of fat mass and fat percentage than the controls. Children born preterm also had lower BMC and a tendency to lower BMD than controls. The difference in BMC partially remained significant after correction for height. The children with a history of NEC had lower weight than their matched controls.

The results from paper II and III suggest that preterm infants diagnosed with NEC need special attention at follow-up during childhood regarding growth and bone health compared to preterm infants without NEC.

Paper IV: Five-year-old children with NEC during infancy had a fecal microbiota with lower alpha diversity than children born preterm without NEC. These differences were more pronounced when comparing children with surgically treated NEC to controls. In terms of relative abundance, on bacterial genus and species level, we found significant differences in profiles between NEC and control cases with more significant differences in the surgically treated NEC group.

Dysbiosis of the gut microbiota may be important not only at the onset of NEC but also in the long-term perspective among NEC survivors.

8 FUTURE PERSPECTIVES

As modern neonatal intensive care develops, survival of preterm infants is increasing. Preterm infants are at risk of developing morbidities related to preterm birth such as NEC. As clinicians and researchers, it is our responsibility to focus not only on the short-term survival of these individuals but also on the long-term consequences of preterm birth and related morbidities.

In the future there is an increasing need for long term follow up studies aiming to improve the lives of individuals with morbidities related to being born preterm.

One aim is to focus on nutrition during the neonatal period in relation to long term effects on microbiota, growth and bone health. What impact does breastmilk, fortifiers, probiotics, fatty acid supplementation and parenteral nutrition have on growth, bone health and microbiota during childhood and beyond?

Furthermore, it would be interesting to look at the effects of antibiotic use, and overuse, during the neonatal period on long-term development of intestinal microbiota.

Most likely we need to look beyond five years of age, both in our clinical follow up and in our research, of these individuals. By better identifying infants at risk for long-term morbidities, for example individuals with surgically treated NEC, we can provide better follow-up programs. There is a need for better understanding how the actions during the first days and weeks in the NICU might have an impact for life for these individuals and their families.

NEC during infancy might have lifelong consequences.

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