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With a focus on early nutrition and initial gut microbiota

Henni Hiltunen

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To my loved ones

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ABSTRACT

Preterm birth is a global health challenge. Even though the survival rates of preterm infants have been increasing, these fragile patients face the risks of significant short- and long-term morbidity. Growth failure is a common clinical problem. Successful nutritional management and early enteral feeding result in improved growth and developmental outcomes. The gut microbiota participates in energy harvest and has been linked to health outcomes in term infants. The colonization process and the contribution of common perinatal exposures on preterm gut microbiota composition remain fairly unknown.

The aim of this study is to focus on the relationship among early nutrition, early gut microbiota composition and growth in preterm infants. First, early nutritional management and its long-term effects on growth outcomes were studied, and an independent association between first-week energy intake and growth outcomes throughout the first two years of life was detected. Then, the early gut microbiota composition of preterm neonates was compared to that of early-term neonates. The preterm gut microbiota composition was characterized as lower diversity with greater individuality compared to term microbiota composition. The contribution of numerous perinatal exposures on the preterm gut colonization process was studied, and the cause of prematurity was found as the sole significant contributor. Finally, an experimental animal model was developed to investigate whether a fecal microbiota transplant would affect growth and metabolism in germ-free mice. The results revealed impaired growth, increased inflammatory activation, and metabolic changes in mice with a very preterm meconium transplant.

In conclusion, early nutritional management, the initial gut microbiota composition and perinatal exposures emerge as potential determinants of preterm infant growth and health and may offer new intervention targets to improve the long-term outcomes of preterm children.

KEYWORDS: preterm infant, growth, energy intake, gut microbiota

TURUN YLIOPISTO
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TIIVISTELMÄ

Ennenaikainen syntymä on maailmanlaajuinen kansanterveysongelma. Vaikka keskosten selviämisennuste on parantunut, ovat he edelleen suuressa riskissä sairastua vakaviin sairauksiin lyhyellä ja pitkällä aikavälillä. Kasvuhäiriö on yleinen kliininen ongelma keskoslasten hoidossa. Onnistunut ravitsemushoito ja aikainen suun kautta toteutettu ravitsemus parantavat sekä kasvua että kehitysennustetta. Suolistomikrobisto osallistuu osaltaan ravinnon hyödyntämiseen, ja sen koostumus on yhdistetty moniin terveysvaikutuksiin täysaikaisilla lapsilla. Keskosen suolistomikrobiston muodostumisesta sekä siihen vaikuttavista raskauden ja synnytyksen aikaisista tekijöistä tiedetään varsin vähän.

Tutkimuksen tavoitteena oli tutkia ennenaikaisena syntyneiden lasten ravitsemuksen, suolistomikrobiston ja kasvun välisiä yhteyksiä. Varhaisen ravitsemuksen pitkäaikaisvaikutuksia kasvutuloksiin selvitettiin, ja ensimmäisen elinviikon ravitsemuksen osoitettiin vaikuttavan lapsen kasvuun kahden vuoden korjattuun ikään asti. Keskoslapsen suolistomikrobiston koostumusta verrattiin täysiaikaiseen lapseen, ja vertailussa havaitsimme keskosen mikrobiston olevan yksipuolisempi sekä hyvin yksilöllinen. Erilaisten varhaisten altisteiden vaikutusta suoliston varhaiseen mikrobikolonisaatioon tutkittiin, ja ennenaikaisen syntymän syy oli tutkimuksessa ainoa mikrobiston koostumukseen vaikuttava tekijä. Lopuksi kokeellisessa eläinmallissa tutkittiin ulosteensiirron vaikutusta koe-eläinten kasvuun ja aineenvaihduntaan. Keskosille tyypillinen kasvuhäiriö, tulehdusvaste ja aineenvaihduntahäiriö saatiin siirrettyä erittäin ennenaikaisen keskosten ulosteensiirrolla steriileissä olosuhteissa kasvaneisiin hiiriin.

Yhteenvetona varhainen ravitsemus, varhainen suolistomikrobisto ja perinataaliset altisteet näyttäytyvät potentiaalisina keskosen kasvuun ja terveyteen vaikuttavia tekijöitä, ja voivat tarjota uuden terveydellisen interventiokohteen.

AVAINSANAT: keskonen, kasvu, energiansaanti, suolistomikrobisto

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Abbreviations

AGA	Appropriate for gestational age
ANCOM	Analysis of composition of microbiomes
ANOVA	Analysis of variance
BM	Breastmilk
BMI	Body mass index
BPD	Bronchopulmonary dysplasia
CPM	Count-per-million
CS	Caesarean section
DADA2	Divisive Amplicon Denoising Algorithm 2
ELBW	Extremely low birth weight
ESPGHAN	The European Society for Paediatric Gastroenterology, Hepatology and Nutrition
EUGR	Extrauterine growth restriction
FDR	False discovery rate
FI	Feeding intolerance
FMT	Fecal microbiota transfer
FT	Full-term
FTVDBF	Full-term, vaginally delivered, exclusively breastfed
GA	Gestational age
GF	Germ-free
GW	Gestational weeks
HLMM	Hierarchical linear mixed model for repeated measures
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IUGR	Intrauterine growth restriction
IVH	Intraventricular hemorrhage
LBW	Low birth weight
LEfSe	Linear discriminant analysis Effect Size
LGA	Large for gestational age
LOS	Late-onset sepsis
MOD	Mode of delivery

MOM	Mother's own milk
M.FT	Mouse, full-term
M.PT	Mouse, moderately preterm
M.VPT	Mouse, very preterm
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care unit
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PDA	Patent ductus arteriosus
PERMANOVA	Permutational multivariate analysis of variance
PPROM	Preterm premature rupture of membranes
PT	Moderately preterm
PVL	Periventricular leukomalacia
RDS	Respiratory distress syndrome
ROP	Retinopathy of prematurity
SEM	Standard error of the mean
SGA	Small for gestational age
TEA	Term-equivalent age
TNF	Tumor necrosis factor
VLBW	Very low birth weight
VPT	Very preterm
WHO	World Health Organization

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Hiltunen H**, Löyttyniemi E, Isolauri E, and Rautava S. Early nutrition and growth until the corrected age of 2 years in extremely preterm infants. *Neonatology*, 2018; 113(2): 100-107.

II **Hiltunen H**, Collado MC, Ollila H, Kolari T, Tölkkö S, Isolauri E, Salminen S, and Rautava S. Spontaneous preterm delivery is reflected in both neonatal and maternal gut microbiota. *Pediatric Research*, 2021; Aug 4.

III **Hiltunen H***, Hanani H*, Luoto R, Turjeman S, Ziv O, Isolauri E, Salminen S, Koren O, and Rautava S. Preterm infant meconium microbiota transplant induces growth failure, inflammatory activation and metabolic disturbances in germ-free mice. *Cell Reports Medicine*, 2021;Nov 16;2(111):10047.

* Equal contribution

 In addition, unpublished data not included in the original publications are presented.

1 Introduction

Preterm birth, defined as being born before 37 weeks of gestation, is a global health concern affecting approximately 10% of pregnancies (Blencowe *et al.*, 2013). It is the leading cause of death in children under five years of age (Liu *et al.*, 2015), and it increases the risk for long-term health consequences (Luu, Rehman Mian and Nuyt, 2017). Preterm birth is also a significant health and economic concern (Behrman and Butler, 2007), since these fragile infants are often treated in intensive care units and require potentially lifelong rehabilitation. Although efforts have been made to prevent premature births, these efforts have not been successful (Matei *et al.*, 2019). Many theories have been proposed for the etiology of preterm birth. However, in the majority of cases, the actual cause remains unknown (Ferrero *et al.*, 2016). A microbial etiology has been suggested (Goldenberg, Hauth and Andrews, 2000), and disturbances in the gut, vaginal and oral microbiome of the mother have been studied as the potential initiator of the events that lead to a premature birth.

Growth failure is a common problem in preterm infants that has been linked to other adverse outcomes, including neonatal morbidity and poor neurodevelopmental results (Horbar *et al.*, 2015). Preventing postnatal growth failure is considered a universal challenge. Many of the common neonatal problems often cluster in preterm infants, since they face the risk of infection and neonatal morbidity. Sufficient nutrition may not always be prioritized, and the nutrient intake remains suboptimal (Uthaya and Modi, 2014). Early starting enteral nutrition is linked to better growth (Dinerstein *et al.*, 2006) and better developmental outcomes (Lechner and Vohr, 2017). However, little is still known about the optimal nutritional component ratio, and the role of specific nutrients. In addition, the long-term outcomes of the early nutritional management have not been extensively studied.

Preterm infants exhibit an aberrant gut microbiota composition compared to term infants (Underwood and Sohn, 2017). Although the establishment and modulators of the initial term gut microbiota are relatively well known, the understanding of the foundations of the preterm gut microbiota is still inadequate. For instance, it is still unknown whether the specific composition of the preterm gut microbiota reflects the mere immaturity of the infant or is mainly directed by the perinatal exposures. Studying the colonization process of the preterm gut is considered a challenge, since

the study cohorts are relatively small, and there are numerous confounding factors, such as the feeding procedures, antibiotics, and differences in skin-to-skin contact and environmental bacteria.

Focusing on the initial gut microbiota and meconium can provide a better understanding of the early colonization process and minimize the contribution of confounding factors. Meconium is defined as the first stool passed after birth. According to the current knowledge, meconium is nonsterile, suggesting that the colonization process may already begin *in utero* (Taddei *et al.*, 2018). The meconium microbiota composition reflects the circumstances that the neonate has faced during pregnancy and labor. However, there is only little evidence linking the specific preterm gut microbiota composition to the potential health consequences, and in addition, the health outcomes related to the microbiota colonization are still mostly unknown.

The gut microbiota participates in the energy harvest from enteral nutrition and plays an important role in the development of the host immune system (Dominguez-Bello *et al.*, 2019). Preterm gut microbiota is often described as proinflammatory and low in diversity (Underwood and Sohn, 2017). Moreover, neonatal morbidity is characterized by an adverse immunological response, and inflammation plays a major role in the pathology of these diseases such as bronchopulmonary dysplasia (BPD) (Morty, 2018), retinopathy of prematurity (ROP) (Rivera *et al.*, 2017), brain damage (Van Steenwinckel *et al.*, 2014; Galinsky *et al.*, 2018), and necrotizing enterocolitis (NEC) (Neu and Walker, 2011a). Inflammation also contributes to poor growth outcomes (Mestan *et al.*, 2010; Trevisanuto *et al.*, 2013; Cuestas *et al.*, 2019), which often occur together with high morbidity. However, no causal connection among inflammatory gut microbiota, immunological development, neonatal morbidity, and growth retardation has yet been established.

The aim of this study is to focus on the relationship among early nutrition, gut microbiota composition and growth in preterm infants. First, early nutritional management and its association with long-term growth outcomes in an extremely preterm infant cohort were studied. Then, the microbial composition of early gut microbiota samples from two different preterm study cohorts was examined and compared to that of term neonates, evaluating the impact of gestational age. The possible perinatal causes modulating initial preterm gut microbiota colonization were also investigated. Finally, an experimental animal model was developed to investigate whether a fecal microbiota transplant (FMT) would affect the growth outcomes and metabolism in germ-free (GF) mice.

2 Review of the Literature

2.1 Premature birth

2.1.1 Public health perspective

According to the World Health Organization (WHO), preterm birth is defined by as a birth occurring before 37 gestational weeks (GW) (WHO, 1977). A premature infant can be classified as extremely preterm or low gestational age (less than 28 GW), very preterm (28–32 GW), or moderately preterm (32–37 GW), which can be further divided into moderate (32–34 GW) and late (34–37 GW) preterm (Howson *et al.*, 2013). Preterm birth affects nearly 15 million births every year (Blencowe *et al.*, 2013). For example, in 2010, the worldwide rate of preterm birth was reported to be 11.1% of livebirths (Blencowe *et al.*, 2013). In developed countries, the preterm birth rate amounts to 8.6% of livebirths, with the globally highest percentage (13.3%) being in South Asia at 13.3% (Blencowe *et al.*, 2012). According to the perinatal statistics of the Finnish Institute for Health and Welfare (statistical report 49/2019, 19.12.2019), 5.8% of the Finnish livebirths are preterm, and this trend has not changed throughout the 21st century. In contrast to many other developed countries, in the United States, the rate of preterm births has recently increased (Lawn *et al.*, 2010), reaching approximately 12.0% (Blencowe *et al.*, 2012).

Preterm birth can be classified by obstetric precursors, which are currently defined as (1) a spontaneous delivery with intact membranes, (2) a spontaneous preterm premature rupture of membranes (PPROM) with either vaginal or caesarean-section delivery, and (3) a delivery for an iatrogenic maternal or fetal cause with either induced delivery or by prelabor caesarean section. Classification can also be simplified to spontaneous (1 and 2) and iatrogenic (3) preterm delivery (Goldenberg *et al.*, 2008). Among preterm deliveries, approximately 40%–45% are spontaneous (1), 25%–30% are related to PPROM (2), and 30%–35% are iatrogenic (3). According to a nationwide Swedish study with more than one million infants, different labor onset types are associated with different adverse outcomes (Morken, Källen and Jacobsson, 2007). In this thesis, the obstetric precursors are called as “the cause of prematurity”.

Preterm birth represents a significant socioeconomic burden. These fragile infants are often treated in intensive care units for weeks, and some of them even require potentially lifelong rehabilitation. In the United States in 2005, the Institute of Medicine estimated the annual costs of prematurity as $26 billion (Behrman and Butler, 2007). Similar calculations were made in Germany (Jacob *et al.*, 2017) and the Netherlands (Van Baaren *et al.*, 2015), where extensive costs have been associated with early preterm deliveries. Therefore, from a health economical perspective, preventing preterm births is considered a public health priority.

Preventing preterm deliveries is challenging because there are multiple etiologies. Although some of the risk factors for a spontaneous preterm birth are known, such as a family history of preterm birth, a low socioeconomic status and maternal age (Simhan, Iams and Romero, 2018), two out of every three preterm births occur without a known risk factor (Ferrero *et al.*, 2016). Moreover, although preventive measures have been devised, only a few have been successful (Matei *et al.*, 2019). Therefore, a more thorough understanding of the etiology and phenotypes of preterm birth (Frey and Klebanoff, 2016) and a standardization of the current definitions (Vogel *et al.*, 2018) may help further understand and ameliorate this global burden.

Table 1. Relevant terminology connected to preterm birth.

TERM	EXPLANATION
PRETERM BIRTH	A birth occurring before 37 gestational weeks
EXTREMELY PRETERM	Gestational age less than 28 weeks
VERY PRETERM	Gestational age from 28 to 32 weeks
MODERATELY PRETERM	Gestational age from 32 to 37 weeks
SPONTANEOUS PRETERM BIRTH WITH INTACT MEMBRANES	A commenced labor without the rupture of the membranes
SPONTANEOUS PRETERM PREMATURE RUPTURE OF MEMBRANES	A commenced labor with the rupture of the membranes
IATROGENIC PRETERM BIRTH	A terminated pregnancy due to a maternal or fetal reason i.e., pre-eclampsia or intrauterine growth restriction
LOW BIRTH WEIGHT (LBW)	Birth weight less than 2500 grams
VERY LOW BIRTH WEIGHT (VLBW)	Birth weight less than 1500 grams
EXTREMELY LOW BIRTH WEIGHT (ELBW)	Birth weight less than 1000 grams

2.1.2 Health consequences

Prematurity is globally the leading cause of death in children under five years of age (Liu *et al.*, 2015). The survival rate increases with gestational age. While only 40% of infants born at 23 GW survive, the survival rate at 25 GW and onwards is greater than 90%, according to the registries of the Finnish Institute for Health and Welfare (*Official Statistics of Finland (OSF): Perinatal statistics - parturients, delivers and newborns [e-publication]*, referred 31.8.2020). According to a recent report, the very preterm infant survival rate in Finland has improved over the last decades from approximately 75% in the late 1980s to 92% in 2012 (Helenius, Gissler and Lehtonen, 2019). The preterm survival rate in Finland is overall notable in international comparison, ranking the second best worldwide, with Japan having the highest survival rate (Helenius *et al.*, 2017).

Preterm infants have a unique spectrum of neonatal morbidities. Common disorders include respiratory distress syndrome (RDS), BPD, NEC, and ROP. Preterm infants are also at a high risk for severe infections such as neonatal sepsis, pneumonia, or meningitis, since their immunological system and skin and mucosal barriers are still in a developing state. They may also develop neurological conditions, such as intraventricular hemorrhage (IVH) or periventricular leukomalacia (PVL), and cardiac conditions, such as patent ductus arteriosus (PDA).

Some studies suggest that preterm infants exhibit excessive inflammatory responsiveness (Nanthakumar *et al.*, 2000; Olin *et al.*, 2018). This may reflect the conditions leading to a premature birth or the immature immunologic state of the infant. Generally, the inflammatory response in preterm infants is usually multifactorial (Olin *et al.*, 2018), and inflammatory responsiveness has been associated with poor growth outcomes. (Mestan *et al.*, 2010; Trevisanuto *et al.*, 2013; Cuestas *et al.*, 2019). Inflammatory responsiveness is also regarded as a contributor to the pathogenesis of neonatal morbidities such as BPD (Morty, 2018), ROP (Rivera *et al.*, 2017), and brain damage (Van Steenwinckel *et al.*, 2014; Galinsky *et al.*, 2018). Intestinal inflammation is a significant component in the development of NEC (Neu and Walker, 2011a). In addition, gastrointestinal dysbiosis, with a higher abundance of proinflammatory bacteria, plays an important role in the pathogenesis of this severe and deadly disease (Neu and Pammi, 2017).

Studies have shown that prematurity increases the risk for a number of chronic diseases. For example, preterm infants exhibit a higher incidence of cardiovascular risk factors (Sipola-Leppänen *et al.*, 2015a; Markopoulou *et al.*, 2019), including metabolic alterations such as metabolic-like syndrome (Heidemann, Procianoy and Silveira, 2019a) and obesity (Thomas *et al.*, 2011; Breukhoven *et al.*, 2012). They also have an elevated risk for developing asthma (Jaakkola *et al.*, 2006). In general, prematurity is associated with an increased risk for neurodevelopmental impairment (Luu, Rehman Mian and Nuyt, 2017), as well as hearing or visual defects (Duncan

and Matthews, 2018). Although all the long-term outcomes of prematurity are still unknown, the health burden of prematurity seems to be potentially lifelong.

2.1.3 Growth and neurodevelopment

Growth is of particular interest in preterm infants, as poor growth is linked to other adverse outcomes. Preterm infant growth can be expressed as absolute grams/centimeters or as a Z-score, which compares an individual child to the population according to gestational age and sex. In addition, in the context of preterm growth failure, both intrauterine growth restriction (IUGR) and extrauterine growth restriction (EUGR) are used to determine the timing of inadequate growth. Newborns are categorized as low birth weight (LBW) at 2500 grams, very low birth weight (VLBW) at less than 1500 grams, or extremely low birth weight (ELBW) at less than 1000 grams. These classifications, however, do not consider gestational age. According to their Z-scores, newborns can be classified as small (SGA), appropriate (AGA), or large (LGA) for gestational age. AGA infants have a birth weight Z-score between –2.0 and +2.0 SD, whereas LGA infants have a birth weight Z-score greater than +2.0 SD. According to a consensus statement, SGA infants have a Z-score less than –2.0 SD (Clayton *et al.*, 2007). Furthermore, SGA infants can be classified with a birth weight in the lowest 10th percentile, which corresponds to a Z-score of –1.282 SD or less (WHO, 1993, 1995).

Growth classification is used to recognize and assess infants with a high risk for certain morbidities and high mortality rates. Although nearly 15% of the infants born worldwide in 2015 were LBW, the trend is decreasing compared to the 17% in 2000 (Blencowe *et al.*, 2019). This study, however, did not distinguish between preterm and term births, and it is worth noting that LBW accounts for 80% of neonatal deaths worldwide, of which two-thirds are preterm (Katz *et al.*, 2013; Blencowe *et al.*, 2019).

Postnatal growth failure is a common problem in all preterm infants that has been studied especially in the VLBW population. According to a study by Horbar et al., although the rate of severe postnatal growth restriction is decreasing, half of the VLBW infants still experience poor postnatal growth outcomes, with one-quarter experiencing severe growth restriction (Horbar *et al.*, 2015). In another study on ELBW infants, Diekmann et al. found that the postnatal median growth fell below the 10th percentile at the third trimester and term-equivalent age (TEA) despite early enteral feeding (Diekmann *et al.*, 2005). It has also been highlighted that although the neonatal morbidity and mortality rates are decreasing, the growth outcomes of the VLBW population have not improved (Fanaroff *et al.*, 2007).

The optimal growth velocity in preterm infants is unknown. According to a recent review, since there are many different calculation methods, standardization is

required (Fenton *et al.*, 2017). In the 2000s, some studies suggested growth velocity improvement and better growth outcomes in preterm infants (Bloom *et al.*, 2003; Andrews *et al.*, 2019), indicating an improvement in uniform clinical practice and overall neonatal intensive care.

