BMJ Open is committed to open peer review. As part of this commitment we make the peer review history of every article we publish publicly available.

When an article is published we post the peer reviewers' comments and the authors' responses online. We also post the versions of the paper that were used during peer review. These are the versions that the peer review comments apply to.

The versions of the paper that follow are the versions that were submitted during the peer review process. They are not the versions of record or the final published versions. They should not be cited or distributed as the published version of this manuscript.

BMJ Open is an open access journal and the full, final, typeset and author-corrected version of record of the manuscript is available on our site with no access controls, subscription charges or pay-per-view fees (http://bmjopen.bmj.com).

If you have any questions on BMJ Open's open peer review process please email info.bmjopen@bmj.com

BMJ Open

A Protocol for a Cross Sectional Observation Study Exploring the Gut Microbiota of Infants with Complex Congenital Heart Disease Undergoing Cardiopulmonary Bypass (the GuMiBear study)

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2022-067016
Article Type:	Protocol
Date Submitted by the Author:	02-Aug-2022

Complete List of Authors:	Magner, Claire; University College Dublin, School of Nursing, Midwifery and Health Systems Jenkins, Dominic; Children's Health Ireland at Crumlin Koc, Fatma; University College Cork, School of Microbiology; Teagasc Food Research Centre, Food Biosciences Tan, Mong Hoi; Children's Health Ireland at Crumlin O’Toole, Molly; Children's Health Ireland at Crumlin Boyle, Jordan; Children's Health Ireland at Crumlin Maguire, Niamh; Children's Health Ireland at Crumlin Duignan, Sophie; Children's Health Ireland at Crumlin Murphy, Kiera; University College Cork APC Microbiome Institute; Teagasc Food Research Centre, Food Biosciences Ross, Paul; University College Cork College of Science Engineering and Food Science Stanton , Catherine ; University College Cork APC Microbiome Institute; Teagasc Food Research Centre McMahon, Colin J.; Children's Health Ireland at Crumlin, Department of Paediatric Cardiology; University College Dublin School of Medicine

Keywords:	Congenital heart disease < CARDIOLOGY, Cardiac surgery < SURGERY, Paediatric cardiology < CARDIOLOGY, Quality in health care < HEALTH SERVICES ADMINISTRATION & MANAGEMENT, Biochemistry < NATURAL SCIENCE DISCIPLINES

SCHOLARONE™ Manuscripts

Title: A Protocol for a Cross Sectional Observation Study Exploring the Gut Microbiota of Infants with Complex Congenital Heart Disease Undergoing Cardiopulmonary Bypass (the GuMiBear study)

Authors: Claire Magner[1], Dominic Jenkins[2], Fatma Koc[3,4,5], Mong Hoi Tan[2], Molly O'Toole[2], Jordan Boyle[2], Niamh Maguire[2], Sophie Duignan[2], Kiera Murphy[4,5], R. Paul Ross[4] , Catherine Stanton[4,5], Colin J. McMahon[6]

[1] School of Nursing, Midwifery & Health Systems, University College Dublin, Ireland.

[2] Children's Health Ireland at Crumlin, Dublin, Ireland

[3] School of Microbiology, University College Cork

[4] APC Microbiome Ireland, University College Cork, Co Cork, Ireland

[5] Food Biosciences, Teagasc Food Research Centre, Moorepark, Co Cork, Ireland

[6] Department Paediatric Cardiology, Children's Health Ireland at Crumlin, Dublin, Ireland

Please address all correspondence to Dr Claire Magner, B3.10 Health Sciences Building, University College Dublin. Email: claire.magner@ucd.ie.

Abstract

Introduction

The gut microbiota develops from birth and matures significantly during the first 24 weeks of life, playing a major role in infant health and development. The composition of the gut microbiota is influenced by several factors including mode of delivery, gestational age, feed type and treatment with antibiotics. Alterations in the pattern of gut microbiota development and composition can be associated with illness and compromised health outcomes.

Infants diagnosed with 'Congenital Heart Disease' (CHD) often require surgery involving cardiopulmonary bypass (CPB) early in life. The impact of this type of surgery on the integrity of the gut microbiome is poorly understood. In addition, these infants are at significant risk of developing the potentially devastating intestinal condition Necrotising Enterocolitis (NEC).

Methods and Analysis

This study will employ a prospective cross-sectional observational methodology to investigate the gut microbiota and urine metabolome of infants with CHD undergoing surgery involving CPB. Stool and urine samples, demographic and clinical data will be collected from eligible infants based at the national centre for paediatric cardiac surgery in

Ireland. Shotgun metagenome sequencing will be performed on stool samples and urine metabolomic analysis will identify metabolic biomarkers. The impact of the underlying diagnosis, surgery involving CPB, and the influence of environmental factors will be explored. Data from healthy age matched infants from the INFANTMET study will serve as a control for this study.

Ethics and Dissemination

This research study is ethically approved (REC REF No: GEN/826/20). Study results will be available to patients with CHD and their families, carers, support networks, paediatric cardiology and microbiome societies and other researchers. Study findings will provide a deeper understanding of the gut microbiota of infants with CHD and inform perioperative management options including strategies to prioritise the integrity of the gut microbiota.

Abbreviations:

APGAR Score Newborn assessment score rating: Appearance, Pulse, Activity, Grimace, Respiration

AVSD Atrioventricular Septal Defect

CHD Congenital Heart Disease

CHC Children's Heart Centre

CHI Children's Health Ireland

CPB Cardiopulmonary Bypass

CRF Case Report Form

EBD Epithelial Barrier Dysfunction

GDPR General Data Protection Regulation

HLHS Hypoplastic Left Heart Syndrome

HRHS Hypoplastic Right Heart Syndrome,

HSE Health Service Executive

ICF Informed Consent Form

IV	Intravenous
NEC	Necrotising Enterocolitis
PICU	Paediatric Intensive Care Unit
PIM3	Paediatric Index of Mortality
RBB	Repeated Beat Beating
TGA	Transposition of the Great Arteries
TP	Time Point

Keywords: Congenital Heart disease, Microbiota, Infant, Antibiotic, Development, Cardiac Surgery, Cardiopulmonary Bypass

Word Count: 3,184

Article Summary

Strengths and Limitations of this Study

- GuMIBear is the first study to investigate the gut microbiome of infants with CHD undergoing surgery with CPB over a 24 week timeframe
- Comparison with age matched healthy controls allow deeper insight into clinically relevant microbiome alterations throughout the surgical course
- The study involves urine metabolomic analysis providing novel insight into the metabolite profile of study participants compared with healthy age matched infants
- The primary limitation of GuMIBear is that it is a single centre study limiting the generalisability of the findings

Introduction

What is currently known

The establishment of gut microbiota begins at birth and continues over the first years of life. Continued evolution of the gut microbiome after birth is governed by host factors such as both the adaptive and innate immune system, as well as external factors such as diet, medication and toxin exposure, and illness.[1] Understanding the role of the gut microbiome in metabolism, immune function and nutrition is gaining increasing recognition, as it is accepted that an altered colonisation has been associated with a higher risk of diseases later in life.[2] In the critical first weeks and months of life, perturbations to the infant gut microbiome have implications for growth development and health.[3]

The Microbiome and Systemic Inflammation

It is evident that under certain conditions, disruption of the normal microbiota that colonise the intestinal tract can occur.[4] These conditions include systemic inflammatory processes, which can result in intestinal inflammation, where proinflammatory bacteria can flourish, interacting with the intestinal epithelium to cause cytokine release, activating key inflammatory pathways increasing morbidity and prolonging critical illness.[5] The pattern of cytokine release in patients undergoing CPB is described as comparable to those released in systemic inflammation such as trauma and sepsis.[6] However, the nature of gut microbiota compositional changes in infants undergoing surgery with CPB remains understudied. This research aims to address this knowledge gap to enhance our understanding and inform care practices.

CHD and Necrotising Enterocolitis (NEC)

CHD affects approximately 1 in every 100 babies born throughout the US every year,[7] and is the most common congenital defect worldwide.[8] A diagnosis of 'Complex Congenital Heart Disease' can include conditions such as Hypoplastic Left Heart Syndrome (HLHS); Hypoplastic Right Heart Syndrome (HRHS), Transposition of the Great Arteries (TGA) requiring intervention in the first week of life while other cardiac conditions such as Atrioventricular Septal Defect (AVSD), Tetralogy of Fallot may require corrective surgery in the first few months of life. Complex CHDs such as those requiring surgery involving cardiopulmonary bypass (CPB) present a greater risk to patients. This increased risk is not limited directly to the surgery, compromised ventricular function or low cardiac output state, but includes the risk of developing NEC.

There is a well established connection between CHD and NEC, a potentially devastating intestinal condition of infancy.[9] NEC carries a reported incidence of between 3 and 9% in infants with congenital heart disease (CHD) with all-cause mortality rates as high as 38% in children with 'cardiogenic' NEC.[10] While CHD remains one of the most common risk factors for NEC, the underlying pathophysiology of this association is complex.[11] Growing evidence suggests that perturbations in the early-life gut microbiota composition increase the risk for NEC.[3,12] Given the likelihood that haemodynamic and intrinsic gut factors predispose this group to NEC, acute and chronic circulatory changes have been investigated. An association between hypoperfusion and episodes of shock and diastolic flow reversal in the abdominal aorta causing impaired mesenteric blood flow has been identified.[13] Furthermore, a significant association between episodes of low cardiac output and shock in the development of NEC was identified.[14] It is reported that infants with certain types of CHD, mainly HLHS, may possess abnormal systemic vasculature contributing to the increased risk for NEC.[15]. Whether it is those, or other causes of impaired perfusion to the gut, the resulting damage to the mucosal barrier can provide an entry point to bacteria provoking an inflammatory cascade, and the devastating consequences that can ensue.[16] The vulnerability of infants with CHD is enhanced during the course of surgical intervention involving CPB, and the role of the gut microbiome has received little research focus in this context.

Surgery involving CPB

Infants diagnosed with CHD are at risk of alterations to their intestinal homeostasis, a further threat is presented in the context of surgery involving CPB. There is evidence to suggest intestinal ischaemia reperfusion injury occurs after CPB and contributes to epithelial barrier dysfunction (EBD) potentially exposing the bloodstream to bacteria or bacterial products.[17] Although alterations in gut barrier integrity and resident microbiota have been demonstrated,[3] it is not fully understood what changes to the microbiome occur following CPB, and the nature and severity of EBD. While the gut microbiota in infants with CHD following CPB remains understudied, a small single centre case control study recently identified significant gut microbiota perturbations in patients with CHD.[18] This case-control study highlighted that children with CHD had a disrupted gut microbiome at baseline with an over-representation of pro-inflammatory bacteria, this was further exacerbated by CPB. Samples were collected pre-operatively and in a limited 24 and 48 hour time frame postoperatively. The significance of intraoperative variables including aortic cross clamp time and duration of CPB was not determined.

