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Colonisation of the proximal intestinal remnant in newborn infants with enterostomy: a longitudinal study protocol

Inês Barreiros Mota,[1,2] Cláudia Marques,[1,2] Ana Faria,[1,2,3] Maria Teresa Neto,[4,5] Gonçalo Cordeiro-Ferreira,[4] Daniel Virella,[4,6] Ana Pita,[4] Luís Pereira-da-Silva,[4,5,6] Conceição Calhau[1,2,7]

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For numbered affiliations see end of article.

Correspondence to
Professor Conceição Calhau; ccalhau@nms.unl.pt

ABSTRACT

Introduction The gut microbiota plays a main role in the maintenance of host's health. Exposure to different conditions in early life contributes to distinct 'pioneer' bacterial communities in the intestine, which shape the newborn infant development. Newborn infants with congenital malformations of the gastrointestinal tract (CMGIT), necrotising enterocolitis (NEC) and spontaneous intestinal perforation (SIP) commonly require abdominal surgery and enterostomy. The knowledge about the colonisation of these newborns' intestine by microorganisms is scarce. This protocol is designed to explore the microbial colonisation over time of the proximal intestinal remnant in newborn infants who underwent surgery for CMGIT, NEC or SIP and require enterostomy.

Methods and analysis The literature about microbiota colonisation in newborn infants with enterostomy was reviewed and an observational, longitudinal, prospective study was designed. The infants will be recruited at the Neonatal Intensive Care Unit of the Hospital Dona Estefânia, Centro Hospitalar Universitário de Lisboa Central. Samples of the enterostomy effluent will be collected every 3 days, through 21 days after the first collection. The microorganisms colonising the proximal intestinal remnant will be identified using the 16S rRNA sequence analysis and a subset of microorganisms will be quantified using real-time PCR. This protocol may serve as basis for future observational and interventional studies on the modulation of the intestinal microbiota (eg, probiotics) on short and long-term outcomes in this population.

Ethics and dissemination This study protocol was approved by the Ethics Committee of Centro Hospitalar Universitário de Lisboa Central (441/2017) and by the Ethics Committee of NOVA Medical School, Universidade Nova de Lisboa (n°50/2018/CEFCM). The results will be spread through peer-reviewed publications and presentations at international scientific meetings.

Trial registration number NCT03340259.

Strengths and limitations of this study

- This is the first longitudinal study assessing the colonisation of the proximal intestinal remnant in infants requiring enterostomy.
- The enterostomy allows the study of the remnant proximal intestine microbiota.
- Data obtained may be useful for future preventive and therapeutic interventions.
- It is a single-centre study.
- The exposure to the previous neonatal unit environment may condition the newborn infants' microbiota.

BACKGROUND

The human microbiota is a collection of microbes living in the human body. It contains approximately 10^{14} cells and is mainly composed of bacteria. Most of these microorganisms reside in the gastrointestinal tract, constituting the gut microbiota.[1–3]

The gut microbiota, which is increasingly regarded as an 'organ', plays a major role in the maintenance of the host health, including intestinal health and function.[4]

The intestinal colonisation is dynamic and the microbial population develops rapidly from birth.[5 6] Host-microbiome interaction in early life is crucial for the development of the barrier function and integrity as well as the mucosal and systemic immune functions.[7]

The type and diversity of intestinal microorganisms differ widely among neonates and are influenced by factors such as mode of delivery, gestational age, type of feeding, antibiotic exposure, infant postnatal age and surrounding environment.[4 5 7–9] Under physiological conditions, the rapid evolution of the infant microbiota depends on the initial contact with microbes by exposure to amniotic fluid, passage through the vaginal canal, intake of mother's milk and skin-to-skin contact.[10] In contrast, infants requiring intensive care are usually nursed in high-sanitary incubators, receive antibiotics, have restricted mother's milk intake and limited contact

Table 1 Studies and case reports addressing colonisation of proximal intestinal remnant in infants with enterostomy