Some studies have shown that SGA infants in particular may face metabolic alterations as adults, if the catch-up growth is too extensive. According to a hypothesis by David Barker in the 1980s, early and even intrauterine conditions may determine to the growth trajectory and metabolic outcomes later in life (Barker, 2007). With suboptimal *in utero* growth conditions and LBW, the body's metabolic system is hypothesized to be programmed to utilize nutrients more efficiently, which may in the modern society with abundant nutrition result in increased adiposity, obesity, and adverse cardiovascular outcomes. This phenomenon may explain the preterm metabolic phenotype, as similar long-term outcomes exist in preterm infants. Balancing between immediate, adequate growth and later, possibly adverse metabolic health outcomes is an emerging challenge in neonatal care.

Many studies have shown that better postnatal growth improves the neurodevelopmental outcomes. These findings have been confirmed in studies observing neurodevelopmental performance in children who are 18 months (Belfort *et al.*, 2011), 18–22 months (Ehrenkranz *et al.*, 2006), and five years (Franz *et al.*, 2009; Leppänen *et al.*, 2014; Guellec *et al.*, 2016) of age. SGA infants face neurodevelopmental and behavioral problems at school age (Vollmer and Edmonds, 2019). It has also been suggested that the early neurodevelopmental outcomes do not improve as well as the overall preterm outcomes do (Rogers and Hintz, 2016). Therefore, ensuring adequate growth is a top priority in the treatment of preterm infants, since it is connected to potentially adverse outcomes. From a long-term perspective, poor neurodevelopmental outcomes may be the most harmful consequences of preterm birth. However, the causalities and exact relationships among nutrition, growth, and neurodevelopment have not yet been clearly established.

2.2 Nutritional management of preterm infants

2.2.1 Clinical practice

Nutritional management of preterm infants can be performed with a combination of parenteral nutritional solutions and enteral feeding. Parenteral nutrition is provided via an intravenous catheter (either peripheral or central), whereas enteral feeding is usually provided via an enteral tube, until the child is healthy enough to eat on their own. The parenteral solutions include carbohydrates (glucose), proteins (amino acids), and fats (lipid emulsion). In addition, electrolytes, minerals, and vitamins are

included in the parenteral nutrition. Enteral feeding usually relies on of either the mother's own milk (MOM), donor milk, or formula. This milk may be fortified with cow-milk-based products. Tolerance to enteral feeding is considered a good indicator of the child's status, since most of the fragile and ill patients require longer parenteral feeding and thriving infants achieve full enteral feeding earlier. Daily feeding is closely monitored, and as the child grows, the amount is gradually increased.

Neonatal nutritional management has many practical challenges. A catabolic state is especially harmful to a small preterm infant with a nonexistent energy reserve and may have potential long-term consequences (Embleton and Simmer, 2014). In addition, newborns require extra energy compared to unborn children, since they may face infections and other stress factors (Hay, Brown and Denne, 2014). Small preterm infants cannot tolerate an excessive amount of fluid, and medications may be prioritized, resulting in an often insufficient actual amount of nutrition. Eventually, the most critically ill patients, who would have the highest need for nutrition in order to recover, may receive the least amount of nutrition. Gastrointestinal immaturity also restricts the amount of enteral nutrition prescribed, and administering parenteral nutrition is vital. A central or peripheral venous catheter is an infection risk, and even thrombophlebitis or sepsis may occur. Moreover, abnormal levels of cholesterol, triglycerides, ammonium and aluminum, related to parenteral administration, are a concern (Embleton and Simmer, 2014).

2.2.2 Guidelines on nutrition

The European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) publishes pediatric nutritional guidelines regularly.

For enteral nutrition, the current guidelines were updated in 2005 (Tsang *et al.*, 2005) and 2010 (Agostoni *et al.*, 2010). The recommended amount for a fully enterally fed preterm VLBW infant includes a fluid intake of 135–200 mL/kg/day, updated from 150–200 mL/kg/day. The recommended fluid intake results in 110–135 kcal/kg/day.

The current guidelines for parenteral nutrition were updated in 2018 (Mihatsch *et al.*, 2018) and from those of 2005 (Koletzko *et al.*, 2005). Moreover, individual ESPGHAN recommendations were published for energy (Joosten *et al.*, 2018), lipids (Lapillonne *et al.*, 2018), amino acids (van Goudoever *et al.*, 2018), and carbohydrates (Mesotten *et al.*, 2018).

As shown in Table 1, the current guidelines have not considerably changed as compared to the previous ones. Currently, the ESPGHAN recommends caloric intake of at least 45 kcal/kg/day for preterm infants, even in the most acute, ill phase, along with a recommendation for a recovery phase to optimize growth. The recommendations for individual macronutrients have remained relatively constant,

with only the recommended maximum amount of protein intake becoming more accurate.

Table 2. Recommendations for daily parenteral nutrition for preterm infants. The recommendations are based on the guidelines of the European Society for Paediatric and Gastroenterology, Hepatology and Nutrition (ESPGHAN) as presented by Mihatsch et al. (2018) and Koletzko et al. (2005).

RECOMMENDATION	CURRENT (2018)	EARLIER (2005)
ENERGY	45–55 kcal/kg (acute phase) 90–120 kcal/kg (recovery phase)	110–120 kcal/kg
PROTEIN	Min. 1.5 g/kg on day one 2.5–3.5 g/kg on day two onwards Max. 3.5 g/kg	Min. 1.5 g/kg on day one Max. 4 g/kg onwards
LIPIDS	Min 0.25 g/kg Max. 4 g/kg	Min. 0.25 g/kg Max. 3 (3.5–4) g/kg
CARBOHYDRATES	4–8 mg/kg/min on day one 8–10 mg/kg/min on day two onwards, increased gradually Min 4 mg/kg/min, max. 12 mg/kg/min	4–8 mg/kg/min Max. 8.3 mg/kg/min

2.2.3 Parenteral nutrition

Very preterm infants have an energy storage of approximately 200 kcal, and preterm infants have been reported to face a notable energy deficit during the first days of life (Embleton, Pang and Cooke, 2001). A few decades ago, neonatologists were hesitant to provide early parenteral nutrition, due to concerns of potential harm. During that time, the consequences of catabolism during the first days of life were not completely understood. Some quite recent studies have revealed poor growth outcomes associated with early, high-dose parenteral nutrition (Blanco *et al.*, 2012). However, the current scientific evidence emphasizes the importance of an early start to improve the neonatal outcomes (Moyses *et al.*, 2013). The growth outcomes seem to particularly improve with higher early energy intake (Lapointe *et al.*, 2016) and with both enteral and parenteral nutrition (Dinerstein *et al.*, 2006). At the nutrient level, early lipid intake has been found to be associated with better growth (Fischer *et al.*, 2014) and neurodevelopmental outcomes (dit Trolli *et al.*, 2012). Similar results in terms of growth outcomes were also observed with early protein (amino acid) intake (Poindexter *et al.*, 2006).

Continuous glucose infusion is usually prescribed to preterm neonates. However, the order of priority after glucose is still under debate, with varying clinical practices. In earlier studies, protein has been highlighted as the most important nutrient and has often been prioritized over lipids in clinical practice. In an observational study, first-week protein intake has been positively associated with neurodevelopmental outcomes at 18 months of age (Stephens *et al.*, 2009). Moreover, in a retrospective study, protein intake during the first three weeks in preterm individuals has been associated with a higher resting energy expenditure and a more favorable body composition at 20 years of age (Matinolli *et al.*, 2015). Several prospective observational studies have established a connection between high protein intake and growth (Maggio *et al.*, 2007; Cormack and Bloomfield, 2013). However, randomized studies have yielded conflicting evidence (Vlaardingerbroek *et al.*, 2014; Bellagamba *et al.*, 2016), and even harmful results with high protein intake have been published in one randomized trial (Uthaya *et al.*, 2016). A randomized study reported enhanced growth outcomes at two years of corrected age, but no differences in survival or neurodevelopmental outcomes after the early administration of high-dose protein and fat emulsions (Roelants *et al.*, 2016).

2.2.4 Enteral nutrition

Breastmilk (BM), which is the form of enteral feeding, is considered the most important part of nutrition for a preterm newborn. The recommended amount of BM ranges from 135 mL to 200 mL/kg/day (Agostoni *et al.*, 2010). Apart from its nutritional content, BM is an important modulator of the preterm, immature immunological system (Lewis *et al.*, 2017). Starting full enteral feeding at an early stage has been associated with better growth outcomes and lower morbidity rates even in extremely preterm infants (Maas *et al.*, 2017). Human milk feeding is also associated with better cognitive outcomes in preterm infants, according to a systematic review (Lechner and Vohr, 2017).

Gastrointestinal immaturity is typical for preterm infants and is considered a crucial factor in the development of the adverse consequences related to enteral feeding: NEC and feeding intolerance (FI). Some researchers have raised concerns that starting enteral feeding too early or increasing enteral feeding might increase the risk for these complications. According to a recent systematic review, early total enteral feeding is safe for stable VLBW infants (Alshaikh *et al.*, 2019). Moreover, according to a recent randomized controlled trial, there are no differences in survival or neurodevelopmental outcomes with elevated enteral feeding volumes, along with no elevated risk for late-onset sepsis (LOS) or NEC elevated (Dorling *et al.*, 2019).

Although there are diverse nutritional strategies for preventing FI and NEC, none of them has proven effective in adequate clinical trials (Fanaro, 2013). To prevent

NEC, there is a clear preference in the feeding choice. In 1990, a prospective study showed that formula-fed infants are at a higher risk for developing NEC when compared to BM-fed infants (Lucas and Cole, 1990). Vast evidence has shown that MOM helps to prevent NEC and has other beneficial effects, making it the recommended source for enteral nutrition (Maffei and Schanler, 2017). Donor milk is the second-best option, since it helps prevent NEC (Patel and Kim, 2018). However, no other beneficial effects, including better neurodevelopment, have been established. Although donor human milk is recommended over formula, it is quite often poorer in nutritional quality with low levels of protein and energy, and needs to be pasteurized (Arslanoglu *et al.*, 2013). Complete donor milk nutrition may even result in undernourishment. Therefore, it is recommended that milk fortifiers be used to improve the nutritional value of both MOM and donor milk in order to improve infants' short-term growth outcomes (Brown *et al.*, 2020).

2.3 The microbiome and preterm birth

2.3.1 The microbiome

The microbiome is defined as the genome of all microorganisms found in the human body, including bacteria, viruses, archaea and fungi. This study focuses on the bacterial microbiota.

In microbiome research, the microbiota is described by alpha diversity and beta diversity. Alpha diversity describes the diversity of species in a particular ecosystem and is expressed as the number of species ("species richness"), whereas beta diversity describes the diversity of species between two or more ecosystems and is expressed as the total number of species that are unique to each ecosystem being compared (Park, 2007). Furthermore, the microbiota can be described at different taxonomic levels: phylum, class, order, family, and genus. The bacterial nomenclature is dictated by the International Code of Nomenclature of Prokaryotes.

Overall, the current microbiota research is mainly based on 16S rRNA gene sequencing, which was also used in this study. The 16S rRNA is a part of the small subunit of one 70S RNA molecule, and it is considered to be highly conserved between bacterial species, and yet contains variable regions for phylogenetic (bacterial) identification. Therefore, it is usually preferred in genetic identification over other rRNA genes (Fraher, O'Toole and Quigley, 2012).

Preterm microbiota research is an emerging area of study, that currently concentrates mainly on the bacterial microbiota. Notably, in the second phase of the Human Microbiome Project, preterm birth was chosen as one of the three main clinical problems, together with inflammatory bowel disease and prediabetes (Proctor *et al.*, 2019), highlighting its importance.

2.3.2 Microbial etiology of prematurity

A microbial etiology has been suggested as the leading cause for spontaneous preterm birth, even though in a majority of cases, the exact cause remains unknown. Certain microbial organisms have been suggested to cause infection and inflammation, with therefore the cascade terminating at preterm birth (Gibbs *et al.*, 1992). Intrauterine infection may account for 25%–40% of preterm births, and several potential infection routes and foci have been suggested, including chorioamnionitis (Goldenberg, Hauth and Andrews, 2000). A hematogenous inflammatory route has also been suggested (Jefferson, 2012). Furthermore, various genitourinary infections increase the risk for preterm birth, and antibiotic treatment does not lower the risk. Systemic inflammation, such as a high-fever respiratory infection (e.g., influenza), is also considered a risk factor. For a long time, the intrauterine environment was considered sterile, and therefore preterm birth was thought to result from either an invasive infection or systemic inflammation. However, new evidence has shown that the amniotic fluid, placenta, and placental membranes may contain microbes, a notion that challenges the sterile-womb theory (Taddei *et al.*, 2018). In addition, the preterm birth cascade may be a result of microbial imbalance. These findings shifted the focus from studying just pathogenic microorganisms to studying the whole microbiome and its contribution to the etiology of prematurity. One great challenge, however, in studying the microbial etiology is the highly individual microbiota composition varying between healthy individuals. Therefore, creating a uniform microbial profile for preterm birth is considered a challenge. More studies defining healthy and "normal" microbiota are, hence, required to assess the microbial etiology of preterm birth (Chu *et al.*, 2018).

Among the studies concentrating on the microbial etiology of preterm birth, the vaginal microbiome appears to be the most studied microbial community. *Lactobacillus* is considered the most dominant genus in the vaginal microbiota (Mendling, 2016), which may help prevent the invasion of inflammatory bacteria. In the vaginal microbiota, proinflammatory bacterial taxa (Fettweis *et al.*, 2019) and higher bacterial richness and diversity (Freitas *et al.*, 2018) have been suggested to be risk factors for spontaneous preterm delivery. However, the available evidence is conflicting. For example, Romero et al. found no differences in the vaginal microbiota in mothers who have experienced preterm and term deliveries (Romero *et al.*, 2014). According to a systematic review, there is a great heterogeneity among studies on this topic, and molecular-based, culture-independent studies are required in this area of research (Peelen *et al.*, 2019).

Other possible maternal bacterial communities have also been studied. For example, some researchers have studied the cervix, cervical dysfunction in particular, and found no clear microbial environment (Vinturache *et al.*, 2016). Placental microbiota has been a topic of debate, and there is emerging evidence of a

bacterial community (Mysorekar and Cao, 2014; Taddei *et al.*, 2018) that may be associated with adverse pregnancy outcomes (Prince *et al.*, 2014). Periodontal diseases and oral microbes (Goldenberg and Culhane, 2006) have also been linked to preterm birth, suggesting a hematogenous transmission route of microbes or a systemic inflammatory response.

The gut microbiota composition changes during pregnancy, during which a shift toward increased diversity, decreased richness, and a higher abundance of *Proteobacteria* and *Actinobacteria* is observed (Koren *et al.*, 2013). Currently, however, there is little evidence regarding the link between maternal gut microbiota and the risk of preterm birth (Vinturache *et al.*, 2016). In a study by Shiozaki et al., mothers experiencing premature delivery were found to harbor a different gut microbiota composition, with a high abundance of *Clostridium* genus and a low abundance of *Lactobacillus* (Shiozaki *et al.*, 2014). Using a logistic regression model, Dahl et al. showed that mothers experiencing a preterm delivery exhibit reduced alpha diversity and a lower abundance of *Bifidobacterium* and *Streptococcus* compared to mothers with term pregnancies (Dahl *et al.*, 2017). Overall, research into the gut microbiota has faced the same challenges as in microbiota research as a whole. This is because the gut microbiota composition is highly individual and is affected by several factors, such as the body mass index (BMI), medications, ethnicity, and lifestyle. However, no clear scheme for how systemic inflammation, local infections, and possible transmission routes are connected to the onset of preterm labor has yet been established. Therefore, identifying a pathogen or specific microbiota composition with a high risk for preterm birth would offer a new target for preventing preterm deliveries.

2.3.3 Neonatal gut microbiota

2.3.3.1 Colonization process

The colonization process of a vaginally delivered term infant is characterized by different phases. In the first phase, the infant is colonized with microbes found in the birth canal, skin, and gut of the mother, as well as in the maternal BM. The first stable gut microbiota of a term infant is usually composed of maternal vaginal and enteric bacteria. Subsequently, the number of facultative bacteria and anaerobes starts to increase in the distal gastrointestinal tract. Term infants delivered by caesarean section exhibit an initial gut microbiota that resembles the skin microbiota of the mother, and the development of a more diverse gut microbiota is often delayed. The gut microbiota acquires the characteristics of an adult microbiota at nearly three years of age (Groer *et al.*, 2014).

The gut colonization process of a full-term, vaginally delivered, exclusively breastfed (FTVDBF) infant, without antibiotic exposure and with normal growth is considered the gold standard of gut microbiota development. Preterm infants often differ from the FTVDBF infants in many characteristics, as they may be delivered by cesarean section, are SGA, are often exposed to antibiotics, and have delayed breastfeeding and skin-to-skin contact. Prematurity affects the maturation of the intestinal tract and the immune system, contributing also to the colonization process. Prematurity itself independently affects the gut microbiota composition (Forsgren *et al.*, 2017). Hence, studying the early colonization patterns in preterm infants requires an adequate understanding of the potential confounding factors. Therefore, it is important to focus on early stool samples, in which the effect of the potential confounding factors is less significant.

For decades, it was believed that the colonization process starts in the birth canal, where the neonate is exposed to maternal bacteria. However, according to recent evidence, the colonization process may already commence during pregnancy. Collado et al. suggested that the human gut microbiota may start to develop *in utero,* as shared microbial features were found in the amniotic fluid, placenta, and meconium, suggesting a maternal-to-fetal transmission (Collado *et al.*, 2016). Similarities in the microbiota of the amniotic fluid and meconium have also been reported in another study on preterm infants (Ardissone *et al.*, 2014). These hypotheses, as well as the intrauterine microbial communities, are currently the focus of scientific research and debate. However, no clear consensus regarding whether the placenta harbors a microbial community has yet been established. It is possible that the microbes found in the thought-to-be-sterile environments are mere contamination. Most studies reporting the existence of intrauterine or meconium microbiota are based on the detection and identification of microbial DNA, which does not necessarily reflect the presence of live bacteria.

Studying the *in utero* environment in humans is challenging, since sterility is often breached during sample collection. Moreover, the study sizes are typically small in microbial studies, affecting the generalizability of the results. Acquiring an intrauterine stool sample from a prior birth is likely impossible. On the other hand, recent animal experiments have revealed bacterial DNA, resembling placental DNA, in murine fetal intestines (Martinez *et al.*, 2018) and low-abundance bacteria from rectal and mucosal bovine samples directly after birth (Alipour *et al.*, 2018). Shared microbial profiles between uterine, placental, and meconium samples have also been observed in sterile caesarean-section deliveries (N. Younge *et al.*, 2019). This topic, however, remains an open question and is considered a research priority.

2.3.3.2 Preterm gut microbiota composition

Preterm gut microbiota is characterized by a decreased diversity, a high abundance of *Gammaproteobacteria* and *Firmicutes*, and a low abundance of common commensal microbes (Underwood and Sohn, 2017). As shown in Table 3, some specific bacteria are typical in preterm infants. A high variation in microbiota composition between individuals has been suggested in numerous studies (Barrett *et al.*, 2013; Patel *et al.*, 2016; Wandro *et al.*, 2018), suggesting an individual-specific, heterogenous composition. The colonization process of *Gammaproteobacteria*, a class of bacteria with known proinflammatory properties, has also been suggested to be dichotomous, as some preterm infants have a high abundance of *Gammaproteobacteria* in early in life, and some have a low abundance that increases over time (Ho *et al.*, 2018). ELBW neonates have been reported to exhibit a low diversity microbiota with a high abundance of facultative anaerobes, and a low abundance of beneficial bacteria, with the greatest alterations in the gut microbiota composition occurring during the first weeks of life (Drell *et al.*, 2014). Another study showed that VLBW infants with IUGR exhibit differences in the early gut microbiota composition compared to AGA infants, mainly in terms of the relative abundance of specific taxa (Li *et al.*, 2019).

In another study, Arboleya et al. found an increased abundance of facultative anaerobic microbes and a reduced abundance of *Bifidobacterium, Bacteroides*, and *Atopobium* in a preterm cohort (Arboleya *et al.*, 2012) compared to term infants,. In a 24-week follow-up, spontaneously born full-term infants were found to maintain a relatively stable gut microbiota, whereas the preterm infant gut microbiota was found to change and its diversity improved (Hill *et al.*, 2017). In another follow-up study by the same research group, these perinatal factors were found to influence the microbiota until up to four years of age, and the preterm infant gut microbiota was found to be characterized by *Lactobacillus, Streptococcus*, and *Carnobacterium* (Fouhy *et al.*, 2019).

Table 3. Key bacterial taxa in the preterm infant microbiota. Modified from Underwood and Sohn, 2017.

