The impact of AMniotic Fluid on the development and microBIal colonization of the prEterm intestinal tract: the AMFIBIE study

SUMMARY

Rationale: Preterm birth remains a major global health issue. Extremely preterm infants, born with gestational age below 28 weeks, are especially vulnerable to adverse health outcomes. Necrotizing enterocolitis, an inflammatory disease of the neonatal intestines, and sepsis, a systemic response to microbial invasion of the bloodstream, are more common in this population. The pathogenesis of both conditions has been linked to the immaturity of the preterm intestines and preclinical alterations in the gut microbiome. It has been hypothesized that amniotic fluid (AF) impacts the intra-uterine development of the fetal gastrointestinal tract as the intestines are exposed to amniotic fluid through swallowing in utero. Previous studies on the characterization of AF have demonstrated that the constitution of AF gradually changes over the course of pregnancy. However, current literature still lacks knowledge on the, potentially, critical changes, that occur in the content of AF between the 24th and 28th week of pregnancy, their effect on the development of the fetal gastrointestinal tract and impact on the microbial colonization of the neonatal gut.

Objective: The aim of this study is to characterize the composition of AF, collected during preterm delivery, of extremely preterm infants (24-28 weeks), using novel molecular techniques. These approaches include microbial (e.g. IS-pro analysis) and metabolomic (e.g. (un)targeted metabolomics) techniques. The impact of AF on neonatal gut colonization will be assessed through analyses of neonatal stool samples, which are collected according to the ongoing eMINDS study.

Study design: In this multicenter observational cohort study, AF will be isolated from mothers delivering their infants extremely preterm. Two subgroups are of particular interest due to their association with morbidity and mortality in the neonatal phase: 1) preterm infants exposed to intra-uterine inflammation, and 2) preterm infants with fetal growth restriction (FGR). To assess the impact of the composition of AF on the colonization of the neonatal gastrointestinal tract, neonatal stool samples are collected daily for the first 28 days of life, according to the ongoing eMINDS study.

Study population: This study involves the inclusion of mother-infant pairs. Obstetric patients expected to deliver extremely preterm and their infants are eligible to participate in the study. Obstetric patients with pregnancies complicated with major fetal congenital or chromosomal abnormalities will be excluded. A reference cohort consisting of AF samples derived during amniocentesis (< 24 weeks) or during planned cesarean sections (C-sections) or vaginal

delivery is included to allow for adequate interpretation of findings. For this reference cohort, AF samples are collected from early midtrimester (< 24 weeks), very early and moderate to late preterm (28 + 0 – 36 + 6/7 weeks), and full term pregnancies (37 + 0 – 41 + 6/7 weeks).

Study parameters: The main outcome is the characterization of the bacterial and metabolic composition of AF derived from obstetric patients delivering their infants extremely preterm using advanced biomedical techniques. Secondary outcomes include analysis of AF profiles of extremely preterm infants exposed to, respectively, inflammation in utero and FGR; assessment of key metabolites found in AF of infants exposed to inflammation in utero or FGR over the course of gestation; correlation of AF profiles to neonatal inflammatory diseases and identification of potential biomarkers in AF for premature infants at risk to develop these diseases; correlation of AF profile to microbial colonization of neonatal intestinal gut microbiome in first month of life.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: Collection of AF, which is generally discarded as biological waste, is non-invasive, posing no additional strain on the obstetric patient. AF can safely be isolated without interfering with the practice of a C-section or a vaginal delivery.

1. INTRODUCTION AND RATIONALE

Introduction

Preterm birth, defined as gestational age (GA) < 37 weeks, is the leading cause of neonatal death and the second leading cause of death in children under five years old worldwide (1). Infants born extremely preterm (GA < 28 weeks) are most susceptible to developing adverse short-term and long-term health outcomes (2). Clinical outcomes of extremely preterm infants have significantly improved over the past decade due to major advancements in medical care. Studies demonstrated that a shift has taken place in the causes of neonatal mortality and morbidity. While previously death and disease was mostly related to pulmonary illness, extremely preterm infants are now more likely to survive the first days of life and are challenged by other, non-pulmonary, diseases, including necrotizing enterocolitis (NEC) and sepsis (3). These developments highlight the need to focus our efforts on improving the identification of preterm infants at risk of developing NEC and sepsis and optimizing diagnosis and treatment.

NEC is a potentially life-threatening disease of the neonatal intestines typically affecting preterm infants and infants with extremely low birth weight (BW). Clinical presentation ranges from abdominal distension and bloody stools to intestinal perforation, sepsis, and circulatory insufficiency. Severe cases NEC can also have a fatal outcome (4). While early detection is essential in determining the course and prognosis of NEC, clinical signs are often subtle and non-specific, particularly at disease onset. Radiological findings and biochemical alterations are often only evident in more advanced stages (5). The pathogenesis of NEC is multifactorial, most likely caused by an inappropriate inflammatory response of the immature intestinal epithelial cells as a result of enteral feeding in the presence of intestinal hypoxia-ischemia-reperfusion combined with intestinal dysbiosis (6-9). While changes in the intestinal microbiome composition, characterized by increased relative abundances of *Proteobacteria* and decreased relative abundances of *Firmicutes* and *Bacteroidetes*, are associated with the pathogenesis of NEC, a distinctive microbial signature for NEC has not yet been characterized [9]. Besides BW, NEC incidence largely depends on GA: a lower GA is associated with higher incidence of NEC. In other words, evidence suggests that each gestational week spent *in utero* reduces the risk of NEC (10).

Another important cause of neonatal morbidity and mortality is neonatal sepsis, which refers to microbial invasion of the bloodstream in infants less than 28 days old. Signs and symptoms of sepsis are often non-specific, while failure to recognize sepsis and commence

antibiotic treatments in an early stage may lead to serious complications such as disseminated intravascular coagulation, congestive heart failure, shock, and death (11). Preterm infants are more susceptible to develop LOS than term neonates due to their immature immune system and exposure to invasive devices including vascular access, endotracheal tubes, and feeding tubes (12, 13). The pathogenesis of LOS, similar to NEC, is associated with alterations in the gut microbiome. While intravascular catheters are considered the most important source for LOS, recent studies have shown genetic similarities between the causative pathogens of LOS, as detected through blood culture, and bacterial isolates derived from the gastrointestinal tract (GIT). Several days prior to clinical onset of LOS, the causative pathogens can be detected in the GIT. The finding that the GIT serves as a reservoir for pathogens responsible for, at least a part of LOS cases, is known as the “gut-derived sepsis” hypothesis (14-16).

The immaturity of the intestines and dysbiosis plays a prominent role in the pathogenesis of both NEC and sepsis. An important yet understudied player in the development of the fetal intestines is amniotic fluid (AF) (17). Reduced exposure to trophic factors in AF, as is the case for preterm infants, may compromise healthy development of the intestines and impact intestinal colonization. However, little is still known about the constitution of AF in extremely preterm infants and its effect on the initiation of the gut microbiome. We hypothesize that elucidating the composition of AF and discerning its effect on the fetal intestines and its potential impact on the initiation of the gut microbiome will increase our understanding of NEC and LOS and aid in the identification of biomarkers and clinical interventions.