Underlying condition	Type of study	Aim	Sample size	Results	Reference
CMGIT, NEC and SIP	Randomised controlled trial	To determine the effect of an enteral oil supplementation on the intestinal microbiome	n=32 preterm infants (n=16 in each group)	Enrichment of many genera from Enterobacteriaceae family, including *Escherichia*, *Pantoea*, *Serratia* and *Citrobacter* over time, in infants receiving standard nutritional therapy. Enteral oil supplementation increased bacterial diversity and decreased the abundance of pathogenic bacteria.	13
SIP and NEC	Case report	To study microbiota diversity according to the length of remnant intestine	n=2 preterm infants	Human infant ileum and colon are dominated by *Bifidobacterium*.	16
CMGIT and SIP	Case report	To quantify Lactobacillus and Bifidobacterium probiotic strains in the neonatal ileum	n=2 (1 preterm and 1 term infant)	*Lactobacillus* and *Bifidobacterium* strains were identified in the neonatal ileum.	17
CMGIT	Case report	To study the effect of probiotic therapy after CMGIT surgery	n=2 (1 preterm and 1 term infant)	Probiotic therapy with *Lactobacillus casei* and *Bifidobacterium breve* was effective and these strains became well established in the intestine.	14
CMGIT, NEC and SIP	Observational study	To compare the microbiota composition in fresh intestinal tissue collected during surgery vs faecal samples	n=7 preterm or term infants	Intestinal bacteria diversity was higher in the intestinal tissue and in faecal samples adherent to the intestinal mucosa.	15

CMGIT, congenital malformation of the gastrointestinal tract; NEC, necrotising enterocolitis; SIP, spontaneous intestinal perforation.

with mother's skin.[11] In addition, infants undergoing surgery of the gastrointestinal tract commonly require some period of fasting, and often the use of gastric acid suppressant.[12] These factors can cause early life dysbiosis, a delayed and suboptimal colonisation of the intestine, which has been associated with long-term morbidities in adulthood.[4 6]

Preterm infants are immunologically immature and especially sensitive and responsive to bacteria colonising the intestine.[11] The type of colonising bacteria may influence their risk of life-threatening morbidities, including late onset sepsis or necrotising enterocolitis (NEC).[7]

Newborn infants with congenital malformations of the gastrointestinal tract (CMGIT), NEC and spontaneous intestinal perforation (SIP) commonly require abdominal surgery and enterostomy.[13] While intestinal microbiota has been extensively studied in infants with anatomically uninterrupted intestine, an extensive review of the literature retrieved only five studies or case reports addressing intestinal colonisation in infants with enterostomy (table 1).[13–17] Data from these studies and reports lack for clarification on to what extent the surgical interruption of intestine affects the developing intestinal microbiota.

In this regard, this is the first study that explores the intestinal colonisation over time in newborn infants with enterostomy.

The primary aim of this study is to obtain prospective data on the microbiota colonisation of the proximal intestinal remnant in newborn infants with enterostomy undergoing surgery due to CMGIT, NEC and SIP. The secondary objective is to explore associations between the colonisation, and perinatal and postnatal factors.

Methods and analysis

Study design

This is an observational, longitudinal and prospective study, implemented at the Neonatal Intensive Care Unit (NICU) of the Hospital Dona Estefânia, Centro Hospitalar Universitário de Lisboa Central, and the NOVA Medical School, Universidade NOVA de Lisboa.

Patient and public involvement

Patients were not involved in the development of this protocol.

Recruitment criteria

Patients admitted to the NICU of the Hospital Dona Estefânia during the neonatal period, to whom an enterostomy has been performed due to CMGIT, NEC or SIP, are eligible (table 2).

Sample size

No published data are available to contribute to estimate the needed sample size. Therefore, a convenience,

Table 2 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
► Newborn infants with enterostomy after surgery for CMGIT, NEC or SIP. ► Newborn admitted up to the 28th day after birth, if a term neonate, or admitted up to 42 weeks postconceptional age if born preterm.	► Diagnosis of inborn errors of metabolism.