PHYLUM	CLASS	ORDER	FAMILY	GENUS
FIRMICUTES	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*
		Lactobacillales	Streptococcaceae	*Streptococcus*
			Enterococcaceae	*Enterococcus*
			Lactobacillaceae	*Lactobacillus*
	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*
	Negativicutes	Selenomonadales	Veillonellaceae	*Veillonella*
	Mollicutes	Mycoplasmatales	Mycoplasmataceae	*Ureaplasma*
PROTEO-BACTERIA	Gammaproteo-bacteria	Enterobacteriales	Enterobacteriaceae	*Klebsiella*
				Escherichia
				Proteus
				Serratia
				Enterobacter
				Cronobacter
		Pseudo-monadales	Pseudo-monadaceae	*Pseudomonas*
			Moraxellaceae	*Acinetobacter*
BACTEROIDETES	Bacteroidetes	Bacteroidales	Bacteroidaceae	*Bacteroides*
ACTINOBACTERIA	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*
		Propioni-bacteriales	Propioni-bacteriaceae	*Propioni-bacterium*

2.3.4 Modulators of the early gut microbiota

In term infants, delivery mode and feeding have been established as the most important modulators of the gut microbiota composition. In contrast, in preterm infants, gestational age has been suggested to be the most important factor modulating the preterm gut microbiota (Ficara *et al.*, 2020). In an observational study, a negative correlation was observed between inflammatory bacteria and gestational age (Ardissone *et al.*, 2014). La Rosa et al. showed that preterm gut colonization is mostly affected by gestational age, in which the process was the most delayed by prematurity (La Rosa *et al.*, 2014). Korpela et al. reported similar results, showing that gestational age is the main driver of preterm colonization (Korpela *et al.*, 2018).

In term infants, mother-to-infant microbial transmission plays a significant role in neonatal gut colonization (Ferretti *et al.*, 2018). However, there is little evidence regarding the contribution of the maternal gut microbiota to the gut composition of preterm neonates. According to a recent study, in preterm infants born after 28 GW, there is a positive correlation between the abundance of *Firmicutes* and *Bacteroides* in the gut and their abundance in the maternal gut composition. No correlation was

found in infants born before 28 GW and the mode of delivery. This suggests that gut bacterial transmission increases after 28 GW (Morais *et al.*, 2020).

Many studies have highlighted the profound influence of the mode of delivery on term neonatal gut microbiota, with reduced diversity and delayed colonization in infants delivered by caesarean section (Ficara *et al.*, 2020). In preterm infants, evidence suggests that the mode of delivery may have a limited effect. In a study performed in 2015, a high abundance of *Bacteroidetes* was observed in vaginally delivered preterm infants (Gregory *et al.*, 2015). Similar results were reported in other studies, and a high abundance of *Bacteroides* was observed in vaginally delivered infants (Arboleya *et al.*, 2015). Ardissone et al. reported a high abundance of *Firmicutes* in preterm infants delivered by caesarean section (Ardissone *et al.*, 2014). In a study by Korpela et al., mode of delivery had a minor effect on gut microbiota (Korpela *et al.*, 2018). In another study by Dahl et al., no effect on preterm gut microbiota by delivery mod was reported (Dahl *et al.*, 2018). This suggests that the mode of delivery may affect the abundance of individual species in preterm infants but does not have the same profound effect on the overall composition and diversity as in term infants.

Antibiotics are commonly prescribed to the mother as an intrapartum prophylaxis and/or to the preterm neonate during the first days of life. Several studies have established that antibiotic treatment lowers the gut microbial diversity. Early antibiotic exposure may also favor proinflammatory bacteria, such as those belonging to the genus *Enterobacter* (Greenwood *et al.*, 2014). It is also worth noting that the duration of antibiotic treatment is linked to a decrease in the richness of the gut microbiota (Rooney *et al.*, 2020). Both intrapartum and early neonatal antibiotics have been reported to have a profound effect on the gut microbiota of VLBW infants, with a reduced abundance of *Bacteroidaceae* in preterm infants compared to term infants (Arboleya *et al.*, 2015). Moreover, some studies have highlighted that a longer antibiotic treatment duration seems to perturb the microbial gut colonization and diversity, although the gut microbiota seems to recover later (Dardas *et al.*, 2014) (Zwittink *et al.*, 2018).

BM, especially MOM, has its own microbiota, which may contribute to the gut colonization in term infants (Pannaraj *et al.*, 2017). In preterm infants, however, the effect is still unknown. Although fortifiers are routinely added to BM, the effect of fortification on the BM or neonatal gut microbiota composition is still unknown. According to a systematic review, MOM is the best option for the preterm gut microbiota community (Xu *et al.*, 2018). MOM-fed infants have been reported to have increased gut microbiota diversity and increased abundance of *Bifidobacterium* and *Bacteroidetes*, whereas donor-milk-fed infants have been reported to have an increased abundance of *Staphylococcus* (Ford *et al.*, 2019). Similar results with gut microbiota diversity and an increased abundance of *Clostridiales* and

Lactobacillales in MOM-fed infants have been reported (Cong *et al.*, 2016) . In a preterm cohort, it was found that the BM microbiota changes over time and that the gut microbiota of preterm infants starts to resemble an FTVDBF gut microbiota after breastfeeding is commenced (Biagi *et al.*, 2018). Formula-fed infants were also found to exhibit decreased gut microbial diversity when compared to MOM-fed infants (Cai *et al.*, 2019), even if they were partially formula-fed (Zanella *et al.*, 2019). In a randomized controlled trial with a group of moderately preterm infants, infants receiving enhanced nutrition supply, both parenteral and enteral, were found to have increased gut microbial diversity, which is associated with better growth outcomes (Blakstad *et al.*, 2019). Notably, donor BM needs to be pasteurized, which eradicates the bacteria and may decrease the beneficial microbial effects of BM.

Evidence shows that the abundance of pathogenic bacteria increases in preterm infants during their neonatal intensive care unit (NICU) stay (Patel *et al.*, 2016). Infants staying for a longer duration in the NICU were found to have a gut microbiota composed of *Enterobacteriaceae* and *Enterococcaceae* (Patel *et al.*, 2016; Stewart *et al.*, 2016), which often resembles the NICU environment, and several possible explanations have been suggested in this regard (Henderickx *et al.*, 2019). In a follow-up study on two-year-old preterm-born children, the gut microbiota of these children was found to resemble a healthy adult's gut microbiota, with higher diversity and lower individual variance. It was also found that the hospital-associated bacteria were replaced (Gómez *et al.*, 2017). Rozé et al. characterized different clusters according to the gut microbiota composition at four weeks of age in preterm infants, and they connected these clusters to NICU treatments and two-year outcomes, suggesting that the microbiota is a noninvasive marker of immaturity (Rozé *et al.*, 2020).

2.3.5 Infant gut microbiota and health

Research has extensively focused on the health consequences of gut microbiota perturbations. In term infants, certain gut microbial features have been linked to long-term health consequences, such as allergic diseases (Kalliomäki and Isolauri, 2003), asthma (Van Nimwegen *et al.*, 2011), obesity (Kalliomaki *et al.*, 2008b), and inflammatory bowel disease (Dominguez-Bello *et al.*, 2019). In preterm infants, however, the understanding of the health consequences of the gut microbiota is still very limited. Studies on preterm gut microbiota and health focus mainly on the development of NEC and LOS, in which disturbed gut microbiota is considered a risk factor together with an immature gastrointestinal tract. However, no specific NEC-related bacterial species or microbial features have yet been established (Groer *et al.*, 2015; Stewart *et al.*, 2016). Gut colonization and maturation also contribute to

the immunological development of infants, and perturbation in the gut microbiota with antibiotics is a known risk factor for both NEC and LOS.

Growth is an important indicator of preterm infants' survival and health. Blanton et al. suggested a link between immature gut microbiota and growth disturbances in children (Blanton *et al.*, 2016), stimulating a discussion regarding a possible connection between the gut microbiota and growth. Younge et al. reported delayed gut microbiota maturation among extremely preterm infants with poor postnatal growth (N. E. Younge *et al.*, 2019). In another study, Grier et al. suggested three different phases for gut microbial development and growth, which may provide a basis for targeted nutritional management (Grier *et al.*, 2017). Another two studies, with one-month (Arboleya *et al.*, 2017) and NICU discharge follow-up (Yee *et al.*, 2019), revealed a link between a specific intestinal microbial composition and growth outcomes. However, despite these studies, extensive evidence regarding preterm gut microbiota and growth outcomes is still lacking. In addition, other possible modulators of preterm gut microbiota and their association with growth outcomes remain unknown.

Currently, several possible associations and causal connections between preterm gut microbiota and later health may be hypothesized on the basis of data from studies performed on term infants. This, however, may be suboptimal, since preterm infants follow different colonization patterns than those of term infants. Some researchers have linked a specific infant gut microbiota composition to obesity later in life (Kalliomaki *et al.*, 2008a; Vael *et al.*, 2011; Dogra *et al.*, 2015). Maternal obesity is a major risk factor for later adverse metabolic health in term infants, and disrupted maternal gut microbiota has been suggested to be a risk factor for childhood obesity (Gohir, Ratcliffe and Sloboda, 2015). Although the contribution of maternal gut microbiota to initial gut microbiota establishment in term infants is currently quite known, an understanding of the contribution of maternal gut microbiota to preterm gut colonization and health outcomes is still lacking.

3 Aims

The aim of this study is to understand the relationship between early exposures and growth in preterm infants. Particularly, it focuses on early nutritional management and the early gut microbiota composition. The first objective was to study whether the early nutritional management influenced long-term growth outcomes in preterm infants. Furthermore, the early preterm gut microbiota composition was investigated and compared according to gestational age between preterm and term infants. Next, the possible perinatal causes modulating the initial preterm gut microbiota composition were studied. Finally, the effects of an FMT from extremely preterm neonates on growth outcomes and metabolism in an experimental animal model were investigated.

The specific goals of this study were as follows:

1. To investigate whether the nutritional management during the first week of life is associated with growth outcomes until two years of age in extremely preterm infants (I);
2. To study whether there are differences in the initial preterm gut microbiota composition (II, III) and immunological profiles among preterm and full-term neonates (III);
3. To assess the possible perinatal factors contributing to the initial gut colonization process in preterm neonates (II);
4. To examine whether an FMT affects the growth outcomes or metabolism in an experimental mouse model (III).

4 Materials and Methods

4.1 Subjects and study design

This study is based on three independent clinical studies of newborns treated at the Department of Paediatrics and Adolescent Medicine, Turku University Hospital, between 2004 and 2018. A flowchart depicting the subject recruitment process followed in these studies is shown in Figure 1.

The first (I) cohort originally comprised all extremely preterm infants (less than 28 GW) treated at the NICU of Turku University Hospital between 2004 and 2012. Among the primary study sample (n=155), outborns (n=7) were excluded. The exclusion criteria were infants with severe congenital malformations (n=7), infants who died before the age of two years of age (n=30), infants with incomplete medical data (n=9), and infants who were followed up for growth and development at another hospital (n=24). This resulted in a final study sample comprising 78 preterm infants. The study was retrospective in nature, and its aim was to investigate, whether nutritional management during the first seven days of life affects the growth patterns until the corrected age of two years. The hypothesis was that early nutrition has long-term effects on the growth outcomes.

The second (II) study comprised 79 preterm infants born before 35 GW and their mothers, who were admitted to the Turku University Hospital between the years 2014 and 2018. We recruited both the mothers (n=65) and their infants (n=79) after delivery. The study subjects were originally recruited to a probiotic intervention study, but the baseline samples not affected by the intervention were used in this study. The inclusion criteria included a duration of pregnancy less than 35 GW and infant age less than three days. Infants with severe congenital anomalies or asphyxia were not recruited. Among the primary sample, B-twins (n=14) were excluded to ensure the independence of the study subjects. In addition, infants (n=10) and mothers (n=14) without adequate biological samples were excluded, resulting in a total of 55 infants and 51 mothers. Moreover, 25 fecal samples from spontaneously born term newborns were collected for comparison. The study was prospective in nature, and its aim was to investigate whether perinatal exposures or the maternal gut microbiota affects the early gut microbial composition of infants. Stool samples were collected from preterm neonates and their mothers at the latest three days after

delivery, and from term infants at the latest four days postpartum. The hypothesis was that perinatal exposures affect the early gut colonization in preterm infants.

The third (III) study comprised 23 infants born between 2013 and 2014. We recruited 44 pregnant women with a duration of pregnancy ranging between $22^{0/7}$ and $34^{6/7}$ GW, who were hospitalized for symptoms or signs suggesting a risk of spontaneous preterm delivery. These symptoms included premature contractions, cervical changes, and PPROM. Eligible women were approached, and 43 provided written and oral informed consent to participate in the study. Because the inclusion criteria included the risk of preterm delivery, all mothers received antenatal corticosteroid treatment. Ultimately, eight women delivered very preterm (VPT, before 32 GW), 15 women delivered moderately preterm (PT, 32–36 GW), and 16 women delivered at full-term (FT, after 37 GW), with five women lost to follow-up. Adequate samples were collected from a total of 23 infants (5 VPT, 7 PT, and 11 FT) and included in the study. The study was prospective in nature, and its aim was to characterize the differences in the meconium microbiota and markers of neonatal immune responsiveness in neonates by collecting meconium stool and umbilical cord blood samples directly after birth. The hypothesis was that the meconium microbiota composition and cord blood CD4+ cell immune responses are distinct in these three groups. Moreover, with an experimental mouse model, we also used an FMT with meconium samples to investigate the causal contribution of the meconium microbiota to the inflammatory immune state, growth failure, and metabolic disturbances often encountered in very preterm neonates. We collected serial stool samples, terminal ileum biopsies and blood samples from the experimental animals at the end of a five-week follow-up period. The hypothesis was that there are differences in the growth, inflammation, and metabolism patterns in the experimental mouse model groups with respect to the duration of gestation of the FMT donors.

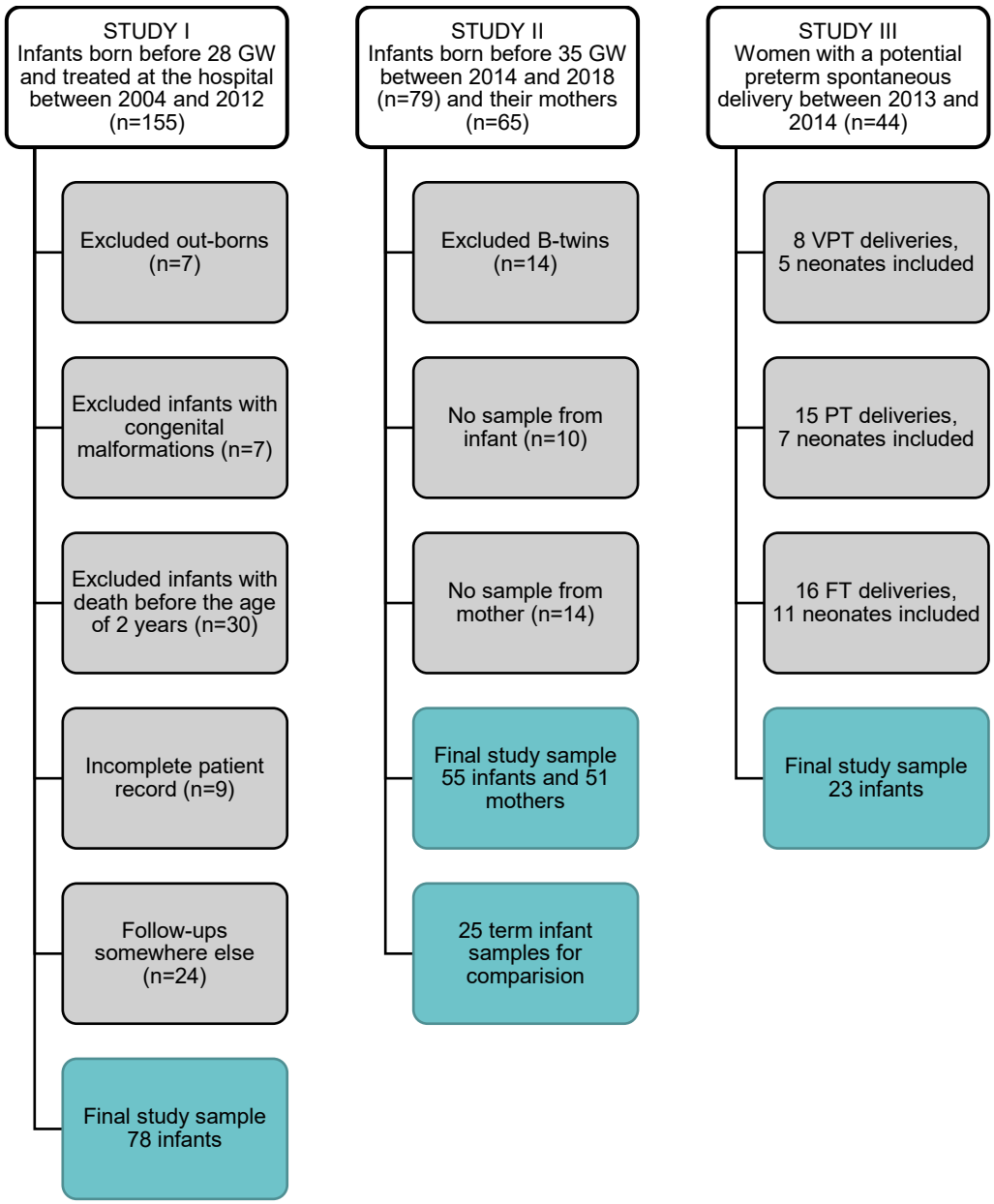


Figure 1. A flowchart of the recruiting process of the three study cohorts. (GW = gestational weeks, VPT = very preterm, PT = moderately preterm, FT = full-term)

4.2 Data and sample collection

4.2.1 Patient data (I, II, and III)

Patient data were collected from the electronic patient records at Turku University Hospital. For all studies, relevant characteristic data were defined as gestational age, sex, birth weight, birth weight Z-score (according to a recalculation by Sankilampi et al. (Sankilampi *et al.*, 2013) or an automatic calculation from the patient records), mode of delivery, and the diagnosis of SGA. For studies I and III, a threshold of birthweight of less than –2.0 SD was used, whereas for Study II, the least 10th percentile was used.

Among the cohort in Study I, detailed growth measures, including weight, height, and head circumference, with Z-scores at birth, at the term-equivalent age (TEA), and at 12 and 24 months of corrected age, were collected. The starting day of enteral nutrition and the total duration of parenteral nutrition were recorded. Moreover, umbilical cord blood pH and Apgar scores at the time of birth were collected. In addition, the diagnoses for relevant neonatal morbidities (RDS, pneumothorax, PDA, NEC, early-onset culture-positive sepsis or meningitis, LOS, bacterial infection, fungal infection, cystic PVL, BPD grades I–IV, IVH grades I–IV, and ROP grades I–IV) were collected. The duration of respiratory treatment and the need for additional oxygen at the age of 28 days and 36 GW were obtained, and the use of maternal antenatal corticosteroids and neonatal corticosteroids, indomethacin, or ibuprofen and the need for surgery were recorded. We adopted the variables that were clinically relevant and sufficiently prevalent among the population in our final statistical model. The tested variables included gestational age, the diagnosis of SGA and IVH, and the use of neonatal corticosteroids. We recalculated the Z-scores for weight, height, and head circumference were recalculated at birth and the TEA using the reference data from Sankilampi et al. (Sankilampi *et al.*, 2013) and at the corrected ages of 12 and 24 months using the reference data from Saari et al. (Saari *et al.*, 2011).

Study II offers a detailed description of the cause of prematurity (spontaneous, PPROM, or iatrogenic). It also provides detailed information on intrapartum and neonatal antibiotic treatment, growth outcomes at the TEA, and the diagnosis of neonatal morbidities (RDS, BPD, PDA, NEC, ROP, IVH, PVL, convulsions, and early or late infections), the use of indomethacin, the need for surgery, maternal BMI, maternal chorioamnionitis or fever, the initial day of enteral feeding, the initial day of MOM feeding, the use of BM fortifiers, the duration of parenteral nutrition, the administration of a central intravenous catheter, respiratory treatment, and blood transfusions were collected. Similar data for term neonates were also collected.

According to the literature, we selected the most relevant variables (mode of delivery, gestational age and cause of prematurity) for a microbiota analysis.

Study III, besides the basic characteristics, focused on collecting early neonatal and maternal data, including the maternal prepregnancy BMI, antibiotic treatment of the mother and infant, whether the mother had PPROM, the Apgar score, and umbilical cord blood pH. All the infants selected for this study were born spontaneously.

4.2.2 Nutritional data (I)

In Study I, nutritional data were collected from patient documents and electronic patient records. Generally, nutritional management in the NICU of Turku University Hospital is planned in 24-hour periods beginning at 2 PM. Therefore, nutritional data were collected from the first incomplete day with the corresponding hours and then from the following seven complete 24-hour periods. The individual nutrient composition of human milk was not assessed, and estimated concentrations for proteins (1.5 g/100 mL), lipids (2.6 g/100 mL), and carbohydrates (6.2 mg/100 mL) were used for calculations regarding human milk, as recommended by Cormack et al. (Cormarck *et al.*, 2016). These calculations yielded as an estimated energy content of 53 kcal/100 mL.