Amniotic fluid impacts development of fetal intestines

Amniotic fluid (AF) is a dynamic biological fluid, which plays a pivotal role in fetal development. AF has multiple functions, ranging from functioning as a protective fluid against physical trauma, to contributing to fetal nutrition and aiding in the protection against fetal infection through the presence of immune cells, microbial peptides, and enzymes (18). AF is also known to be an important contributor to the development of the fetal GIT as the fetal intestines are exposed to AF through swallowing, starting around the 12th week of gestation. Trophic factors impacting the fetal intestinal epithelium include epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-1, erythropoietin (EPO), and various interleukins (IL) (18, 19). AF reflects the health of both mother and fetus, highlighting the value of AF as a tool in the diagnosis of a wide spectrum of both maternal and fetal clinical conditions (20, 21). Previous studies on the characterization of AF through metabolomic, proteomic, and transcriptomic analyses have demonstrated that the constitution of AF gradually changes over the three trimesters of pregnancy (22, 23).

However, current literature still lacks knowledge on the, potentially critical, changes that occur in the content of AF between the 24th and 28th week of pregnancy, their effect on the development of the fetal intestines and correlation to morbidity and mortality in the neonatal period. Our aim is to address this gap in knowledge through the thorough characterization of AF of premature infants using various novel molecular techniques. To accomplish this we aim to isolate AF from mothers delivering their infants extremely preterm (24 to 28 weeks). Two subgroups are of particular interest due to their association with morbidity and mortality in the neonatal phase: 1) preterm infants exposed to intra-uterine inflammation (IUI), and 2) preterm infants with fetal growth restriction (FGR).

Background: Intra-uterine inflammation

The first group of interest consists of preterm infants exposed to chorioamnionitis (CAM). While the intra-uterine environment is thought to lack a microbiome in a physiological state (24), microbial invasion of the amniotic cavity (MIAC) can cause CAM or intra-uterine inflammation (IUI), which is defined as an inflammatory or infectious disorder of either the chorion, the amnion or both. MIAC is the most commonly found cause of IUI but other causes such as sterile inflammation have also been described. Various factors are associated with IUI, including preterm premature rupture of membranes (PPROM), prolonged rupture of membranes (PROM), and maternal colonization of group B streptococcus (GBS) (25).

CAM is often challenging to diagnose due to non-specific clinical presentation. Manifestation of CAM can also occur subclinically without any clinical signs and present as preterm labor with PROM or even with intact fetal membranes. Suspicions of CAM can be confirmed by histopathology of the placenta by a trained pathologist. The placenta can demonstrate signs of neutrophilic infiltration in the decidua or even the presence of micro-abscesses as a result of IUI. However, obtaining the placenta report may take up to six weeks (26). In a subset of patients with CAM, pathogens can be isolated from AF. In up to 65% of microbially proven cases two or more pathogens can be isolated from AF. The most commonly isolated microorganisms (MO) from AF and the placenta are *Ureaplasma* spp. but also *Gardnerella vaginales*, *Bacteroides*, GBS and *Escherichia coli.* The presence of *Ureaplasma* spp. is associated with chronic and clinically silent intra-uterine infections. From the second trimester onwards *Ureaplasma* spp. can be isolated from AF and can remain clinically silent for up to several months (27). Traditional culturing of many of these pathogens has proven to be difficult. Novel molecular methods, such as 16S-based sequencing, yield more promising results (28). Diagnosis of CAM can be difficult due to a variety of challenges, while identification of infants exposed to IUI may provide novel insights on various neonatal diseases, highlighting the need to study the composition of AF of infants exposed to CAM.

IUI is one of the major causes of preterm birth. IUI can induce a fetal inflammatory response (FIRS), which can initiate preterm labor (29, 30). Fetal exposure to IUI is associated with neonatal morbidity in various organ systems, including NEC and sepsis (31, 32). *In vivo* studies in animal models have shown that *in utero* exposure of the fetal GIT to pathogens and inflammatory mediators in AF is associated with altered development of the intestinal barrier, including increased intestinal permeability and increased bacterial translocation (33). Another study using a mice model also underlined these findings, while demonstrating that exposure to IUI results in increased susceptibility to injury later in life (34), providing a possible explanation for the correlation found between NEC and CAM. Noteworthy, a recent study in a rat model demonstrated that exposure to IUI not only resulted in intestinal injury but also in significant changes in the neonatal intestinal microbiome. These changes were characterized by increased *Proteobacteria* and decreased *Firmicutes* 7 days after birth (35). To our knowledge so far only one study focused on identifying the impact of CAM on neonatal gut colonization in humans. The fecal samples of preterm infants < 28 weeks exposed to CAM with funisitis demonstrated a higher relative abundance of *Mycoplasmataceae*, *Prevotella* and *Sneathia* compared to a healthy control group. In addition, exposure to CAM was associated with higher incidence of LOS, which correlated with the presence of *Fusobacteria*, *Sneathia,* and *Mycoplasmatacea* (36).

Background: Fetal growth restriction

The second group of interest consists of preterm infants with fetal growth restriction (FGR). FGR is defined as an inability of the fetus to grow to its expected biological potential *in utero* (estimated fetal weight (EFW) or abdominal circumference (AC)) < 10th percentile for GA) and is one of the most common pregnancy complications. FGR has various known causes and can be multifactorial. Causes include maternal factors, fetal factors, and causes involving placental insufficiency (37, 38). FGR can be classified depending on the time of onset; early FGR commences < 32 weeks, while late FGR is diagnosed ≥ 32 weeks. Early onset FGR is associated with preterm birth and a variety of neonatal morbidities, including increased incidence of LOS and NEC (39, 40).

In response to the lack of oxygen and nutrients, fetal cardiovascular adaption will take place to ensure brain sparing and altered development of various organ systems may take place, including altered development of the fetal GIT (41). Animal studies have shown that FGR not only results in decreased BW but also impacts the development of the small intestines and may result in increased susceptibility to gastrointestinal disorders (42). Infants with FGR are characterized by the disrupted constitution of gut microbiota and metabolic profiles compared

to non-growth restricted infants. The gut microbiome of growth-restricted infants is characterized by decreased relative abundance of *Enterococcus* and *Acinetobacter*, while the metabolic profile is characterized by downregulated methionine and cysteine levels (43).

Previous studies on the composition of AF have shown that various metabolites demonstrate a significant change in abundance associated with the second-to-third-trimester transition. These changes are hypothesized to correlate with the stabilization of fetal growth during this period (23), raising our interest in the characterization of AF in growth-restricted infants. However, studies on composition and characterization of AF of infants diagnosed with FGR, specifically in the context of preterm birth, are still lacking from current literature and of particular interest due to the impact of AF on the development of the intestines, the initiation of the gut microbiome and correlation with dysbiosis-related disease in the neonatal period.

