**PrePhage**

# A Research Protocol:

# PrePhage – Faecal bacteriophage transfer for enhanced gastrointestinal tract maturation in preterm infants

**PrePhage**

# 1. General information

## 1.1. Protocol title, version number, date, list of abbreviations

Title: PrePhage - Faecal bacteriophage transfer for enhanced gastrointestinal tract maturation in preterm infants

VEK submission number: 78259

Version: Version 02

Date: 27/07/2021

**List of abbreviations:**

AE: adverse events

CMV: Cytomegalovirus

DBS: dried blood spot

DSMB: data safety monitoring board

FFT: faecal filtrate transfer

FMK: det fælles medicinkort

FMT: Faecal microbiota transplantation

GA: gestational age

GI: gastrointestinal

GM: gut microbiota

HAV, HBV, HCV: Respectively Hepatitis A, B, and C virus

HIV: Human immunodeficiency viruses

IBD: inflammatory bowel disease

ITT: intention-to-treat

NEC: necrotizing enterocolitis

NICU: neonatal intensive care unit

VEK: De Nationale Videnskabsetiske Komitéer

rCDI: recurrent clostridoides difficile infection

RCT: randomized control trial

SAE: serious adverse events

SSI: Statens Serum Institut

UCPH: university of Copenhagen

VEGF: vascular endothelial growth factor

**PrePhage**

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### 1.2. Good clinical practice

We will consult the good clinical practice board of University Hospital of Copenhagen before initiation of the study.

### 1.3. Time plan

| | ASAP | 2022 | 2023 | 2024 | 2025 | 2026 |
|---|---|---|---|---|---|---|
| **Start invitations** | | | | | | |
| **Donor screening** | | | | | | |
| **Clinical study** | | | | | | |
| **Followup** | | | | | | |

### 1.4. Signature

Lise Aunsholt:

Gustav Riemer Jakobsen:

### 1.5. Study Location

The clinical trial takes place at RH NICU. If the participants wish, some of the data collection can be performed at their homes.

Biochemical and microbiological analysis is performed at the Department of Clinical Biochemistry at both RH and Odense University Hospital, as well as Statens Serum Institute (SSI).

Analysis of the fecal microbiota will take place at Microbiology and Fermentation, at UCPH. A portion of the donated feces will be used in a piglet study at Comparative Pediatrics and Nutrition at UCPH.

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## 2. Summary

This pilot study aims to investigate if faecal filtrate transfers (FFT) to preterm infants is safe and tolerable. To investigate this, we will recruit 10-20 donor infants and their mothers from time of delivery, and both will be subjected to a novel screening program including blood, urine, breastmilk, faecal screening and standard clinical investigation. Donor faecal samples will be collected from time of birth and with variating intervals for consecutive 3 years for 3 purposes: 1) to conduct safety studies in preterm piglets before transfer to preterm recipient infants, 2) to conduct FFT to preterm infants, and 3) to map normal microbiota development in healthy infants.

The faeces used for donation will be collected between 2-4 weeks after birth. After one year, donated feces will be released for FFT to preterm, but only if the donor infant at this time has been healthy and normally developed. Donors are followed up for consecutive 3 years after birth. Maternal faecal samples will be compared to infant samples, to investigate maternal to infant transfer of microbiome. 20 preterm infants with gestational age (GA) between 26+0 – 30+6 weeks + days, are block randomized to either FFT or saline placebo within 24 hours after birth and the following 3 days, in total 4 donations. The recipients are clinically and biochemically close monitored by attending staff and the group of research team according to best clinical practice and predefined clinical observation. The recipients are followed up for consecutive 3 years to evaluate potential late side-effects and to monitor change in faecal microbiome after transplant or placebo.

The primary endpoint is to assess safety of FFT to preterm infants with expected no increase in necrotizing enterocolitis (NEC), sepsis and death in the intervention group. The secondary endpoint is to assess if, FFT treatment will reduce incidence of feeding tolerance and improve healthy gut development in recipient preterm infants. We expect to find FFT safe and with less cases of NEC and sepsis, though not powered to show the latter. We aim to follow up with a double-blinded multicenter randomized control trial, powered to document our hypothesis, that when colonizing with a healthy microbiome, we can decrease incidence of NEC in premature infants.

# 3. Background

**Preterm birth**

Preterm births below gestational week 37 occur in 11% of all pregnancies worldwide and in 6% of Danish, with an incidence of very preterm pre-term births (< 32 weeks of gestation) of 1% in Denmark [1]. Survival rates have improved over the years, due to improved obstetric and neonatal treatment, but a substantial fraction very preterm infants still experience subsequent comorbidities.

**Necrotizing enterocolitis**

Serious infections are common in preterm newborns due to aberrant microbial exposure and impaired host defense mechanisms. While the majority of these patients survive, many are left with chronic handicaps due to inflammation-related injuries including their developing brain [2]. Necrotizing enterocolitis (NEC) is among the most prominent life-threatening infection of preterm newborns. NEC is a severe bowel inflammation associated with imbalanced gut microbiota (GM) development and impaired gut barrier function. Mortality is high (~30%), and treatment often involves surgical removal of the necrotized intestine with life-long consequences [3]. The incidence of NEC in the Copenhagen region is at present 9% (for infants born <1500g), with a maximum risk of 20.5% for infants with birth weight 500-599g [4,5].

**NEC and the gut microbiome**

Microbial colonization of the gut begins immediately after birth. Gestational age at birth, is the major determinant of the gut microbiome (GM) colonization pattern, exceeding the effects of both delivery mode, nutrition and antibiotic treatment [6,7]. The GM of very preterm infants is dominated by Bacilli, Clostridia and Gamma-proteobacteria, with lowered bifido-bacteria presence, but with substantial intra-individual variability and significant fluctuations over time [8–10]. NEC has been linked to GM dysbiosis and NEC related GM patterns have been identified [8,10,11].

**Bacteriophages and the early life gut virome**

Viruses are as abundant as bacteria in the human gastrointestinal (GI) tract. Of these, only few infect human cells, whereas the vast majority attack and kill bacteria. Such viruses are called bacteriophages (phages) and are specialized in targeting bacteria in a highly host specific manner, shaping microbial community structure, and maintaining bacterial diversity [12].

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The role of phages during early life microbiome establishment is poorly understood, but recent findings support that they play important roles in shaping the GM [12,13]. Already during the first month of life the term infant gut is colonized by eukaryotic viruses such as Adenoviridae, and Annelloviridae as well as vast amounts of phages, with bacteria and phages being present in a ratio of 1:1 in the infant gut [13,14]. In adults virome dysbiosis plays a pivotal role in the development of inflammatory bowel disease (IBD) [15], but the role of virome dysbiosis for diseases manifesting in early life is unknown.

**Microbial therapies for NEC prevention**

Probiotics are administered to preterm infants at many (but far from all) hospitals to prevent NEC and Meta-analysis supports implementation of probiotics as NEC prophylaxis in routine care of preterm infants [16]. However, the NEC incidence reduction is rather modest and the large English PiPS trial did not find any beneficial effect of probiotic administration to preterm infants with respect to NEC [17,18].

Faecal microbiota transplantation (FMT) has recently gained much interest due to efficient correction of GM dysbiosis and clinical improvement in patients suffering from recurrent Clostridioides difficile infection (rCDI) [19]. In a preterm pig NEC model we recently showed beneficial effects of FMT on gut dysbiosis and NEC incidence [20]. However, FMT also had adverse effects, increasing the risk of bacterial translocation, inner organ colonization and sepsis. Hence, FMT based on crude donor feces should not be tested in preterm infants. Despite of that, it should be noted that in a study in Helsinki, Finland, mother-to-infant low-dose FMT for term infants delivered by Caesarean section showed no adverse events and a significant improvement in gut microbiome maturation [21].
Interestingly, it has been shown that removal of all intact bacteria from a donor faecal slurry by sterile filtration (leaving only viruses and small-size molecules) before administration to rCDI patients has similar efficacy as regular FMT [22]. Inspired by this, we tested donor faecal filtrate transfer (FFT) in comparison to regular FMT in the preterm pig NEC model. Orally administered FFT was superior to conventional FMT, consistently preventing NEC and reducing gut permeability with no obvious adverse effects [23]. Our data indicate that the donor-derived phages specifically inhabit the viscous mucus layer, where they eliminate NEC-related pathogens.