Daily macronutrient data were converted into grams per kilogram per day using the known composition of each nutritional product and the infant's birth weight. For parenteral nutrition, the energy content (parenteral proteins 4 kcal/g, lipids 10 kcal/g, and carbohydrates 3.4 kcal/g) was calculated for each nutrient as recently recommended by Cormack et al. (Cormarck *et al.*, 2016). Moreover, the total amount of energy was calculated for parenteral nutrition by the macronutrients and from the enteral nutrition by estimating 65 kcal/100 mL for human milk.

4.2.3 Biological samples (II, III)

4.2.3.1 Stool samples (II, III)

In Study II, the first stool sample was collected after birth, at the latest three days postpartum for preterm and at the latest four days postpartum for term infants. For some of the neonates in Study II, this sample was not the actual first stool sample, because some neonates had defecated before enrollment. The sample collection procedure was conducted with the same protocol for every neonate. Maternal stool samples were collected within three days postpartum. All samples were frozen immediately after collection and kept at –80°C until analysis. In Study III, meconium

samples were collected from 20 subjects at 1–3 days after birth, at the latest five days postpartum.

4.2.3.2 Umbilical cord blood samples (III)

Umbilical blood samples were collected directly after birth from 14 neonates in the study (2 VPT, 4 PT, and 8 FT), and CD4+ T cells were isolated. First, mononuclear cells were enriched using a Ficoll-Paque gradient centrifuge (GE Healthcare Biosciences AB) followed by CD4+ T cell isolation using positive magnetic separation (Dynal, Invitrogen, MA, USA). Then, the cells were snap-frozen in liquid nitrogen and stored at –80°C before being processed for gene expression and DNA methylation analysis.

4.2.3.3 Murine samples (III)

GF Swiss Webster female mice (8–10 weeks old) were used for the animal experiment. FMT defined the first day of the experiment. The mice were initially weighed on day 0 and then serially weighed during the 35-day experiment. Mouse stool samples were collected on days 7 and 35 of the experiment, and terminal ileum specimens were collected after sacrificing the mice by CO_2 inhalation on day 35 of the experiment. Then, a 1-cm section of the terminal ileum was immediately removed using sterile scissors, placed in a tube containing RNAlater, and stored at –80°C. Blood samples were obtained from the heart using thoracotomy on day 35, and blood was separated by centrifugation and the plasma was stored at –80°C.

4.3 Methods

4.3.1 Sample preparation

4.3.1.1 DNA extraction and sequencing of human stool samples (II, III)

In cohort II, total DNA was extracted from the neonatal and maternal stool samples as previously described (Nylund *et al.*, 2010). Briefly, 100–125 mg of feces was weighed and homogenized in the presence of a lysis buffer via bead beating with FastPrep-24 (MP Biomedicals, Irvine, CA, USA). DNA was then extracted with the commercial kit InviMag Stool DNA Kit (STRATEC Molecular, Berlin, Germany) using an automated KingFisher DNA system (Thermo Fisher Scientific Oy, Vantaa, Finland). For this system, the protocol steps included nucleic acid binding on magnetic beads, five-step washing, and elution. Then, the total DNA concentration

was measured using a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and normalized. A specific 16S rRNA gene region (V3-V4 region) was amplified following the 16S rDNA gene Metagenomic Sequencing Library Preparation (Illumina, CA, USA) protocol (Cod. 15044223 Rev. A). Then, after 16S rDNA gene amplification, a multiplexing step was performed using a Nextera XT Index Kit (FC-131-2001). Then, 1 μL of the PCR product was run on a DNA 1000 Bioanalyzer chip to verify the size, with an expected size on a bioanalyzer trace of approximately 550 bp. Next, the libraries were sequenced using a 2 x 300 bp paired-end run (MiSeq Reagent Kit v3) on an Illumina MiSeq platform according as per the manufacturer's instructions.

For the meconium samples in the cohort III, DNA was extracted together with appropriate positive and negative controls using a PureLink Microbiome DNA Purification Kit (Invitrogen). Then, the V4 region of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the 515F (AATGATACGGCGACCACCGAGATCTACACGCT) barcoded and 806R (TATGGTAATTGTGTGYCAGCMGCCGCGGTAA) primers. Next, PCR was performed, including 35 cycles of denaturation (95°C), annealing (55°C), and extension (72°C), with a final elongation step at 72°C. The PCR products were then purified using AMPure magnetic beads (Beckman Coulter, CA, USA) and quantified using a Quant-iT PicoGreen dsDNA quantitation kit (Invitrogen). Next, samples (30 ng), were loaded on 2% agarose E-Gel (Thermo Fisher Scientific, MA, USA), purified, and sequenced using an Illumina MiSeq platform (Genomics Center, Azrieli Faculty of Medicine, Bar-Ilan University, Safed, Israel). A meconium sample from one subject was excluded from the analyses because the isolated DNA was not PCR-amplifiable. The analyses were, therefore, performed on samples from five VPT, seven PT, and seven FT neonates (n=19).

4.3.1.2 RNA/DNA isolation and sequencing of human umbilical cord blood samples (III)

Total RNA and genomic DNA were isolated from CD4+ T cells using an AllPrep DNA/RNA Micro Kit (Qiagen Inc.) and stored at –80°C until used. The quality of the isolated RNA was verified using Nano RNA chips in a 2100 Bioanalyzer (Agilent, CA, USA). Library construction was performed using TruSeq RNA Library Prep Kit v2 as per the manufacturer's instructions, and sequencing was performed at the Finnish Molecular Sequencing Center (Turku, Finland) using a HiSeq 2500 with single-end reads of length 50 bp. The quality of the isolated DNA was then verified using a DNA 1000 kit in a 2100 Bioanalyzer (Agilent), and 2 μg of DNA was used as input for reduced representation bisulfite sequencing, as described previously (Smith *et al.*, 2010). Briefly, DNA was digested with an MspI

restriction enzyme to cut the DNA at CCGG sites independent of the methylation status. Next, end repair and ligation of adapters for sequencing were performed, followed by gel-based selection of DNA fragment sizes between 40 and 220 bp. Finally, single-end sequencing of purified libraries with a 50 bp read length was performed at the Finnish Molecular Sequencing Center using a HiSeq 2500 (Illumina).

4.3.1.3 RNA extraction of murine ileum samples (III)

Total RNA was extracted from tissues using a Total RNA Purification Kit (Norgen Biotek Corp., Canada). In brief, terminal ileum tissue samples were homogenized in 2 mL of a cold RL buffer (Norgen Biotek Corp.) using a Bio-Gen PRO200 tissue homogenizer (PRO Scientific, CT, USA). The homogenates were then centrifuged, and the supernatant was purified as per the manufacturer's protocol (Total RNA Purification Kit; Norgen Biotek Corp.). Next, the RNA quantity was determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and then reverse-transcribed using 5x All-in-One RT MasterMix (Applied Biological Materials, Canada). In brief, 200 µg of total RNA was added into a 10 µL reaction volume, and then the mixture was incubated at 25°C for 10 minutes, followed by synthesis at 42°C for 15 minutes, and a termination reaction at 85°C for 5 minutes. Next, 190 µL of nuclease-free water was added, and cDNA was stored at –80°C until needed for real-time PCR. Real-time PCR amplification was performed with cDNA in a reaction containing with a final concentration of 0.1 µM for each primer and 5 µL of Fast SYBR Green Master Mix (Applied Biosystems) with 80 ng of cDNA in 10 µL total volume. The reactions were run on a ViiA™ 7 Real-Time PCR System (Applied Biosystems, MA, USA) with the following cycle conditions: 95°C for 20 minutes, 40 cycles at 95°C for 1 minute, and 60°C for 20 minutes.

4.3.2 Fecal microbiota transplant experiment (III)

First, the GF status of mice was verified using 16S rRNA PCR on fecal samples, as described above for human meconium stool samples in Study II. Meconium, FMTs from neonates were introduced to 8–10-week-old GF Swiss Webster female mice, defining day 0 of the experiment. Each meconium sample was suspended in 800 µL of sterile phosphate-buffered saline and dissociated by vortexing for 1 minute. Then, 200 µL of the suspension from each meconium sample was used as oral gavage for one GF mouse. A total of 18 meconium specimens were transferred to 18 mice divided into three groups: VPT (n=4), PT (n=6) and FT (n=8). The mice were housed in isolated cages and maintained on a 12-hour light/dark cycle. All mice were handled uniformly and fed with the same diet (Maintenance Diet 1324; Altromin

International, Germany). DNA was then extracted from stool samples using a MO BIO PowerSoil DNA extraction kit (MO BIO, CA, USA). The remaining analysis steps were performed in a manner similar to that used for the human meconium stool samples. The weight of each mouse was monitored during the experiment, and the weight fold change was calculated in the mice as a measure of growth after FMT.

4.3.3 Bioinformatics and statistical analysis

4.3.3.1 Nutritional multivariate model (I)

In Study I, the patient characteristic and nutritional numerical data were described as means and ranges, whereas categorical data were described as counts and percentages. The mean changes in weight, height, and head circumference over two years (absolute values expressed as absolute Z-scores) were analyzed using a hierarchical linear mixed model for repeated measures (HLMM). Moreover, the Z-scores were calculated using the reference values provided by Sankilampi et al. at birth and the TEA (Sankilampi *et al.*, 2013), and by Saari et al. for the corrected ages of 12 and 24 months (Saari *et al.*, 2011). Both the Kenward-Roger correction was and compound symmetry covariance structure were used. In addition, potential confounding factors (gestational age, diagnoses of SGA and IVH, and corticosteroid treatment) were tested, and statistically significant ones were selected for the final model (gestational age, SGA, and IVH). The explanatory variables included in the model were the energy intake (kcal/kg/day) during the first seven days of life, gestational age, SGA, IVH, and age (at birth as the baseline, at the TEA, and at the corrected ages of 12 and 24 months). In addition, the interactions between these explanatory variables and age were evaluated in the model to study whether the mean change differs depending on the explanatory variable's value. Pearson's correlation (r) was calculated between the energy intake and growth factors separately for each age. A similar model was developed in which the energy intake was replaced by protein, lipid, and carbohydrate intake. A p-value less than 0.05 was considered statistically significant (two-tailed). All statistical analyses were performed using SAS software (version 9.3 for Windows; SAS Institute Inc, Cary, NC, USA).

4.3.3.2 Microbiota analysis (II, III)

In Study II, raw sequences were analyzed using the QIIME2 (version 2019-7) pipeline (Bolyen *et al.*, 2019). Data were imported using the Phred33 importing tool for paired-end data and then quality-filtered using the Divisive Amplicon Denoising Algorithm 2 (DADA2) method (Callahan *et al.*, 2016). The taxonomy was developed using the Greengenes v. 13.8 database and a 99% amplicon sequence variant

taxonomic classifier, creating a phylogenetic tree. Alpha diversity was assessed using of Faith's phylogenetic diversity (Faith, 1992), in addition, alpha diversity evenness and Shannon index (Shannon, Weaver and others, 1949) were used. Beta diversity was assessed using the Bray–Curtis (Sorenson, 1948) and unweighted UniFrac (Lozupone and Knight, 2005) distance matrices. Both analysis of composition of microbiomes (ANCOM) (Mandal *et al.*, 2015) and Linear discriminant analysis Effect Size (LEfSe) (Segata *et al.*, 2011) were used to study the differences in the taxonomical abundance between the groups. Moreover, group comparison was performed with either analysis of variance (ANOVA) or permutational multivariate analysis of variance (PERMANOVA). Statistical significance was determined as a corrected p-value of less than 0.05. Calypso software (version 8.24, http://cgenome.net/calypso/) was used, along with total sum normalization for the statistical analysis. In addition, the contribution of the maternal gut microbiota to the neonatal gut microbiota was assessed with SourceTracker using QIIME version 1.9 (Knights *et al.*, 2011).

In Study III, data analysis was also performed using QIIME2. Sequence reads were demultiplexed by per-sample barcodes and Illumina-sequenced amplicon read errors were corrected using the DADA2 method. A phylogenetic tree was generated. Alpha diversity was calculated using Faith's phylogenetic diversity, and beta diversity was analyzed using UniFrac. principal coordinate analysis (PCoA) was performed on the basis of unweighted UniFrac distance matrices. Finally, LEfSe was performed to identify the features that significantly differ between samples according to the relative abundance.

4.3.3.3 Maternal and perinatal factor contribution (II)

The contribution of the maternal gut microbiota to the preterm gut microbiota was selected as a primary response variable. The relationships between the percentage of microbes and continuous variables (gestational age, birth weight Z-score, and maternal and neonatal alpha diversity assessed using Shannon index) were examined using Spearman's rank correlation because of the nonnormal distributions. Associations between the relative abundances of microbial taxa and categorical variables were analyzed using Wilcoxon's rank sum test. The cause of prematurity (iatrogenic/spontaneous), mode of delivery (vaginal delivery/caesarean section), intrapartum antibiotic exposure (yes/no), and neonatal antibiotic exposure (yes/no) were treated as categorical variables. The level of significance was set at a p-value less than 0.05. All analyses were performed using SAS software (version 9.4 for Windows),

4.3.3.4 RNA sequencing and gene expression (III)

RNA sequencing data were processed as described previously (Kumar *et al.*, 2017). Briefly, data were cleaned using Trimmomatic (Bolger, Lohse and Usadel, 2014) for low quality, Illumina adapters, and short read length. Only reads that passed all filters were considered for mapping and were aligned to the human genome (GRCh38) using STAR (Dobin *et al.*, 2013) with the guidance of EnsEMBL v82 gene models. Alignment was performed using the default two-pass per-sample parameters, except that the overhang on each side of the splice junctions was set to 49. Picard tools were used to sort alignments and mark PCR duplicates, SubRead was used to construct feature counts (Liao, Smyth and Shi, 2013), and trimmed mean of M-values normalization (Robinson and Oshlack, 2010) was used to convert feature counts into normalized expression estimates. Default parameters were used, except that feature counting reads were assigned to overlapping genome features. Poorly expressed genomic features with a count-per-million (CPM) value of ≤1.00 in less than three samples were then removed, and differential expression was studied using limma-voom (Law *et al.*, 2014) with and without $\log_2$ of sample preparation time as a covariate. Significance values corrected by the false discovery rate (FDR) were obtained using Storey's FDR method (Benjamini, Hochberg and Benjamini, Yoav, 1995), and genomic features with an adjusted q-value of ≤0.05 and a $\log_2$ fold-change of ≥1.00 were reported as significant. Hierarchical clusters were generated using the Euclidean distance and average linkage method. Principal component analysis was performed on the filtered CPM values using prcomp in R. Pathway enrichment and network analysis of identified differentially expressed genes with known gene symbols and their corresponding expression values were performed using the Ingenuity Pathway Analysis software (Qiagen Inc.) (Krämer *et al.*, 2014). In addition, DAVID (Huang, Sherman and Lempicki, 2008a, 2008b) was used for gene-annotation enrichment and functional annotation clustering.

4.3.3.5 Murine sample analysis (III)

The results were analyzed using the ViiA™ 7 software and exported to Microsoft Excel (Microsoft Corp., Redmond, WA, USA) for further analysis. Each sample was processed in technical triplicate. In total, 15 terminal ileum samples were analyzed (mouse, very preterm M.VPT n=3; mouse, moderately preterm M.PT n=5; and mouse, full-term M.FT n=7). One terminal ileum from each group was not included because of technical problems. Plasma samples were used for measuring the levels of metabolic hormones (insulin, leptin) using a MILLIPLEX MAGPIX system (Merck Millipore, MA, USA). A total of 17 samples (M.VPT n=3, M.PT n=6, and M.FT n=8); were analyzed, and one blood sample was not included in the analyses because of technical problems. Independent-samples t-tests were used to identify the

differences between the fold changes in mouse weight from day 0 and at day 35 and between the study groups. In addition, t-tests were performed to examine the differences between plasma leptin and insulin levels and between the expression levels of the inflammatory cytokines interleukin-1β (IL-1β) and interleukin-6 (IL-6) in the mouse terminal ileum via quantitative PCR analysis between the groups.

4.3.4 Ethical consideration

Since the Study I was retrospective in nature and employed already existing patient records, it did not cause harm or discomfort to the study subjects. The study was approved by the Department of Pediatrics, Turku University Hospital. All patient records were handled confidentially, and the patients remained anonymous.

Studies II and III were prospective in nature, and both of them were approved by the Ethics Committee of the Hospital District of Southwest Finland. Oral and written informed consent was obtained from the caregivers.

Regarding Study III, all mouse experimental protocols were approved by the Ethics Committee of Bar-Ilan University, where the animal experiments were performed.

5 Results

5.1 Clinical characteristics of study subjects (I, II, and III)

The clinical characteristics of the subjects in the different study populations are shown in Table 4.

For Study I, RDS was the most common diagnosis in 76 (97%) infants, along with NEC (10/13%), BPD (46/59%), IVH (21/27%), and PDA (67/86%). The study comprised eight twins. One child had an early bacterial infection, and 23 (29%) were diagnosed with a bacterial infection overall. Moreover, 13 infants received postnatal corticosteroids (17%).

For Study II, vaginal birth was more common in the subjects with spontaneous prematurity ($p<0.001$), whereas the neonates born iatrogenically preterm had an lower birth weight ($p=0.046$) and were more often SGA ($p=0.002$). All neonates in the study received human milk at the time of sample collection, and none of the subjects had received formula. Three cases of chorioamnionitis were observed. These neonates were born at 30, 31, and 34 GW. Of these cases, two newborns and one mother participated in the analyses.

As for Study III, none of the neonates exhibited symptoms or signs of perinatal asphyxia, as reflected by their Apgar scores at 5 and 15 minutes of age. Only mothers with symptoms and signs suggesting a high risk of spontaneous preterm delivery were recruited. Therefore, all the neonates included in this study had been exposed to antenatal corticosteroid treatment. The FT neonates were all vaginally born, whereas in the preterm groups, caesarean section delivery occurred in approximately half of the cases. Mothers were recruited during pregnancy, and the mode of delivery could not be predicted. For the statistical analysis, extremely preterm neonates (<28 GW) and very preterm neonates (28 to 32 GW) were combined as a single group.

Table 4. The clinical characteristics of the infants. The values are shown in either percentages or means and range. (For Study III: VPT= very preterm, PT = moderately preterm, FT = full-term, SGA = small for gestational age)

	STUDY I	STUDY II PRETERM	STUDY II TERM	STUDY III VPT	STUDY III PT	STUDY III FT
N	78	55	25	5	7	11
SEX (MALE)	41 (53 %)	22 (40 %)	9 (36%)	2 (40 %)	4 (57 %)	5 (45 %)
GESTATIONAL AGE	26+2 (23+3–27+6)	32+2 (27+5–34+5)	40+6 (38+1–42+0)	28+2 (25+0–30+4)	34+4 (33+4–36+1)	39+1 (37+0–41+5)
SGA	10 (13%)	13 (24 %)	1 (4%)	0 (0)	1 (14 %)	0 (0)
BIRTH WEIGHT	843 (530–1320)	1885 (755–3050)	3479 (2580–4650)	1278 (850–1730)	2511 (1645–3840)	3420 (2820–4440)
BIRTH WEIGHT Z-SCORE	–0.3 (–4.1–3.8)	–0.3 (–3.5–3.9)	0.9 (–2.2–1.8)	0.2 (–0.5–0.6)	0.0 (–2.7–5.4)	–0.3 (–1.4 –1.7)
APGAR 5 MIN / 15 MIN	6.5 / 6.9 (1–9) / (3–9)	N/A	9.2 / 9.3 (8–10)	6.8 / 8.3 (2–10) / (7–10)	8.9 / 9 (8–10) / (8–10)	8.7 / 9 (6–9) / (9–9)
VAGINAL DELIVERY	35 (49 %)	34 (62 %)	23 (92%)	3 (60 %)	4 (57 %)	11 (100 %)
STARTING DAY OF ENTERAL FEEDING	2 (1–15)	1 (1–1)	1 (1–1)	N/A	N/A	N/A
DURATION OF PARENTERAL FEEDING	21 (3–176)	4 (0–20)	N/A	N/A	N/A	N/A
INTRAPARTUM ANTIBIOTIC	N/A	32 (59 %)	5 (20%)	4 (80 %)	7 (100 %)	2 (18 %)

5.2 Nutritional intake during the first week of life in extremely preterm neonates (I)

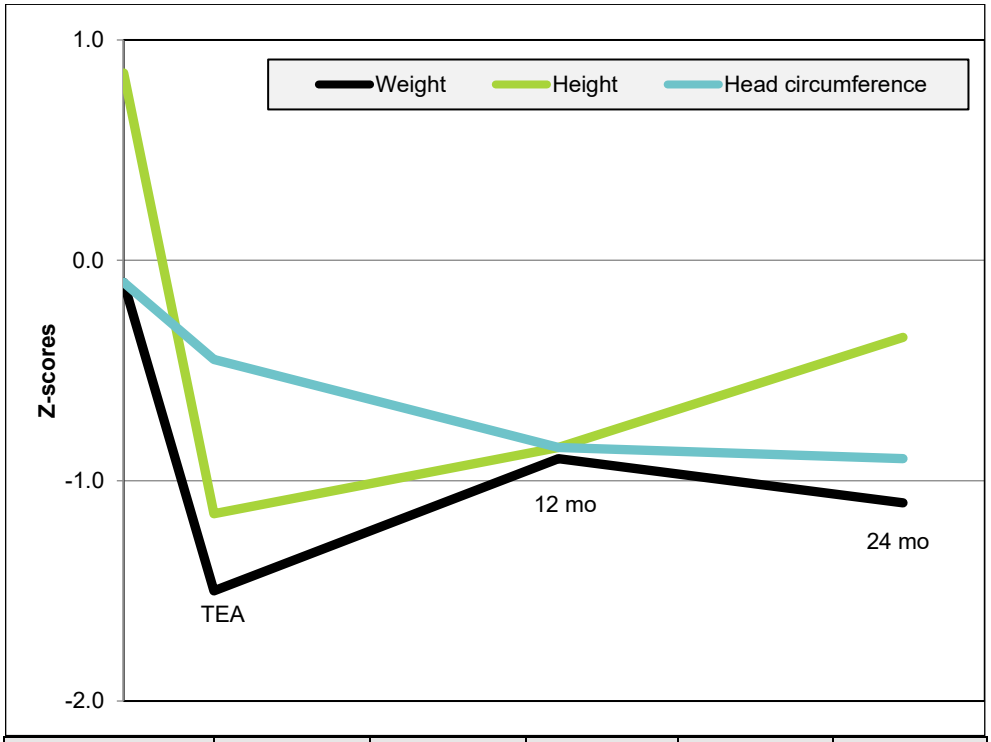
The mean intake of energy during the first week of life in the study population of Study I was found to be 46.5 kcal/kg/day (ranging from 24.8 kcal/kg/d to 80.7 kcal/kg/day) and did not reach the level of 120 kcal/kg/d recommended by the ESPGHAN at the time (Koletzko *et al.*, 2005) (Table 5). Moreover, when each macronutrient was independently investigated, the mean intake of lipids and carbohydrates was found to not reach the recommended amounts. However, some of the neonates reached the recommended levels of protein and energy intake from the first day. In addition, it is notable that the lipid intake during the first two days of life in the majority of the neonates was virtually zero.