Conclusion

Being born extremely preterm lays a pivotal foundation for short-term and long-term consequences, in which diseases due to inflammation and infection play an important role. We argue that we must increase our understanding of the role of microbiota and inflammation before and after preterm birth to ensure better outcomes for preterm infants in the long run. To achieve this we must cross the bridge between fetal and neonatal research. We argue that this exploratory cohort focusing on the characterization of AF in preterm pregnancies between 24 and 28 weeks of gestation is uniquely suited to make a step in the right direction. Thoroughly studying the content of AF in relationship to the initiation of the neonatal gut microbiome and metabolome in extremely preterm infants will not only increase our understanding of the pathogenesis of NEC and sepsis but will also aid in the identification of subgroups of infants at risk to develop such morbidities. In the future, this research will potentially guide strategies to prevent NEC and sepsis and associated long-term morbidities.

2. OBJECTIVES

Primary objectives:

To assess the bacterial and metabolic composition of AF derived from mothers delivering extremely preterm infants (duration of pregnancy 24 + 0 – 27 + 6/7 weeks) using advanced biomedical techniques (e.g. 16S-based sequencing, IS-pro, (un)targeted metabolomics).

Secondary objectives:

1. To analyze and compare the AF profiles of extremely preterm infants exposed to inflammation *in utero* vs. infants who were not exposed to inflammation, taking into account clinical signs and symptoms of CAM as well placental histopathology reports.
2. To analyze and compare the AF profiles of extremely preterm infants diagnosed with FGR, according to the consensus definition by Gordijn *et al.* (2016) vs. non-growth restricted infants.
3. To assess the course of key metabolites in AF identified in objective 2 (infants exposed to inflammation) and 3 (infants with FGR) over the course of gestation.
4. Relate AF profiles to neonatal disease in which inflammation plays a role (including NEC, sepsis, and bronchopulmonary dysplasia (BPD) and identify potential biomarkers in AF for premature infants at risk to develop these diseases.
5. To correlate the bacterial and metabolic composition of AF to microbial colonization of neonatal intestinal gut microbiome in the first month of life.

3. STUDY DESIGN

This prospective observational cohort study will be conducted in the Amsterdam UMC (Amsterdam, the Netherlands) and Máxima Medisch Centrum (Veldhoven, the Netherlands), commencing in 2024 until the desired amount of mother-infant pairs (pregnancy duration between 24 + 0 and 27 + 6/7 weeks) have been included (n = 125). In addition, AF will be collected from a reference cohort (n = 150).

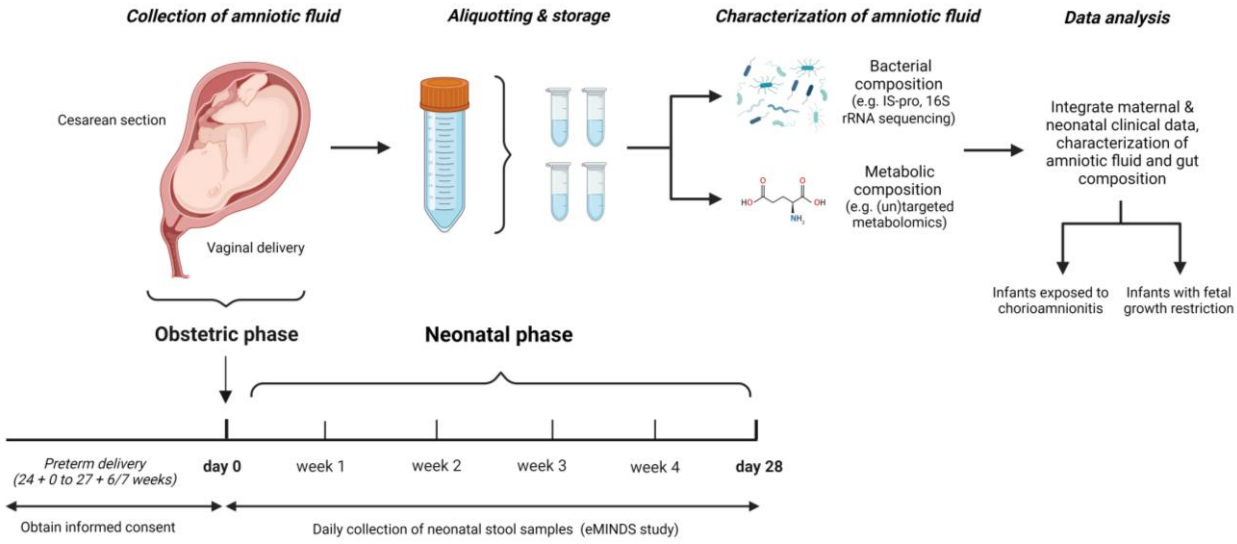

Figure 1 – schematic overview of the study population (24 + 0 to 27 + 6/7 weeks), consisting of the obstetric phase and the neonatal phase. Created with Biorender.com.

4. STUDY POPULATION

4.1 Population (study population)

AF will be collected from the study population (pregnancy duration of 24 + 0 – 27 + 6/7 weeks) and from a reference cohort (respectively, early midtrimester (≤ 23 + 6/7 weeks), very and moderate to late preterm (28 + 0 – 36 + 6/7 weeks), and full-term pregnancies (37 + 0 – 41 + 6/7 weeks). **Table 1** contains an overview of the inclusions for each category.

Study population*:* Patients admitted to the obstetric unit of the participating center, either the Amsterdam-UMC (Amsterdam, The Netherlands) or the Máxima Medical Center (Veldhoven, The Netherlands), who are expected to deliver extremely preterm (pregnancy duration 24 + 0 – 27 + 6/7 weeks) will be briefed on participation the study. In short, patients will be asked for permission for the following: 1) collection of AF during extremely preterm delivery (24 + 0 – 27 + 6/7 weeks of gestation), and 2) collection of clinical data from the electronic patient record (EPR) by researchers, as will be elaborated below. To be included in the AMFIBIE study, an AF sample at time of delivery must successfully be collected. In case of a failed attempt, participants will not be included in the study.

Inclusion criteria study population: If the obstetric patient is > 16 years of age and mentally competent, AF can be collected at time of delivery between 24 and 27 + 6/7 weeks of gestation and informed consent is obtained from the obstetric patient, the participant can be included.

Exclusion criteria study population: Obstetric patients < 16 years of age and/or mentally incompetent or with pregnancies complicated with major fetal congenital or chromosomal comorbidities will be excluded from the study.

Reference cohort: Patients admitted to the obstetric unit of the participating center, either the Amsterdam-UMC (Amsterdam, The Netherlands) or the Máxima Medical Center (Veldhoven, the Netherlands), who are delivering their infants between 28 + 0 – 36 + 6/7 weeks (very and moderate to late preterm) or 37 + 0 – 41 + 6/7 weeks (full-term) can be included in the reference cohort. Patients will be asked for permission for collection of AF during delivery and the collection of clinical data from the EPR. Patients can also be included in the reference cohort if there is a clinical indication for amniocentesis, which are generally performed before the 24th week of gestation (usually around the 15th/16th week) and a few milliliters of additional AF can be collected during this procedure. In the unlikely event that diagnostic amniocentesis is performed > 24 weeks, the sample will not be included in the

reference cohort. The reference cohort is explained in more detail in section 8.3 “Study procedures – reference cohort”.