Our study proposes to address the knowledge gap and advance existing research by examining the gut microbiome of infants with CHD pre-operatively, and at defined time points up to 24 weeks postoperatively. This timeline will account for the recovery phase post cardiac surgery, including time to re-establish full feeds, wean from mechanical ventilation and circulatory support, and allow for surveillance of NEC postoperatively. Comparisons will be made with healthy age-matched infants recruited as part of the INFANTMET study.[19] As well as collecting intraoperative variables such as duration of CPB, and aortic cross clamp time, a novel aspect of this research will be to profile the metabolites in urine to assess potential metabolic biomarkers and pathway changes. Our research will recruit patients in a National Centre for Paediatric Cardiac Surgery, where 40 open cardiac surgeries are performed on infants annually. We therefore anticipate active recruitment will ensure the proposed target sample of 50 participants is achievable. No additional invasive procedures will be required for sample collection, enhancing the acceptability of the research for consenting parents or carers. This project will investigate the subdivisions of the gut microbiota of infants with CHD, and environmental factors such as the influence of mode of delivery, pre-operative fasting states and mode of feeding, and use of pre-operative antibiotics. Understanding the status of the intestinal microbiome of infants with CHD and the effects of undergoing surgery with CPB is vital in informing best care practices to enhance patient outcomes.

Methods

Study design

This study is a cross-sectional observational study of infants with Complex CHD undergoing CPB at the National Centre for Paediatric Cardiac Surgery at Children's Health Ireland at Crumlin, Dublin, Ireland. This single-site study will investigate the differences in the microbiome, metabolomics readouts, and stress levels between infants with CHD undergoing CPB and healthy age matched controls.

Participant selection

This study will involve the collection of demographic and clinical maternal and infant data from those meeting eligibility criteria with complex CHD scheduled for surgery involving CPB (see Table 1 for participant inclusion and exclusion criteria). Infant stool and urine samples will be collected at defined time points set out in the protocol (See Appendix 1 for Case Report Form).

Public and Patient Involvement Statement

The mother of a child who had cardiac surgery as a baby was involved in reviewing the study literature.

Inclusion and exclusion criteria

To be eligible for the study, the participants must meet the terms of the inclusion and exclusion criteria as presented in Table 1.

Table 1. Inclusion and Exclusion Criteria.

Inclusion criteria	Exclusion criteria
Infants born full term (≥37 weeks) weeks gestation)	Stillbirth or live birth where the baby is born alive but dies shortly after
Infants diagnosed with *CHD and scheduled for surgery involving CPB	Infants who are born healthy with no underlying illness, syndrome, or chronic disease
Infants born in Ireland to allow sample follow up	Participation in another study
Ability of the participant's parent/carers (in the investigator's opinion) to comprehend the full nature and purpose of the study	Infants not undergoing surgery involving cardiopulmonary bypass

Consent to participate in the study and willingness to comply with the protocol and study restrictions by the participant's parent/carers	Infants where parents/carers do not give consent to participate in the study
Upper age limit of less than 6 months	Gastrointestinal pathology or intestinal surgery, excluding gastrostomy tube

*CHD includes Hypoplastic left heart syndrome (HLHS); Hypoplastic Right Heart Syndrome (HRHS), Transposition of the Great Arteries (TGA), Atrioventricular Septal Defect (AVSD), Tetralogy of Fallot.

Recruitment

Participants meeting inclusion criteria will be selected after admission to the hospital, outpatient clinic or cardiac day unit. Study-related information will be given in written form as well as explained by a member of the project team. No study-related activities will begin before the potentially eligible participants' parents/carers have signed the Informed Consent Form (ICF). Participants parents/carers will be asked to refer to the Privacy Notice on the hospital website or they can receive a hardcopy if they wish. Signed ICFs will be stored safely in a locked cabinet in the research office.

Compensation

No compensation will be provided to the participants. There are no cost implications for the Health Service Executive (HSE) or to the participants. The management of patients and investigative tests will comply with current standards of care.

Study timeline

After completing recruitment procedures, i.e., determining whether the patient meets the study inclusion criteria, discussing the study with the parents/caregivers and obtaining informed consent, clinical and demographic data will start to be collected.

The study will be undertaken for a period of 24 weeks after the infant is initially recruited.

Demographic Data

The infants' diagnosis, co-morbidities, date of birth, gestational age, sex, mode of delivery, APGAR scores, birth weight, head circumference and antibiotics administered post-delivery and antenatal events will be recorded.

Maternal Data

Maternal history including age, antibiotics received, smoking status and significant antenatal events will be recorded.

Surgical course

The type of surgery performed, duration of cardiopulmonary bypass and cross-clamp time will be recorded. Antibiotic use and any intraoperative events will be recorded as well as clinical information including arterial blood gas data.

Post-operative Data

Paediatric Index of Mortality (PIM) score, duration of mechanical ventilation, renal and cardiovascular support and duration of stay in paediatric intensive care unit will be recorded.

Feeding Information Data

Feeding information including the type of feed and duration of the feed prior to surgery will be recorded. The date the patient is established on full feeds will be recorded. Full feeding is defined as when the patient no longer requires parenteral nutrition or intravenous fluids.

Discharge Information Data

Discharge information data: This will include the patient's status on discharge from PICU, as well as length of PICU and hospital stay.

Complications

The occurrence of complications will be recorded, for example the development of NEC. The timeline for recording NEC onset will be based on the initiation of triple antibiotic therapy, based on a full surgical review including clinical presentation, radiological and laboratory data.

Subject withdrawal/exclusion

Under the Declaration of Helsinki, the research nurse will explain to the consenting adult that they have the right to withdraw from the study at any time and that this will in no way prejudice their future treatment. The reason for withdrawal will be recorded in the source documents and on the appropriate CRF. Consenting adults will be made aware that stored samples from individuals withdrawing from the study may have undergone processing and may be analysed in the study.

Regulatory procedures

The study is conducted following the version Fortaleza, Brazil, October 2013 of the Declaration of Helsinki 1964. The Protocol and the ICF have been approved by the Clinical Research Ethics Committee of Children's Health Ireland GEN/826/20. As biological samples will be procured in one institution and sent to another, a data sharing agreement is in place between The Cardiology Department, Children's Health Ireland at Crumlin Hospital, and APC Microbiome in Cork. This research is fully compliant with the guidelines as set out in The General Data Protection Regulation (GDPR), the Irish Data Protection Acts 1988 to 2018 including Protection Act 2018 (Section 36(2)) (Health Research) Regulations 2018.

Data Statement

Once collected, the anonymised demographic, clinical and laboratory analysis data as well as statistical codes will be uploaded to the open access Research Repository University College Dublin.

Objectives and Outcomes

The primary study objectives and outcomes are-

- To characterise the gut microbiota composition of infants with specified CHD undergoing surgery with CPB at specific time points perioperatively.
- To determine any differences in gut microbiota composition of infants who take part in this study compared with the microbiota of healthy babies from the INFANTMET study at matching time points.[19]
- To characterise the urine metabolite profile of infants with specified CHD undergoing surgery with CPB and compare with healthy infants from the INFANTMET study.[19]

Secondary objectives and outcomes

- To explore the influence of maternal and environmental factors on gut microbiome composition.

Sample collection and analysis

Faecal samples

Stool samples will be collected at the following time points: within 24 hours after birth (TP 1), within 24 hrs pre surgery (TP 2) 1 week post-surgery (TP 3) 4 weeks post-surgery (TP 4), 24 weeks post-surgery (TP 5). Information about antibiotic therapy administered before or during the stool collection will be recorded.

The sample will be collected by the bedside nurse or the parent/carers and transferred to the laboratory upon receipt of the sample during the weekdays or weekends. At night, the sample will be kept in the dedicated fridge prior to transfer to the laboratory the following day by the bedside nurse. Samples will be stored at -80°C until further analysis.

Urine Samples

Urine samples will be collected at 4-8 weeks post-surgery for metabolomic analysis using Sterisets Uricol Urine Collection Pack (MedGuard, Ireland). The urine sample will be collected from the urinary catheter if the participant is catheterised. Alternatively, a pad will be placed in the diaper and used to collect an unsoiled urine sample from the infant. The pad will then be placed in a biohazard bag and frozen immediately at -80 °C prior to processing. After all the sample collections are complete, they will be shipped to Teagasc Food Research, Moorepark, Ireland, using DHL overnight service for microbiome and metabolomics analyses. Styrofoam Saf-T-Pak STP-309 shipper box or equivalent will be used. DNA extraction will be performed on stool samples using the modification of the Repeated Bead Beating Plus Column (RBB+C) method.[20] LC-MS will be utilised for metabolomics analysis of urine.[19] Sample collection for discharged participant

Parents/carers will receive a sample collection discharge pack and a parent diary/instruction served as reminder to collect due samples at different time-point prior to discharge home. They will receive a follow up text message or phone call to remind them on the due sample. The sample collection discharge pack consists of urine/stool collection containers with study code, sterile pad, syringe, zip-lock bag, gloves, biohazard bag and cooler bag. Parents/carers are asked to keep the collected sample at the dedicated section of their home freezer. They will transport the collected sample in the cooler bag provided when attending out-patient department for appointments. They will ask a member of the project team to transfer the collected sample to the laboratory upon arrival at the hospital. The study researcher is available at the dedicated contact number for any queries.

Adverse events and participant well-being

There are no expected safety concerns related to the study. All study participants will be under the care of the cardiology team at the hospital with access to psychological support, as well as nursing and medical professionals, social workers and chaplaincy.

Data collection and management

The study diaries, study dataset, and paper/digital CRF systems will be used for recording data from each study participant. All the data collected in this study is pseudonymised, as each of the participants will be assigned a specific study code and upon receipt, data will be referred to the study code. All study staff responsible for entering data into the CRF system received training in advance of the study commencement. This training included familiarity with the study diaries, study dataset, and paper/digital CRF system and have completed good clinical practice in research training. They have individual access to the password protected study shared drive within the hospital. The study team will monitor the data/sample collection process. Any inconsistencies identified during the study will be presented as queries at the regular project team meeting.

Comparison Data

Data collected as part of the INFANTMET Study will serve as a healthy control comparison for this study. INFANTMET compared the gut microbiota development of breastfed infants born via C-section or vaginally at full –term or preterm at Cork University Maternity Hospital. Ethical approval for sample collection by Cork University Hospital Research Ethics Committee (reference number ECM(w) 07/02/2012). One hundred and ninety two infants were recruited to the INFANTMET study and stratified according to delivery mode and gestational age at birth. Faecal samples were collected from the infants at 1, 4, 8 and 24 weeks of age and stored under controlled conditions. Urine samples were collected at 4 weeks of age for metabolomic analysis and stored in a freezer at -80°C prior to processing. Samples were analysed in accordance with the analysis proposed for the GuMIBear study.