Table 5. The mean nutritional intake during the first seven days of life and range. The recommended daily intakes were 120 kcal/kg for energy, 3–4 g/kg for protein, and 2–3 g/kg for lipids. Modified from original publication I.

	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	MEAN
ENERGY KCAL/KG	22.4 (0–43.4)	34.5 (7.0–64.1)	49.0 (21.4–88.3)	53.5 (18.0–96.2)	53.0 (17.4–116.0)	54.5 (15.9–119.0)	58.9 (21.4–116.8)	46.5 (24.8–80.7)
PROTEIN G/KG	1.2 (0–4.0)	2.2 (0–4.2)	2.7 (1.1–4.4)	3.0 (1.6–5.0)	3.0 (1.4–5.6)	3.0 (1.4–4.1)	3.0 (1.4–4.4)	2.6 (1.7–4.2)
LIPIDS G/KG	0.0 (0–0.3)	0.7 (0–2.2)	1.5 (0.0–3.4)	1.8 (0–3.4)	1.6 (0–4.0)	1.6 (0–4.6)	1.8 (0–4.4)	1.3 (0.3–2.8)
CARBO-HYDRATE G/KG	6.3 (0–13.5)	7.0 (1.5–12.0)	8.7 (4.7–13.8)	8.9 (4.9–14.1)	9.0 (5.3–16.2)	9.4 (5.5–16.6)	10.1 (3.0–16.0)	8.5 (5.7–12.3)

5.3 Growth outcomes until the corrected age of two years (I)

Modest growth outcomes were observed in the patient population during the first two years of life (Figure 2). The age-specific Z-scores for weight, height, and head circumference all decreased from birth until the TEA, with weight being the most affected. From the TEA onwards, height seemed to be the growth parameter that recovered the best. Both weight and head circumference remained at the –1.0 SD Z-score at the corrected age of two years.



Variable	Age	Median	Mean	Lower 95%	Upper 95%
Weight	Birth	–0.10	–0.33	–0.66	0.00
	TEA	–1.50	–1.59	–1.87	–1.30
	12 months	–0.90	–0.98	–1.26	–0.71
	24 months	–1.10	–0.98	–1.27	–0.68
Height	Birth	0.85	0.39	–0.04	0.82
	TEA	–1.15	–1.50	–1.86	–1.14
	12 months	–0.85	–0.92	–1.21	–0.64
	24 months	–0.35	–0.57	–0.85	–0.29
Head circumference	Birth	–0.10	0.18	–0.19	0.54
	TEA	–0.45	–0.54	–0.81	–0.26
	12 months	–0.85	–0.86	–1.17	–0.55
	24 months	–0.90	–1.04	–1.36	–0.73

Figure 2. The median Z-score growth parameters (weight, height, head circumference) at birth, at the term equivalent age (TEA) and at the corrected ages of 12 and 24 months. Mean values and confidence intervals are presented in the table. The Z-scores have been recalculated using the references by Sankilampi et al. (2013) and Saari et. al (2011). Modified from original publication I.

5.4 Early energy intake is associated with growth until the corrected age of two years (I)

As shown in Figure 3, a statistically significant positive correlation was observed between the first week energy intake and the age-specific Z-score growth parameters until the corrected age of 24 months. This was also observed in the weight Z-score results at the TEA ($r=0.55$; $p<0.0001$) and at the corrected ages of 12 months ($r=0.33$; $p=0.0028$) and 24 months ($r=0.25$; $p=0.0255$). Moreover, a positive correlation was observed between the first week energy intake and the birth weight ($r=0.48$; $p<0.0001$). Similar positive correlations were observed between early energy intake and height (at the TEA $r=0.57$; $p<0.0001$, at 12 months $r=0.38$; $p=0.0007$ and at 24 months $r=0.26$; $p=0.0191$) and head circumference (at the TEA $r=0.57$; $p<0.0001$, at 12 months $r=0.29$; $p=0.0100$ and at 24 months $r=0.26$; $p=0.0234$) at the corresponding ages.

After the potential confounding factors (gestational age, sex, and a diagnosis of SGA and IVH) were adjusted for, the energy intake during the first seven days of life was found to be positively associated with the absolute Z-scores of weight ($\beta=0.03149$; $p=0.0008$), height ($\beta=0.03924$; $p=0.0003$), and head circumference ($\beta=0.02571$; $p=0.0271$) from birth until the corrected age of two years (Table 6). However, the intake of individual macronutrients was not statistically significantly associated with the Z-scores of the weight ($p=0.26$ for proteins, $p=0.15$ for lipids, and $p=0.72$ for carbohydrates), height ($p=0.19$ for proteins, $p=0.13$ for lipids, and $p=0.77$ for carbohydrates) or head circumference ($p=0.64$ for proteins, $p=0.58$ for lipids, and $p=0.68$ for carbohydrates) of infants during the first two years of life.

Table 6. The association of individual factors on growth parameters in a linear mixed model for repeated measures. *Individual age and growth from birth until the term-equivalent age, the corrected ages of 12 and 24 months. The effect column shows the direction of association for each explanatory variable and the p-value describes, whether the association is significant between the explanatory variable and the growth parameter Z-score. Energy*Age describes that the association between the total energy intake during the first week and the growth parameter Z-scores remain over two years while the change of association is not statistically significant. Modified from original publication I. (GA = gestational age, SGA = small for gestational age, IVH = intraventricular hemorrhage)

	Weight		**Height**		**Head circumference**	
	Effect	p-value	Effect	p-value	Effect	p-value
Energy	**Positive**	**0.0008**	**Positive**	**0.0003**	**Positive**	**0.027**
Energy*Age	None	0.25	None	0.061	None	0.18
GA	None	0.46	None	0.21	None	0.60
SGA	**Negative**	**0.0076**	**Negative**	**0.0020**	None	0.056
IVH	None	0.26	None	0.73	None	0.26
Age	**Negative**	**0.015**	**Negative**	**<0.0001**	**Negative**	**0.035**

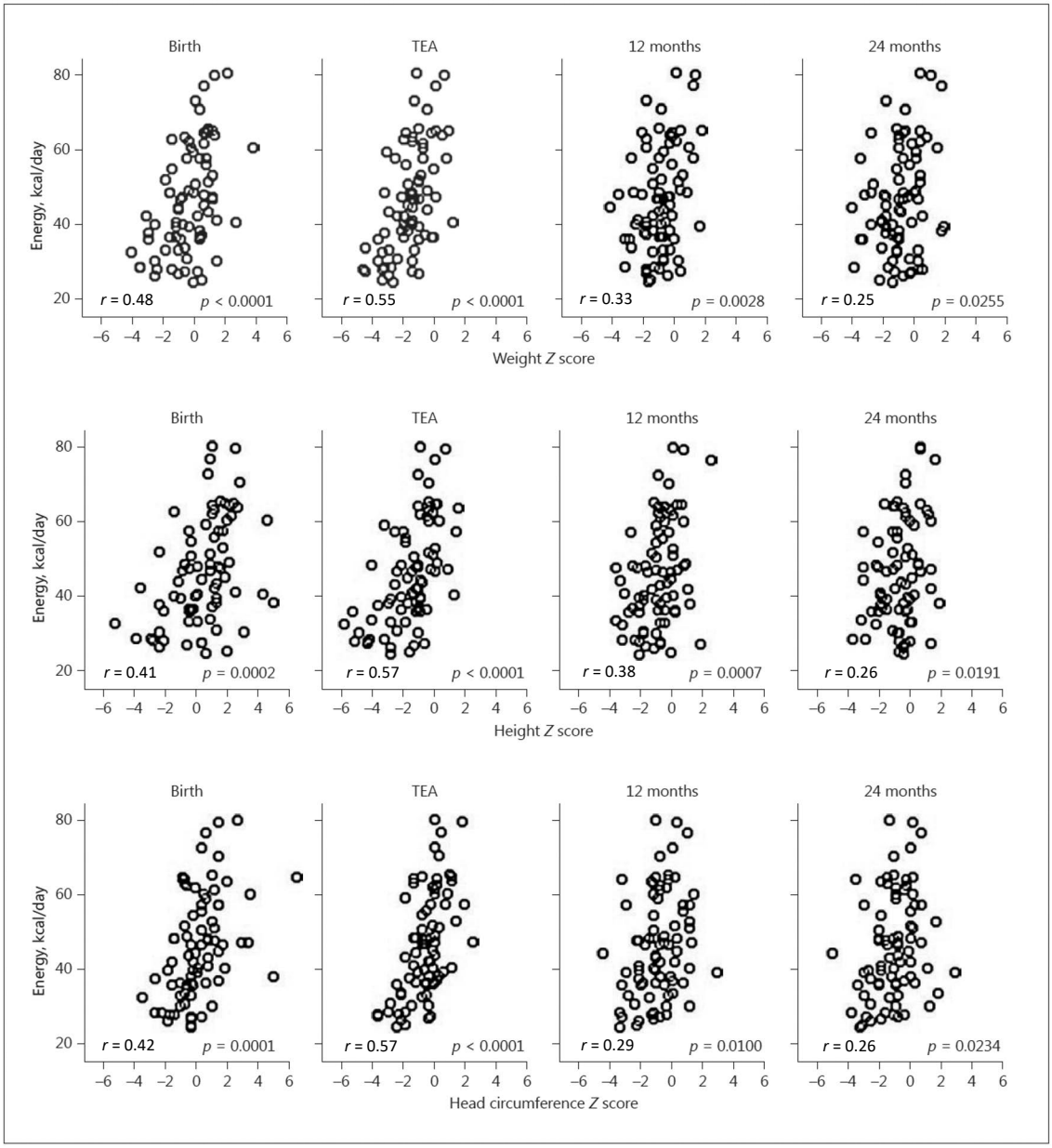


Figure 3. Scatter plots representing the association between the mean energy intake during the first week of life (y-axis) and weight, height, and head circumference (x-axis) Z-scores at birth, at the TEA (calculated with the Sankilampi et al. 2013 reference) and the corrected ages of 12 and 24 months (calculated with the Saari et al. 2011 reference). P-values are based on Pearson correlation analyses. Modified from original publication I. (TEA = term-equivalent age)

5.5 The preterm neonatal gut microbiota is distinct from that of term neonates and is highly individual (II, III)

In Study II, the initial gut microbiota composition in preterm neonates (n=51) was compared to that in spontaneously born, term, neonatal samples (n=25). Significant differences were observed in the microbiota composition between preterm and term samples, in terms of both alpha and beta diversity and the relative abundance of specific taxa. Compared to the preterm population, significantly higher alpha diversity was observed in the term population, as assessed by alpha diversity evenness ($p=0.02$) and Shannon index ($p=0.03$) (Figure 4A). Significant clusters were also observed using the Bray–Curtis ($p=0.001$) and unweighted UniFrac ($p=0.001$) beta diversity matrices according to the two groups (Figure 4B).

Comparing the relative abundances at the phylum level showed that Actinobacteria were more abundant in the term group (W=13). At the family level, the term neonates were found to exhibit higher levels of *Bifidobacteriaceae* (W=104), *Streptococcaceae* (W=103), and *Bacteroidaceae* (W=102) than in preterm neonates. At the genus level, statistically higher levels of *Bifidobacterium* (W=179), *Streptococcus* (W=178), and *Bacteroidetes* (W=177) were observed in term than in preterm microbiota. The most abundant phyla in the preterm samples were *Firmicutes* (60.4%), *Proteobacteria* (26.3%), and *Bacteroidetes* (8.8%) (Figure 5A). At the family level, the three most abundant bacterial families were *Planococcaceae* (28.2%), *Lactobacillaceae* (20.8%), and *Enterobacteriaceae* (11.7%) (Figure 5B). A wide variation was also observed among the preterm subjects in terms of the taxonomic gut microbiota composition. At the phylum level, the term gut microbiota was mainly composed of *Proteobacteria* (32.5%), *Firmicutes* (30.7%), and *Actinobacteria* (29.5%). The three most abundant bacterial families were *Bifidobacteriaceae* (30.6%), *Enterobacteriaceae* (24.9%), and *Planococcaceae* (15.4%).

In Study III, significant differences in the meconium microbiota composition were observed by gestational age. When assessed using Faith's phylogenetic diversity, both VPT and PT neonates were found to exhibit significantly lower alpha diversity than that of FT neonates (Figure 6A, $p<0.05$). Significant clustering of the meconium microbiota was observed between VPT, PT, and FT neonates when beta diversity was analyzed using unweighted UniFrac (Figure 6B, $q=0.003$).

Analyzing the relative abundance at the phylum level revealed that *Firmicutes* was the dominant phylum in 95% of all meconium samples (highest abundance observed in the VPT neonates as compared to PT and FT neonates). The second most dominant phylum was *Bacteroidetes*, in 50% of all meconium samples. Figure 6C summarizes the 12 most abundant bacterial families in each sample as taxa plot

figures. LEfSe was performed to investigate the differences in the community composition between the groups. The features differentiating the groups are summarized in Figure 6D.

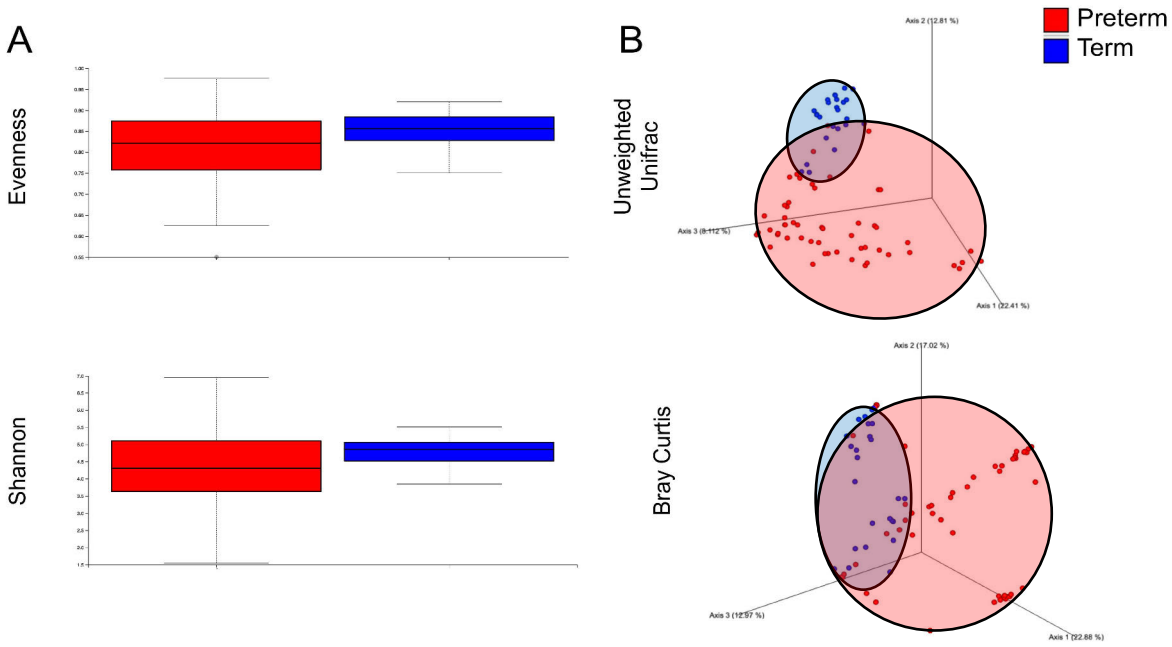
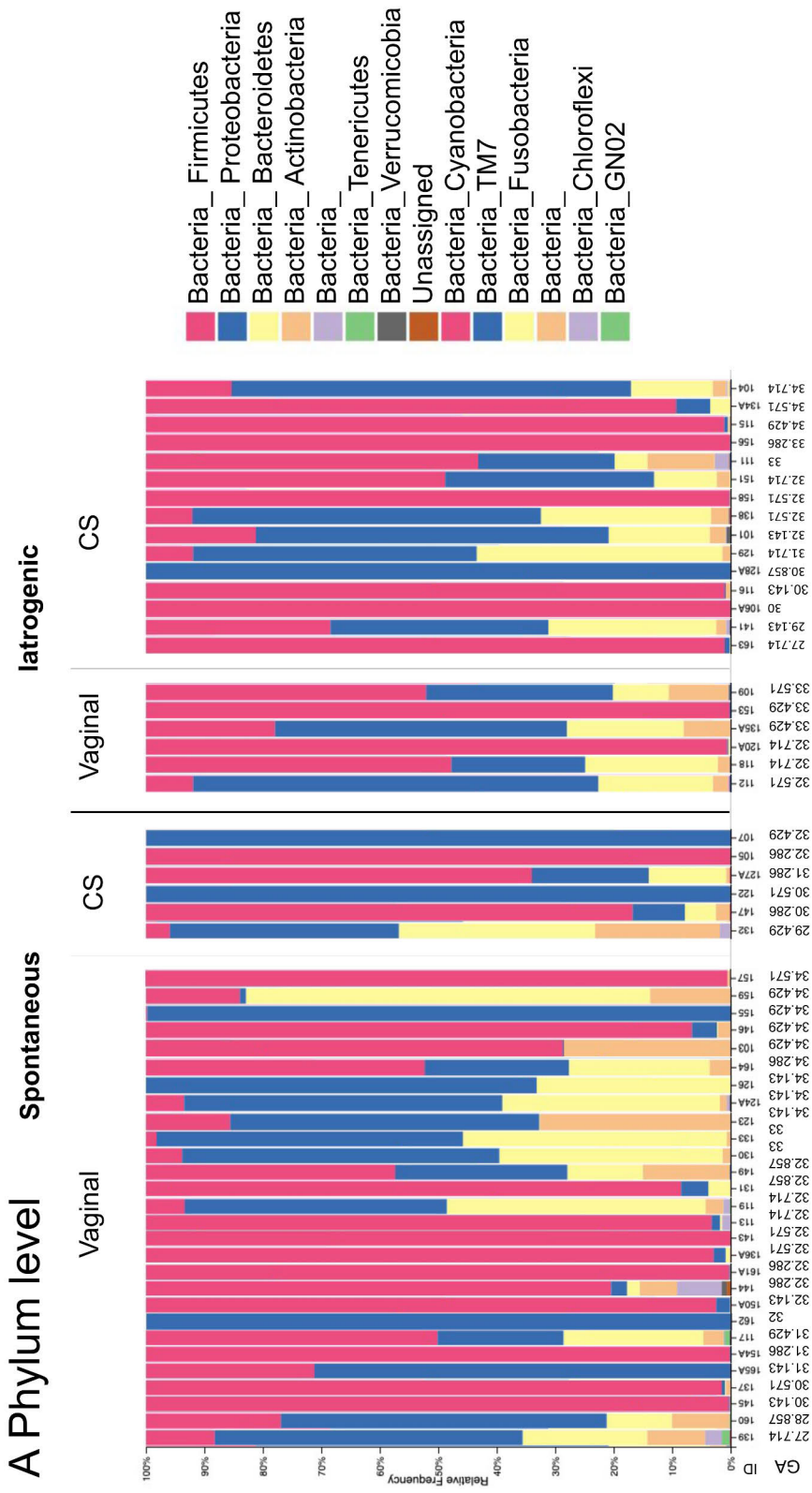


Figure 4. Comparison between the preterm and the term gut microbiota composition. Significant differences were observed in (**A**) alpha diversity evenness (p=0.02) and alpha diversity Shannon index (p=0.03) (Kruskal-Wallis test) and (**B**) beta diversity as assessed by unweighted UnifFac (p=0.001) and Bray-Curtis (p=0.001) analyses (PERMANOVA). Modified from original publication II.