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The term infant gut bacterial microbiome undergoes significant fluctuations during the first years of life [24], and the optimal age of a faecal donor for FFT to preterm infants is unknown. However, a few weeks into life the GM of term infants' transits from a very dynamic, unstable phase into a more stable. Colonization of the GI-tract by bacteria begins in the first few weeks and a primitive flora is formed at 1 month of age [13]. Further, in piglets we have observed that 10-day old piglets' function well as donors for FFT to preterm piglets [23]. Based on our findings from the preterm pig model, we hypothesize that 2-4 weeks after birth is optimal time for donation.

Given these encouraging preliminary findings, PrePhage aims to develop a phage-based prophylactic treatment, assess its safety and NEC-preventive efficacy in preclinical piglet experiments, and ultimately test its safety and feasibility in a phase 1 clinical trial on preterm infants in a danish neonatal care unit.

## 4. Objectives, hypothesis and feasibility

The primary purpose of this study is to demonstrate the safety and investigate the feasibility of administering FFT to preterm infants.

At first, a carefully designed donor screening program is developed, selected donors are screened, and faecal samples collected. Donors and the mothers of donors are followed for 3 years to ensure that the donor is and remains healthy.
Faecal samples are thoroughly screened for known pathogens, filtrated and manipulated to remove known eukaryotic vira associated with risk of infection. The product is then transplanted to preterm piglets. If no adverse effects are observed, validated FFT will be transplanted to preterm infants in a progressive clinical pilot study.
The study will be blinded. Statistical and laboratory analyses will be performed blindly.

**Stop/go decision**

The decision to continue to the clinical pilot trial depends on the results of an in-vivo evaluation of safety in preterm piglets. Pre-term piglets are given human FFT, followed for 2 weeks and euthanized. The safety in piglets is evaluated in terms of growth impact, feeding tolerance, stool patterns, gut histopathology, complete blood count, hepatic and renal function tests as well as blood culture and arterial blood gas analysis in case of suspected infection. If the study gives

any indication that it would be unsafe to continue the trial, the study will be either discontinued or a contingency plan will be developed and submitted to “De Videnskabsetiske Komitéer” (VEK) before continuation.

*Our primary aim* is to demonstrate that administering FFT from donor term infants is safe and tolerable when used from the first 24 hours of life in preterm infants born between 26+0 and 31+6 weeks of gestational age, hence the pilot trial.

*Our secondary aim* is to examine if FFT can improve gastrointestinal outcome for preterm infants in terms of:

- Reduced time to development of balanced gut microbiome and virome
- Increased feeding tolerance
- Reduced time to full enteral feeding
- Improved infant thriving
- Reduced incidence of NEC

*The tertiary purpose* of this trial is to map development of faecal virome over time and to determine donor characteristics for optimal outcome of FFT, hence the donor study.
We will map the development of the infant virome in conjunction with child feeding patterns, GI-development and thriving as well as in relation to maternal microbiome.

Finally, we wish to examine a broad range of explorative outcomes to study mechanism-of-action of FFT as well as effects of differences in gut microbiome and virome on normal gastrointestinal development and lastly to identify effect modifying factors.

- Faecal calprotectin
- Key inflammatory markers
- Total DNA and virus-like particles will be extracted from faecal samples
- Absolute bacterial abundance of faecal samples
- Determination of gut metagenome in faecal samples using shotgun high-throughput sequencing

As the clinical study is a feasibility study, with only 10 infants receiving FFT and 10 infants receiving placebo, sample size is not likely to be large enough to demonstrate above effects. Therefore, it is our intention to follow up with a larger randomized clinical trial, as well as develop a biobank for use in further studies if safety and tolerability is confirmed in this study.

**Feasibility**

Motivations to participate in clinical trials are altruism along with the hope of personal gain [25]. For infants, the informed consent is given by the parents, who can be in a conflict of interest since their role is to protect and care for the infant.

To gain more knowledge about motivation to participate in a clinical trial like PrePhage a nurse-run feasibility study of FFT to preterm infants will be carried out. Using ethnographic fieldwork in the NICU and semi-structured interviews with parents and the NICU staff, their response and reactions toward the study will be investigated.

The aim is to generate qualitative and quantitative data to identify factors of importance for later larger clinical trials.

Feasibility outcomes:

1) Consent rate (willingness to participate)
2) Adherence sample collection
    a. Proportion of missing blood and fecal sample
    b. Proportion of incomplete questioners
3) Qualitative evaluation of source documentation and procedures
    a. Informed consent procedures
    b. Data collection procedures and tools
    c. Sample collection of biological material (sample collection, freezing)

Based on data material, combined with ethnographic fieldwork in the NICU, the group of coordinating investigators will create an overview (see below) to help the clinically responsible and the project leader (in correspondence with the DSMB) to decide whether it is feasible to go ahead with the planning of a larger RCT based on results and practical experience from the pilot study.

To generate knowledge about informed consent we plan to do interviews of parents both given consent and declined consent (informed consent will be collected before). To generate knowledge about data collection procedures, handling of the FVT product, procedures for

reporting adverse events and sample collections we plan to do an ethnographic fieldwork of the staff members involved in the project. In case of concerns corrections will be made before a larger RCT is initiated.

| | 🟢 | 🟡 | 🔴 |
|---|---|---|---|
| Consent rate | >70% | 50-70% | <50% |
| Proportion of completed blood and faecal samples | >50% | 20-50% | <20% |
| Proportion of incomplete questioners | <20% | 20-50% | >50% |

Pre-defined criteria used to decide whether, or how, to proceed with a later, larger RCT. Green color: fully acceptable and feasible. Yellow color: feasibility concern, changes to be decided. Red color: serious feasibility concerns. Clear actions must be taken before a larger RCT is planned.

# 5. Donor study

## 5.1. Design and recruitment

10-20 eligible donor infants will be recruited while intrauterine for 3 purposes: 1) to donate feces for a safety trial in piglets, 2) to donate feces for the pilot FFT trial 3) to study development of gut virome and microbiome over time. The piglet safety trial is described in a separate protocol, and thus not described in detail in this protocol.
Infants included will be followed up one year before donation can be executed and consecutive for three years (2022 – 2024). Faecal samples and questionnaires will be collected throughout the period. A novel donor screening program, including blood, faecal and urine samples from up to 20 term infant donors is applied.

The mothers of infants will be recruited from in-patient maternity clinics in The Capital Region of Denmark (Region Hovedstaden) and will be included in the study, for three purposes. 1) To screen for preventable transmittable disease. 2) To compare development of the infant faecal microbiome to the microbiome of their mothers. 3) To identify external factors that might lead to

unforeseen changes in donor microbiome development. They will be followed up for 1 year or until the child is no longer breastfed, whichever comes first. They will be asked to answer questionnaires, provide blood- milk- urine- and faecal samples as well as provide access to their obstetric and medical record (Fælles Medicin Kort (FMK)).

Mother and child cannot be enrolled separately. If either mother or child does not fulfill inclusion/exclusion criteria, neither can participate.
Some maternal samples may be collected before birth. If complications arise during or after birth so they no longer fulfill inclusion criteria, mother and child are excluded from the study and all data and biological material collected up till this exclusion will be destroyed.