Bioinformatics and Statistical analysis

Sample size justification

Published research in this area is lacking. However one case control study by Salomon et al (2021) included 17 cases and 12 control participants and was sufficiently powered to determine a statistically significant difference in beta diversity in cases versus controls ($F=5.6$, $p<0.001$). Our study proposes to include 50 patients with CHD undergoing surgery with CPB and age matched controls, almost three times the Solomon et al (2021) study. We therefore anticipate that the proposed sample size is justified.

Demographic and Clinical Data

Demographic and clinical data and laboratory information will be tested for normality using the Shapiro-Wilk test. Descriptive statistics will be used to describe normally distributed data, and expressed as mean $\pm$ SD. Continuous data not normally distributed will be reported as median and interquartile ranges. Categorical variables will be expressed as counts and percentages. Groups will be compared using chi square tests for categorical variables and independent-samples student's *t*- tests for normally distributed continuous variables. For variables not normally distributed, the Mann-Whitney *U* test will be used.

Microbiome analysis

Metagenomic shotgun sequencing data will be analysed using bioBakery suite of tools (https://huttenhower.sph.harvard.edu/biobakery_workflows/). Trimmed and human reads filtered using KneadData (v0.7.2) with the default parameters. Quality controlled data will be taxonomically profiled at the species level with relative abundance by MetaPhlAn2. Functional profiling will be performed using HUMAnN and strain profiling using StrainPhlAn.

For alpha diversity analysis, samples will be rarefied to even depth and phyloseq::estimate richness will be used to calculate Chao1, Shannon and Simpson indices. Alpha diversity indices between groups will be univariately compared using the Wilcoxon rank sum test. A beta-diversity ordination will be generated using the Aitchison distance and visualised using Principal Component Analysis (PCA) plot. The Adonis function in the vegan package will be used to implement a permutational multivariate analysis of variance to test whether samples cluster beyond that expected by sampling variability. Differentially abundant features will be identified using MaAsLin2.

Discussion

Despite an increasing awareness of the early-life critical window of microbiome development on the health and wellness of infants, there remains much to learn about the interactions of the microbiome with the infant host with CHD undergoing surgery involving CPB. This study is designed to address this knowledge gap, and incorporates a sound methodology with particular strengths enhancing the value of its findings. The specimen collection strategy occurring at multiple time points over a 24 week period in the proposed study will deepen our understanding of the temporal dynamics of the colonising microbiota, and their interactions with host physiology. The study design will account for maternal and perioperative variables to determine changes to the microbiome. Access to existing microbiome data from healthy age matched infants provides a valuable opportunity to present high quality comparative information. While multi-centre trials capturing sufficient case numbers of NEC cases will offer robust conclusions, this study will offer valuable evidence to support the influence of CHD and CPB on the microbiome and intestinal EBD. Future research can build on existing studies, and explore treatment strategies including recommendations for efficacious probiotic strain administration, including the supplements to promote a diverse gut microbiota to improve outcomes for this vulnerable population.

Status of Study

The trial is ongoing and as of July 20th 1st 2022, 70 % of the participants have been recruited. Laboratory analysis has been carried out on 20% of study samples.

Funding Statement

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors

Competing interests

The authors declare no association with commercial entities, either financial or non-financial.

Author contributions

CM, CJMcM, CS, KM devised the project, and the main conceptual ideas. CJMcM, CM, CS, RPR, FK, DJ, MHT were involved in the study design and writing of the manuscript. MOT, JB, NM, SD, CJMcM are involved in consenting participants, collecting samples and acquiring data. DJ, CS, FK and RPR are responsible for analysing study samples. All authors read manuscript revisions, approved the final manuscript and accept accountability for the accuracy and integrity of the work.

Acknowledgements

All Paediatric Intensive Care Unit/ Children's Heart Centre nurses for assisting in sample collection. All laboratory staff including Ms Irene Regan who have facilitated tracking and secure storage of study samples. Dr Adam James, Dr Ross Foley and the Consultant Paediatric Consultants, CHI at Crumlin for their support with this study.

Appendix 1: Case Report Form PDF Version

References

1. Skillington O MS, Gupta A, Mayer EA, Gill CIR, Del Rio D, O'Riordan KJ, Cryan JF, Ross RP and Stanton C The contrasting human gut microbiota in early and late life and implications for host health and disease. Nutrition and Healthy Ageing. 2012;6:157-78.
2. Cilieborg MS, Boye M, Sangild PT. Bacterial colonization and gut development in preterm neonates. Early human development. 2012;88 Suppl 1:S41-9.
3. Healy DB, Ryan CA, Ross RP, Stanton C, Dempsey EM. Clinical implications of preterm infant gut microbiome development. Nature microbiology. 2022;7(1):22-33.
4. Ohland CL, Jobin C. Microbial activities and intestinal homeostasis: A delicate balance between health and disease. Cellular and molecular gastroenterology and hepatology. 2015;1(1):28-40.
5. Cabrera-Perez J, Badovinac VP, Griffith TS. Enteric immunity, the gut microbiome, and sepsis: Rethinking the germ theory of disease. Experimental biology and medicine. 2017;242(2):127-39.
6. Halter J, Steinberg J, Fink G, Lutz C, Picone A, Maybury R, et al. Evidence of systemic cytokine release in patients undergoing cardiopulmonary bypass. The journal of extra-corporeal technology. 2005;37(3):272-7.
7. Centers for Disease Control and Prevention (CDC). Data and Statistics on Congenital Heart Defects. 2022 [updated January 24th 202226th July 2022]. Available from: https://www.cdc.gov/ncbddd/heartdefects/data.html Accessed 26th July 2022.
8. van der Linde D, Konings E, Slager M, Witsenburg M, Helbing W, Takkenburg J, Roos-

Hesselink J. Birth Prevalence of Congenital Heart Disease Worldwide: A Systematic Review and Meta-Analysis. Journal of the American College of Cardiology. 2011;58(21):2241-2247.
9. Martin R, Makino H, Cetinyurek Yavuz A, Ben-Amor K, Roelofs M, Ishikawa E, et al. Early-Life Events, Including Mode of Delivery and Type of Feeding, Siblings and Gender, Shape the Developing Gut Microbiota. PloS one. 2016;11(6):e0158498.
10. Siano E, Lauriti G, Ceccanti S, Zani A. Cardiogenic Necrotizing Enterocolitis: A Clinically Distinct Entity from Classical Necrotizing Enterocolitis. European journal of pediatric surgery : official journal of Austrian Association of Pediatric Surgery. 2019;29(1):14-22.
11. Kelleher ST, McMahon CJ, James A. Necrotizing Enterocolitis in Children with Congenital Heart Disease: A Literature Review. Pediatric cardiology. 2021;42(8):1688-99.
12. Murphy K, O'Donovan AN, Caplice NM, Ross RP, Stanton C. Exploring the Gut Microbiota and Cardiovascular Disease. Metabolites. 2021;11(8).
13. Carlo WF, Kimball TR, Michelfelder EC, Border WL. Persistent diastolic flow reversal in abdominal aortic Doppler-flow profiles is associated with an increased risk of necrotizing enterocolitis in term infants with congenital heart disease. Pediatrics. 2007;119(2):330-5.
14. McElhinney DB, Hedrick HL, Bush DM, Pereira GR, Stafford PW, Gaynor JW, et al. Necrotizing enterocolitis in neonates with congenital heart disease: risk factors and outcomes. Pediatrics. 2000;106(5):1080-7.
15. Miller TA, Minich LL, Lambert LM, Joss-Moore L, Puchalski MD. Abnormal abdominal aorta hemodynamics are associated with necrotizing enterocolitis in infants with hypoplastic left heart syndrome. Pediatric cardiology. 2014;35(4):616-21.
16. Nino DF, Sodhi CP, Hackam DJ. Necrotizing enterocolitis: new insights into pathogenesis and mechanisms. Nature reviews Gastroenterology & hepatology. 2016;13(10):590-600.
17. Typpo KV, Larmonier CB, Deschenes J, Redford D, Kiela PR, Ghishan FK. Clinical characteristics associated with postoperative intestinal epithelial barrier dysfunction in children with congenital heart disease. Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies. 2015;16(1):37-44.
18. Salomon J, Ericsson A, Price A, Manithody C, Murry DJ, Chhonker YS, et al. Dysbiosis and Intestinal Barrier Dysfunction in Pediatric Congenital Heart Disease Is Exacerbated Following Cardiopulmonary Bypass. JACC Basic to translational science. 2021;6(4):311-27.
19. Hill CJ, Lynch DB, Murphy K, Ulaszewska M, Jeffery IB, O'Shea CA, et al. Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. Microbiome. 2017;5(1):4.
20. Yu Z, Morrisson M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques. 2004;36(5):808-12.

GuMIBear Study

Study ID: GMB: ______

^**999** – missing data; **0** – no data in medical notes

CASE RECORD FORM

Stool Sample Collections

Period/ Time Frame		Projected Date	Sample Date	Sample Collected	Comments
1.	Within 24h of Birth			☐ YES ☐NO	
2.	Pre-operatively			☐ YES ☐NO	
3.	Week 1 of life/Post-Op			☐ YES ☐NO	
4a.	Week 4 to 8 life/Post-op			☐ YES ☐NO	
4b.	**Urine** Sample Week 4 to 8			☐ YES ☐NO	
5.	Week 24 of life/Post-Op			☐ YES ☐NO	

PATIENT DEMOGRAPHIC

Date of Enrolment:		Date of Birth:	
Gender:	☐ Male ☐ Female ☐ Other/Ambiguous	Gestational Age: APGAR at ① ⑤ min	Multiple ☐ Yes ☐ No Birth Order:
Reason for admission:			
Comorbidities:			
Mode of Delivery: SVD: ☐Yes ☐No LSCS: ☐Yes ☐No, if Yes: ☐Elective/ ☐Emergency		Weight at Birth: ________ . ________ KGs Head Circumference: ________ cms	
Antibiotics to Infant post-delivery; ☐ Yes ☐ No If Yes, list:			
Significant Antenatal Events:			

MATERNAL INFORMATION

Maternal Age (years) at Birth:	Gestational Age at Booking Appt:
Antibiotics given Pre-Delivery: ☐ Yes ☐ No	List:
Maternal Smoking during Pregnancy: ☐ Yes ☐ No	Other Household Members Smoking during Pregnancy: ☐ Yes ☐ No