Inclusion criteria reference cohort: If the obstetric patient is > 16 years of age and mentally competent and AF can be collected at time of clinically indicated amniocentesis (during early midtrimester pregnancies) or during delivery in very preterm and moderate to late preterm pregnancies or full-term pregnancies, the participant can be included in the study.

Exclusion criteria reference cohort: Obstetric patients < 16 years of age and/or mentally incompetent or with pregnancies complicated with major fetal congenital or chromosomal comorbidities will be excluded from the study.

Table 1: overview of collection of amniotic fluid samples

	Study population	**Reference population**	**Methods**
≤ 23 + 6/7 weeks (early midtrimester)	-	*60*	*Amniocentesis (clinical indication)*
24 + 0 – 27 + 6/7 weeks (early preterm pregnancies)	125 infants*	-	Vaginal delivery, cesarean section
28 + 0 – 36 + 6/7 weeks (very, moderate and late preterm pregnancies)	-	60	Vaginal delivery, cesarean section
37 + 0 – 41 + 6/7 weeks (full-term pregnancies)	-	30	Vaginal delivery, cesarean section
Total	125	150	
*Amniotic fluid is collected from all obstetric patients during extremely preterm delivery. The study group is expected to include approximately 30 infants exposed to CAM and 25 infants exposed to FGR based on incidence of respective pregnancy complications.			

Neonatal population: Both parents and/or legal guardians of extremely preterm infants (GA 24 + 0 – 27 + 6/7 weeks) born to mothers included in the study will be asked for permission for the collection of both clinical data and neonatal fecal samples during the first month of life, in line with the eMINDS study (reference number: Metc2014.386(A2020.190)). eMINDS is an acronym for “*Enteral Microbiota and metabolomics in Infants for prediction of Necrotizing enterocolitis, neuromotor Development and late-onset Sepsis*”. The eMINDS study is a research project that has been ongoing in seven intensive care units (NICUs) in the Netherlands since March 2021. The overarching aim of this project is the characterization of the intestinal microbiome and metabolome for the early detection of NEC and LOS in extremely preterm infants. For a detailed explanation of the eMINDS study, including a comprehensive description of collection, storage, and analysis of fecal samples as well as

collection of clinical neonatal data, the eMINDS study protocol can be consulted. The most recent version of the protocol is added as attachment.

If permission is obtained for the eMINDS study and AF is adequately collected during delivery, both parents and/or legal guardians will sign an additional form to give permission to link the data from the AMFIBIE study to the data from the eMINDS study. In the following sections, we will refer to the participants included in the study population and their infants as the "mother-infant pairs" (**Table 2**). In both the Amsterdam-UMC as well as the Máxima Medical Center, the eMINDS study has a very high inclusion rate (>95%) and the expectation is that the vast majority of patients that will give permission for the AMFIBIE study, will also give permission for participation in the eMINDS study. In the unlikely case that no permission is granted on behalf of the infant for participation in the eMINDS study but informed consent has been provided for the AMFIBIE study, the obstetric patients will not be excluded from the study and the sample can be included in the analyses. Noteworthy, the eMINDS study also includes a follow-up study (known as generation P), which requires additional consent. However, follow-up data will not be linked to the data of the AMFIBIE study, so consent for the generation P study is not required in our study setup.

Tabel 2: overview mother-infant pairs in the study population

Study group: 24 + 0 – 27 + 67 weeks (very preterm pregnancies)		
	Obstetric participants	**Neonatal participants**
Number of inclusions	125	125*
Sample type	Amniotic fluid sample during delivery	Daily fecal sample during the first 28 days of life
Clinical data	Extensive clinical and demographic data (8.3 Study procedures)	Extensive clinical and demographic data (8.3 Study procedures)
*In the unlikely case that no informed consent is provided by parents and/or guardians for participation in the eMINDS study and therefore fecal samples cannot be collected, obstetric patients will not be excluded from the AMFIBIE study and microbial and metabolic analysis on AF will still be performed.		

4.2 Sample size calculation

A formal power analysis cannot be performed as there is currently insufficient literature available on the composition of AF in extremely preterm infants and its impact on the development of the neonatal gut microbiome. The incidence of CAM and FGR varies greatly in literature, respectively ± 25-40% for CAM and ± 20-30% for FGR in preterm infants (44, 45). Due to the exploratory nature of this study, we aim to include approximately 30 infants

exposed to inflammation *in utero* and 25 infants with FGR. Taking into account the incidence of CAM and FGR in preterm infants, we aim to collect AF samples from 125 mother-infant pairs with a pregnancy duration between 24 and 28 weeks. In this cohort, we expect to include 31 infants with a sepsis episode and 9 infants with a NEC episode, based on the incidence of 25% of sepsis and 7% of NEC in extremely preterm infants (46, 47).

5. TREATMENT OF SUBJECTS

Not applicable

6. INVESTIGATIONAL PRODUCT

Not applicable

7. NON-INVESTIGATIONAL PRODUCT

Not applicable

8. METHODS

8.1 Study parameters/endpoints

8.1.1 Main study parameter/endpoint

Characterization of bacterial and metabolic composition of AF derived from mothers delivering extremely preterm infants (duration of pregnancy 24 + 0 – 27 + 6/7 weeks) using advanced biomedical techniques (e.g. 16S-based sequencing, IS-pro, (un)targeted metabolomics).

8.1.2 Secondary study parameters/endpoints

- Analysis of microbial constitution of AF by 16S rRNA sequencing and/or IS-pro.
- Analysis of neonatal intestinal microbiota by 16S rRNA sequencing and/or IS-pro.
- (Un)targeted metabolomics of isolated AF samples.
- Assessment of longitudinal course throughout gestation of key metabolites.
- Correlation of demographic and clinical maternal as well as neonatal data to identified AF profile, as elaborated on above.
- Correlation of neonatal intestinal microbiome to AF profile.

8.1.3 Other study parameters

Not applicable

8.2 Randomisation, blinding and treatment allocation

Not applicable

8.3 Study procedures

The study consists of two phases for each mother-infant pair in the study group (extremely preterm pregnancies 24 + 0 – 27 + 6/7 weeks, n = 125): phase 1 (*the obstetric phase*) and phase 2 (*the neonatal phase*) (**Figure 1**). Additionally, a reference database will be constructed of AF collected from early midtrimester (≤ 23 + 6/7 weeks), very preterm and moderate to late preterm (28 + 0 – 36 + 6/7 weeks), and full -term pregnancies (37 + 0 – 41 + 6/7 weeks) (n = 150).

Phase 1: the obstetric phase

The obstetric phase consists of the following components: collection of maternal clinical data, isolation of AF during delivery, and subsequent short-term storage, laboratory analyses of AF samples and long-term storage for future research.