### 5.2. Inclusion criteria for donors

- The donor must be of term (>37+0 weeks GA, < 41+0 weeks GA),
- Be born vaginally with no maternal pre-birth infection,
- Be exclusively breastfed un till fulfilled donation at 4 weeks of age,
- Have no known predisposition for disease.

### 5.3. Exclusion criteria for donors

- Antibiotic exposure before collection of faecal material for donation,
- Disease between time of birth and collection of feces for donation,
- Major congenital anomalies or birth defects, perinatal asphyxia, need for mechanical ventilation or cardiovascular support before time of inclusion.
- Positive stool sample for *C. difficile* toxin, parasites or other pathogens
- Positive HIV, HBV, or HCV or CMV
- Parents who do not want to know the HIV, HBV or HCV status of the child

### 5.4. Inclusion criteria for mothers of donors

- Women aged 18-45 and currently healthy
- No continuous medical consumption with effects on microbiome
- Non-smoking
- Ability to give informed consent

### 5.5. Exclusion criteria for mothers of donors

- Known or high risk of infectious disease such as HIV, HBV, or HCV
- Positive CMV IgM during pregnancy
- Positive stool sample for *C. difficile* toxin, parasites or other pathogens
- Systemic antibiotic treatment < 1 months prior to study
- New tattoo < 1 month prior to study
- Risky sexual behavior
- Gestational diabetes
- Family history of inflammatory bowel disease

### 5.6. Primary endpoints

Characterization of faecal virome and microbiome over time in the term infant to map early gut development and relation to feeding patterns, growth, normal infant development and association to maternal faecal microbiome.
Further we find it important to identify if any donors develop serious gastrointestinal disease (not including infectious diarrhea) during the study period, to ensure their feces is not used for human FFT.

### 5.7. Secondary endpoints

Clinical evaluation of normal development, anthropometrics from birth to end of observation (3 years of age), time to establish breastfeeding, length of hospital stay related to birth, days to regain birth weight, stool characteristics and frequency (combining Amsterdam Stool Scale [26] and Diapered Infant Stool Scale [27] at first, switching to a combination of Bristol stool scale and Diapered Infant Stool Scale after full solid nutrition), feeding pattern (feeding intolerance, frequency, type of nutrition,) and time switch from fluid to full solid food as well as frequency of infection from birth to end of observation.

### 5.8. Overview screening, data collection and fecal sample collection, donor study

Potential donors are screened at Rigshospitalet according to age-adjusted international standards for FMT treatment [28,29] as well as clinical guidelines [30]. Mothers of potential donors

are likewise screened at Rigshospitalet according to adjusted international standards for FMT treatment [28,29].

Donors are screened before sampling for donation, then donor stool is quarantined for one year till successful first year follow-up. Following a flawless screening and normal development of the donor the collected stool is released for use in the human safety study. Our preclinical experiments suggest that the NEC-reducing efficacy of FFT is maintained despite 2-3 years storage of donor stool at -80°C.

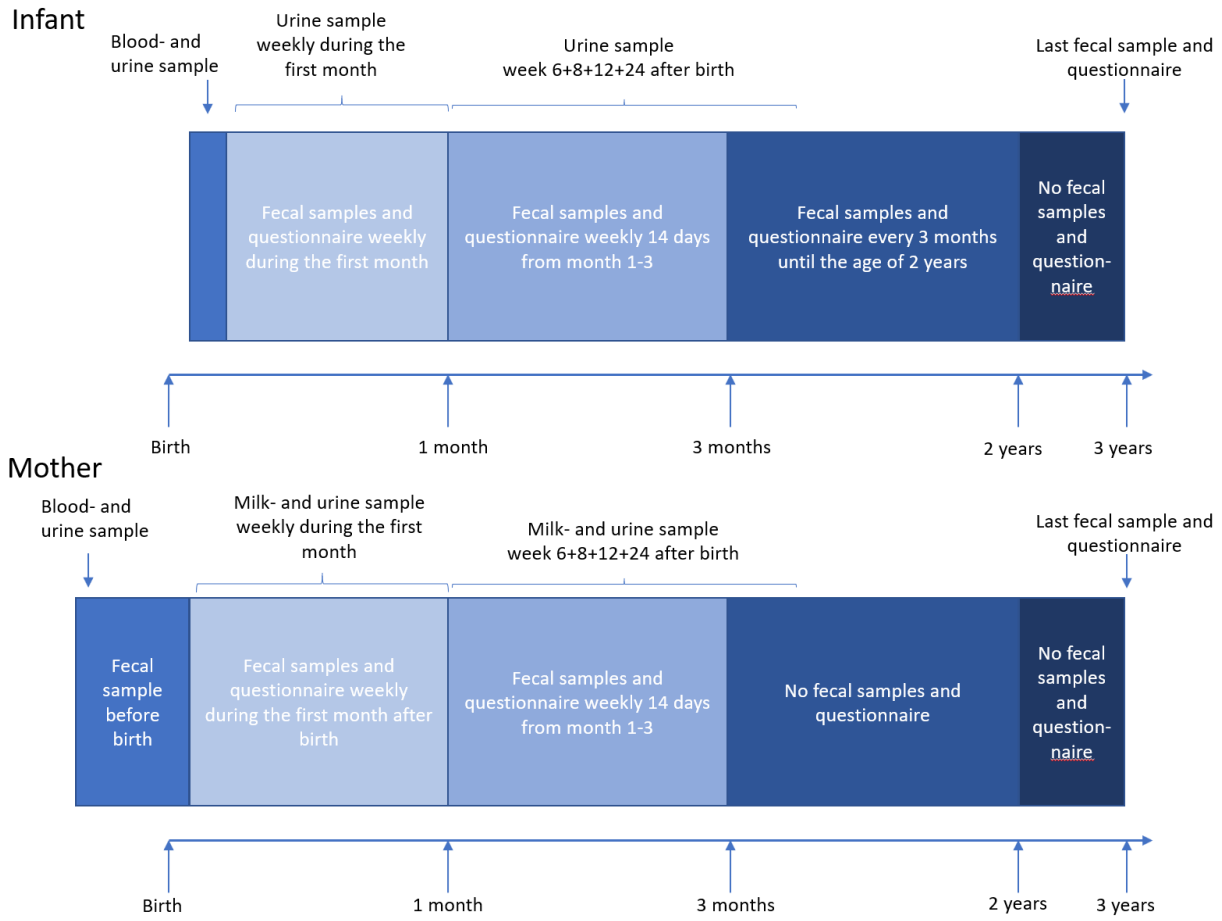


*Figure 1 Overview of data collection, donor study*

### 5.8.1. Donor infant screening and data collection

- Blood sample for screening is taken at between birth and 14 days:

- Serological test for hepatitis A (HAV-IgM), B (HbsAg), and C (anti -HCV); HIV-1 and HIV-2 (anti-HIV-1,2); syphilis (screening: WR and RPR), CMV (CMV-IgM and CMV-IgG) and EBV (VCA IgM, VCA-IgG and EBNA IgG), as well as NAT screening for HIV, HBV, and HCV.
    - HbsAg, Anti-HCV, HIV-1 and 2, NAT-Screening for HIV, HBV and HCV will be performed on dried blood spots (DBS) on the PKU at Department of Clinical Biochemistry at Odense University Hospital
    - CMV will be analyzed as DBS from the PKU at Statens Serum Institut (SSI)
    - EBV antibodies as well as syphilis reactions analyses are currently neither available as capillary blood samples nor analyses on dried blood spots.
        - We are in correspondence with collaborators to develop a possibility for this before initiation of the trial. If this proves impossible to do, we will omit these samples from the infant. The probability of the infant having syphilis while the mother does not (as she is screened at inclusion) is extremely low. In terms of screening for EBV, it is improbable, that the donor infant will contract and become infected the virus without displaying symptoms of it in the 2-4 weeks after birth, where feces for donation is collected. Therefore, we do not believe the benefit of this screening outweighs the discomfort of taking a venous blood sample, nor can we justify drawing the large blood volume currently needed to perform the analysis.
- Blood samples will be collected as capillary samples by trained personnel at RH NICU.
- Donor infants are expected have 80 ml of blood/kg and to weigh at least 3000 g, thus having a total blood volume of at least 240 ml. Current regional guidelines advise that blood volume taken at a given time is not to exceed 1% of total blood volume and 3% over a week [31].
- The total volume will be 2.45 ml, 1 % of minimally expected blood volume in total.