CMGIT, congenital malformation of the gastrointestinal tract; NEC, necrotising enterocolitis; SIP, spontaneous intestinal perforation.

non-probabilistic, consecutive sample, limited in time, will be recruited. The study is planned to recruit participants over 2 years. Based on the 11 patients admitted to the same NICU from March 2016 to March 2017, who fulfil the eligibility criteria, it is estimated that approximately 20 infants will be recruited.

Intestinal effluent sampling and storage

The first sample will be collected when a sufficient enterostomy effluent is available (approximately 2 mL). The periodicity of sampling is determined by the every 3-day routine change of ostomy bags. Any spill or leak from the stoma collection bag will be considered as lost for sampling due to potential contamination by skin flora. In these cases, the next effluent will be considered for collection. The period of 21 days scheduled for the longitudinal study was based on the average stay in the NICU after surgery of the aforementioned conditions (figure 1).

Samples will be transported conditioned in a thermal bag with ice and stored at −20°C at the NOVA Medical School until being analysed.

Microbiota analysis

Bacterial and fungi DNA will be extracted directly from intestinal effluent samples using a NZY Tissue gDNA Isolation Kit (NZYTech, Lisbon, Portugal) as previously described.[18] Some modifications to this protocol were made according to the protocol by Zoetendal *et al*.[19] In brief, 350 µL of buffer NT1 will be added to the enterostomy effluent (2–5 mL) and incubated in a shaking bath at 80°C for 15 min. Samples will be centrifuged at 1500 x *g* for 1 min. RNAse (4 mg/mL) will be added to 200 µL of supernatant for incubation at room temperature for 5 min. Subsequently, 25 µL of proteinase K will be added for incubation in a shaking bath at 70°C for 10 min. The remaining steps will follow the manufacturer instructions.[18 19] DNA quantification will be assessed with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). The microorganisms colonising the proximal intestinal remnant will be identified using the 16S rRNA sequence analysis, as described.[20] A subset of microorganisms will be quantified using real-time PCR.[18] Primer sequences will be used to target bacterial 16S rRNA gene and fungi 18S rRNA gene[18 21–24] (table 3). The set of microorganisms quantified was chosen based on previous reports of microorganisms found in premature infants, infants under intensive care, infants with NEC[5 11 25–28] and in adults subjected to gastrointestinal surgery.[29] The microbiota analysis will be performed at NOVA Medical School.

Independent factors

The following factors that may influence gut microbiota composition will be recorded:

Perinatal factors: gestational age, mode of delivery (vaginal or caesarean section), Apgar scores at 1 and 5 min, prenatal antibiotic exposure, sex, body weight, length and head circumference at birth.

Surgical factors: surgical condition (CMGIT, NEC and SIP), antimicrobial therapy previous to surgery, age at the enterostomy surgery, intestinal level of the proximal enterostomy, estimated length of proximal intestinal remnant, type of feeding before surgery.

Postsurgery factors: daily body weight, days of fasting, days on parenteral nutrition, central catheter and type, type of feeding, volume and mode of administration; antimicrobial therapy and prophylaxis, H_2-receptor antagonists therapy, days on invasive ventilation; acute events, such as sepsis, daily characteristics of enterostomy effluent and date of discharge.