A Phylum level
Spontaneous
Iatrogenic
Vaginal
CS
Vaginal
CS
Bacteria_ Firmicutes
Bacteria_ Proteobacteria
Bacteria_ Bacteroidetes
Bacteria_ Actinobacteria
Bacteria_
Bacteria_Tenericutes
Bacteria_Verrucomicobia
Unassigned
Bacteria_Cyanobacteria
Bacteria_TM7
Bacteria_Fusobacteria
Bacteria_
Bacteria_Chloroflexi
Bacteria_GN02
Relative Frequency
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
GA ID
27.714 139
28.857 160
30.143 145
30.571 137
31.143 165A
31.286 154A
31.429 117
32 162
32.143 150A
32.286 144
32.286 161A
32.571 136A
32.571 143
32.714 113
32.714 119
32.857 131
32.857 149
33 130
33 133
34.143 123
34.143 124A
34.143 126
34.286 164
34.429 103
34.429 146
34.429 155
34.429 159
34.571 157
29.429 132
30.286 147
30.571 122
31.286 127A
32.286 105
32.429 107
32.571 112
32.714 118
32.714 120A
33.429 135A
33.429 153
33.571 109
27.714 163
29.143 141
30 106A
30.143 116
30.857 128A
31.714 129
32.143 101
32.571 138
32.571 158
32.714 151
33 111
33.286 156
34.429 115
34.571 134A
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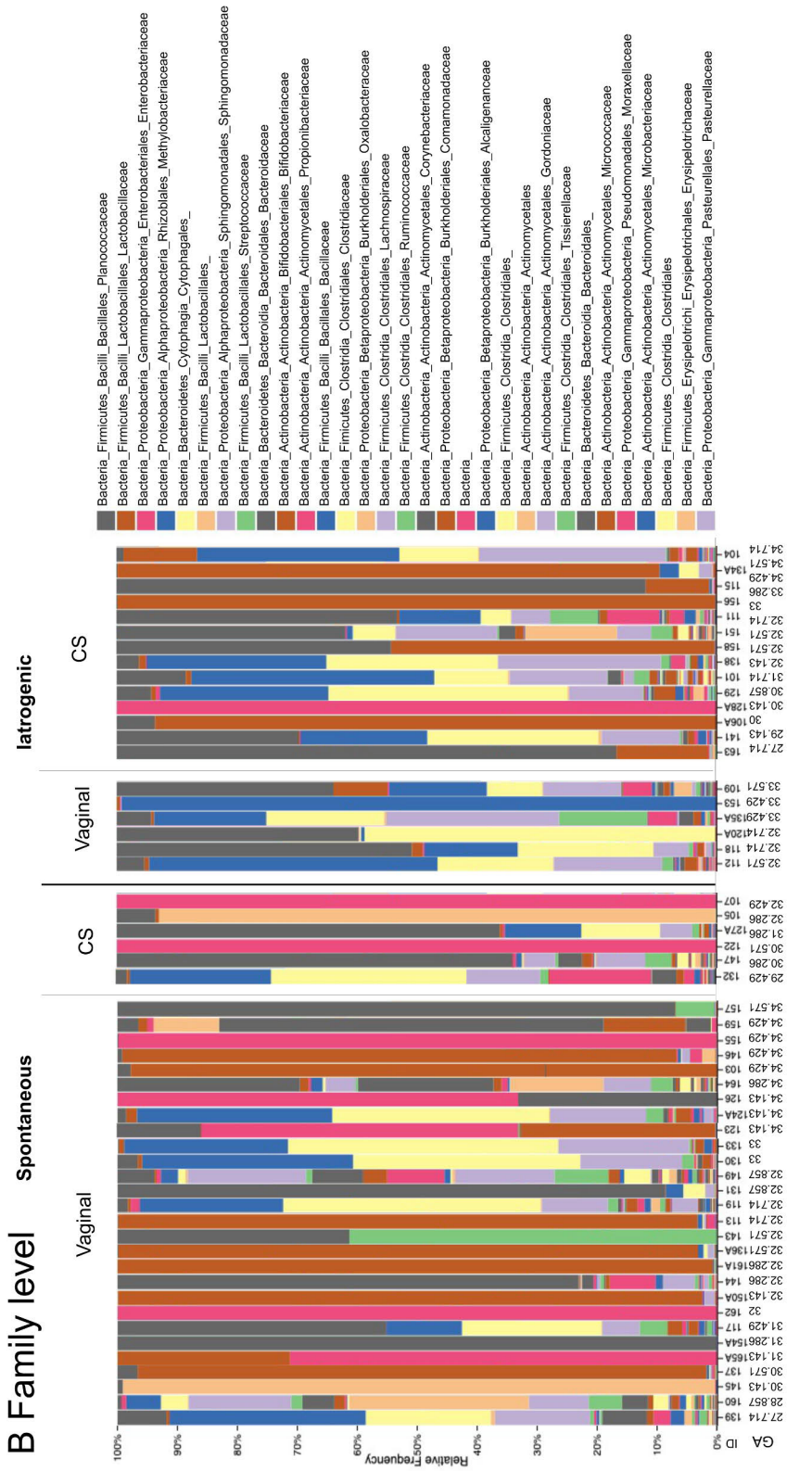


Figure 5. Preterm gut microbiota taxa plots (**A**) at the phylum and (**B**) at the family levels grouped by the cause of prematurity and mode of delivery and organized by gestational age. The most abundant phyla were *Firmicutes*, *Proteobacteria* and *Bacteroidetes*, and the most abundant families were *Planococcaceae*, *Lactobacillaceae* and *Enterobacteriaceae*. The 31 most abundant families are shown. Modified from original publication II. (CS = caesarean section)

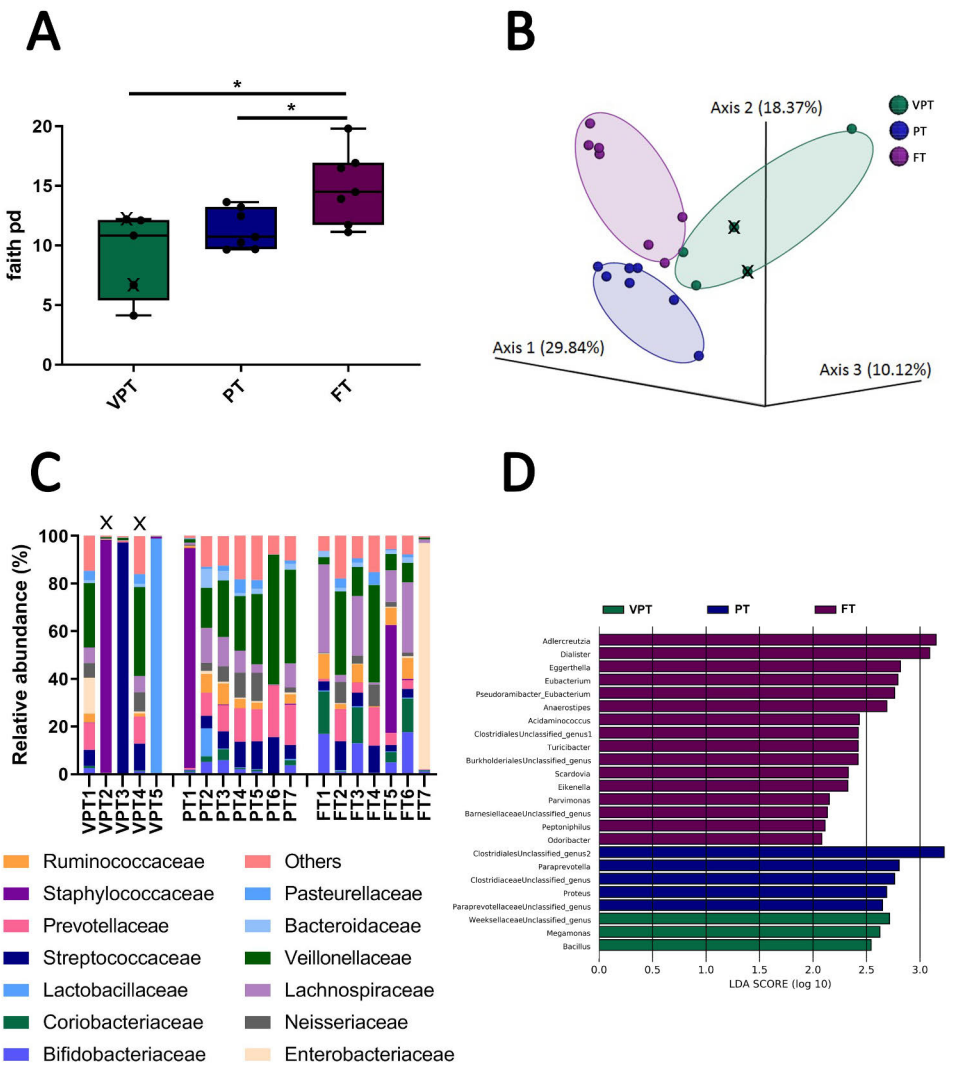


Figure 6. The meconium composition of the VPT, PT, and FT neonates. (**A**) Alpha diversity among the VPT, PT, and FT neonates using Faith's phylogenetic diversity index, compared using t-tests (p-value<0.05). Data are mean ± standard error of the mean (SEM). (**B**) PCoA of unweighted UniFrac distances between the microbiota of the three groups (PERMANOVA, q-value=0.003). (**C**) Relative abundance at the family level. (**D**) The greatest differences in taxa between the three groups are presented according to the LDA-scores (log10), as determined using the LEfSe method. From original publication II. *P< 0.05; VPT, very preterm (n=5); PT, moderately preterm (n=7); FT, full-term (n=7). X denotes extremely preterm neonates (gestational age less than 28 weeks) within the VPT group. (PCoA = principate coordinate analysis).

5.6 The cause of prematurity is associated with the gut microbiota composition in preterm neonates (II)

Significant clustering was observed depending on the cause of prematurity (p=0.047, Figure 7A) and postnatal exposure to antibiotics (p=0.045, Figure 7B), as assessed with the Bray–Curtis beta diversity distance matrix. No differences in the alpha diversity or unweighted UniFrac beta diversity were observed in relation to these two factors. Moreover, the gestational age, mode of delivery, intrapartum maternal antibiotic exposure, and intrauterine growth retardation did not affect the initial neonatal gut microbiota composition.

LEfSe showed that SGA neonates had a higher score for *Planococcaceae* (p=0.022; ANOVA, LD-score=5.14) when compared to the neonates with an appropriate birth weight. Other than that, no statistically significant differences were found in LEfSe.

Moreover, no consistent patterns or significant differences were detected in the relative abundance of specific taxa at the phylum or family levels in neonates born to spontaneous and iatrogenic preterm delivery, nor were the gestational age, mode of delivery, intrapartum antibiotic exposure, and intrauterine growth retardation associated with taxonomic differences.

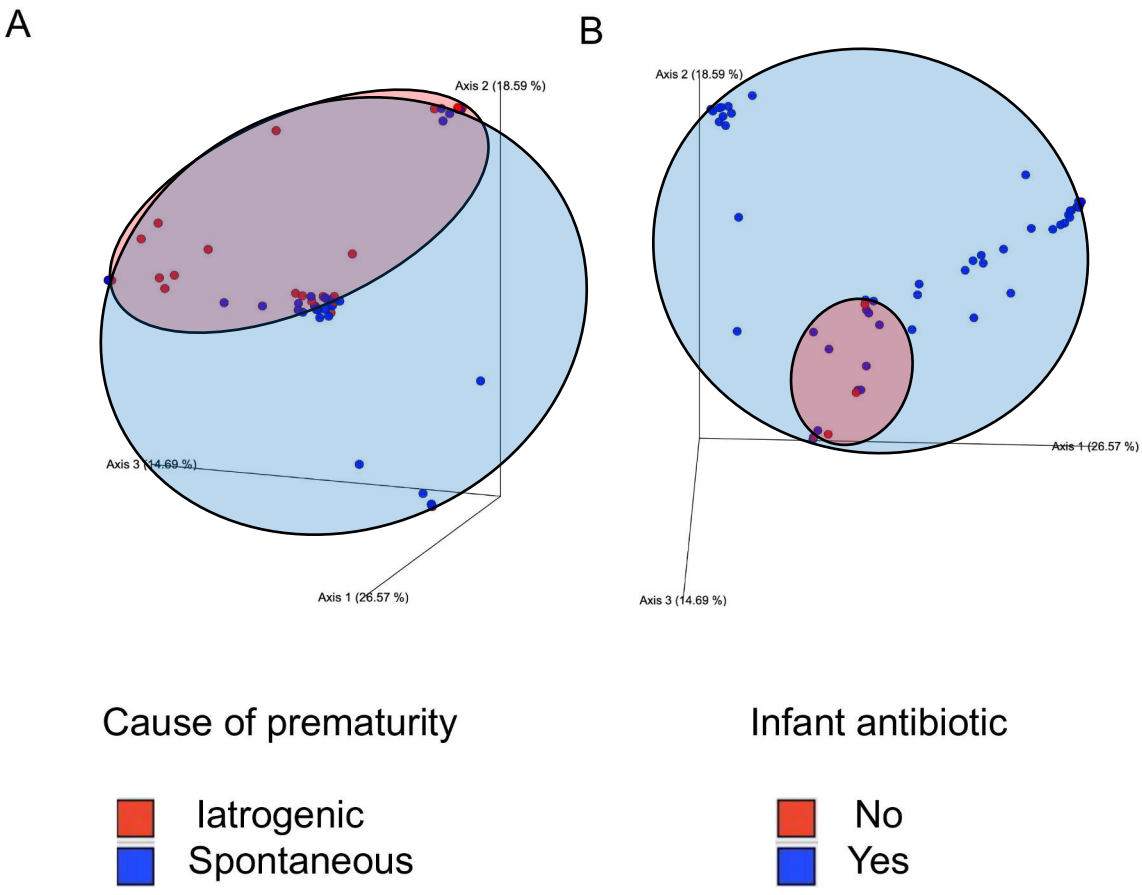


Figure 7. Significant clustering in the PCoA plots of preterm neonate gut microbiota beta diversity grouped by (**A**) the cause of prematurity (p=0.047) and (**B**) neonatal antibiotic exposure (p=0.045). The p-values correspond to the Bray–Curtis analysis (PERMANOVA). Modified from original publication II. (PCoA = principal coordinate analysis)

5.7 Gut microbiota in mothers of preterm neonates (II)

Firmicutes (62.4%) was found to be the dominant taxon at the phylum level in the maternal microbiota. The second and third most abundant phyla were *Bacteroidetes* (29.6%) and *Actinobacteria* (4.3%). At the family level, *Bacteroidaceae* (22.5%), *Lachnospiraceae* (22.4%), and *Ruminococcaceae* (21.6%) were the most abundant. In addition, mothers who did not undergo antibiotic treatment had an increased abundance of *Porphyromonadaceae* (W=37). At the genus level, the mothers who experienced vaginal deliveries were found to have a greater abundance of *Roseburia* (W=118) than in mothers who experienced caesarean-section deliveries. Moreover, mothers who did not undergo antibiotic treatment exhibited an increased abundance of *Macellibacteroides* (W=63). Distinct and significant clustering was detected among mothers by the cause of prematurity (spontaneous, iatrogenic, and PPROM delivery) by Bray–Curtis beta diversity analysis (p=0.041, Figure 8). The maternal gut microbiota composition was found to be associated with intrapartum antibiotic use, which was to be expected in samples collected postpartum. Statistically significant differences with regard to intrapartum antibiotic use were observed in alpha diversity (Faith PD, p=0.039; evenness, p=0.003), and beta diversity (Bray–Curtis, p=0.004; unweighted UniFrac, p=0.002). However, no statistical differences were observed in alpha or beta diversity in relation to gestational age or mode of delivery.

5.8 Maternal gut microbiota significantly contributes to the gut colonization of spontaneously born preterm neonates (II)

First, the contribution of the maternal gut microbiota to the neonatal gut microbiota was assessed with SourceTracker using QIIME version 1.9 (Figure 9). The rate of the maternal gut microbiota contribution to the neonatal gut microbiota varied markedly between individuals. The maternal gut microbiota contributed more in neonates born spontaneously as compared to those born to iatrogenic preterm delivery (p=0.007). Notably, gestational age and mode of delivery did not significantly affect the rate of contribution. Interestingly, however, a manifest contribution from the maternal gut microbiota was also observed in the gut microbiota of caesarean-section born neonates. The SourceTracker analysis included all infants, since the analysis predicts the source of microbial communities from a set of source samples.

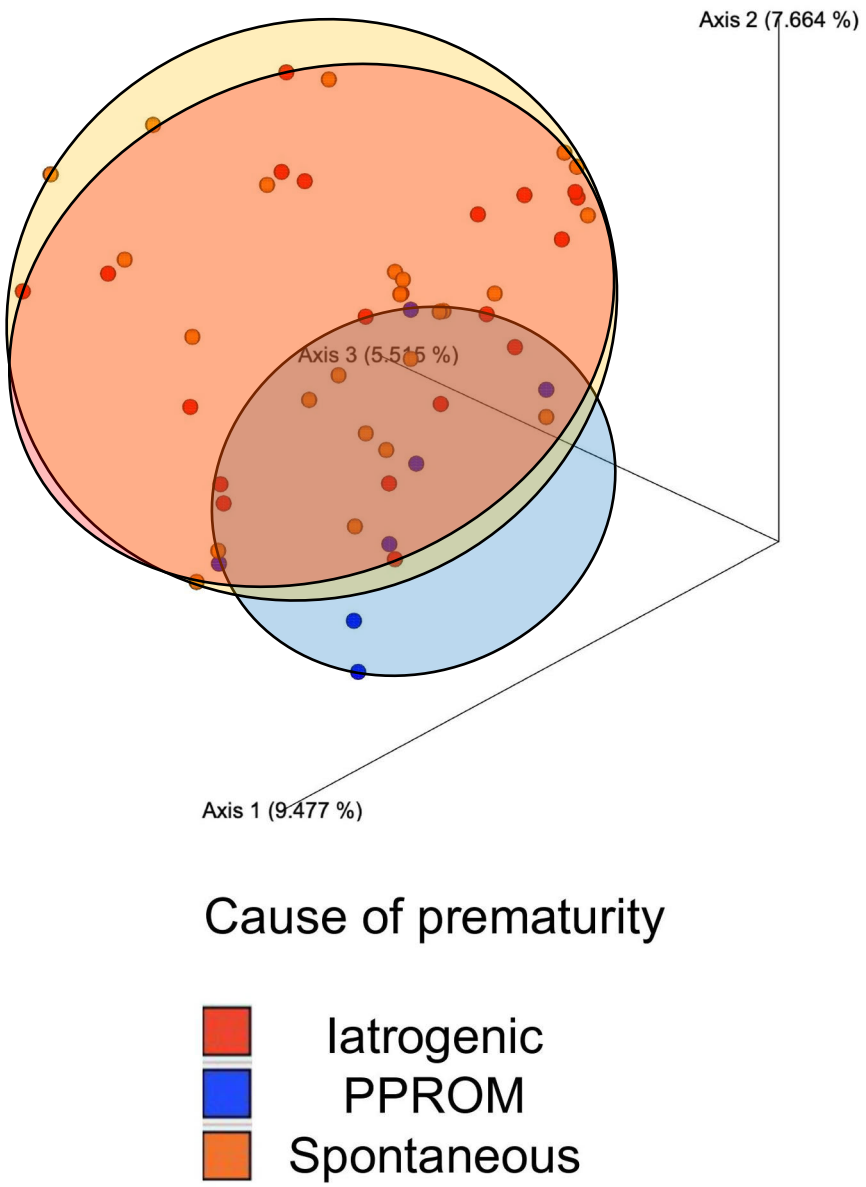


Figure 8. Principal coordinate analysis plots of the maternal microbiota beta diversity grouped by the cause of prematurity. There was a statistically significant difference between the mothers with a spontaneous and PPROM delivery (p=0.041) as assessed using the Bray–Curtis analysis (PERMANOVA). Modified from original publication II. (PPROM = preterm premature rupture of membranes)