- Nasal swab: if donor infants are symptomatic of an upper respiratory tract infection at sample collection or clinical examination, a nasal swab for RS virus as well as influenza

A and B virus will be performed

- *Urine sample* taken at inclusion and week 2, 3, 4, 6, 8, 12 and 24 to screen for CMV
    - Urine samples are collected using a urine collection bag, as they are exclusively used to screen for CMV
- *Faecal samples, growth measurement and:* weekly during the first month, every 14 days from month 1-3, every 3 months until the age of 2 years, once at the age of 3 years.
    - Faecal samples are collected from diapers. If donor is potty-trained, they will be provided with standard collecting kits. Feces is removed from the diaper/potty, as soon as possible after defecation. As soon as removed, feces are stored in a container and kept until picked up by research team at home. During week 2-4, feces are divided in to 3 at the hospital, two small proportion and one larger. Feces is then stored in containers marked with each donor's ID, until used for 1) faecal sample screening as described beneath, 2) testing in preterm piglets and 2) donation for preterm infants.
    - Faecal samples collected from week 2-4 after birth are potentially used for transplantation and are screened for:
        - *Clostridioides difficile, Salmonella* spp., *Shigella* spp., *Campylobacter coli / jejuni, Yersinia enterocolitica, Aeromonas* spp.
        - Diarrhea-causing *Escherichia coli* (E. coli): (Verocytotoxin producing *E. coli* (VTEC), Enteropathogenic *E. coli*). Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), and Attaching and Effacing *E. coli* (A / EEC)
        - Intestinal pathogenic viruses: *Adenovirus, Atrovirus, Sapovirus, Enterovirus, Parechovirus, Rotavirus* and *Norovirus*; intestinal pathogenic parasites: *Entamoeba histolytica, Cryptosporidium parvum/hominis, Giardia lamblia* and worms
        - Multiresistant bacteria: Extended-spectrum beta-lactamase (ESBL) producing *E. coli*, *Klebsiella pneumoniae* and *Proteus mirabilis,* Carbapenemase-producing organism (CPO), vancomycin-resistant enterococci (VRE)
        - In addition, they are tested for *Helicobacter pylori* antigen, SARS-CoV-2 RNA, as well as RS-virus and Influenza A and B virus

- Faecal samples collected during week 2-4 are prepared for FFT to preterm piglets and preterm infants, as described in section 6.9
- Faecal samples collected outside week 2-4 are only used for microbial characterization.
- Gut microbiome and virome are analyzed as described in section 7.
- At the first donation, approx. 10 mL of faeces will be stored. These are stored at -80 ° C in case a recipient is later diagnosed with known or currently unknown infectious disease, where it is desired to investigate whether the donor material contained the microbiological agent.
- Screening of faecal samples will be performed at RH Department of Microbiology

- *Questionnaires:* are performed at the same time intervals as faecal samples. Questions are arranged in 5 domains: stool characteristics, feeding pattern, infection and, and behavior (Questionnaire included in appendix 7)
- *Clinical examination:* at inclusion, at 2 weeks, at 4 weeks, 6 months, 1, 2 and 3 years
  - Clinical examination is performed according to best clinical practice to ensure normal development of the infant. The clinical examinations will be documented in the patient record.
- *Access to patient record and FMK*
  - FMK access is required throughout the data-collection period to adjust for any antibiotic use, when analyzing composition of faecal mikrobiota. Further, time since last antibiotic treatment is required, to adjust for impact on faecal microbiota. All medication that potentially could affect the flora of faeces must be identified via FMK.
  - Patient record access is required throughout the data-collection period to identify any donors who develop significant gastrointestinal, autoimmune or acute diseases that would prohibit the infant from being a donor. (i.e., inflammatory bowel disease or major congenital disease requiring surgical treatment, diabetes). Records will be accessed in conjunction with the clinical examinations.

#### 5.8.2. Maternal screening and data collection

- *History at inclusion:* ethnicity, number of siblings in the household, socioeconomic status (level of education and current income), smoking

- *Blood samples:* taken at inclusion for screening purposes, lipid profile, Hba1c as well as complete blood count (i.e. leukocytes, differential count, platelets, hemoglobin), immunoglobulin A (IgA) and serological test for hepatitis A (HAV-IgM), B (HbsAg and Anti-Hbc), and C (anti -HCV); HIV-1 and HIV-2 (anti-HIV-1,2); syphilis (screening: WR and RPR), CMV (CMV-IgM and CMV-IgG) and EBV (VCA IgM, VCA-IgG and EBNA IgG), as well as NAT screening for HIV, HBV and HCV.
    - All maternal serological screening will be analyzed as venous blood samples at RH Department of Microbiology
- *Urine samples* at weeks 1, 2, 3, 4 to screen for CMV. *Milk samples* at weeks 1, 2, 3, 4, 6, 8, 12 and 24 to screen for CMV. Milk samples are collected until cessation of breastfeeding.
    - Both urine and milk samples are collected according to standard procedure
- *Faecal samples:* before birth, weekly during the first month after birth, at 6 months and 12 months.
    - After birth faecal samples are used for microbial characterization. Gut microbiome and virome are analyzed as described in section 7
    - Faecal samples before birth are testet for *Clostridioides difficile*, *Salmonella* spp., *Shigella* spp., *Campylobacter coli* / *jejuni*, *Yersinia enterocolitica*, *Aeromonas* spp., diarrhea-causing *Escherichia coli* (*E. coli*): (Verocytotoxin producing *E. coli* (VTEC), Enteropathogenic *E. coli*). Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), and Attaching and Effacing *E. coli* (A / EEC); intestinal pathogenic viruses: *Adenovirus, Atrovirus, Sapovirus, Enterovirus, Parechovirus, Rotavirus* and *Norovirus*; intestinal pathogenic parasites: *Entamoeba histolytica, Cryptosporidium parvum/hominis, Giardia lamblia* and worms; multiresistant bacteria: Extended-spectrum beta-lactamase (ESBL) producing *E. coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*, Carbapenemase-producing organisms (CPO), vancomycin-resistant enterococci (VRE). In addition, the samples will be tested for *Helicobacter pylori* antigen and SARS-CoV-2 RNA.

- *Questionnaire:* Monthly during study period until the child is primarily eating solid foods. Questions are asked regarding diet, smoking, alcohol intake, gastrointestinal symptoms, stool characteristics, and use of medicine (appendix 7)

- *Obstetric record and FMK:*
    - Before inclusion: Access to obstetrical information and FMK, is a allowed to ensure the participant is eligible for inclusion, according listed inclusion criteria..
    - At inclusion and until the mother stop breastfeeding the child: Access to FMK to adjust for any antibiotic use or other drugs that could affect the faecal microbiota. Knowledge is used when analyzing data of microbiota and especially in comparison between mother and infant microbiota
- *Throat swab:* to test for SARS-CoV-2 and Respiratory Syncytial Virus (RSV) . Testing will follow current local guidelines at Rigshospitalet NICU.

# 6. Clinical Trial

In a double-blinded, block randomized study, 20 preterm infants (GA 26+0 to 30+6) will be randomized to either FFT or saline solution.