Primary outcome

The primary outcome is the longitudinal characterisation of postsurgical microbial colonisation of the proximal intestinal remnant. The set of the bacteria and fungi that will be searched (table 3) was chosen based on previous reports,[8 13–17 27 30–32] considering the limited amount of

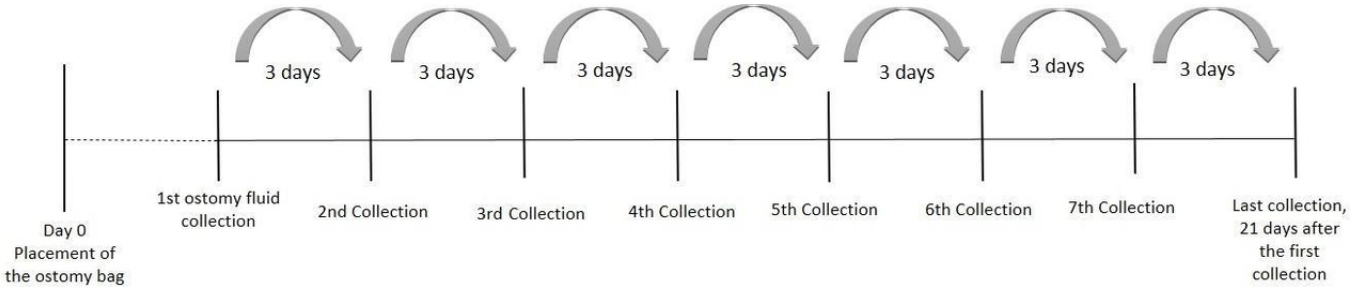

Figure 1 Schedule of effluent sampling during study period.

Table 3 Primer sequences and real-time PCR conditions used for microbiota analysis

Target group	Primer sequence (5'–3')	Genomic DNA standard	AT	Reference
Universal	F: AAACTCAAAKGAATTGACGG R: CTCACRRCACGAGCTGAC	*Bacteroides vulgatus* ATCC 8482	62	18
Staphylococcus	F: GAT GTG CGA AAG CGT GGG GAT R: GAA CTG AGA ACA ACT TTA TGG GA	*S. aureus* ATCC 12600	60	24
Bifidobacterium	F: CGC GTC YGG TGT GAA AG R: CCC CAC ATC CAG CAT CCA	*B. longum* subspecies *infantis* ATCC 15697	60	18
Bacteroides fragilis	F: TCRGGAAGAAAGCTTGCT R: CATCCTTTACCGGAATCCT	*B. fragilis* ATCC 25285	60	21
Escherichia coli	F: *GTTAATACCTTTGCTCATTGA* R: ACCAGGGTATCTAATCCTGTT	*E. coli* ATCC 25922	60	23
Candida	F: TTGGTGGAGTGAT TTGTCTGCT R: TCTAAGGGCATCACAGACCT G	*C. albicans* ATCC10231	60	22

AT, anneling temperature (°C).

enterostomy effluent collected in the pilot cases, which in turn restricts the amount of DNA available for extraction and subsequent identification.

Secondary outcomes

The secondary outcomes are the associations between the identified microorganisms, the timing of first identification and the considered independent factors, based on previously reported drivers of intestinal colonisation in infants.[4–12]

Statistical analysis

Descriptive subgroup analysis is planned to be performed taking into account the cause for the enterostomy: CMGIT, NEC or SIP. Additional descriptive subgroup analysis is planned to be performed taking into account other main independent variables: gestational age (term, 37–41 weeks; moderate to late preterm, 32–36 weeks; very preterm, 28–31 weeks and extremely preterm, <28 weeks), age at the enterostomy, intestinal level of the proximal enterostomy (duodenostomy, jejunostomy, ileostomy or colostomy) and mode of feeding history. Inference multilayered subgroup analysis and multivariable analysis will depend on the sample size and heterogeneity.

Ethics and dissemination

Parents or legal representative of eligible infants will be asked for written informed consent to participate.

This study was registered at ClinicalTrials.gov with the title 'Intestinal colonisation in newborn infants with enterostomy' (NCT03340259), on 13 November 2017.

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. In addition, the results will be accessible from updates at the ClinicalTrials.gov registry. The results will be disseminated through peer-reviewed publication and presentation at international scientific meetings.