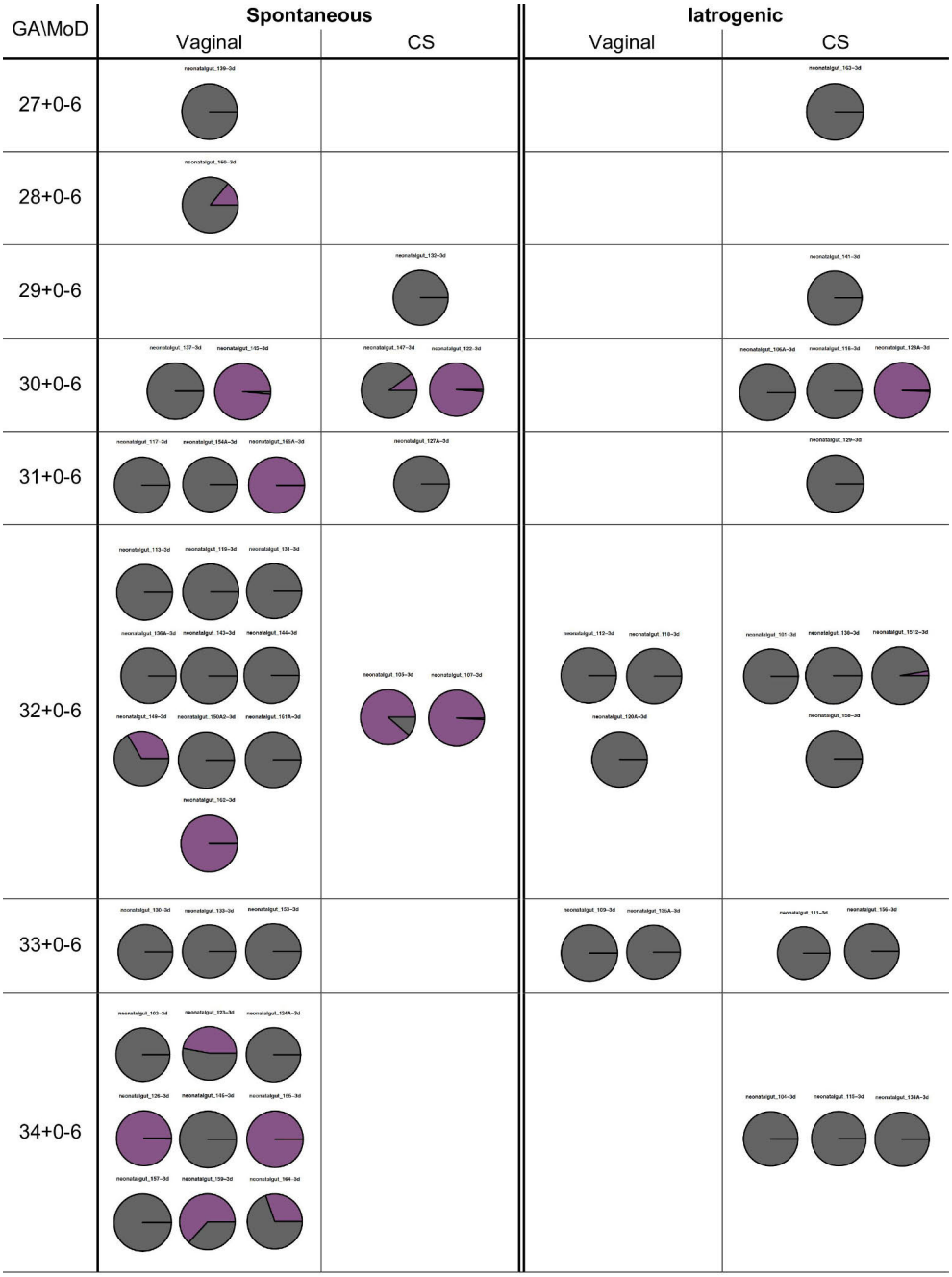
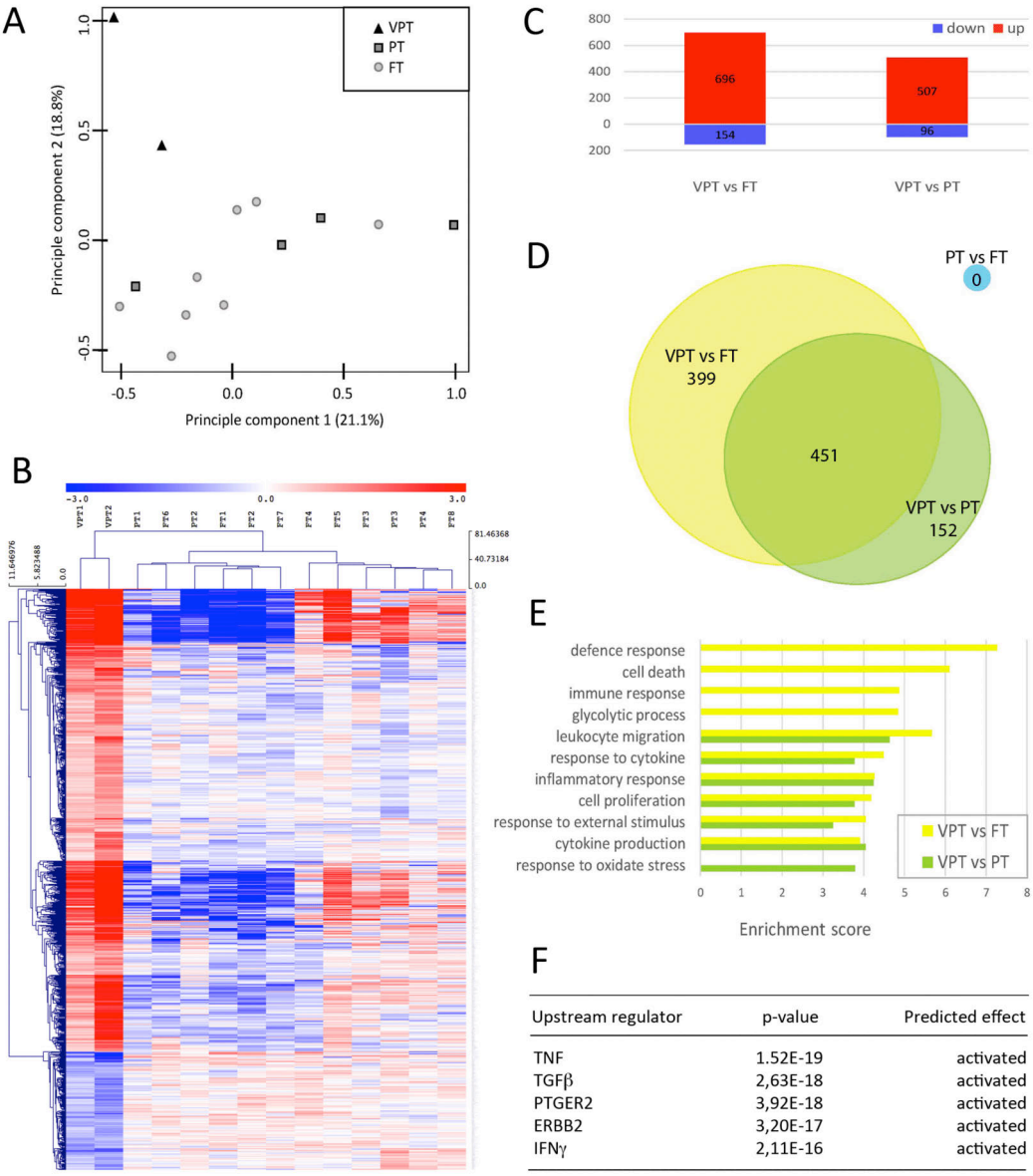


Figure 9. The contribution of the maternal gut microbiota to the initial neonatal gut microbiota composition as assessed using a SourceTracker analysis and grouped by gestational age (GA), the cause of prematurity and the mode of delivery (MoD). Each chart represents one study subject. The maternal contribution is calculated as percentage and shown in purple. Modified from original publication II. (CS = caesarean section).

5.9 Very preterm neonates exhibit an inflammatory tone in umbilical cord blood immune cells (III)

Very preterm birth was found to be associated with distinct fetal immune cell gene expression profiles and particularly fetal inflammatory responsiveness, as assessed by the RNA sequencing of cord blood CD4+ cells. Principal component analysis (PCA) showed that the CD4+ cell gene expression patterns in VPT neonates differ significantly from both PT and FT neonates (Figure 10A). Interestingly, no significant differences were observed between PT and FT neonates. Altogether, 850 genes were differentially expressed with an FDR threshold of 0.05 and a $\log_2$ fold-change threshold of 1.00 between VPT and FT neonates, and 603 genes were differentially expressed between VPT and PT neonates. Moreover, the gene expression signature changes in VPT neonates were mostly upregulated compared to PT- and FT-delivered neonates (Figures 10B and 10C) and overlapped largely among these comparison groups (Figure 10D). Investigation of the physiological role of these observed differences with the DAVID functional annotation tool revealed that inflammation and defense responses were among the most significantly enriched processes among differentially expressed genes, as shown in the Venn diagram in Figure 10E. The Ingenuity Pathway Analysis tool (Qiagen Inc.) was used to further identify the cascades of upstream regulators potentially causing the observed gene expression changes in the VPT neonates. The most enriched upstream regulator in the prediction analysis was the proinflammatory cytokine, tumor necrosis factor (Figure 10F).



F

Upstream regulator	p-value	Predicted effect
TNF	1.52E-19	activated
TGFβ	2,63E-18	activated
PTGER2	3,92E-18	activated
ERBB2	3,20E-17	activated
IFNγ	2,11E-16	activated

Figure 10. Umbilical cord blood CD4+ cell gene expression patterns in very preterm (VPT, n=2), moderately preterm (PT, n=4), and full-term (FT, n=8) neonates. (**A**) The overall gene expression patterns in VPT neonates were distinct from those of PT or FT neonates as determined by principal component analysis. (**B** and **C**) VPT neonates exhibited mostly up-regulation of all gene expression as compared to PT and FT. (**D**) The overlapping genes in VPT, PT, and FT neonates are indicated by a Venn diagram. (**E**) Enriched responses, as assessed by functional annotation tool (DAVID); inflammation and defense responses were among the most significantly enriched physiological processes in VPT neonates. (**F**) Table listing the most enriched upstream regulators identified by the Ingenuity Pathway Analysis tool.

5.10 The initial gut microbiota composition may be causally linked to a preterm phenotype in germ-free mice (III)

An FMT with meconium from VPT (n=4), PT (n=6) or FT (n=8) neonates was performed in 18 eight-week-old GF mice to establish whether the altered meconium microbiota in VPT neonates may play a role in the development of a preterm infant phenotype. Mouse stool samples were collected seven and 35 days after the FMT. At seven days, at the genus level, *Akkermansia* and *Faecalibacterium* were found to be overrepresented in mice receiving an FMT from VPT neonates (n=4). An unclassified *Clostridiales* was found to be overexpressed in mice that received an FMT from PT neonates (n=6) 35 days after the transplant.

To assess the association between the meconium microbiota and growth, the weight of each mouse was monitored, and the weight fold change to the baseline at the time of FMT was calculated in the mice as a measure of growth. After 35 days, the mice that received the meconium microbiota of VPT neonates (n=4) were found to gain significantly less weight compared to those that received the meconium microbiota of FT neonates (n=8) (Figure 11A), whereas the mice that received an FMT from PT neonates (n=6) exhibited intermediate growth. These results suggest that the distinct microbiota in VPT meconium is associated with growth failure in mice.

We next investigated the impact of FMT from VPT, PT, and FT neonates on intestinal immune activation. The expression of mRNA encoding the inflammatory cytokines IL-1β and IL-6 was measured in mouse terminal ileum samples collected after sacrificing the mice on day 35 of the experiment. The mice receiving the microbiota from FT neonates (n=7) were found to exhibit significantly higher levels of IL-1β compared to mice with transplants from VPT (n=3) and PT (n=5) neonates (Figure 11B). Moreover, the mice that received meconium microbiota from VPT and PT neonates displayed significantly higher intestinal inflammatory activation, as measured by the IL-6 mRNA (Figure 11C) levels, as compared to the mice that received meconium microbiota from FT neonates. These data indicate that mice receiving an FMT with very premature meconium exhibit altered intestinal inflammatory activation.

Finally, we assessed the impact of FMT from VPT, PT, and FT neonates on mouse metabolism. The plasma levels of the metabolic hormones insulin and leptin were measured in blood samples collected after sacrificing the mice on day 35 of the experiment. The mice that received the meconium of VPT neonates (n=3) were found to exhibit statistically significant differences in the metabolic state, as demonstrated by the significantly decreased plasma levels of both insulin and leptin, as compared to those that received the meconium of FT neonates (n=8) (Figures 11D and 11E). Moreover, the mice that received an FMT from PT neonates (n=6) exhibited intermediate levels of metabolic hormones.

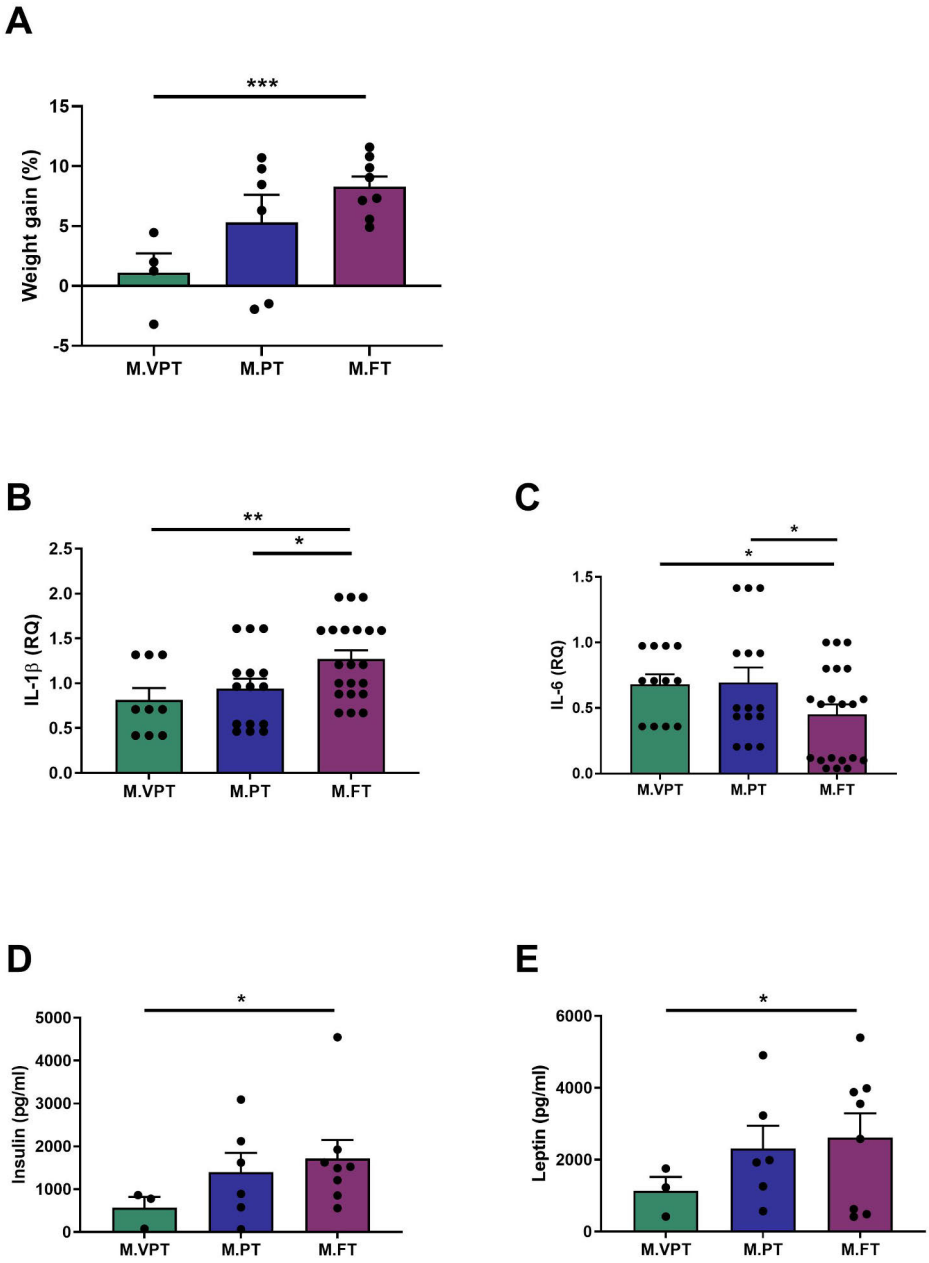


Figure 11. The effect of a meconium FMT on growth, metabolic hormones and inflammatory cytokines in germ-free mice. (**A**) Percent weight gain (M.VPT n=4, M.PT n=6, M.FT n=8); **B-C**, Expression of the inflammatory cytokines in terminal ileum, determined by quantitative PCR analysis. Every sample was processed in technical triplicates. (**B**) Expression of IL-1β (M.VPT n=3, M.PT n=5, M.FT n=7) (**C**) Expression of IL-6 (M.VPT n=3, M.PT n=5, M.FT n=7); **D-E**, Concentration of the metabolic hormones in mouse blood determined by Multiplex assay. (**D**) Concentration of insulin (M.VPT n=3, M.PT n=6, M.FT n=8). (**E**) Concentration of leptin (M.VPT n=3, M.PT n=6, M.FT n=8). *P< 0.05 (t-test); Data represent the mean ± standard error of the mean (SEM). From original publication III.

6 Discussion

In this study, we focused on elucidating the association between early nutritional management and gut microbiota composition on growth. The main results suggest that early exposure and treatment should be considered when investigating the different factors influencing preterm infant growth. The association between early nutritional management of extremely preterm neonates and growth patterns has been found to extend through the first two years of life. Preterm neonates exhibited a lower-diversity initial gut microbiota composition when compared to term neonates, and a great variation was detected between preterm individuals. Several potential perinatal exposures, such as intrapartum antibiotic treatment or mode of delivery, did not affect the initial gut microbiota composition in this study. However, spontaneous preterm birth was associated with gut microbiota composition changes in neonates and their mothers as compared to iatrogenic preterm delivery. Finally, a characteristic unfavorable preterm infant phenotype, including growth restriction, inflammatory activation, and metabolic changes, was induced in GF mice with very preterm neonatal FMT, suggesting a causal connection between very preterm meconium and these typical characteristics of a preterm infant.

6.1 Early nutrition is associated with long-term growth outcomes

Energy intake during the first week of life was found to be associated with growth outcomes until two years of corrected age in extremely preterm infants. Our results may be interpreted to suggest that early, insufficient nutrition and the ensuing catabolic state impact the growth trajectory of infants and, therefore, prevent reaching the expected growth outcomes during the first two years of life. It has previously been shown that early caloric intake is associated with growth outcomes during the first eight weeks of life (Berry, Abrahamowicz and Usher, 2015). The data obtained in this study extend these findings up to the corrected age of two years. These observations are in line with the latest ESPGHAN recommendation, according to which a minimum of 45 kcal/kg should be provided to preterm neonates from the first day of life (Joosten *et al.*, 2018; Mihatsch *et al.*, 2018). To match fetal growth *in utero*, an energy intake value up to 120 kcal/kg should be used, and the ESPGHAN

recommends rapidly increasing the energy intake to 90–120 kcal/kg to approximate the intrauterine lean body mass accretion and growth after the first days of life. The modest changes made in the ESPGHAN 2018 recommendation compared to the 2005 recommendation reflect the accumulating evidence regarding rapid catch-up growth and adverse metabolic changes, especially for protein intake recommendations.

The neonates in Study I did not receive the recommended amount of energy during the first week of life. In general, not following the nutritional recommendations is a common problem encountered in NICUs (Lapillonne *et al.*, 2013). Over the years, the importance of early nutrition has been highlighted and the situation among preterm infants seems to be improving (Uthaya and Modi, 2014). Our first data are from the year 2004, and when we calculated the mean intake values in our NICU on an annual basis, we found that the mean energy intake seemed to improve. Notably, during the early 2000s, the nutritional recommendations were different, and various clinical factors may affect suboptimal nutritional administration, as discussed earlier. Following the recommendations remains a clinical challenge. It is also notable that, according to our results, the smallest infants received the least amount of energy. This may reflect their poor clinical status and prioritization of other treatments.

In our statistical model, SGA was independently associated with growth outcomes until two years of corrected age. Earlier studies have highlighted that the link between IUGR and later growth outcomes (Clayton *et al.*, 2007; Kramer *et al.*, 2014). These infants are considered to be at an increased risk for continuous poor growth, potentially resulting in further neurodevelopmental impairments. On the other hand, rapid catch-up growth has raised some concerns regarding the suboptimal body composition and adverse metabolic alterations later in life. Further interpretation of these data suggests that the intrauterine growth conditions may affect later growth outcomes. This emphasizes the importance of understanding the perinatal circumstances in order to optimize the growth outcomes.