## 6.1. Design

**Block-Randomization**

The block randomization will be performed before treatment is initiated. Infants who meet the inclusion criteria will have FFT or saline solution administered within the first 24 hours after birth and treatment will be double-blinded. We aim to give first treatment during daytime.
Treatment will be stepwise, and the Data Safety Monitoring Board will receive information about acceptance of treatment, suspected adverse events or other observations, after each step.
Each step will be monitored for 4 weeks before moving onwards to the next. Steps will be as:

1) 1+1
2) 1+1
3) 1+1
4) 7+7

All cases will be followed-up for at least 4-weeks or until discharge, whichever comes first.
Long-term follow-up is performed till 3 years after study completion.

### 6.2. Inclusion criteria for participant preterm infants

- Preterm infants born between GA 26+0 and 30+6
- Delivery at RH or transferred to RH NICU within 24 hours of delivery
- Signed parental consent

### 6.3. Exclusion criteria for participant preterm infants

- Major congenital anomalies or birth defects
- Congenital infection
- Extremely SGA infant (weight SD score < -3 SD)
- Need for mechanical ventilation or cardiovascular support before first FFT treatment

### 6.4. Inclusion criteria for mothers of participants

- Women aged 18-45
- Ability to give informed consent

### 6.5. Exclusion criteria for participant mothers

- Mothers who have severe infection, defined by need for other treatment to support infection-related comorbidities, besides from antibiotics (e.g. inotropic treatment, iv fluid resuscitation)

### 6.6. Endpoints, clinical trial

The trial is designed as a feasibility study. We are aware, that it is not powered to investigate any of the endpoints with statistical significance.

#### 6.6.1. Primary endpoints

To demonstrate the safety and tolerability to FFT in terms of no serious adverse events: specifically, no increased incidence of serious infections including NEC, sepsis, and death.

#### 6.6.2. Secondary endpoints

We wish to examine the potential benefits of FFT in terms of

**PrePhage**

- Characterization of faecal virome and microbiome over time in the term infant to map early gut development determine gut microbiome maturation relative to the known progression in term infants
- feeding intolerance
- time to full enteral feeding
- stool characteristics
- paraclinical data (e.g. CRP, leukocytes, and capillary acid/base status)
- anthropometric data
- days of hospitalization

## 6.7. Overview of data collection from participants

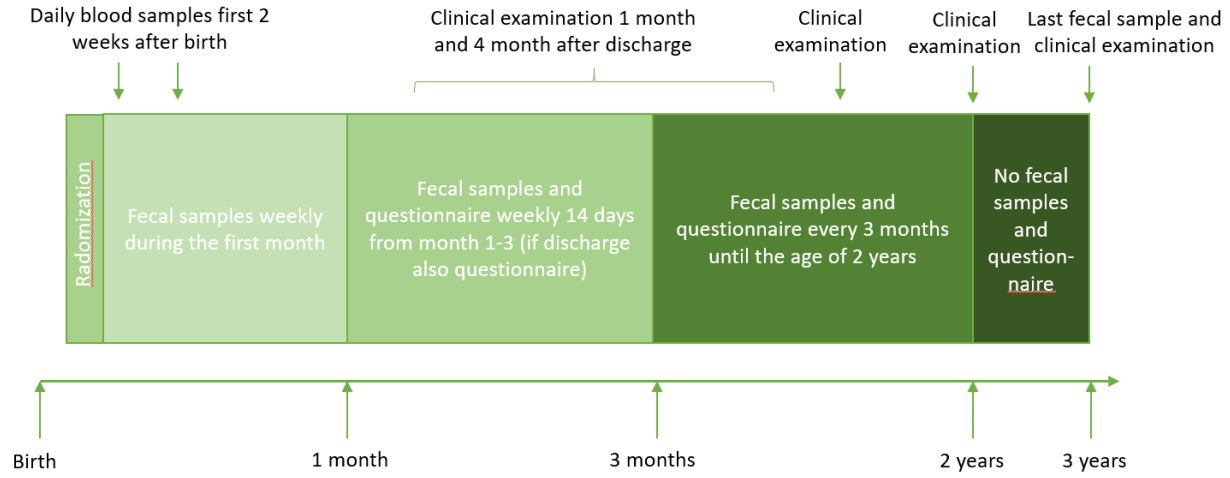


*Figure 2 Overview of data collection, clinical trial*

- *Blood samples in the neonatal period:* routine blood samples taken for clinical purposes are used in the study (capillary acid/base, thrombocytes, CRP, total leucocytes and coagulation factors). These are standard blood tests taken at birth, after 12 hours and daily for the first week for the vast majority of infants admitted to Rigshospitalet NICU
    - After the first week we ask for permission to continue the use of daily routine samples (capillary acid/base, CRP and in case CRP increase, then thrombocytes and total leucocytes) for one more week and in case of suspicion of adverse events.
        - Recipient infants are expected to have 80 ml of blood/kg and expected weights between 600 and 1500 g at inclusion, leading to a total blood

volume of 48-120 ml of blood. [32,33] Current regional guidelines on clinical samples advise that blood volume taken at a given time does not exceed 3% of total blood volume, and that total volume collected during a 30-day period does not exceed 10% of total blood volume [31].

- Required volumes for samples:
  - Acid/base-status: 0.1 ml
  - CRP: 0.01 ml
  - White blood cell count and platelets: 0.25 ml
- Daily samples are maximally 0.36 ml of blood, 0.8% of expected minimum blood volume. Planned samples comprise 0.77 ml, 1.6 % of expected minimum blood volume. If it is deemed necessary to collect white blood cell count and platelets for clinical reasons (suspected infection), this is considered to be of clinical need and is not counted in total blood volume for research purposes.

- *Urine samples:* 1 sample each week for 4 weeks, the first urine is sampled after 1 week
  - Urine samples are collected using a collecting bag
  - Urine samples are used only to detect CMV
- *Faecal samples:*
  - Feces is removed from the diaper as soon as possible after defecation. Feces is removed from the diaper, as soon as possible after defecation. As soon as removed, feces are stored in a container and is given to either RH NICU staff or research team while admitted to the NICU or collected by research team if discharged.
  - Sample collection: Daily until 7 samples are collected, then weekly during the first month, every 14 days from month 1-3, every 3 months until the age of 2 years, and once at the age of 3 years.
  - Gut microbiome and virome are analyzed as described in section 7
  - Faecal calprotectin is analyzed in all samples during the first month as a marker of gut inflammation.

- *Growth:* Daily measurement of weight till regained birth weight then weekly along with length and head circumference while admitted to the NICU. After discharge,

anthropometrics will be measured at the same times as faecal sample collection.

- *Evaluation of feeding tolerance:* Clinical evaluation every second hour according to standardized procedure. Parameters include ventricle content from nasogastric tube, abdominal appearance, and quantity and characteristics of stool (appendix 8)

- *Questionnaire on feeding, faecal pattern, and well-being:* Once the infant is on full enteral feeds and discharged from the NICU, participants will fill out the same questionnaires as the donors (Questionnaire included in appendix 7).

- *Clinical examination by research team in addition to routine examinations performed at RH NICU:* daily for the first week, at 2 weeks, 3 weeks, 4 weeks, 6 months, 1, 2 and 3 years
    - Clinical examination is done according to best clinical practice for examining normal health and development

- *Access to patient record and FMK*
    - Before inclusion: patient record of mothers, who are at risk for preterm birth, will be accessed to ensure mothers fulfil inclusion criteria and that infants are not intrauterine diagnosed with known congenital diseases that will led to exclusion. If mother and infant before birth are eligible for inclusion, this information will be passed on to the research team.
    - After birth patient records of both mother and infant is accessed to ensure all inclusion criteria is still fulfilled
    - Infant FMK access is required throughout the data-collection period to adjust for any use of antibiotics or other drugs with potential effects on analysis of faecal microbiota composition ..
    - Infant patient record access is required throughout the data-collection period to identify positive effects and potential side effects to intervention during admission and till termination of the study. This is part of the safety follow-up, to ensure that treatment does not cause long-term side-effects. Patient records will be accessed in conjunction with the clinical examinations.