GuMIBear Study

Study ID: GMB: ______

^**999** – missing data; **0** – no data in medical notes

SURGERY INFORMATION

Surgery Date:				
Surgery Performed:				
Pre-Op Antibiotics[1]? List:			Post-Op Antibiotics[1]? List:	
ABG	pH	PO^2		
First Pre-Op ABG:			Cardiopulmonary Bypass Duration:	
First Post-Op ABG:			Aortic Cross Clamp Duration:	
Significant Intraoperative Events:				

POST-OPERATIVE INFORMATION

PIM3 Score:		No. of days in ICU (1^{st} adm):	
Mechanical Vent:	☐ Yes ☐ No	No of Days on ECLS:	
No of Days Vent:		No of Days on RRT:	
Agent	Day 1	Day 2	Day 3
Milrinone			
Epinephrine			
Norepinephrine			
Vasopressin			
Midazolam			
Morphine			
Clonidine			
Others			

FEEDING INFORMATION

Mode of Feeding (note ***date initiated*** and ***date d/c***): Breastmilk: Infant Formula: Other:	
Prebiotics given to Infant: ☐ Yes ☐ No Type and Date Given:	
Excessive Infantile Crying (cried for >3 Hrs for 3 Days in one week): ☐ Yes ☐ No	
Date Trophic Feeds Commenced: Type of Feed Used:	
Time to Establishment of full feed [2]:	
Development of NEC[3]: Days post-op when developed NEC?	
~~Gut stasis:~~	N/A
Management Strategy:	

DISCHARGE INFORMATION

Date of Discharge

Ward: Home: RIP:

GuMIBear Study

Study ID: GMB: ______

^**999** – missing data; **0** – no data in medical notes

READMISSION TO ICU

Total ICU Readmission days:				
	Date of Admission	Date of Discharge	Total ICU stays	Reason for admission
1.				
2.				
3.				
4.				
5.				

DATA ENTRY BY (NAME)

Admission	Date	Paper	Date	G-Drive
First				
Second				
Third				

[1]Antibiotic treatment at time of stool sampling as below. Important not to include antibiotics which were started post the stool sampling

1) Abs < 48hrs pre sampling
2) Abx < 72 hrs pre sampling
3) Abx in previous 7 days

[2]Full feed – No longer requires parenteral nutrition or intravenous fluids supplement regardless the TFI.

[3]NEC –Initiation of triple IV antibiotic therapy and nil by mouth for at least 5 days, based on a full surgical review including clinical presentation, radiological and laboratory data.

A Protocol for a Prospective Cohort Study Exploring the Gut Microbiota of Infants with Congenital Heart Disease Undergoing Cardiopulmonary Bypass (the GuMiBear study)

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2022-067016.R1
Article Type:	Protocol
Date Submitted by the Author:	11-Nov-2022
Complete List of Authors:	Magner, Claire; University College Dublin, School of Nursing, Midwifery and Health Systems Jenkins, Dominic; Children's Health Ireland at Crumlin Koc, Fatma; University College Cork, School of Microbiology; Teagasc Food Research Centre, Food Biosciences Tan, Mong Hoi; Children's Health Ireland at Crumlin O'Toole, Molly; Children's Health Ireland at Crumlin Boyle, Jordan; Children's Health Ireland at Crumlin Maguire, Niamh; Children's Health Ireland at Crumlin Duignan, Sophie; Children's Health Ireland at Crumlin Murphy, Kiera; University College Cork APC Microbiome Institute; Teagasc Food Research Centre, Food Biosciences Ross, Paul; University College Cork College of Science Engineering and Food Science Stanton , Catherine ; University College Cork APC Microbiome Institute; Teagasc Food Research Centre McMahon, Colin J.; Children's Health Ireland at Crumlin, Department of Paediatric Cardiology; University College Dublin School of Medicine
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Paediatrics
Keywords:	Congenital heart disease < CARDIOLOGY, Cardiac surgery < SURGERY, Paediatric cardiology < CARDIOLOGY, Quality in health care < HEALTH SERVICES ADMINISTRATION & MANAGEMENT, Biochemistry < NATURAL SCIENCE DISCIPLINES

Title: A Protocol for a Prospective Cohort Study Exploring the Gut Microbiota of Infants with Congenital Heart Disease Undergoing Cardiopulmonary Bypass (the GuMiBear study)

Authors: Claire Magner[1], Dominic Jenkins[2], Fatma Koc[3,4,5], Mong Hoi Tan[2], Molly O'Toole[2], Jordan Boyle[2], Niamh Maguire[2], Sophie Duignan[2], Kiera Murphy[4,5], R. Paul Ross[4] , Catherine Stanton[4,5], Colin J. McMahon[6]

[1] School of Nursing, Midwifery & Health Systems, University College Dublin, Ireland.

[2] Children's Health Ireland at Crumlin, Dublin, Ireland

[3] School of Microbiology, University College Cork

[4] APC Microbiome Ireland, University College Cork, Co Cork, Ireland

[5] Food Biosciences, Teagasc Food Research Centre, Moorepark, Co Cork, Ireland

[6] Department Paediatric Cardiology, Children's Health Ireland at Crumlin, Dublin, Ireland

Please address all correspondence to Dr Claire Magner, B3.10 Health Sciences Building, University College Dublin. Email: claire.magner@ucd.ie.

Abstract

Introduction

The gut microbiota develops from birth and matures significantly during the first 24 months of life, playing a major role in infant health and development. The composition of the gut microbiota is influenced by several factors including mode of delivery, gestational age, feed type and treatment with antibiotics. Alterations in the pattern of gut microbiota development and composition can be associated with illness and compromised health outcomes.

Infants diagnosed with 'Congenital Heart Disease' (CHD) often require surgery involving cardiopulmonary bypass (CPB) early in life. The impact of this type of surgery on the integrity of the gut microbiome is poorly understood. In addition, these infants are at significant risk of developing the potentially devastating intestinal condition Necrotising Enterocolitis (NEC).

Methods and Analysis

This study will employ a prospective cohort study methodology to investigate the gut microbiota and urine metabolome of infants with CHD undergoing surgery involving CPB. Stool and urine samples, demographic and clinical data will be collected from eligible infants based at the National Centre for Paediatric Cardiac Surgery in Ireland. Shotgun metagenome sequencing will be performed on stool samples and urine metabolomic analysis will identify

metabolic biomarkers. The impact of the underlying diagnosis, surgery involving CPB, and the influence of environmental factors will be explored. Data from healthy age matched infants from the INFANTMET study will serve as a control for this study.

Abbreviations:

APGAR Score Newborn assessment score rating: Appearance, Pulse, Activity, Grimace, Respiration

ASD	Atrial Septal Defect
AVSD	Atrioventricular Septal Defect
BUN	Blood Urea Nitrogen
CHC	Children's Heart Centre
CHD	Congenital Heart Disease
CHI	Children's Health Ireland
CPB	Cardiopulmonary Bypass
CRF	Case Report Form
D-TGA	Dextro-Transposition of the Great Arteries
EBD	Epithelial Barrier Dysfunction
ECG	Electrocardiogram
GCP	Good Clinical Practice
GDPR	General Data Protection Regulation
HLHS	Hypoplastic Left Heart Syndrome
HRHS	Hypoplastic Right Heart Syndrome
HSE	Health Service Executive
ICF	Informed Consent Form

IV	Intravenous
IVS	Intra Ventricular Septum
L-TGA	Levo-Transposition of the Great Arteries
LVOT	Left Ventricular Outflow Tract
MaAsLin2	Multivariate Associations with Linear Models
NEC	Necrotising Enterocolitis
PCA	Principal Component Analysis
PICU	Paediatric Intensive Care Unit
PIM3	Paediatric Index of Mortality
PPI	Public and Patient Involvement
PS	Pulmonary Valve Stenosis
RBB+C	Repeated Beat Beating Plus Column (RBB+C) method
RVOT	Right Ventricular Outflow Tract
sCCA	Sparse canonical correlation analysis
SD	Standard Deviation
SOP	Standard Operating Procedure
TGA	Transposition of the Great Arteries
TP	Time Point
VSD	Ventricular Septal Defect

Keywords: Congenital Heart disease, Microbiota, Infant, Antibiotic, Development, Cardiac Surgery, Cardiopulmonary Bypass

Word Count: 3,824

Article Summary

Strengths and Limitations of this Study

- GuMIBear is the first study to investigate the gut microbiome of infants with CHD undergoing surgery with CPB over a 2 year timeframe
- Comparison with age matched healthy controls allow insights into clinically relevant microbiome alterations throughout and beyond the surgical course
- The study involves urine metabolomic analysis providing novel insight into the metabolite profile of study participants compared with healthy age matched infants
- The primary limitation of GuMIBear is that it is a single centre study limiting the generalisability of the findings

Introduction

What is currently known

The establishment of gut microbiota begins at birth and continues over the first years of life. Continued evolution of the gut microbiome after birth is governed by host factors such as both the adaptive and innate immune system, as well as external factors such as diet, medication and toxin exposure, and illness.[1] Understanding the role of the gut microbiome in metabolism, immune function and nutrition is gaining increasing recognition, as it is accepted that an altered colonisation has been associated with a higher risk of disease later in life.[2] In the critical first weeks and months of life, perturbations to the infant gut microbiome have implications for growth development and health.[3]

The Microbiome and Systemic Inflammation

It is evident that under certain conditions, disruption of the normal microbiota that colonise the intestinal tract can occur.[4] These conditions include systemic inflammatory processes, which can result in intestinal inflammation, where proinflammatory bacteria can flourish, interacting with the intestinal epithelium to cause cytokine release, activating key inflammatory pathways increasing morbidity and prolonging critical illness.[5] The pattern of cytokine release in patients undergoing CPB is described as comparable to those released in systemic inflammation such as trauma and sepsis.[6] However, the nature of gut microbiota compositional changes in infants undergoing surgery with CPB remains understudied. This research aims to address this knowledge gap to enhance our understanding and inform care practices.

CHD and Necrotising Enterocolitis (NEC)

CHD affects approximately 1 in every 100 babies born throughout the US every year.[7] It is the most common congenital defect worldwide.[8] A diagnosis of 'Complex Congenital Heart Disease' can include conditions such as Hypoplastic Left Heart Syndrome (HLHS); Hypoplastic Right Heart Syndrome (HRHS), Transposition of the Great Arteries (TGA) requiring intervention in the first week of life, while CHD such as Atrioventricular Septal Defect (AVSD), Tetralogy of Fallot may require corrective surgery in the first few months of life. CHD requiring surgery involving cardiopulmonary bypass (CPB) present a greater risk to patients. This increased risk is not limited directly to the surgery, compromised ventricular function or low cardiac output state, but includes the risk of developing NEC.