DISCUSSION

Early life dysbiosis plays a significant role in the pathogenesis of NEC.[33] The relative abundance of γ-proteobacteria and the paucity of strictly anaerobes have been described prior to diagnosis of NEC.[4 7 33] In addition, low bacterial diversity and potential pathogenic microorganisms such as *Clostridium* spp, *Escherichia coli*, *Klebsiella pneumoniae*, torovirus, astrovirus, cytomegalovirus and *Candida* spp were found in infants with NEC.[11 26] In the aetiopathogenesis of SIP the role of the intestinal microbiota has not been elucidated.[34] Infants with enterostomy have specific factors that may affect the development of intestinal microbiota, including the interruption of the intestine and the intraluminal contact with air through the stoma.[29 35] Nevertheless, enterostomy allows to study the remnant intestine microbiota located closer to the intestinal mucosa.[16 27] We assume that identification of bacteria in enterostomy effluents may reflect colonisation in remnant intestine. This may be useful to orient preventive and therapeutic approaches, including the use of specific probiotics. Little is known about the effects of surgery on the microbiota composition of the intestine as a large number of microorganisms might be removed along with the intestine.

This study is the first assessing the longitudinal colonisation of the proximal intestinal remnant in three distinct surgical conditions, addressing the first 3 weeks after the first feasible collection of enterostomy effluent. The 16S rRNA sequence analysis used has the advantage of identifying the whole microbiota. The targeted quantitative PCR with specific primers to genus/species allows the quantification of microbiota. A novel aspect of this study includes the 18S rRNA gene analysis to examine fungal elements that has been overlooked in similar studies. Finally, the comparison of the microbiota identified in different points of assessment will give an insight of changes over time.

We acknowledge some limitations of this study. First, in a single-centre study it may be difficult to attain a sufficient sample size to assure heterogeneity as the practice of antibiotics prescription may be different from the protocols of other NICUs. Second, as a tertiary referral centre for neonatal surgery, the NICU of the Hospital Dona Estefânia receives several infants from other NICUs where they were originally admitted and previously exposed to their environments. The microbiological characteristics of those original NICUs are not contemplated in this study. Nevertheless, this issue will probably be of minor importance when enterostomy is performed within the first postnatal hours. Finally, the contact with air through the ostomy may affect the colonisation by anaerobic bacteria.

This pioneer study is expected to be useful for future research in the target population as it may serve as a basis for observational and interventional studies evaluating the modulation of the intestinal microbiota (eg, prebiotics and probiotics) on short-term and long-term outcomes.

Author affiliations
[1]Nutrition and Metabolism, NOVA Medical School | Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Lisbon, Portugal
[2]CINTESIS - Center for Health Technology and Services Research, Porto, Portugal
[3]Comprehensive Health Research Centre, Universidade NOVA de Lisboa, Lisbon, Portugal
[4]Neonatal Intensive Care Unit, Hospital Dona Estefânia, Centro Hospitalar Universitário de Lisboa Central, Lisbon, Portugal
[5]Medicine of Woman, Childhood and Adolescence, NOVA Medical School | Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Lisbon, Portugal
[6]Research Unit, Centro Hospitalar Universitário de Lisboa Central, Lisbon, Portugal
[7]Unidade Universitária Lifestyle Medicine, José de Mello Saúde by NOVA Medical School, Lisbon, Portugal

Contributors IBM, LP-da-S, DV and CC were responsible for the study conception and design. CM and AF will be responsible for gut microbiota analysis. MTN, GCF, DV, AP and LP-da-S contributed to the implementation and development of this study protocol in the NICU. All authors critically revised the manuscript and approved the final version.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval This study protocol was approved by the Ethics Committee of Centro Hospitalar Universitário de Lisboa Central (441/2017) and by the Ethics Committee of NOVA Medical School, Universidade Nova de Lisboa (n°50/2018/CEFCM). The study will be conducted in accordance with the ethical principles of the Declaration of Helsinki, the Portuguese law, and the Good Clinical Practice guidelines.

Provenance and peer review Not commissioned; externally peer reviewed.

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