Clinical data collection

Maternal clinical data to be collected include:

- Standard demographic information (e.g. maternal age, medical history, gravidity, parity, date of delivery, mode of delivery (e.g. vaginal delivery, primary or secondary C-section), duration of pregnancy).
- Maternal complications during pregnancy and/or delivery, including the occurrence of infectious diseases or inflammatory diseases.
- Medication before, during, and after delivery, including oral/intravenous antibiotics use, insulin, and antihypertensives.
- Vaccinations received during pregnancy.
- Clinical data (e.g. signs of intra-uterine infection, including maternal fever (> 38^{O}C), tachycardia, abdominal pain, elevated white blood cell count and/or elevated C-reactive protein, positive blood culture results, fetal tachycardia or other signs of fetal distress in cardiotocography (CTG)).
- Placenta pathology reports (e.g. signs of maternal vascular malperfusion, fetal vascular malperfusion, acute or chronic chorioamnionitis, and villitis of unknown etiology (VUE)).
- Fetal ultrasound reports (e.g. head circumference (HC), AC, EFW, femur length (FL), HC/AC and FL/HC ratios, uterine artery Doppler velocimetry (UADV)).

- Results of any microbial swabs that were taken prior/after delivery (e.g. vaginal swab to assess maternal colonization with GBS).

Relevant definitions: chorioamnionitis and fetal growth restriction

Maternal clinical data will be collected for all participants included in the study. To study the primary and secondary research objectives, participants in the study group of extremely preterm pregnancies will be assigned to three groups: 1) CAM, 2) FGR, and 3) no suspicion of CAM or FGR. The definition used for FGR is the consensus definition by Gordijn *et al.* (2016), which agreed upon three solitary parameters (AC < 3rd centile, EFW < 3rd centile, and absent end-diastolic flow in the umbilical artery (UA)) for early FGR (48, 49). The definition for CAM is more complex. For example, CAM can be defined as a clinical or histopathologic entity (50). For clinical CAM, the definition is based on the clinical signs of CAM (e.g. maternal fever, uterine tenderness, odorous discharge, and maternal-fetal tachycardia) as well as laboratory findings (e.g. leukocytosis) (49). For histologic CAM, the diagnosis is based on histopathologic examination of the placenta assessing the morphologic features of acute histologic CAM, which are characterized by diffuse infiltration of neutrophils into the chorioamniotic membranes (50). In both the Máxima Medical Center and Amsterdam-UMC assessment of the placenta by a pathologist is standard practice, so pathologic reports of the placenta will be available. Since there is no single consensus definition, both definitions will be utilized in the analyses of the samples. All information required to adequate assign participants to the three groups are collected during standardized data collection, as explained in detail above.

Collection of amniotic fluid during (preterm) delivery

AF will be collected from a cohort of obstetric patients delivering their infants extremely preterm by the obstetrician of the participating center. AF can be easily, safely, and non-invasively collected by the treating doctor during C-section or vaginal delivery, posing no additional strain on the obstetric patient.

Vaginal delivery

While AF is often lost during vaginal delivery, inclusion of mothers delivering vaginally is encouraged, if AF can be collected by the obstetric health care provider, for example when the amniotic membranes are manually ruptured in the case of induction of delivery or during routine cervical exams. The different methods of collection are described in detail in **Appendix A**. Noteworthy, when AF is collected during vaginal delivery, there is a high chance of contamination of the sample by the maternal urogenital microbiome. When

assessing the samples with the various microbial and metabolomic approaches, the mode of collection should be taken into account when interpreting the findings.

C-section

The rate of C-section for delivery of preterm infants is between 31.0% and 36.7%. If a C-section is indicated, AF, which is generally discarded as biological waste, can be safely collected in multiple ways. After abdominal and uterine incision, which will be performed according to standard practice, approximately 5 mL AF can be collected in a sterile conical tube. AF can be drained from the amniotic cavity before rupturing of the membranes using a sterile catheter, for example, a blunt plastic canula or catheter, which is inserted through the amniotic membranes and attached to a needleless sterile syringe. If collection of AF before rupturing of the amniotic membranes is not deemed feasible by the obstetrician, AF can be collected in a sterile tube after rupturing of the amniotic membranes. The different methods of collection are described in the Standard Operating Procedure (SOP) in **Appendix A**.

The collection of AF through intact amniotic membranes has been described in various publications, none of which have reported complications (such as damaging the skin or eyes of the neonate or hampering the progress of the C-section). The amniotic membranes are transparent, making the position of the neonate in the uterus clearly visible. A selection of these studies, a description of their cohort, the used techniques for AF collection, and any reported complications is provided below (**Tabel 3**).

The various approaches to collect AF during C-section were extensively reviewed with the obstetric department of the Amsterdam-UMC and the Máxima Medical Center. The staff members of the Amsterdam-UMC prefer using a cannula to collect AF due to previous positive experiences, while the staff members of the Máxima Medical Center prefer to use a container to collect AF after rupturing of the membranes, due to less experiences with cannula collection and a preference of “en caul” delivery of the infant. Both methods are deemed safe according to the available literature. Therefore, the physician performing the C-section can choose their preferred method. Arguably the collection through intact amniotic membranes minimizes contamination risk the most. The success rate of collection of AF (for both C- section and vaginal delivery) has to be closely monitored and evaluated at least yearly. If necessary, the number of intended inclusions may be altered based on the percentage of successful AF collection.

Table 3: an overview of studies that collected amniotic fluid through intact amniotic membranes prior to or during C-section.

Referentie	Cohort	Methode	Sample volume	Complications
(51)	n = 17 samples, healthy term pregnancies.	"Blunt end insertion with a catheter into amnion membrane"	Median 70 mL, range 10-815 mL	1 failed attempt, no complications
(52)	n = 79 samples, GA 37-42 weeks (planned C-section = 35 samples, secondary C-section = 44 samples)	"Direct syringe aspiration at time of cesarean section""	Unknown	5 attempts without enough material, no complications
(53)	n = 47 samples (healthy pregnancies = 40 samples, mean GA 37.9 ± 1.8; preeclampsia = 7 samples, mean GA 36.1 ± 2.6).	"Sterile acupuncture after myometrial incision before incision of amniotic membranes"	Unknown	No complications
(54)	n = 8 samples, GA 37 – 39 weeks	"Collected at cesarean delivery after entry into the uterus prior to rupture of amniotic membranes with blunt plastic cannula attached to 20 mL syringe'"	10 mL	No complications
(55)	N = 50 samples (preterm pregnancies = 25 samples, mean GA 32.6 ± 3.2; term pregnancies = 25 samples, mean GA = 39.9 ± 0.5).	"Puncture of intact membranes using a 22-gauge needle through dilated cervical cavity prior to artificial rupture of dilated cervical cavity"	Unknown	No complications
(56)	n = 24 samples, healthy term pregnancies.	"At time of C-section by aspirating through intact amniotic membranes"	Unknown	No complications
(57)	n = 65 samples, GA 35 – 41 weeks.	"After uterotomy, by aspiration of amniotic fluid through intact amniotic membranes using a sterile 19G needle and 10mL syringe"	Unknown	No complications

All methods of collecting AF during preterm delivery have been described in the designated informed consent form (respectively, E1-E2b_PIF_1_preterm delivery) in both the Dutch and English language. AF samples will immediately be stored at 4-8 ^{o}C. Within 7 days after collection, AF will be aliquotted in 2 mL tubes and stored at -20^{o}C for short term storage before being transferred to -80^{o}C for permanent storage.