### 6.8. FFT treatment

FFT will be administered in a suspension via a nasogastric tube, which the preterm infants will have as part of their treatment in all cases. The first dose will be given within 24 hours and repeated every 24 hours for 3 days for a total of 4 doses. The volume of each dose will be 1 ml. The small volume ensures that the process of administering the transplant will not disturb enteral feeding of the infant.

#### 6.8.1. Faecal viral transplant – Risk assessment

The viral transplant will be screened for known pathogens as described in the donor study. Furthermore, the risk of translocation of bacteria to the blood stream seen with full faecal transplants will be avoided, since the filtrate will contain virtually no bacteria.

As a nasogastric tube is already necessary for nutritional purposes in the premature infant, this will provide no additional discomfort to the infant. Administration of faecal microbiome transplantation by nasogastric tube has been seen to be well-tolerated and safe [21]. It should be noted that the dosage administered was relatively low (0.7–16 3 10^6 live bacterial cells).

### 6.9. Preparation of faecal filtrate transplantation

The faecal virome used for transplantation will be isolated from approved donors (10-20 donors) after the thorough screening process. Donor faeces are processed without mixing faeces from several donors, i.e. each recipient will receive faecal virome transplantation from the same donor. This is done to ensure traceability between donor and recipient.
The faecal donor material is collected in anaerobic sterile zip-bag. The faecal samples will be dissolved in buffer (stabilizing phages). Bacteria and other larger fragments are removed by centrifugation followed by sterile filtration (0.45 µm filter) after which the virome will be concentrated using Centrisart® tubes to the same volume, as the original sample [34–36]. The final mixture will be stored at -80 °C until use. The faecal virome will be administered to the infants mixed in their daily feed. A total volume of 1 ml of faecal virome will be administered to the infants on day 0, 1, 2 and 3. The faecal virome will contain the same number of bacteriophage particles, as the original faecal (approximately 10(9) bacteriophages/ml).

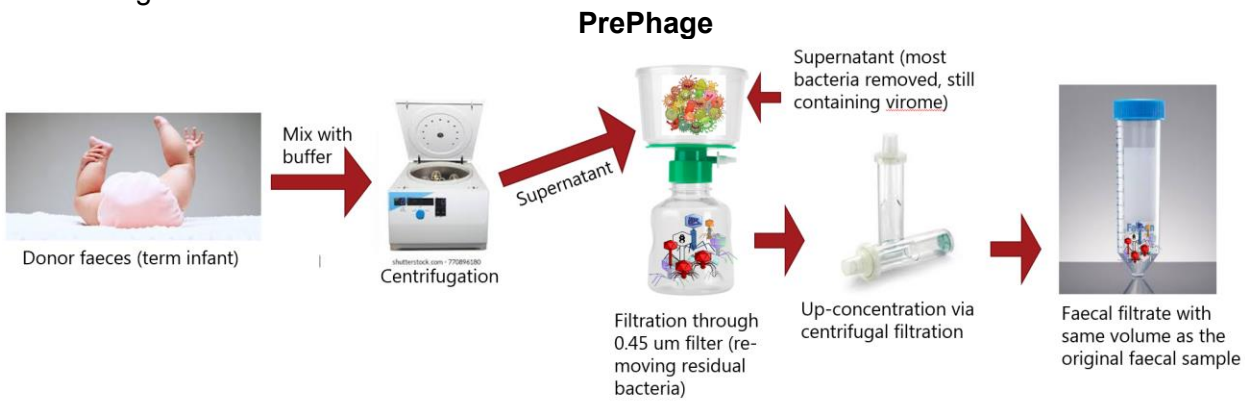


*Figure 3 Illustration of the FFT preparation*

### 6.10. Clinical observation and safety during admission

Participants in the study will be admitted to RH NICU. They will be monitored and treated according to best clinical practice. Participation in the study will not influence other treatment given while admitted to the NICU. NICU personnel will have access to routine samples taken during the study as well as study evaluation of feeding tolerance (appendix 8).
Management of any potential complications will be done in collaboration with NICU doctors.

### 6.11. Long term follow-up after discharge

Participants are followed clinically until the age of 3 as described in overview of data collection. Parents have direct access to project staff throughout the study period and are encouraged to contact research team with any potential questions or unforeseen complications.

### 6.12. Randomization code

Randomization code is kept securely. Access to the code is done if serious adverse events are suspected.

## 7. Analysis of microbiome and virome in faeces

Faecal samples will be collected according to standard operation procedures for subsequent standardized total DNA extraction as well as total virome DNA/RNA extraction. All analysis will

be performed at University of Copenhagen, Food Science, Department of Microbiology and Fermentation.

Total genomic DNA will be subjected to deep metagenome sequencing and related to the study outcomes. When extracting faecal DNA as well as viral DNA/RNA, physical fractionation or selective lysis will be employed to ensure host DNA is kept to a minimum. Remaining host DNA material will be removed during bioinformatics filtering and mapping of the shotgun metagenomics data. These procedures will ensure that no human genome data end up in the microbiome data. The content of metabolites in faeces will be analysed based on metabolomics at Department of Food Science, UCPH using a combination of proton NMR spectroscopy and LC-MS.

## 8. Biobank and storage of samples

Biological material that is not immediately sent for analysis (feces and milk samples) is stored in a research-biobank created for this study and registered centrally at NEXS, UCPH, until it is analyzed. In this, biological material is stores until analyzed. Further mothers and parents of participants are asked, for permission to donate excess biological material, in case of any. This excessed material will be stored in a registered biobank at NEXS, UCPH in accordance to Datatilsynets regulations as decribed in section 5.3 (https://www.nvk.dk/emner/biobanker/vejledning-om-bio-mat). If “Datatilsynet” does not give its consent to create the new biobank, all excess material will be destroyed at the conclusion of the study in 31/12/2030.

Consent for donation of material to the biobank is part of the signed consent form (appendix 4, 5 and 6), and patients are informed about this in the participant information (appendix 2 and 3).

The material will only be used in new and related research projects after these have been approved by the VEK, unless otherwise specified by the guidelines of VEK. If a participant agrees to donate possible excess material to the biobank and later regret it, they can contact either the research team or **NEXS** and have their material destroyed, unless the samples have been anonymized. This means that any ID-log that connects study participants and biologic material is destroyed.

Capillary blood samples and dried blood spots from the clinical study are not stored, as we expect to have no excess material. Blood samples are sent for analyses directly after collection. Urine samples are analyzed for CMV directly after collection. Excess urine is not stored.
Part of the milk samples are sent directly to analysis for CMV after collection. Excess material, if any, is stored as described above.

The PI is aware of the fact, that collection of biologic material for a biobank is not covered by the committee law, but by the GDPR/Databeskyttelsesloven. All biological samples in the Biobank are handled confidentially and stored in accordance with applicable law, GDPR and "Databeskyttelsesloven".

# 9. Recruitment, consent, and participant information

## 9.1. Recruitment strategy

### 9.1.1. Recruitment for Donor Study

Participants for the donor study are recruited in 2 different ways. The first is using posters placed at strategic locations at the campuses for medicine and veterinary medicine at UCPH, as well as uploading digital versions of the posters to relevant online study platforms. The second is through the obstetric out-patient clinics in Region Hovedstaden (RH, Herlev and Hvidovre, but not Nordsjælland), where clinical staff will inform briefly about the study when the family is there for routine checkups. They will be given a small pamphlet with information regarding the study and contact information of the research team. When families contact the research team, an information meeting will be planned directly.