6.2 Early preterm gut colonization and perinatal exposures

In our two separate study cohorts (II and III), we found that the preterm meconium microbiota presented high individuality and had a distinct composition when compared to term neonatal microbiota. These results are consistent with previously reported data (Barrett *et al.*, 2013; Patel *et al.*, 2016; Underwood and Sohn, 2017; Wandro *et al.*, 2018) and strengthen the evidence regarding differences in initial gut microbiota colonization between preterm and term neonates. Generally, gut microbiota with low diversity may be favorable for pathogenetic bacteria and pose a

health risk. While the colonization process of an FTVDBF infant is well established, according to our study, it seems that the preterm early colonization process not only differs from term colonization but also is highly individual. It may also be suggested that preterm infants do not share a common initial colonization pattern. However, it seems that proinflammatory bacteria are overrepresented in the preterm neonate gut compared to term gut colonization. Some key bacterial taxa have been established in the literature, suggesting that there is at least some similarity in the preterm colonization process between the different individuals. The microbial environment in which a term, vaginally delivered neonate is born differs markedly from the one that a cesarean-section-delivered, extremely preterm neonate is born in. Different colonization patterns may result, at least in part, from exposures known to perturb normal gut colonization, including caesarean-section delivery (Stokholm *et al.*, 2016), antibiotic exposure (Arboleya *et al.*, 2015; Gibson *et al.*, 2016), reduced breastfeeding, and skin-to-skin contact. These exposures often coexist in preterm neonates. Besides these exposures, earlier studies have shown that prematurity independently affects the early gut colonization patterns (Forsgren *et al.*, 2017). According to Korpela et al., the gut microbiota of preterm neonates is initially dominated by only a few bacterial taxa. However, over the first postnatal weeks, the gut microbiota composition gradually evolves to be primarily composed of bifidobacteria, resembling the microbiota of term infants (Korpela *et al.*, 2018). Interestingly, while the overall microbiota composition seemed to be strongly correlated with postnatal age in the study of Korpela et al., gestational age was found to be the strongest determinant of gut microbiota composition in our cohort (Study III). This is because significant differences were observed in the meconium gut microbiota composition among VPT, PT, and FT neonates. These results are in line with a previous study indicating that the meconium microbiota in preterm neonates differs not only from that in term neonates, but also from the gut microbiota in the same individuals later during the neonatal period and infancy (Gómez *et al.*, 2017). It has also been suggested that the altered meconium microbiota in preterm neonates may be proinflammatory and causally linked to maternal and fetal inflammatory responses associated with preterm delivery (Ardissone *et al.*, 2014). However, the question of the causal relationships among preterm birth, gut microbiota, and inflammatory responsiveness remain an important future research topic.

In general, the meconium reflects the *in utero* circumstances, since it is formed before birth and is, thus, shaped by the events that occurred during fetal life. In our two independent study cohorts, we collected stool samples during the very first days of life to obtain the most representative samples. Even though there is conflicting evidence regarding the meconium's colonization and sterility, according to our results, manifest colonization and differences between preterm and term early gut microbiota were detected. We also studied the microbial etiology and inflammatory

activation as initiators of spontaneous preterm birth. We found that spontaneously born preterm neonates and mothers who have experienced spontaneous delivery have a different gut microbiota composition from that of those who have experienced preterm birth for an iatrogenic cause. However, whether these changes in the gut microbiota are a cause or a consequence of preterm delivery remains unknown. Our results suggest that the events leading to preterm birth may be linked to the gut microbiota composition before birth, since the differences were observed in the meconium and early maternal samples.

We focused on the association between perinatal exposures, including gestational age, mode of delivery, cause of prematurity, intrapartum maternal antibiotic use, IUGR, and maternal gut microbiota, and the initial colonization of the preterm gut. We found that the cause of prematurity is, as a sole factor, associated with changes in the initial preterm neonate gut microbiota among these perinatal exposures. In addition, we found that the maternal gut microbiota contributed more to gut colonization in spontaneously born neonates than in those delivered preterm for an iatrogenic cause. Although the role of the maternal gut microbiota in the colonization process of preterm infants remains poorly characterized , it is considered a major contributor in term infant gut colonization (Ferretti *et al.*, 2018). Notably, we also found in this study that the mode of delivery did not affect the initial gut microbiota composition or the maternal contribution. This finding is consistent with studies by Dahl et al. (Dahl *et al.*, 2018) and Ardissone et al. (Ardissone *et al.*, 2014). Interestingly, in our SourceTracker analysis revealed a distinct maternal contribution after the caesarean-section delivery of preterm neonates. In addition, the caesarean-section born infants were found to be partly colonized by bacteria that are usually associated with birth canal and vaginal delivery (*Lactobacillaceae* family). We therefore believe that preterm infants represent a unique, individual, initial colonization process in which known perinatal exposures do not contribute to the same measure as in term infants. Importantly, while we have focused on the initial gut microbiota, the longer-term effects of these perinatal exposures need to be studied, which is undoubtedly an important area of future research.

6.3 Unfavorable preterm infant phenotype

An unfavorable preterm infant phenotype is characterized by growth restriction, exaggerated inflammatory activation, and metabolic alterations. The results obtained in this study show a potential connection between VPT meconium microbiota and this phenotype. With the experimental FMT model, a possible causal connection between the initial gut microbiota and growth failure, inflammatory activation, and metabolic changes was detected in GF mice. Therefore, our results may suggest that

preterm infant growth restriction, inflammatory activation, and metabolic alterations may be modulated by the early gut microbiota.

In our study, we found that the mice with a VPT meconium microbiota transplant exhibited a statistically significant restricted weight gain as compared to mice that received an FT meconium microbiota transplant, despite the uniform nutritional management of the experimental animals. Generally, postnatal growth restriction is a substantial clinical problem in VLBW preterm infants (Fanaroff *et al.*, 2007), and with inadequate nutrition often serving as a contributing factor (Lapointe *et al.*, 2016). Recently, the potential link between the gut microbiota and later growth outcomes has also been studied. An association between the gut microbiota composition and suboptimal postnatal growth has been reported by Arboleya et al., who studied the link between early gut microbiota composition assessed at two and 10 days of age and weight gain in preterm neonates born between 28 and 33 GW (Arboleya *et al.*, 2017). Younge et al. reported defective gut microbiota maturation in extremely preterm infants with growth failure as compared to those exhibiting normal growth (N. E. Younge *et al.*, 2019). Interestingly, lower intestinal microbiota diversity was found to precede the development of impaired growth and was detectable already during the first week of life. Many clinical and experimental studies focusing on overweight and obesity later in life have established a link between the gut microbiota composition and growth (Kalliomaki *et al.*, 2008b; Tremaroli and Bäckhed, 2012). An association between the first stool and obesity at the age of three years has also been recently reported (Korpela *et al.*, 2020). In general, intestinal microbes contribute to the energy harvest from the diet and modulate the host metabolism associated with growth and energy storage. Blanton et al. reported that defective gut microbiota maturation is associated with growth failure. They found that a microbiota transplant from malnourished Malawi children resulted in growth impairments in an experimental mouse model, compared to healthy donor transplants (Blanton *et al.*, 2016). In the present study, the potential causal role of aberrant initial gut colonization in subsequent growth restriction may be explained by the inflammatory activation observed in both the VPT neonates and the experimental animals receiving an FMT. However, the potential direct connection between preterm gut microbiota and growth outcomes requires further examination in future studies.

In earlier studies, inflammation has been associated with impaired growth outcomes in preterm infants in previous studies (Mestan *et al.*, 2010; Trevisanuto *et al.*, 2013; Cuestas *et al.*, 2019). In general, inflammation is a known contributor to numerous preterm morbidities, such as NEC, BPD, and poor neurodevelopmental outcomes. In the experimental animals, elevated IL-1β mRNA levels signaled inflammatory activation, and increased inflammatory activation was observed in mice with a VPT transplant compared to FT-transplanted mice. The VPT neonates

in this study also exhibited an inflammatory response, as assessed by their umbilical cord blood CD4+ cell gene expression patterns. While our sample size was limited, similar results have been published by Olin et al., who observed inflammatory activation in preterm umbilical cord blood samples (Olin *et al.*, 2018). Currently, however, it is still unknown whether inflammatory activation reflects the immaturity of a preterm infant or whether it may be the cause or consequence of premature delivery. According to Vora et al., fetal inflammatory gene expression patterns are present in the amniotic fluid before spontaneous preterm birth (Vora *et al.*, 2017). In our study, we found that the upregulated functions of CD4+ cell gene expression are related to inflammation, immunity, and defense responses to external stimuli in the VPT samples compared to the PT or FT samples. While inflammation is a major contributor to the onset of spontaneous preterm birth, our data suggest that inflammatory activation may reflect the cause of preterm birth. According to a report by Schreurs et al., the fetal gut harbors naive CD4+ T cells, which support mucosal development (Schreurs *et al.*, 2019). Dysregulation of this process by preterm birth and premature exposure to antigens is suggested to contribute to intestinal inflammation. In cohort III, we observed an intestinal inflammatory tone, reflected by a modest but statistically significant increase in baseline IL-6 expression in the mice that received an FMT from VPT neonates but not in those that received a PT or FT meconium FMT. We also observed a decrease in intestinal IL-1β in mice receiving VPT neonate meconium microbiota. Despite its proinflammatory role, this cytokine has been reported to also play a role in intestinal repair (Voronov and Apte, 2015). It has also been well established that the propensity for an exaggerated intestinal inflammatory response in preterm neonates plays a role in the pathogenesis of NEC (Neu and Walker, 2011b). Using experiments involving a gnotobiotic mouse model, Yu et al. have reported that an FMT with early gut microbiota from preterm infants exhibiting poor growth resulted in defective intestinal maturation as compared to infants receiving an FMT from preterm infants with normal weight gain (Yu *et al.*, 2016). Taken together, these data suggest that an aberrant initial gut microbiota composition may impair growth and increase the risk of NEC by inducing inflammation and perturbing gut maturation. Interventions aiming to modulate gut colonization and intestinal immune responses, including early human milk feeding (Walker and Iyengar, 2015; Lewis *et al.*, 2017) and probiotics (Sohn and Underwood, 2017), have been shown to reduce both intestinal inflammation and adverse clinical outcomes (Bhatia, 2013; Lechner and Vohr, 2016; Dermyshi *et al.*, 2017), including NEC (Maffei and Schanler, 2017). Our present data may suggest new preventive approaches, involving interventions aiming to influence early gut colonization, that can be initiated prenatally.

Studies have shown that mice receiving a meconium FMT from VPT neonates exhibit reduced levels of insulin and leptin, which are both considered fetal growth

factors (Fant and Weisoly, 2001; Özdemir and Akşit, 2018). Fetal and neonatal exposure to leptin has also been suggested to protect against adverse metabolic outcomes in later life (Stocker and Cawthorne, 2008). Other studies have shown that the cord blood concentration of leptin is lower in preterm than in term neonates (Matsuda *et al.*, 1999; Özdemir and Akşit, 2018). In addition, it has recently been suggested that the aberrant gut microbiota observed in extremely preterm infants with growth retardation may contribute to a persistent metabolic state resembling fasting, characterized by defective anabolic glucose metabolism (N. E. Younge *et al.*, 2019). In the present study, we found that the mice that received an FMT from VPT neonates exhibited significantly decreased plasma concentrations of insulin and leptin, which may explain the growth restriction observed in these mice. Generally, metabolic alterations are common in preterm infants during the neonatal period and beyond. According to epidemiological and cohort studies, preterm birth is associated with an increased incidence of metabolic syndrome and a significantly increased incidence of risk factors for later metabolic and cardiovascular disease, including a lower lean body mass, and higher body fat content, as well as impaired glucose metabolism (Sipola-Leppänen *et al.*, 2015b; Heidemann, Procianoy and Silveira, 2019b). This is the first demonstration of a potential causal connection between initial gut colonization patterns and the adverse metabolic consequences of very preterm birth.

The results obtained in this study provide evidence of a possible causal relationship between an aberrant initial gut microbiota composition and the unfavorable preterm infant phenotype in the experimental mouse model. We observed a significant change in the growth trajectory of the mice after the meconium transplant, and we also found that the mice with a VPT transplant exhibited restricted growth in addition to an increased inflammatory tone and metabolic alterations. These results may suggest that the specific VPT meconium may initiate an inflammatory activation process that contributes to poor postnatal growth outcomes. Furthermore, these findings may suggest new approaches to ameliorate the adverse consequences of very preterm birth, including interventions aimed at influencing early gut colonization. These results should be confirmed in a human research setting, however, since many of the phenomena observed in preterm infants are confounded by the perinatal exposures.

6.4 Limitations and strengths

Among all individual studies, one shared limitation was the relatively small sample size. Preterm infants represent a limited group, and our exclusion criteria also ruled out several patients, possibly limiting the generalizability of the results. A shared strength among our studies, however, was that all the subjects were treated at the

same hospital, at the same NICU, by the same staff, and with the same clinical protocols every year. We also followed uniform nutritional recommendations and medical guidelines with uniform indications, such as for antibiotic administration, and performed extensive reporting for all neonates. Moreover, we followed a structured protocol throughout the sample and data collection phase. Therefore, we believe that the data are reliable and representative.

In Study I, nutritional data were collected only during the first week of life. Therefore, the growth patterns may have been influenced by later nutrition. However, it has been previously reported that a significant variation in the nutritional management of preterm infants seems to occur during the first week of life (Uthaya and Modi, 2014). We therefore believe that the greatest variation in nutritional intake occurred during the first week of life in this group of extremely preterm age infants. Notably, a great proportion of these neonates achieved considerable amounts of enteral feeding during the first week of life, indicating a stable nutritional situation. We adopted a structured follow-up system for the infants at the same university hospital, and the nutritional data that we collected were the actual received nutrition, not an estimate. We estimated the composition of the mothers' milk and did not use analyses based on individual samples. The outcome data collected were elaborate and based on uniform criteria used by the Vermont Oxford Network. This allowed us to control for several confounding factors when analyzing the data.

In Study II, the most important limitation was the time of sample collection. Preterm samples were collected zero to three days postpartum, which may have possibly promoted a higher microbial variation and a lower bacterial biomass. On the other hand, the maternal fecal samples were collected after birth and were, therefore, potentially affected by intrapartum antibiotic exposure, labor, and other external factors. Although prenatal maternal samples could have been more informative, precisely predicting preterm delivery is difficult. Another clear limitation was the differences in the rates of caesarean section and fetal growth restriction between spontaneously born neonates and neonates born preterm for an iatrogenic cause. However, potential confounding was ruled out by the fact that the mode of delivery or being SGA displayed no statistically significant association with the preterm gut microbiota composition in our analyses. The relatively small sample size may also affect the applicability of the results. However, we characterized the study subjects in detail and followed a structured study protocol while collecting the samples and treating the preterm neonates in the NICU. All preterm neonates received BM, either from donors or from their own mothers, from the first day of life. Antibiotics were used according to the unit protocol, with initial empirical antibiotics consisting of a combination of penicillin G and gentamicin. Moreover, the term population chosen for comparison was a uniform group born at the same hospital with no neonatal antibiotic treatment, and the term fecal samples were

collected during the first days of life. Therefore, we consider our results, in which the cause of prematurity emerged as the sole significant factor, to be reliable.

In Study III, the fecal samples were collected from diapers and were, therefore, potentially exposed to environmental microbes. However, uniform sample collection protocols and tools were used to avoid systemic bias. Moreover, a variation was observed among the groups regarding the mode of delivery. However, because of the study design, the mode of delivery could not be predicted. In future research, it is important to minimize the differences in the mode of delivery. Furthermore, in this study, the umbilical cord blood samples were acquired only from a limited subgroup of our original study sample. However, our results regarding the meconium microbiota composition and cord blood immune responsiveness are consistent with larger previously published studies. Therefore, we believe that the samples are were representative. A further limitation was the fact that the weight gain and metabolic outcomes of the neonates in this study were not assessed. This is because we had information on the growth outcomes of only a limited number of the preterm infants: only three out of five infants in the VPT group and two out of seven in the PT group. Therefore, we relied on previously published data from larger studies, which indicate that postnatal growth failure is a frequently encountered problem in very preterm infants and that adverse metabolic consequences are common. Despite these limitations, we believe that our data reliably demonstrate an association among aberrant meconium microbiota, an inflammatory immune tone, and the characteristic unfavorable preterm infant phenotype that involves growth failure and metabolic disturbances. Furthermore, the results obtained from the FMT experiments on GF mice suggest that a causal relationship may underlie these associations. Even though the assumption of interspecies transfer of pathology is prone to criticism and the results should be interpreted with caution (Walter *et al.*, 2020), an FMT from individuals with and without a pathology into GF rodents, followed by a comparative analysis of the pathological phenotypes in the recipient animals, remains the most commonly used model to draw causal inferences regarding gut microbiota aberrancies and pathogenesis of disease. Our experimental animal model displayed common phenomena observed in preterm infants: growth restriction, inflammatory activation, and metabolic alterations. We, therefore, consider the results reliable.

6.5 Future perspectives

For future research, adequately blinded, randomized, controlled trials should be conducted to assess the long-term health outcomes of early nutritional management in preterm infants. Optimal early nutrition should balance between the risks of poor growth outcomes and potentially impaired neurodevelopment on the one hand and later adverse metabolic health outcomes on the other hand. However, no clear

connection between nutritional deficits and cardiovascular disease has yet been established, even though preterm infants have increased potential cardiovascular risk factors, including a lower lean body mass and higher body fat content, as well as impaired glucose metabolism later in life (Sipola-Leppänen *et al.*, 2015a; Markopoulou *et al.*, 2019). It is also worth noting that data regarding the other long-term health outcomes and specific early nutritional patterns are lacking. More information should be provided in adequate trials to optimize the nutritional guidelines to improve preterm infant health.

We believe that more attention should be paid to the perinatal circumstances to optimize the preterm growth outcomes and improve health. In our study, we found that SGA is an independent risk factor for poor postnatal growth. Generally, SGA may have various underlying reasons, and not all of them are even potentially treatable. Therefore, to obtain a better understanding of the underlying perinatal and treatable causes of poor growth, both extra- and intrauterine, more research should be performed on the risk factors. The research should focus on the maternal conditions and intrauterine fetal circumstances. An interesting point of view is that inflammation, which was evident in our umbilical and murine samples, may contribute to the growth outcomes. Therefore, prenatal inflammatory activation and its impact on intra- and extrauterine growth should be further investigated.

In this study, we assessed perinatal exposures and their impact on the initial preterm gut microbiota composition. In the future, after more evidence is gathered and after the colonization process of the preterm gut is described in more detail, we can attempt to modulate these exposures or directly modulate the gut microbiota to a more favorable composition. Still, our knowledge regarding the healthy gut microbiota composition in preterm infants is insufficient. In addition, the long-term health outcomes of specific preterm infant gut microbiota compositions are still unknown. Notably, our experimental animal model suggested a causal relationship among meconium microbiota, and restricted growth, inflammatory activation, and metabolic alterations. In future research, establishing a link between specific microbial taxa and later growth outcomes would be groundbreaking and would offer a new target for intervention. Microbial research is mainly observational, and larger study groups and longer surveillance durations are recommended to obtain more reliable data. Our results suggest that the prenatal events leading to very preterm birth and their impact on initial gut colonization may play an important role in the development of an unfavorable preterm phenotype. Hence, experimental and clinical studies aiming to further elucidate the perinatal exposures and events modulating the meconium microbiota are expected to provide a novel means for improving long-term health. Moreover, appropriate clinical outcomes should be considered in these trials to obtain convincing evidence.

In general, the challenges of microbial research, the great number of confounding factors in colonization of the preterm gut, and, most importantly, the current incomplete understanding of the healthy neonatal gut microbiota set boundaries to this research area. However, preterm infants are a prioritized group, and ensuring favorable growth and health outcomes for this fragile group is vital for their treatment. Moreover, acquiring reliable information regarding the associations among the gut microbiota composition, growth, and later health may offer a new target for personalized interventions. Furthermore, since a link between nutrition and gut microbiota has been established, personalized nutrition and probiotics can offer a treatment potential to enhance growth and later health in preterm infants.

Nutrition modulates the intestinal microbiota, and, in the future, may serve as a mediator between the gut microbiota and growth outcomes. With the development of the understanding of the gut microbiota and its impact on later health, personalized medicine may be applied in the nutritional treatment of preterm infants. Indeed, the goal behind treating this group of patients to ensure normal growth and development, and both appropriate nutrition and a healthy gut microbiota may help achieve this goal.

7 Conclusions

The main conclusions of the present study are as follows:

1. The energy intake during the first seven days of life is independently associated with infant growth until the corrected age of two years in extremely preterm infants. This emphasizes the importance of early nutritional management for preterm neonates.
2. Very preterm neonates harbor distinct, heterogenous, initial gut microbiota composition, which is characterized by lower diversity and significant clustering when compared to term neonates. The gut microbiota composition also differs between very and moderately preterm neonates but is not as evident. This suggests that the early colonization patterns in preterm neonates are individual, and do not share the same known patterns as in term neonates.
3. Spontaneous preterm birth is associated with changes in the initial gut microbiota composition of both preterm neonates and their mothers. The mode of delivery, intrapartum antibiotic use, and intrauterine growth do not affect the gut microbiota composition. Moreover, the contribution of the maternal gut microbiota to the initial neonatal gut colonization is more pronounced after a spontaneous than after an iatrogenic preterm delivery and is not dependent on the delivery mode. This finding offers new evidence regarding the early gut colonization modulators in preterm infants.
4. The initial meconium microbiota in very preterm neonates may be causally associated with the unfavorable very preterm infant phenotype characterized by growth restriction, an inflammatory tone, and altered metabolism, according to fecal microbiota transfer experiments conducted in a germ-free mouse model. These findings may suggest new approaches to ameliorate the adverse consequences of very preterm birth, including interventions aiming to influence early gut colonization.

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