IS-pro analysis

The main approach to assess to the microbial composition of AF is the intergenic space profiling (IS-pro) method, which will be conducted in collaboration with the external party inBiome (Amsterdam, The Netherlands). The IS-pro technique utilizes the length and sequence variations within the 16S-23S rDNA interspace region (58). A standardized procedure will be performed. In short, approximately 1 to 2 ml of aliquotted AF is needed for IS-pro analysis. After bacterial DNA isolation, 10 µl of eluted DNA will be amplified using a standardized PCR reaction. Using two appropriate fluorescently-labeled forward primers and three universal unlabeled reverse primers, the phylum-specific interspace region will be amplified. The forward primers, contained within the FIRBAC mastermix 2.0 (inBiome, Amsterdam, The Netherlands) and PROTEO+IC mastermix 2.0 (inBiome, Amsterdam, the Netherlands), are targeted towards, respectively, the *Firmicutes, Actinobacteria* and *Bacteroidetes* phyla as well as the *Proteobacteria* phylum. Following the amplification process, 2.5 µl of the PCR product is prepared for DNA fragment analysis through addition of 20 µl of eMix (inBiome, Amsterdam, the Netherlands). During the final step, IS fragments are separated and detected by capillary gel electrophoresis (CGE) using ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). The resulting data consists of peak profiles with different colors relating to the different phyla and length signatures corresponding to specific species. Preprocessing and preliminary analysis will be performed using IS-pro software (Spotfire).

16S rRNA analysis

In addition to the IS-pro method, 16S rRNA sequencing will be conducted for a subset of the samples for validation purposes. After preprocessing, consisting of centrifugation at 12.000 x g for 20 min at 4^{o}C to pellet the cells, the pellet will be resuspended in 1 mL sterile PBS, while the supernatant is saved and stored for analysis by other techniques. A standardized procedure will be performed, briefly explained below. Approximately 1 to 2 ml of aliquotted AF is needed for 16S analysis. After DNA extraction, the 16s rRNA gene sequences will be amplified from the DNA samples using polymerase chain reaction (PCR) followed by library

preparation, sequencing, and analysis to identify and classify the bacterial strains present in the AF sample. Noteworthy, taking the main pathogens found in AF from existing literature into account, forward primers with adequate coverage of *Ureaplasma* spp. and *Mycoplasma* spp. will be used (59).

(Un)targeted metabolomics

To characterize the metabolic content, all AF samples will be analyzed using liquid chromatography-mass spectrometry (LC-MS) in collaboration with the Department of Laboratory Medicine (Amsterdam-UMC, location AMC, Amsterdam, The Netherlands). Dependent on the technique, up to 2 mL AF has to be available to perform analysis. Prior to analysis the AF samples are centrifuged at 3000xg for 20 minutes to separate the cells from the supernatant, the latter will be used to assess the metabolic content. The metabolite profiles of AF will be detected using an LC-MS/MS system. Either untargeted metabolomics will conducted on the collected samples or targeted metabolomics on a specific subgroup of interest drawn from current literature, for example amino acids and amino alcohols (20).

Storage

The remainder of the AF samples will be frozen at -80^{o}C and kept for possible future studies, in line with the overarching aim of the present study, to increase our understanding of the impact of AF on the development of the fetal GIT and the initiation of the gut microbiome in extremely preterm infants. AF samples can potentially be studied models of the fetal GIT.

Phase 2: the neonatal phase

Neonatal clinical data and daily fecal samples during the first 28 days of life will be collected according to the protocol of the eMINDS study. In short, for all preterm infants born below 28 weeks informed consent is obtained from both parents or legal guardians. Neonatal stool samples are collected daily in the first month of life. Subsequently, the stool samples are stored at -20^{o}C for short-term storage before they are transferred to -80^{o}C for long-term storage until analysis. The following clinical data, as drawn from the eMINDS study protocol, will be collected during the first 28 days of life.

- Standard demographic data, such as sex, gestational age, mode of delivery, birth month, APGAR score (1 and 5 min).
- Information on maternal complications and medication during gestation.

- Neonatal medication (including antibiotics, surfactant, steroids, enema).
- Total duration and type of parenteral nutrition.
- Feeding type (breastfeeding or formula).
- Invasive medical devices, type and duration (including respiratory tubes, peripheral iv's, umbilical arterial or venous line, central venous catheter and/or arterial lines).
- Anthropometrics (including length, weight, and skull circumference).
- Potential diagnosis of Infant respiratory distress syndrome (IRDS), sepsis, intraventricular hemorrhage (IVH), BPD, and modified Bell's staging for NEC will be noted.

Relevant definitions: necrotizing enterocolitis and sepsis

Of particular interest in this study are infants diagnosed with NEC or sepsis. The definition that is used for NEC is the modified Bell's staging (60). All infants with NEC suspected by the treating clinician will retrospectively be reviewed and staged by two experts. At least the clinical, radiographic, biochemical and, in some cases, pathological data will be thoroughly assessed. All NEC cases with modified Bell stage > 2A will be considered a true NEC case. The definition for neonatal sepsis is used is according to the Vermont Oxford Criteria. Early and late-onset sepsis cases are defined as isolation of pathogen from blood culture drawn, respectively < 72h and ≥ 72h postnatally and pathogen-specific antibiotic treatment that was continued for at least 5 consecutive days. An exception to this definition is sepsis caused by coagulase negative *staphylococci* (CoNS), which is often treated with shortened duration of antibiotics and may include removal of (central) line. CoNS sepsis is defined as a positive blood culture with CoNS species with clinical signs of infectious disease and/or a CRP value of at least 10 mg/L within 24 hours of suspicion of disease.

The fecal samples are analyzed using various methods, including IS-pro and 16S rRNA analysis to assess the composition of the gut microbiome and LC-MS to assess the composition of the gut metabolome. For detailed explanation of the eMINDS study, the study protocol can be consulted (reference number: Metc2014.386(A2020.190)).

Reference cohort

While the goal of this study is not to determine GA-dependent changes in the metabolite composition of AF, the composition of AF is highly dependent on the GA and the construction of a database with AF samples from a reference population is necessary to adequately interpret findings on the composition of AF from extremely preterm infants (< 28 weeks). In

the previously referenced studies by Bhatti *et al.* (2022) and Orczyk-Pawilowicz *et al.* (2016) important components of AF have been described in detail over the course of gestation, including the proteome and the metabolome (22, 23). While these studies serve as important reference publications for our reference cohort, we argue that these studies differ too much from our methodology. For example, no samples from very preterm and moderate to late preterm pregnancies are collected (28 + 0 – 36 + 6/7 weeks) and crucial information regarding maternal-fetal health is lacking. For that reason, we argue that setting up our own reference cohort is required for adequate interpretation of the findings regarding the composition of AF in extremely preterm pregnancies.