There is a well-established connection between CHD and NEC, a potentially devastating intestinal condition of infancy. [9] NEC carries a reported incidence of between 3 and 9% in infants with CHD with all-cause mortality rates as high as 38% in children with 'cardiogenic' NEC.[10] While CHD remains one of the most common risk factors for NEC, the underlying pathophysiology of this association is complex. [11] Growing evidence suggests that perturbations in the early-life gut microbiota composition increase the risk for NEC. [3,12] A significant association between episodes of low cardiac output and shock in the development of NEC is recognised. [13, 14] It is reported that infants with certain types of CHD, mainly HLHS, may possess abnormal systemic vasculature contributing to the increased risk for NEC. [15] Whether it is those, or other causes of impaired perfusion to the gut, the resulting damage to the mucosal barrier can provide an entry point to bacteria provoking an inflammatory cascade, and the devastating consequences that can ensue.[16] The vulnerability of infants with CHD is enhanced during the course of surgical intervention involving CPB (11), and the role of the gut microbiome has received little research focus in this context.

Surgery involving CPB

Infants diagnosed with CHD are at risk of alterations to their intestinal homeostasis, a further threat is presented in the context of surgery involving CPB. [11] There is evidence to suggest intestinal ischaemia reperfusion injury occurs after CPB and contributes to epithelial barrier dysfunction (EBD) potentially exposing the bloodstream to bacteria or bacterial products.[17] Although alterations in gut barrier integrity and resident microbiota have been demonstrated.[3] it is not fully understood what changes to the microbiome occur following CPB, and the nature and severity of EBD. While the gut microbiota in infants with CHD following CPB remains understudied, a small single centre case control study recently identified significant gut microbiota perturbations in patients with CHD.[18] This case-control study highlighted that children with CHD had a disrupted gut microbiome at baseline with an over-representation of pro-inflammatory bacteria, this was further exacerbated by CPB. Samples were collected pre-operatively and in a limited 24 and 48 hour time frame postoperatively. The significance of intraoperative variables including aortic cross clamp time and duration of CPB was not determined.

Our study proposes to address the knowledge gap and advance existing research by examining the gut microbiome of infants with CHD pre-operatively, and at defined time points up to 2 years of life/ postoperatively. This timeline will account for the recovery phase post cardiac surgery, including time to re-establish full feeds, wean from mechanical ventilation and circulatory support, and allow for surveillance of NEC postoperatively. Comparisons will be made with healthy age-matched infants recruited as part of the INFANTMET study.[19] As well as collecting intraoperative variables such as duration of CPB, and aortic cross clamp time, a novel aspect of this research will be to profile the metabolites in urine to assess potential metabolic biomarkers and pathway changes. Our research will recruit patients in a National Centre for Paediatric Cardiac Surgery, where 40 open cardiac surgeries are performed on infants annually. We therefore anticipate active recruitment will ensure the proposed target sample of 50 participants is achievable. No additional invasive procedures will be required for sample collection, enhancing the acceptability of the research for consenting parents or carers. This project will investigate the subdivisions of the gut microbiota of infants with CHD, and environmental factors such as the influence of mode of delivery, pre-operative fasting states and mode of feeding, and use of pre-operative antibiotics. Understanding the status of the intestinal microbiome of infants with CHD and the effects of undergoing surgery with CPB is vital in informing best care practices to enhance patient outcomes.

Objectives and Outcomes

The primary study objectives and outcomes are:

- To characterise the gut microbiota composition of infants with specified CHD undergoing surgery with CPB at specific time points perioperatively.
- To determine any differences in gut microbiota composition of infants who take part in this study pre and post-operatively compared with the microbiota of healthy babies from the INFANTMET study at matching time points.[19]
- To characterise the urine metabolite profile of infants with specified CHD undergoing surgery with CPB and compare with healthy infants from the INFANTMET study.[19]

Secondary objectives and outcomes:
To explore the influence of maternal and environmental factors on gut microbiome composition.

Methods

Study design

This study is a prospective cohort study of infants with CHD undergoing CPB at the National Centre for Paediatric Cardiac Surgery at Children's Health Ireland (CHI) at Crumlin, Dublin, Ireland. This single-site study will investigate the differences in the gut microbiome, metabolomics readouts, and stress levels between infants with CHD undergoing CPB and healthy age matched controls.

Participant selection

This study will involve the collection of demographic and clinical maternal and infant data from infants diagnosed with CHD scheduled for surgery involving CPB. CHD is typically diagnosed antenatally with fetal echocardiography performed routinely at 20-24 weeks gestation. Any child presenting with a murmur or features of cardiac conditions, or symptoms of CHD will be diagnosed using clinical examination, including palpation, auscultation, electrocardiogram (ECG) and echocardiography. Cardiac diagnoses are classified according to cardiovascular physiology, i.e., left to right shunt, cyanosis with biventricular circulation, univentricular circulation and outflow tract obstruction, as presented in table 1.

Table 1. Classification of CHD Subtypes

Group	CHD Group	CHD Subtypes
1	Left to right shunt	AVSD, VSD, large ASD
2	Cyanotic CHD with biventricular circulation	D-TGA/IVS, D-TGA/VSD
3	Cyanotic CHD with univentricular circulation	HLHS, HRHS (Tricuspid Atresia) Pulmonary Atresia
4	RVOT Obstruction	Tetralogy of Fallot, dysplastic PS
5	LVOT Obstruction	Shones Syndrome, Coarctation of Aorta, Interrupted Aortic Arch
6	Others	Ebstein Anomaly, Truncus Arteriosus

To be eligible for the study, the participants must meet the terms of the inclusion and exclusion criteria as presented in Table 2.

Table 2. Inclusion and Exclusion Criteria.

Inclusion criteria	Exclusion criteria
Infants born full term (≥37 weeks gestation)	Stillbirth or live birth where the baby is born alive but dies shortly after
Infants diagnosed with *CHD and scheduled for surgery involving CPB	Infants who are born healthy with no underlying illness, syndrome, or chronic disease
Infants born in Ireland to allow sample follow up	Participation in another study
Ability of the participant's parent/carers (in the investigator's opinion) to comprehend the full nature and purpose of the study	Infants not undergoing surgery involving cardiopulmonary bypass

Consent to participate in the study and willingness to comply with the protocol and study restrictions by the participant's parent/carers	Infants where parents/carers do not give consent to participate in the study
	Gastrointestinal pathology or intestinal surgery, excluding gastrostomy tube

*CHD diagnoses and subtypes are presented in Table 1.

Public and Patient Involvement (PPI) Statement

The mother of a child who had cardiac surgery as a baby was involved in reviewing the research questions, outcome measures and study literature at the study design phase. The PPI representative did not participate in the recruitment or the conduct of the study due to competing demands and availability. The PPI representative has offered to support dissemination of the study results through their involvement in charitable foundations and child and parent support fora.

Recruitment

Participants meeting all inclusion criteria will be selected after admission to the hospital, outpatient clinic or cardiac day unit. Study-related information will be given in written form as well as explained by a member of the project team. No study-related activities will begin before the potentially eligible participants' parents/carers have signed the Informed Consent Form (ICF). Participants parents/carers will be asked to refer to the Privacy Notice on the hospital website or they can receive a hardcopy if they wish. Signed ICFs will be stored safely in a locked cabinet in the research office.

Compensation

No compensation will be provided to the participants. There are no cost implications for the Health Service Executive (HSE) or to the participants. The management of patients and investigative tests will comply with current standards of care.

Study timeline

After completing recruitment procedures, i.e., determining whether the patient meets the study inclusion criteria, discussing the study with the parents/caregivers and obtaining informed consent, clinical and demographic data will start to be collected. The study will be undertaken for a period of 2 years after the infant is initially recruited.

Demographic Data

The infants' diagnosis including whether antenatal or postnatal diagnosis, co-morbidities, date of birth, gestational age, sex, mode of delivery, Appearance, Pulse, Activity, Grimace, Respiration (APGAR) scores, birth weight, head circumference, mode and type of feeding pre and post operatively, antibiotics administered post-delivery, complications and antenatal events will be recorded (case record form located in Appendix 1).

Maternal Data

Maternal history including age, antibiotics received, smoking status use of probiotics and significant antenatal events will be recorded.

Surgical course

The type of surgery performed, duration of CPB and cross-clamp time will be recorded. Antibiotic use and any intraoperative events will be recorded as well as clinical information including arterial blood gas data.

Post-operative Data

Paediatric Index of Mortality (PIM) score, duration of mechanical ventilation, haematology variables including haemoglobin and haematocrit, renal data e.g. blood urea nitrogen (BUN) and creatinine, fluid balance, and cardiovascular support including vasoactive inotrope score, as well as duration of stay in paediatric intensive care unit will be recorded.

Feeding Information Data

Feeding information including the type of feed and duration of the feed prior to and after surgery will be recorded. The date the patient is established on full feeds will be recorded. Full feeding is defined as when the patient no longer requires parenteral nutrition or intravenous fluids.

Discharge Information Data

Discharge information data: This will include the patient's status on discharge from Paediatric Intensive Care Unit (PICU), as well as length of PICU and hospital stay.

Complications

The occurrence of complications will be recorded, for example the development of NEC. The timeline for recording NEC onset will be based on the initiation of triple antibiotic therapy, based on a full surgical review including clinical presentation, radiological and laboratory data. In addition, the occurrence of death, rehospitalisation for heart failure or cardiac problems will be included.

Subject withdrawal/exclusion

Under the Declaration of Helsinki, the research nurse will explain to the consenting adult that they have the right to withdraw from the study at any time and that this will in no way prejudice their future treatment. The reason for withdrawal will be recorded in the source documents and on the appropriate CRF. Consenting adults will be made aware that stored samples from individuals withdrawing from the study may have undergone processing and may be analysed in the study.

Regulatory procedures

The study is conducted following the version Fortaleza, Brazil, October 2013 of the Declaration of Helsinki 1964. The Protocol and the ICF have been approved by the Clinical Research Ethics Committee of Children's Health Ireland GEN/826/20. As biological samples will be procured in one institution and sent to another, a data sharing agreement is in place between The Cardiology Department, CHI at Crumlin Hospital, and APC Microbiome Ireland, in Cork. This research is fully compliant with the guidelines as set out in The General Data Protection Regulation (GDPR), the Irish Data Protection Acts 1988 to 2018 including Protection Act 2018 (Section 36(2)) (Health Research) Regulations 2018.

Data Statement

Once collected, the anonymised demographic, clinical and laboratory analysis data as well as statistical codes will be uploaded to the open access Research Repository University College Dublin.

Sample collection and analysis

Faecal samples

Stool samples will be collected at the following time points: within 24 hours after birth (timepoint (TP) 1), within 24 hrs pre surgery (TP 2), 1 week post-surgery (TP 3) 4 weeks post-surgery (TP 4), 24 weeks post-surgery (TP 5) at 52 weeks (TP 6) and 2 years of age (TP 7). Information about antibiotic therapy administered before or during the stool collection will be recorded.