### 9.1.2. Recruitment for Clinical Trial

Parents admitted to the Department of Obstetrics at Rigshospitalet will be informed briefly verbally and in writing about the study when they are given standard information regarding preterm birth. If they are interested, they will be given the written participant information and will be contacted by the research team to be fully informed before potential inclusion in the study.

## 9.2. Consent

Both parents (if two) must give consent to inclusion of the infant in both donor study and clinical trial for the infant to be enrolled in the study. In the donor study, the mother must also give consent to participating in the study.

## 9.3. Principles for informing participants and obtaining consent

Potential participants identified as described above receive an informative consultation. Prior to this, the investigators will consult the participants medical record and FMK in order to ensure, they fulfill the inclusion criteria.
In both studies the informative consultation will take place in privacy, in a room where it is possible to ensure disturbance is prohibited and with possibility of a bystander. In the donor study, information could be given at the family's homes or at the NICU at RH. In the clinical study, the consultation will include considerations to the condition of the mother. While admitted with risk of preterm birth, the consultation will take place in the room where the mother is admitted and bedridden and it is secured that it is given in privacy.

If the family wishes, they can have access to a bystander of their own choice. If they feel the need for a bystander with neonatal competences, they can have access to one from among the personnel at RH NICU with no direct relation to the research project. In case of single parents, they will be asked to bring along an assessor. Besides written and oral information about the study (Information, appendix 2 and 3), the pamphlet "Før du beslutter dig: Om at være forsøgsperson i sundhedsvidenskabelige forsøg" from VEK, together with the pamphlet "Forsøgspersonens rettigheder i et sundhedsvidenskabeligt forskningsprojekt" reviewed and published by the ethical committee system of 21st Sep 2019, and a copy of the informed consent forms (appendix 4, 5 and 6).

In the clinical trial, we aim to obtain consent from both parents before time of birth in order to allow the treatment to start within 24 hours. In case, this is not possible, we will aim to obtain consent as soon as possible after birth to be able to give treatment as fast as possible. In the donor study, we aim to have consent before the time of birth. If possible, a consideration period of 24 hours will be offered before signing the informed consent, in which case a new meeting

(the screening) will be scheduled. However, as FFT is to be administrated within 24 hours of birth, the consideration period might be shortened due to practical considerations. If participants do not require time for consideration, the declaration of informed consent may be signed immediately after the information meeting by the participant mother or the parents of the participant and the investigator (or research team who have had this responsibility assigned by the investigator). When the informed consent is signed, the participant will be offered a copy. Signed informed consent is a prerequisite for screening or any study-related procedures to start.

They will be informed that the participation is completely voluntary, and that they have the right to withdraw their informed consent at any time without explanation or consequence. Potential participants and assessors will be invited to ask questions with trained research team members. If the research team or the RH NICU staff judge that a participant needs a second explanation of the study, this will be encouraged. Participants will also be informed that data collected before they withdraw their informed consent may be used by the researchers in the overall data analysis. In addition, they will be informed that by signing the informed consents they also give permission for the use of their personal data in accordance with the purpose of this project and controlling authorities to access their data and that a person that carries out these functions is participant to rules of confidentiality.

## 10. Discontinuation and withdrawal

The parents are free to withdraw the participants at any time during the intervention period as well as during the data collection period. The reason(s) will be recorded. When possible, the parents will be asked if they will allow that we use collected data before withdrawal for publication. The parents may ask to have biological material stored in the biobank destroyed at any time. The neonatologists and research staff can discontinue the participants from the study at any time, and the reason(s) will be recorded and reported.

# 11. Adverse events

## 11.1. Definition of adverse events

An adverse event (AE) is defined as any untoward occurrence in a participant in the clinical study, which does not necessarily have a causal relationship with the study intervention. AEs can be illnesses, signs, symptoms, or irregular results of routine laboratory tests occurring during the intervention period, and until discharge.

## 11.2. Definition of serious adverse events

A serious adverse event (SAE) is a fatal or life-threatening event, which causes or may cause death, prolonged hospitalization, persistent or significant disability, or requiring long-term intervention to prevent permanent impairment or damage.

## 11.3. Baseline incidence of Serious Adverse Events

In all cases, being born preterm infant is associated with significant risks. Therefore, we cannot expect zero adverse events during the study. However, it is our expectation that our rate of death, NEC and sepsis does not exceed the latest incidence rates from very low birth weight infants at Rigshospitalet NICU. The current rates are mortality 16.7 %, NEC 7.1% and positive blood cultures 17.1%. The current use of antibiotics at Rigshospitalet NICU, is 33% of all admitted infants. Though not reflecting numbers of infants with bacterial sepsis, we use the incidence of positive blood cultures in Copenhagen as a surrogate measure [4,5].

## 11.4. Follow-up of adverse events

All adverse events will be followed until they have abated, or until they have reached a stable situation. Depending on the event, follow up may require additional tests and/or medical procedures.

## 11.5. Recording and reporting of the adverse events

The very preterm infants in a NICU are often seriously ill and some die. Many of the adverse events will be observed with or without the FFT intervention, and therefore AEs and SAEs will likely occur in the study. Therefore, recording and reporting of all AEs is not realistic and relevant.

In the current study, sepsis, surgical NEC, and death will be recorded by the research team and reported to the chief investigator and the DSMB. The CI will re-evaluate the causal relation to the study. If it is probable, or certain, the CI will report this to the ethical committee. Severe unexpected suspected adverse reactions will be similarly reported.

A safety report will be sent annually to the relevant standing ethical committees. This safety report will include the interim analysis of the DSMB (see below).

### 11.6. Classification of causality

The research team and attending neonatologists will evaluate the possibility of a connection between the FFT intervention and adverse events on the basis of the following criteria:

1) Unrelated: There is an obvious other explanation for the AE, e.g.:
   The AE is clearly explained by the condition of the participant
   The AE is related to the side-effect of one or more medical treatments
2) Unlikely relation: Possible connection with the intake of FFT, but there is another plausible explanation for the occurrence of the AE. Participant does not have a clear response to intervention withdrawal.
3) Probable relation: Reasonable temporal relationship with the intake of FFT, and plausible reasons point to a causal relationship with the study product.
4) Certain relation: Reasonable temporal relationship with the intake of FFT, and there is no other explanation for the AE. Descending and/or disappearing of the AE happen when withdrawing the intervention.

## 12. Side-effects and risks

### 12.1. Faecal and urine samples

Collecting both urine samples and faecal samples is both safe and tolerable. There is a minor practical inconvenience associated with collecting the sample.

### 12.2. Blood samples

Blood samples will be drawn by trained personnel at Rigshospitalet NICU. When possible, project blood samples will be coordinated with blood samples drawn for clinical purposes. It is

anticipated that the needle used, will not cause any harm to the participants besides that associated with the insertion i.e. a small local pain. There is a risk that the skin around the entry point may swell slightly, become sore and blue/brown discoloration emerges on the day after blood sampling.

### 12.3. Safety during trial

To our knowledge, FFT in the preterm has not been performed before. A recent study of maternal faecal microbiota transplant in 10 cesarean-born infants has been shown to be safe [21]. Administration of the FFT will be given while admitted to Rigshospitalet NICU, giving optimal circumstances for managing any adverse event. The families of both donors and participants are given contact information to the research team. They are encouraged to call, text, or write an email with any possible questions about potential side effects, fear or discomfort. Furthermore, clinical examination by trained professionals is planned to ensure that signs of potential disease not observed by family are found.

## 13. Data Safety Monitoring Board (DSMB)

There will be a DSMB instituted for this study. Persons with the following professions will take place in the board:

- Neonatology
- Infections
- Methodology in clinical trials

## 14. Termination of the study

Safety and tolerability of FFT transplantation will be evaluated by the neonatologists in the neonatal departments of RH, as well as the research team. This will happen at least after each of block of randomization. This will be accessed by the clinical outcomes, the routine laboratory analysis, the plasma amino acid profiles, and SAEs. The study will be terminated if FFT is not tolerated or found unsafe. Before termination, the DSMB and VEK are asked for advice.