AF will be collected from early midtrimester (< 23 + 6/7 weeks), very to moderate and late preterm (28 + 0 – 36 + 6/7 weeks), and full-term pregnancies (37 + 0 – 40 + 6/7 weeks). An overview of the samples that will be collected is provided in **Table 1**. In addition to the collection of AF, extensive maternal clinical data will be collected from the EPD to assess whether the sample is collected from a healthy pregnancy or, for example, a pregnancy complicated by FGR. This will be relevant for further analyses of the reference samples explained in more detail in section 10 “Statistical analysis”. No formal power analysis can be conducted due to limited knowledge on this topic. To get an idea of the changing composition over the course of gestation of potential discriminatory metabolites associated with extremely preterm birth, specifically in case of FGR and CAM, we aim to collect approximately 60 early midtrimester, 60 very preterm and moderate to late preterm, and 30 full term AF samples (n = 150). The in- and exclusion criteria are described in detail in section 4 “Study population”.

Prior to collection of AF, the obstetric patient will be informed on the study, ideally by the member of the research team, and informed consent has to be obtained. AF will be withdrawn either transabdominally while monitoring with ultrasound (amniocentesis) or during delivery, as described in more detail in the section 8.3 (Collection of amniotic fluid during (preterm) delivery) and **Appendix A**. If there is a clinical indication to conduct an amniocentesis, a few milliliter of additional AF can be collected for research. In the case of left-over AF, which is not required for clinical diagnostics, the residual fluid can also be used as reference material. Amniocentesis will be performed conform the guideline of the medical center where the collection is taking place, posing no additional stress or risks for the participant of the study. Amniocentesis is generally performed in early midtrimester, between the 16th and 20th week of gestation. All AF samples that can be obtained through amniocentesis before the 24th week of gestation will be included in the reference cohort. After 24 weeks of gestation, amniocentesis is generally not performed anymore as the infant is considered viable and any diagnostic findings do not have clinical implications anymore (for

example, termination of the pregnancy). In the unlikely case that amniocentesis is performed after the 24th week of gestation, this sample will not be included in the reference cohort. For very preterm to moderate and late preterm and full-preterm pregnancies, AF will be collected during vaginal delivery or C-section, similar to the collection in the study group with extremely preterm infants. Noteworthy, the amniocentesis procedure is only performed in the Amsterdam-UMC. Hence, all AF samples in the reference population from the Máxima Medical Center will be collected during vaginal or cesarean delivery. The 150 samples that are included in the reference cohort do not need to be evenly distributed across the two participating centers.

Noteworthy, two separate informed consent forms have been constructed for collection of AF from the reference population as opposed to collection during preterm delivery (respectively, “E1-E2b_PIF_2_delivery reference group” for collection during delivery and “E1-E2b_PIF_3_amniocentesis reference group” for collection by means of amniocentesis). Both forms are available in the Dutch and English language.

8.3.1 Specific criteria for withdrawal

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

8.4 Replacement of individual subjects after withdrawal

Not applicable

8.5 Follow-up of subjects withdrawn from treatment

Not applicable

8.6 Premature termination of the study

Due to the non-invasive nature of this study, we expect that premature termination of the study is highly unlikely. In the case of premature termination, the investigator will notify the accredited METC within 15 days, including the reasons for the premature termination (as described in detail in section 12.5).

9. SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the investigator will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The investigator will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

Due to the non-invasive nature of the study, which is limited to the collection of AF, which is generally discarded as biological waste, AEs or SAEs are not expected.

9.2.2 Serious adverse events (SAEs)

In the unlikely case of SAEs surrounding the collection of AF during delivery, the investigator will report this without undue delay after obtaining knowledge of the events. Since collection of AF is the only procedure in this study, there is no indication for registering SAE's in the neonatal period (such as feeding intolerance or invasive ventilation). The investigator will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life-threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the investigator has first knowledge of the serious adverse events.

9.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Not applicable

9.3 Annual safety report

Not applicable

9.4 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. SAEs need to be reported till end of study within the Netherlands, as defined in the protocol

9.5 Data Safety Monitoring Board (DSMB) / Safety Committee

Not applicable

10. STATISTICAL ANALYSIS

Clinical and demographical characteristics will be analyzed using Statistical Package for the Social Science (SPSS) version 26.0 (IBM, Armonk, NY). When deemed appropriate, either independent t-test (for normally distributed continuous variables), Mann-Whitney U test (for non-normally distributed continuous variables) or χ^2 test (for categorical variables) will be used for the analysis of the clinical and demographical data of both maternal and neonatal data.

10.1 Primary study parameter(s)

For bacterial analysis, relative abundances will be assessed and compared between the groups of interest. Alpha-diversity will be calculated as the Shannon diversity index for each phylum as well as all phyla combined. Beta-diversity will be calculated using analysis of similarity in Bray-Curtis distance. For both bacterial as well as metabolomic data, principle coordinate analysis (PCoA) will be used to assess the variation in the composition of AF between samples and to visualize potential clusters of samples by metadata, specifically for the subgroups of infants exposed to IUI and infants with FGR. To give further insight into the key metabolites and bacterial species that are identified within the study population for FGR and CAM, the levels of these metabolites in the reference cohort will be assessed. The changes in the metabolite composition over the course of duration of pregnancy will be analyzed using mixed-effect models to account for both fixed effects (e.g. time) and random effects (e.g. individual variability). We aim to determine the temporal behavior of the proposed key metabolites in both healthy pregnancies as well as pregnancies complicated with FGR as these are heavily impacted by both duration of pregnancy as well as disease state.

10.2 Secondary study parameter(s)

To determine whether the clinical groups of interest can be distinguished from each other using the principle components an independent t-test will be conducted. To check for potential confounders (e.g. GA, BW, antibiotic use, etc.), the association between potential confounders and outcomes of interest will be assessed. If a potential confounder is identified, the association between the confounder and the content of interest will be tested by Fishers Exact test (for categorical variables) and ANOVA (for continuous variables). Logistic

regression models will be used to adjust for confounding variables. To assess the composition of AF in relation to the neonatal gut microbiome (e.g. Shannon diversity, microbial genera) for both microbial and bacterial components, associations will be tested by fitting generalized linear models of the microbiome feature on each component of interest, adjusting for confounding variables. Noteworthy, multivariate regression analyses require an adequate sample size and should be used cautiously due to the risk of overfitting and reduced statistical power, which can lead to biased findings.

10.3 Other study parameters

Not applicable

10.4 Interim analysis

Not applicable

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

The study will be conducted according to the principles of the Declaration of Helsinki (version, date, see for the most recent version: www.wma.net) and in accordance with the Medical Research Involving Human Subjects Act (WMO).