As the study site is the National Centre for Paediatric Cardiology, all infants diagnosed with CHD antenatally are transferred to the study site from the Maternity Unit for management of CHD. The first study stool specimen is typically obtained after the infant is transferred to the study site and informed consent obtained, which is typically within 24 hours of delivery.

The sample will be collected by the bedside nurse or the parent/carers and transferred to the laboratory upon receipt of the sample during the weekdays or weekends. At night, the sample will be kept in the dedicated fridge and transferred to the laboratory within 4-5 hours for appropriate storage at -80°C until further analysis. A standard operating procedure (SOP) for sample collection when participant is no longer an in-patient is provided in Appendix 2.

Urine Samples

Urine samples will be collected at 4-8 weeks post-surgery for metabolomic analysis using Sterisets Uricol Urine Collection Pack (MedGuard, Ireland). The urine sample will be collected from the urinary catheter if the participant is catheterised. Alternatively, a pad will be placed in the diaper and used to collect an unsoiled urine sample from the infant. The pad will then be placed in a biohazard bag and frozen immediately at -80 °C prior to processing. After all the sample collections are complete, they will be shipped to Teagasc Food Research, Moorepark, Ireland, using DHL overnight service for microbiome and metabolomics analyses. Styrofoam Saf-T-Pak STP-309 shipper box or equivalent will be used. DNA extraction will be performed on stool samples using the modification of the Repeated Bead Beating Plus Column (RBB+C) method.[20] LC-MS will be utilised for metabolomics analysis of urine.[19]

Sample collection for discharged participant

Parents/carers will receive a sample collection discharge pack and a parent diary/instruction served as reminder to collect due samples at different time-points prior to discharge home. They will receive a follow up text message or phone call to remind them on the due sample. The sample collection discharge pack consists of urine/stool collection containers with study code, sterile pad, syringe, zip-lock bag, gloves, biohazard bag and cooler bag. Parents/carers are asked to keep the collected sample at the dedicated section of their home freezer. They will transport the collected sample in the cooler bag provided when attending out-patient department for appointments. They will ask a member of the project team to transfer the collected sample to the laboratory upon arrival at the hospital. The study researcher is available at the dedicated contact number for any queries.

Adverse events and participant well-being

There are no expected safety concerns related to the study. All study participants will be under the care of the cardiology team at the hospital with access to psychological support, as well as nursing and medical professionals, social workers and chaplaincy.

Data collection and management

The study diaries, study dataset, and paper/digital CRF systems will be used for recording data from each study participant. All the data collected in this study is pseudonymised, as each of the participants will be assigned a specific study code and upon receipt, data will be referred to the study code. All study staff responsible for entering data into the CRF system received training in advance of the study commencement. This training included familiarity with the study diaries, study dataset, and paper/digital CRF system and have completed good clinical practice (GCP) in research training. They have individual access to the password protected study shared drive within the hospital. The study team will monitor the data/sample collection process. Any inconsistencies identified during the study will be presented as queries at the regular project team meeting.

Comparison Data

Data collected as part of the INFANTMET Study will serve as a healthy control comparison for this study. INFANTMET compared the gut microbiota development of breastfed infants born via C-section or vaginally at full –term or preterm at Cork University Maternity Hospital. Ethical approval for sample collection by Cork University Hospital Research Ethics Committee (reference number ECM(w) 07/02/2012). One hundred and ninety two infants were recruited to the INFANTMET study and stratified according to delivery mode and gestational age at birth. Faecal samples were collected from the infants at 1, 4, 8 and 24 weeks of age and year 1, 2 and 4 of life and stored under controlled conditions. Urine samples were collected at 4 weeks of age for metabolomic analysis and stored in a freezer at -80°C prior to processing. Samples were analysed in accordance with the analysis proposed for the GuMIBear study. Although INFANTMET study participants did not have CHD, they nonetheless serve as a valuable comparison group. The stool and urine samples collected as part of the GuMIBear study will be as closely time matched as possible to the INFANTMET study samples to capture the major developmental period of the early life gut microbiota.

Bioinformatics and Statistical analysis

Sample size justification

Published research in this area is lacking. However one case control study by Salomon et al. (2021) included 17 cases and 12 control participants and was sufficiently powered to determine a statistically significant difference in beta diversity in cases versus controls ($F=5.6$, $p<0.001$). Our study proposes to include 50 patients with CHD undergoing surgery with CPB and age matched controls, almost three times the Solomon et al. (2021) study. We therefore anticipate that the proposed sample size is justified.

Demographic and Clinical Data

Demographic and clinical data and laboratory information will be tested for normality using the Shapiro-Wilk test. Descriptive statistics will be used to describe normally distributed data, and expressed as mean ± standard deviation (SD). Continuous data not normally distributed will be reported as median and interquartile ranges. Categorical variables will be expressed as counts and percentages. Groups will be compared using chi square tests for categorical variables and independent-samples student's *t*- tests for normally distributed continuous variables. For variables not normally distributed, the Mann-Whitney *U* test will be used. Comparison will include subgroup analysis of participants who experienced post-operative complications including NEC with those that did not. Comparisons will also include cyanotic versus acyanotic heart disease subgroup analysis, as well as mode and type of feeding pre and post-operatively.

Microbiome analysis

Metagenomic shotgun sequencing data will be analysed using bioBakery suite of tools (https://huttenhower.sph.harvard.edu/biobakery_workflows/). Trimmed and human reads filtered using KneadData (v0.7.2) with the default parameters. Quality controlled data will be taxonomically profiled at the species level with relative abundance by MetaPhlAn2. Functional profiling will be performed using HUMAnN3 and strain profiling using StrainPhlAn.

For alpha diversity analysis, samples will be rarefied to even depth and phyloseq::estimate richness will be used to calculate Chao1, Shannon and Simpson indices. Alpha diversity indices between groups will be univariately compared using the Wilcoxon rank sum test. A beta-diversity ordination will be generated using the Aitchison distance and visualised using Principal Component Analysis (PCA) plot. The Adonis function in the vegan package will be used to implement a permutational multivariate analysis of variance to test whether samples cluster beyond that expected by sampling variability. MaAsLin2 (Multivariate Associations with Linear Models) will be used to investigate multivariable associations between sequencing data and clinical metadata. MaAsLin2 performs boosted, additive, linear models to detect associations while adjusting for confounding factors. Sparse canonical correlation analysis (sCCA) will be used to calculate the overall correlation between metabolites and microbes, and to identify strongly associated biomarkers. Pairwise spearman rank correlation analysis will also be performed.

Discussion

Despite an increasing awareness of the early-life critical window of microbiome development on the health and wellness of infants, there remains much to learn about the interactions of the microbiome with the infant host with CHD undergoing surgery involving CPB. This study is designed to address this knowledge gap, and incorporates a sound methodology with particular strengths enhancing the value of its findings. The specimen collection strategy occurring at multiple time points over a 2 year period in the proposed study will deepen our understanding of the temporal dynamics of the colonising microbiota, and their interactions with host physiology. The study design will account for maternal and perioperative variables to determine changes to the microbiome. Access to existing microbiome data from healthy age matched infants provides a valuable opportunity to present high quality comparative information. While multi-centre trials capturing sufficient case numbers of NEC cases will offer robust conclusions, this study will offer valuable evidence to support the influence of CHD and CPB on the microbiome and intestinal epithelial barrier dysfunction (EBD). Future research can build on existing studies, and explore treatment strategies including recommendations for efficacious probiotic strain administration, including the supplements to promote a diverse gut microbiota to improve outcomes for this vulnerable population.

Ethics and Dissemination

This research study is ethically approved (REC REF No: GEN/826/20). Study results will be available to patients with CHD and their families, carers, support networks, paediatric cardiology and microbiome societies and other researchers. Study findings will provide a deeper understanding of the gut microbiota of infants with CHD and inform perioperative management options including strategies to prioritise the integrity of the gut microbiota.

Status of Study

The trial is ongoing and as of November 10th 2022, 80 % of the participants have been recruited. Laboratory analysis has been carried out on 25% of study samples.

Funding Statement

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests

The authors declare no association with commercial entities, either financial or non-financial.

Author contributions

CM, CJMcM, CS, KM devised the project, and the main conceptual ideas. CJMcM, CM, CS, RPR, FK, DJ, MHT were involved in the study design and writing of the manuscript. MOT, JB, NM, SD, CJMcM are involved in consenting participants, collecting samples and acquiring data. DJ, CS, FK and RPR are responsible for analysing study samples. All authors read manuscript revisions, approved the final manuscript and accept accountability for the accuracy and integrity of the work.

Acknowledgements

All study participants, Paediatric Intensive Care Unit/ Children's Heart Centre nurses for assisting in sample collection. All laboratory staff including Ms Irene Regan who have facilitated tracking and secure storage of study samples. Dr Adam James, Dr Ross Foley and the Consultant Paediatric Consultants, CHI at Crumlin for their support with this study.

Appendix 1: Case Report Form PDF Version

Appendix 2: SOP for Obtaining a Stool Sample when Participant is no longer Inpatient

References

1. Skillington O MS, Gupta A, Mayer EA, Gill CIR, Del Rio D, O'Riordan KJ, Cryan JF, Ross RP and Stanton C The contrasting human gut microbiota in early and late life and implications for host health and disease. Nutrition and Healthy Ageing. 2012;6:157-78.
2. Cilieborg MS, Boye M, Sangild PT. Bacterial colonization and gut development in preterm neonates. Early human development. 2012;88 Suppl 1:S41-9.
3. Healy DB, Ryan CA, Ross RP, Stanton C, Dempsey EM. Clinical implications of preterm infant gut microbiome development. Nature microbiology. 2022;7(1):22-33.
4. Ohland CL, Jobin C. Microbial activities and intestinal homeostasis: A delicate balance between health and disease. Cellular and molecular gastroenterology and hepatology. 2015;1(1):28-40.
5. Cabrera-Perez J, Badovinac VP, Griffith TS. Enteric immunity, the gut microbiome, and sepsis: Rethinking the germ theory of disease. Experimental biology and medicine. 2017;242(2):127-39.
6. Halter J, Steinberg J, Fink G, Lutz C, Picone A, Maybury R, et al. Evidence of systemic cytokine release in patients undergoing cardiopulmonary bypass. The journal of extra-corporeal technology. 2005;37(3):272-7.
7. Centers for Disease Control and Prevention (CDC). Data and Statistics on Congenital Heart Defects. 2022 [updated January 24th 202226th July 2022]. Available from: https://www.cdc.gov/ncbddd/heartdefects/data.html Accessed 26th July 2022.
8. van der Linde D, Konings E, Slager M, Witsenburg M, Helbing W, Takkenburg J, Roos-Hesselink J. Birth Prevalence of Congenital Heart Disease Worldwide: A Systematic Review and Meta-Analysis. Journal of the American College of Cardiology. 2011;58(21):2241-2247.
9. Martin R, Makino H, Cetinyurek Yavuz A, Ben-Amor K, Roelofs M, Ishikawa E, et al. Early-Life Events, Including Mode of Delivery and Type of Feeding, Siblings and Gender, Shape the Developing Gut Microbiota. PloS one. 2016;11(6):e0158498.
10. Siano E, Lauriti G, Ceccanti S, Zani A. Cardiogenic Necrotizing Enterocolitis: A Clinically Distinct Entity from Classical Necrotizing Enterocolitis. European journal of pediatric surgery : official journal of Austrian Association of Pediatric Surgery. 2019;29(1):14-22.
11. Kelleher ST, McMahon CJ, James A. Necrotizing Enterocolitis in Children with Congenital Heart Disease: A Literature Review. Pediatric cardiology. 2021;42(8):1688-99.
12. Murphy K, O'Donovan AN, Caplice NM, Ross RP, Stanton C. Exploring the Gut Microbiota and Cardiovascular Disease. Metabolites. 2021;11(8).
13. Carlo WF, Kimball TR, Michelfelder EC, Border WL. Persistent diastolic flow reversal in abdominal aortic Doppler-flow profiles is associated with an increased risk of necrotizing enterocolitis in term infants with congenital heart disease. Pediatrics. 2007;119(2):330-5.