# 15. Statistical analysis

## 15.1. General statistical considerations

The statistical analyses described in this protocol will be carried out by the research team. The level of significance is set at 0.05 and if necessary, data will be transformed to reach normality. The overall statistical modelling approach will the Mann–Whitney U test, as our sample size is likely too small to fit normal distribution. Results from fitted models will be reported with and without control for multiplicity. For control for multiplicity e.g. Bonferroni corrections will be performed.

This study is a feasibility study. Therefore, initial small sample size is made in terms of practical and safety considerations. If this study goes according to plan, we will follow up with a larger randomized clinical trial.

All statistical analysis will be performed using “R” (https://www.r-project.org/).

## 15.2. Effect-modifying factors

- Gender
- Ethnicity
- Parenteral nutrition
- Time till meconium discharge
- Antibiotic consumption
- Infections
- Time on respiratory support

### 15.2.1. Primary outcome

As the main outcome of the study is to have no serious adverse events causally attributed to FFT, no statistical analysis will be performed for the primary outcome. Difference between incidence of NEC and late-onset sepsis will be analyzed, but since the study is not powered enough to show differences, the findings will be descriptive.

### 15.2.2. Secondary outcomes

For testing preliminary effects of FFT, clinical and paraclinical outcomes will be compared between the two treatment groups. Statistical analysis will be performed blindly. Continuous outcomes will be checked for the normal distribution, and if not, they will be transformed to fulfill

the normal distribution. If no proper transformation method can be found, non-parametric analyses will be performed. For continuous outcomes, General Linear Model with or without covariates will be used. For binary outcomes, logistic regression will be used. For repeated measurement, mixed model with repeated measurement will be applied.

### 15.3. Definition of datasets

To allow evaluation of the biological effect of the FFT treatment, complete-case analysis and per-protocol analyses of the primary and secondary outcomes will be carried out. Available-case analysis, utilizing all available data will be used for the exploratory analyses as appropriate. Additionally, Intention-to-treat (ITT) analyses may be carried out for all outcomes.

### 15.4. Handling missing values

For ITT analyses, missing outcome values will be handled using last-observation-carried-forward method.

## 16. Data handling and storage

All information obtained during the study will be handled according to local regulations and the GDPR. All data collected during the study including relevant information from the participants medical record (antibiotic use, infections, presence of gastrointestinal disease) will be stored using a project specific account at REDCap (https://www.project-redcap.org/). The account is protected by security code and is only accessible by registered users. The database has an in-built audit-trail. The project database will be erased after 10 years.

If necessary, in conjunction with mandatory monitoring by the appropriate regulatory authorities or internal quality control, the project ID will be retraced to the participant, who may then gain access to the research database as well as the patient's medical record.

Questionnaires answered at home by the participants (appendix 7) will be answered directly via REDCap, and consent forms will be signed digitally using Nem-ID and REDCap.
The staff at RH NICU will be trained in the use of the feeding tolerance evaluation (appendix 8) before initiation of the study to minimize interrater variability. They will be entered digitally in a standardized manner and stored using REDCap. The physical copies will be stored safely at RH NICU by the research team.

Any biological material not analyzed directly in the clinical setting will be identified only using a specific project ID after collecting the material.

The randomization code is stored by the primary investigator in a locked safe. The intervention code is broken if serious side-effects are suspected that are deemed unacceptable for the participant.

# 17. Ethical aspects

## 17.1. Administering FFT to the preterm infant: potential benefit and harm

We believe that administration of FFT will support beneficial GI tract maturation and decrease the risk of NEC, which must be one of the most cruel diseases related to prematurity.
Based on previous piglet studies of FFT and the steps taken to develop the filtrate with thorough screening for pathogenic vira and exclusion of bacteria, it is our belief that the risk of serious infection because of the procedure is minimal.

Placing a nasogastric tube is a routine procedure all premature infants admitted to RH NICU is exposed to and causes minimal distress to the infant. Administration of FFT via NG-tube has been shown to be equally efficacious to administration using colonoscopy.
Infant feeding pattern is not expected to be disturbed, as the FFT is administered using an already placed nasogastric tube, and the volume is not expected to exceed 1 ml per dosage and 4 ml in total.

# 18. Considerations on research with neonates

### 18.1.1. Study group

We believe that the study can only be performed using premature infants, as they are the primary group affected by NEC. Hence, results from feasibility studies in term infants will not generalize to preterm infants, and as such we believe it inadvisable to perform the study in another population.

#### 18.1.2. Considerations for ensuring minimal physical and emotional distress for participants

Screening samples from donor infants will be coordinated with standard sample screenings for metabolic disease (PKU). Routine blood samples are coordinated with routine clinical care and continued only in the extent we believe it necessary to ensure safety during study protocol. Collecting faecal samples from diapered children does not cause any significant emotional distress. We offer the parents the service of collecting samples at home and measuring growth at home if they so prefer.

### 18.2. Investigator expertise

The group of responsible research team collaborating in this study all have a broad experience within each of their competences (Dennis Sandris Nielsen, microbiome, Andres Brunse, animal studies, Susanne Soendergaard Kappel, ethnographic fieldwork in the neonatal setting, and Lise Aunsholt neonatal clinical studies). Besides from that, people within the group have long-term collaboration with each other. The primary clinical investigator, Lise Aunsholt, has experience in translational studies, combining animal studies with clinical studies, in a classical bench to bedside set-up and has investigated novel interventions previously. Apart from that, she has experience in large-scale national and international (China) multicenter studies involving more neonatal intensive care units and collaborators.
The Ph.D. student involved in this study will acquire competences within translational studies during his Ph.D. under close supervision be the senior team.

## 19. Patient record access and handling of personal data

The patient record will be accessed as described in the observations and measurements. The patient records will not be accessed after the study conclusion.
When consent to participate in the project has been given, based on written and verbal information, then the research team have permission to access patient records including FMK, in order to perform mandatory quality control. Furthermore, consent will allow controlling authorities access to the patient record in order to perform legally mandated control of clinical research projects.

Personal information (including CPR-number) is used in the project. All personal data will be treated in accordance with “Databeskyttelsesforordningen” (EU forordninger 2016/679 af 27. april 2016) and “Databeskyttelsesloven” (lov nr 502 af 23/5/2018).

## 20. Insurance

Both donors and participants are covered by the public patient insurance of Denmark ( https://www.nvk.dk/emner/forsikring-og-erstatning/vejledning-om-forsikring )

## 21. Reimbursement of participants

The participants will not be reimbursed for participation. Treatment and medical advice given during the study will be free of charge.

## 22. SARS-Cov2

Testing of participants and personnel as well as social distancing and use of protective equipment will always follow the current guidelines at RH NICU.

## 23. Economy

This project was initiated by Dennis Sandris Nielsen, Anders Brunse, and Lise Aunsholt and funding for the study which include synergy between 3 institutions was applied as a synergy application. The project is funded by the Novo Nordisk Foundation with 15.000.000 DKr. (public at www.novonordiskfonden.dk). Each institution has a budget of 5.000.000 DKr., which include salary for involved staff, sample analysis and costs of publication as well as congress presentations. For the clinical study, Lise Aunsholt holds an account at Rigshospitalet. Lise Aunsholt has no relation to the Novo Nordisk Foundation.

## 24. Publication plan

The pilot study will be registered on ClinicalTrials.gov. Positive, negative, or inconclusive results are all planned to be published in peer-reviewed international journals. If the amount and quality of obtained results from this pilot study do not meet the normal criteria for publication in international journals, results will be published as a public report available online UCPH.

**PrePhage**

Authorship will be determined according to the International Committee of Medical Journal Editors Guideline.

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