11.2 Recruitment and consent

The study will be conducted in the Amsterdam-UMC (Amsterdam, the Netherlands) and the Máxima Medisch Centrum (Veldhoven, the Netherlands), commencing in 2024 until the desired amount of mother-infant pairs as well as participants of the reference cohort have been included. A member of the research team, including research nurses, will approach the obstetric patients eligible for this study. Besides receiving a participant information form (PIF), patients will receive an oral explanation in either Dutch or English on all aspects deemed necessary to give informed consent. Information on collection of AF during delivery and use of personal and clinical data by researchers will be discussed. If it is not possible to obtain informed consent by a member of the research team, for example when the delivery occurs at night or on the weekends, the obstetric patients will be approached and informed by an obstetric doctor. If feasible, the informed consent will be asked by another obstetric doctor rather than their primary obstetrician.

As preterm delivery is often unexpected and requires acute handling with extensive counseling on various topics to expecting parents, both maternal and neonatal, it may not

always be possible to immediately obtain written informed consent prior to the collection of the AF sample. Due to the non-invasive and safe nature of the study, if it is not possible to obtain full written informed consent prior to delivery, AF may be collected during delivery by the obstetric doctor. However, written informed consent must be obtained from the participant prior to commencing the study, which entails the collection of clinical data or microbial or metabolic analysis of the AF sample. At any time during the study period, the study participant can refuse further continuation of the study and the study will be stopped immediately, which means that no clinical data may be collected anymore and stored AF samples will not be analyzed in the laboratory Stored AF samples will be destroyed unless study participant agrees with further analysis of the samples.

Receiving informed consent from both parents and/or legal guardians regarding the collection of neonatal clinical data and neonatal fecal samples will occur according to the existing protocol of the eMINDS study (reference number: Metc2014.386(A2020.190)). In addition to the form that is signed by both parents and/or legal guardians to consent to participation in the eMINDS study, they will give additional consent to link the data from the eMINDS study to the data from the AMFIBIE study.

11.3 Objection by minors or incapacitated subjects

Not applicable

11.4 Benefits and risks assessment, group relatedness

Through participation in this study, participants can contribute to improving our understanding of the impact of AF composition on the development and colonization of the premature intestines through consenting to the collection of AF during delivery, which would otherwise be discarded as medical waste. AF can safely and non-invasively be collected during the standard care procedures of obstetric patients, without interfering with daily clinical care. Similarly, neonatal feces are normally disposed of when changing diapers and can easily be collected without interfering with standard care of the preterm infant. When deemed feasible by the obstetric doctor, the obstetric patients will receive counseling from their caregiver regarding participating in the study prior to giving birth to their preterm infant.

Part of obtaining consent is informing the obstetric patients delivering their infants extremely preterm about potential neonatal morbidities, such as NEC and sepsis, which may arise during the neonatal period. General practice already consists of a consultation between the obstetric patient and a neonatologist when extreme preterm birth is expected, providing, among other information, an explanation on serious complications such as NEC and sepsis.

Hence, no additional stress is posed on the obstetric patients when informing them prior to delivery about this study. However, thorough and empathetic counseling by the physician obtaining informed consent is always required.

A potential theoretical risk includes delay of delivery due to collection of AF. However, these risks are deemed minimal due to the non-invasive nature of collection of AF. The treating obstetrician is responsible for making a well-informed decision on determining whether it is feasible to safely isolate AF. If deemed necessary by the treating physician due to urgent medical reasons, the subject can always be withdrawn from the study. Additionally, as is the case with all research where access is provided to personal and clinical data, there is a small chance that confidentiality could be compromised. However, adequate precautions will be taken to minimize this risk.

11.5 Compensation for injury

The investigator has a liability insurance which is in accordance with article 7 of the WMO. Because of the non-invasive observational character of the study, no additional risks are expected from participation in this study. Therefore, the sponsor has obtained dispensation from the statutory obligation to provide insurance for the subject participating in the study, because participating in the study is without risks and therefore no insurance for the subjects is necessary

11.6 Incentives

Not applicable

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

Every obstetric patients and their infant participating in this study are assigned a unique study number. The key for description is kept at the center of birth. Only the main researchers are able to access this encrypted information. All pseudo-anonymized data is recorded into an electronic case report form on CASTOR EDC platform. Collection of AF samples will be stored at -80^{o}C, until further analyses. The same is true for feces, in line with the eMINDS study (reference number: Metc2014.386(A2020.190)).

Data will be collected and processed in accordance with the General Data Protection Regulation (EU) 2016/679 and General Data Protection Regulation (GDPR). Data processing will be done coded, as the researchers can only identify the subject by use of a key that is solely available to the local research team. Data will be collected using an accredited electronic data capture system (CastorEDC), which is an application system that enables the collection and clean-up of study data using the Internet. All data will be stored for 15 years in a secured database on a secured computer, to which only the investigators will have access. Coded human material that is identified by the same study code as mentioned above, will be stored temporarily at the participating center after sampling.

Depending on the inclusion rate, samples may subsequently be stored at the study site (Amsterdam UMC), before being shared with an external laboratory (InBiome, Amsterdam), where further analyses will be performed. We intend to perform microbial and metabolomic analyses on a yearly basis, but this may depend on the inclusion rate and could equally affect storage time at either the participating center or at the sponsor site. No other data (e.g. name, date of birth, health data) will be shared with InBiome. Personal data will not be inserted in one of the machines or programs involved in any of the analyses. After the analysis on the collected AF at InBiome, isolated bacterial genetic material will be stored for 5 years locally to allow quality control only within the context of this research project. The results of the analyses on the AF will not be shared with the care providers involved in the treatment of the individual study participants. Coded data can be shared with international authorities (e.g. FDA) when this is requested by these authorities.

12.2 Monitoring and Quality Assurance

Monitoring of the coordinated investigator will be done by a GCP-certified person otherwise not involved in the study. The monitor will provide a yearly written report to the coordinated investigator after each visit including a summary of the significant findings, deviations and deficiencies, conclusions, actions take, or recommendations to secure compliance. The coordinated investigator will run consistency checks on a monthly basis and produce queries to be resolved by the local investigator(s). The final database will be obtained after the resolution of all queries. Due to the nature of our study and the minimal risk associated with the collection of amniotic fluid, the establishment of a Data Safety Monitoring Board (DSMB) is not deemed necessary.

12.3 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC of the Máxima Medical Center that gave a favourable opinion. Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the investigator.

12.4 Annual progress report

The investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, number of subjects included and number of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

12.5 Temporary halt and (prematurely) end of study report

The investigator will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined one the estimated amount of amniotic fluid samples is collected.

The investigator will also notify the METC immediately of a temporary halt of the study, including the reason of such an action.

In case the study is ended prematurely, the investigator will notify the accredited METC within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator will submit a final study report

with the results of the study, including any publications/abstracts of the study, to the accredited METC.

12.6 Public disclosure and publication policy

Publication will be in accordance with the basic principles of Central Committee on Research Involving Human Subjects (CCMO) statement on publication policy. Amsterdam UMC has full clearance to publish the results and no constraints are in place in light of the collaboration with InBiome or any foreign participating centers.

13. STRUCTURED RISK ANALYSIS

Not applicable

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