14. McElhinney DB, Hedrick HL, Bush DM, Pereira GR, Stafford PW, Gaynor JW, et al. Necrotizing enterocolitis in neonates with congenital heart disease: risk factors and outcomes. Pediatrics. 2000;106(5):1080-7.
15. Miller TA, Minich LL, Lambert LM, Joss-Moore L, Puchalski MD. Abnormal abdominal aorta hemodynamics are associated with necrotizing enterocolitis in infants with hypoplastic left heart syndrome. Pediatric cardiology. 2014;35(4):616-21.
16. Nino DF, Sodhi CP, Hackam DJ. Necrotizing enterocolitis: new insights into pathogenesis and mechanisms. Nature reviews Gastroenterology & hepatology. 2016;13(10):590-600.
17. Typpo KV, Larmonier CB, Deschenes J, Redford D, Kiela PR, Ghishan FK. Clinical characteristics associated with postoperative intestinal epithelial barrier dysfunction in children with congenital heart disease. Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies. 2015;16(1):37-44.
18. Salomon J, Ericsson A, Price A, Manithody C, Murry DJ, Chhonker YS, et al. Dysbiosis and Intestinal Barrier Dysfunction in Pediatric Congenital Heart Disease Is Exacerbated Following Cardiopulmonary Bypass. JACC Basic to translational science. 2021;6(4):311-27.
19. Hill CJ, Lynch DB, Murphy K, Ulaszewska M, Jeffery IB, O'Shea CA, et al. Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. Microbiome. 2017;5(1):4.
20. Yu Z, Morrisson M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques. 2004;36(5):808-12.

GuMIBear Study

Study ID: GMB: _______

^**999** – missing data; **0** – no data in medical notes

CASE RECORD FORM

Stool Sample Collections

Period/ Time Frame		Projected Date	Sample Date	Sample Collected	Comments
1.	Within 24h of Birth			☐ YES ☐NO	
2.	Pre-operatively			☐ YES ☐NO	
3.	Week 1 of life/Post-Op			☐ YES ☐NO	
4a.	Week 4 to 8 life/Post-op			☐ YES ☐NO	
4b.	**Urine** Sample Week 4 to 8			☐ YES ☐NO	
5.	Week 24 of life/Post-Op			☐ YES ☐NO	
6.	Week 52 of life/Post-Op			☐ YES ☐NO	
7.	Year 2 of life/Post-Op			☐ YES ☐NO	

PATIENT DEMOGRAPHIC

Date of Enrolment:		Date of Birth:	Cardiac Classification Group No:
Gender:	☐ Male ☐ Female ☐ Other/Ambiguous	Gestational Age: APGAR at ① ⑤ min	Multiple ☐ Yes ☐ No Birth Order:
Reason for admission:			
Comorbidities:			
Mode of Delivery: SVD: ☐Yes ☐No LSCS: ☐Yes ☐No, if Yes: ☐Elective/ ☐Emergency		Weight at Birth: ________ . ________ KGs Head Circumference: ________ cms	
Antibiotics to Infant post-delivery; ☐ Yes ☐ No If Yes, list:		Timing of cardiac diagnosis: Postnatal ☐ Antenatal ☐	
Significant Antenatal Events:			

MATERNAL INFORMATION

Maternal Age (years) at Birth:	Gestational Age at Booking Appt:
Antibiotics given Pre-Delivery: ☐ Yes ☐ No List:	Maternal Probiotics taken during pregnancy: ☐ Yes ☐ No List:
Maternal Smoking during Pregnancy: ☐ Yes ☐ No	Other Household Members Smoking during Pregnancy: ☐ Yes ☐ No

GuMIBear Study

Study ID: GMB: ______

^**999** – missing data; **0** – no data in medical notes

SURGERY INFORMATION

Surgery Date:					
Surgery Performed:					
Pre-Op Antibiotics[1]? List:			Post-Op Antibiotics[1]? List:		
1. Abs <48hrs pre sampling			1. Abs <48hrs pre sampling		
2. Abx <72hrs pre sampling			2. Abx <72hrs pre sampling		
3. Abx in last 7 days/during sample			3. Abx in last 7 days/during sample		
ABG	pH	PO^2			
First Pre-Op ABG:			Cardiopulmonary Bypass Duration:		
First Post-Op ABG:			Aortic Cross Clamp Duration:		
Significant Intraoperative Events:					

[1]Antibiotic treatment at time of stool sampling as below. Important not to include antibiotics which were started post the stool sampling

1) Abs < 48hrs pre sampling
2) Abx < 72 hrs pre sampling
3) Abx in previous 7 days/ during sample collection

POST-OPERATIVE INFORMATION

PIM3 Score:		No. of days in ICU (1st adm):	
Mechanical Vent:	☐ Yes ☐ No	No of Days on ECLS:	
No of Days Vent:		No of Days on RRT:	
Agent	Day 1	Day 2	Day 3
Milrinone			
Epinephrine			
Norepinephrine			
Vasopressin			
Midazolam			
Morphine			
Clonidine			
Others			
Fluid Balance:			
BUN:			
Creatinine:			
HCT:			
Hgb:			

FEEDING INFORMATION

Mode of Feeding (note ***date initiated*** and ***date d/c***):		
Breastmilk:	Infant Formula:	Other:
Prebiotics given to Infant: ☐ Yes ☐ No	Type and Date Given:	
Excessive Infantile Crying (cried for ≥3 Hrs for 3 Days in one week): ☐ Yes ☐ No		

GuMIBear Study

Study ID: GMB: ______

^**999** – missing data; **0** – no data in medical notes

Date Trophic Feeds Commenced:		Type of Feed Used:
Time to Establishment of full feed[2]:		
Development of NEC[3]: Days post-op when developed NEC?		
~~Gut stasis:~~	Not applicable	
Management Strategy:		

[2]Full feed – No longer requires parenteral nutrition or intravenous fluids supplement regardless the TFI.

[3]NEC – Initiation of triple IV antibiotic therapy and nil by mouth for at least 5 days, based on a full surgical review including clinical presentation, radiological and laboratory data.

DISCHARGE INFORMATION

Date of Discharge		
Ward:	Home:	RIP:

READMISSION TO ICU

Total ICU Readmission days:

	Date of Admission	Date of Discharge	Total ICU stays	Reason for admission
1.				
2.				
3.				
4.				
5.				

DATA ENTRY BY (NAME)

Admission	**Date**	**Paper**	**Date**	**G-Drive**
First				
Second				
Third				

Participant Withdrawal from Study: ☐ Yes ☐ No

GCP Procedure Followed: ☐ Yes ☐ No

See Study Folder Appendix 4. Signed:______________ Date: ____________________

The Gut Microbiota of Infants with Complex Congenital Heart Disease Undergoing Cardiopulmonary Bypass

(GuMiBear)

STANDARD OPERATING PROCEDURE FOR STOOL SAMPLE COLLECTION AS AN OUTPATIENT

Purpose: To collect infant stool samples while the infant is an out-patient from CHI at Crumlin

Objective: To collect infant stool samples for the study in a uniform manner and under a set of conditions, so that they can be processed by the laboratories to achieve optimal results.

Procedure:

1. Ensure that at least one legal guardian has provided written informed consent for their infant to participate in the study and that they are happy for their infant to remain in the study.
2. Ensure that in the hospital chart of the infant that it is noted that he/she is participating in the study and contact details of the study team.
3. Ensure the parent/guardian has been supplied with a study pack containing the requisites for the collection of the stool sample
   a. Completed labels
   b. Disposable Gloves
   c. Stool collection containers
   d. Bio-hazard bags
4. Explain to the legal guardian that a minimum of a teaspoon of stool has to be collected. Explain to the legal guardian when the samples have to be collected as close as possible to the next out-patient appointment.
5. Explain to parent/guardian how to collect the sample as follows:
   a. Have requisites for collection at the ready.(Gloves, sample container, red biohazard bag & labels)
   b. Place appropriate label on the sample bottle.
   c. Wear disposable gloves
   d. Unscrew cap of sample bottle

The Gut Microbiota of Infants with Complex Congenital Heart Disease Undergoing Cardiopulmonary Bypass

(GuMiBear)

   e. Spoon in stool sample (1 teaspoon in volume)
   f. Screw cap on tightly
   g. Place in red hazard bag
   h. Remove disposable gloves
   i. Dispose with soiled nappy
   j. Wash hands
   k. Place stool sample in fridge
   l. Text research nurse that sample is ready for collection.

6. Label the sample bottles with Subject Number, Date of Birth, Initials, Sample Number, Date of Collection
7. Ask the parent/guardian to attach the appropriate label to the sample container and immediately place container in freezer.
8. Text the parent/guardian the night before the sample is to be collected. Meet the parent/guardian in out-patients to collect the stool sample and store the sample in the study container in the designated study freezer.
9. Update the study spreadsheet to indicate sample has been obtained.

BMJ Open

A Protocol for a Prospective Cohort Study Exploring the Gut Microbiota of Infants with Congenital Heart Disease Undergoing Cardiopulmonary Bypass (the GuMiBear study)

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2022-067016.R2
Article Type:	Protocol
Date Submitted by the Author:	07-Feb-2023
Complete List of Authors:	Magner, Claire; University College Dublin, School of Nursing, Midwifery and Health Systems Jenkins, Dominic; Children's Health Ireland at Crumlin Koc, Fatma; University College Cork, School of Microbiology; Teagasc Food Research Centre, Food Biosciences Tan, Mong Hoi; Children's Health Ireland at Crumlin O'Toole, Molly; Children's Health Ireland at Crumlin Boyle, Jordan; Children's Health Ireland at Crumlin Maguire, Niamh; Children's Health Ireland at Crumlin Duignan, Sophie; Children's Health Ireland at Crumlin Murphy, Kiera; University College Cork APC Microbiome Institute; Teagasc Food Research Centre, Food Biosciences Ross, Paul; University College Cork College of Science Engineering and Food Science Stanton , Catherine ; University College Cork APC Microbiome Institute; Teagasc Food Research Centre McMahon, Colin J.; Children's Health Ireland at Crumlin, Department of Paediatric Cardiology; University College Dublin School of Medicine
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Paediatrics
Keywords:	Congenital heart disease < CARDIOLOGY, Cardiac surgery < SURGERY, Paediatric cardiology < CARDIOLOGY, Quality in health care < HEALTH SERVICES ADMINISTRATION & MANAGEMENT, Biochemistry < NATURAL SCIENCE DISCIPLINES

SCHOLARONE™ Manuscripts

Title: A Protocol for a Prospective Cohort Study Exploring the Gut Microbiota of Infants with Congenital Heart Disease Undergoing Cardiopulmonary Bypass (the GuMiBear study)

Authors: Claire Magner[1], Dominic Jenkins[2], Fatma Koc[3,4,5], Mong Hoi Tan[2], Molly O'Toole[2], Jordan Boyle[2], Niamh Maguire[2], Sophie Duignan[2], Kiera Murphy[4,5], R. Paul Ross[4] , Catherine Stanton[4,5], Colin J. McMahon[6]

[1] School of Nursing, Midwifery & Health Systems, University College Dublin, Ireland.

[2] Children's Health Ireland at Crumlin, Dublin, Ireland

[3] School of Microbiology, University College Cork

[4] APC Microbiome Ireland, University College Cork, Co Cork, Ireland

[5] Food Biosciences, Teagasc Food Research Centre, Moorepark, Co Cork, Ireland

[6] Department Paediatric Cardiology, Children's Health Ireland at Crumlin, Dublin, Ireland

Please address all correspondence to Professor Colin McMahon, 3rd Floor Medical Tower, Children's Health Ireland at Crumlin, Dublin, Ireland. D12 N512. Email: colin.mcmahon@olchc.ie. Tel: (01) 409 6100

Abstract

Introduction

The gut microbiota develops from birth and matures significantly during the first 24 months of life, playing a major role in infant health and development. The composition of the gut microbiota is influenced by several factors including mode of delivery, gestational age, feed type and treatment with antibiotics. Alterations in the pattern of gut microbiota development and composition can be associated with illness and compromised health outcomes.

Infants diagnosed with 'Congenital Heart Disease' (CHD) often require surgery involving cardiopulmonary bypass (CPB) early in life. The impact of this type of surgery on the integrity of the gut microbiome is poorly understood. In addition, these infants are at significant risk of developing the potentially devastating intestinal condition Necrotising Enterocolitis (NEC).

Methods and Analysis

This study will employ a prospective cohort study methodology to investigate the gut microbiota and urine metabolome of infants with CHD undergoing surgery involving CPB. Stool and urine samples, demographic and clinical data will be collected from eligible infants based at the National Centre for Paediatric Cardiac Surgery in Ireland. Shotgun metagenome

sequencing will be performed on stool samples and urine metabolomic analysis will identify metabolic biomarkers. The impact of the underlying diagnosis, surgery involving CPB, and the influence of environmental factors will be explored. Data from healthy age matched infants from the INFANTMET study will serve as a control for this study.

Abbreviations:

APGAR Score Newborn assessment score rating: Appearance, Pulse, Activity, Grimace, Respiration

ASD	Atrial Septal Defect
AVSD	Atrioventricular Septal Defect
BUN	Blood Urea Nitrogen
CHC	Children's Heart Centre
CHD	Congenital Heart Disease
CHI	Children's Health Ireland
CPB	Cardiopulmonary Bypass
CRF	Case Report Form
D-TGA	Dextro-Transposition of the Great Arteries
EBD	Epithelial Barrier Dysfunction
ECG	Electrocardiogram
GCP	Good Clinical Practice
GDPR	General Data Protection Regulation
HLHS	Hypoplastic Left Heart Syndrome
HRHS	Hypoplastic Right Heart Syndrome
HSE	Health Service Executive

ICF	Informed Consent Form
IV	Intravenous
IVS	Intra Ventricular Septum
L-TGA	Levo-Transposition of the Great Arteries
LVOT	Left Ventricular Outflow Tract
MaAsLin2	Multivariate Associations with Linear Models
NEC	Necrotising Enterocolitis
PCA	Principal Component Analysis
PICU	Paediatric Intensive Care Unit
PIM3	Paediatric Index of Mortality
PPI	Public and Patient Involvement
PS	Pulmonary Valve Stenosis
RBB+C	Repeated Beat Beating Plus Column (RBB+C) method
RVOT	Right Ventricular Outflow Tract
sCCA	Sparse canonical correlation analysis
SD	Standard Deviation
SOP	Standard Operating Procedure
TGA	Transposition of the Great Arteries
TP	Time Point
VSD	Ventricular Septal Defect

Keywords: Congenital Heart disease, Microbiota, Infant, Antibiotic, Development, Cardiac Surgery, Cardiopulmonary Bypass

Word Count: 3,887

Article Summary

Strengths and Limitations of this Study

- GuMIBear is the first study to investigate the gut microbiome of infants with CHD undergoing surgery with CPB over a 2 year timeframe
- Comparison with age matched healthy controls allow insights into clinically relevant microbiome alterations throughout and beyond the surgical course
- The study involves urine metabolomic analysis providing novel insight into the metabolite profile of study participants compared with healthy age matched infants
- The primary limitation of GuMIBear is that it is a single centre study limiting the generalisability of the findings

Introduction

What is currently known

The establishment of gut microbiota begins at birth and continues over the first years of life. Continued evolution of the gut microbiome after birth is governed by host factors such as both the adaptive and innate immune system, as well as external factors such as diet, medication and toxin exposure, and illness.[1] Understanding the role of the gut microbiome in metabolism, immune function and nutrition is gaining increasing recognition, as it is accepted that an altered colonisation has been associated with a higher risk of disease later in life.[2] In the critical first weeks and months of life, perturbations to the infant gut microbiome have implications for growth development and health.[3]

The Microbiome and Systemic Inflammation

It is evident that under certain conditions, disruption of the normal microbiota that colonise the intestinal tract can occur.[4] These conditions include systemic inflammatory processes, which can result in intestinal inflammation, where proinflammatory bacteria can flourish, interacting with the intestinal epithelium to cause cytokine release, activating key inflammatory pathways increasing morbidity and prolonging critical illness.[5] The pattern of cytokine release in patients undergoing CPB is described as comparable to those released in systemic inflammation such as trauma and sepsis.[6] However, the nature of gut microbiota compositional changes in infants undergoing surgery with CPB remains understudied. This research aims to address this knowledge gap to enhance our understanding and inform care practices.

CHD and Necrotising Enterocolitis (NEC)

CHD affects approximately 1 in every 100 babies born throughout the US every year.[7] It is the most common congenital defect worldwide.[8] A diagnosis of 'Complex Congenital Heart Disease' can include conditions such as Hypoplastic Left Heart Syndrome (HLHS); Hypoplastic Right Heart Syndrome (HRHS), Transposition of the Great Arteries (TGA) requiring intervention in the first week of life, while CHD such as Atrioventricular Septal Defect (AVSD), Tetralogy of Fallot may require corrective surgery in the first few months of life. CHD requiring surgery involving cardiopulmonary bypass (CPB) present a greater risk to patients. This increased risk is not limited directly to the surgery, compromised ventricular function or low cardiac output state, but includes the risk of developing NEC.

There is a well-established connection between CHD and NEC, a potentially devastating intestinal condition of infancy. [9] NEC carries a reported incidence of between 3 and 9% in infants with CHD with all-cause mortality rates as high as 38% in children with 'cardiogenic' NEC.[10] While CHD remains one of the most common risk factors for NEC, the underlying pathophysiology of this association is complex. [11] Growing evidence suggests that perturbations in the early-life gut microbiota composition increase the risk for NEC. [3,12] A significant association between episodes of low cardiac output and shock in the development of NEC is recognised. [13, 14] It is reported that infants with certain types of CHD, mainly HLHS, may possess abnormal systemic vasculature contributing to the increased risk for NEC. [15] Whether it is those, or other causes of impaired perfusion to the gut, the resulting damage to the mucosal barrier can provide an entry point to bacteria provoking an inflammatory cascade, and the devastating consequences that can ensue.[16] The vulnerability of infants with CHD is enhanced during the course of surgical intervention involving CPB (11), and the role of the gut microbiome has received little research focus in this context.

Surgery involving CPB

Infants diagnosed with CHD are at risk of alterations to their intestinal homeostasis, a further threat is presented in the context of surgery involving CPB. [11] There is evidence to suggest intestinal ischaemia reperfusion injury occurs after CPB and contributes to epithelial barrier dysfunction (EBD) potentially exposing the bloodstream to bacteria or bacterial products.[17] Although alterations in gut barrier integrity and resident microbiota have been demonstrated.[3] it is not fully understood what changes to the microbiome occur following CPB, and the nature and severity of EBD. While the gut microbiota in infants with CHD following CPB remains understudied, a small single centre case control study recently identified significant gut microbiota perturbations in patients with CHD.[18] This case-control study highlighted that children with CHD had a disrupted gut microbiome at baseline with an over-representation of pro-inflammatory bacteria, this was further exacerbated by CPB. Samples were collected pre-operatively and in a limited 24 and 48 hour time frame postoperatively. The significance of intraoperative variables including aortic cross clamp time and duration of CPB was not determined.

Our study proposes to address the knowledge gap and advance existing research by examining the gut microbiome of infants with CHD pre-operatively, and at defined time points up to 2 years of life/ postoperatively. This timeline will account for the recovery phase post cardiac surgery, including time to re-establish full feeds, wean from mechanical ventilation and circulatory support, and allow for surveillance of outcome measures including NEC, repeat surgery, and mortality postoperatively. Comparisons will be made with healthy age-matched infants recruited as part of the INFANTMET study.[19] As well as collecting intraoperative variables such as duration of CPB and aortic cross clamp time, a novel aspect of this research will be to profile the metabolites in urine to assess potential metabolic biomarkers and pathway changes. Our research will recruit patients in a National Centre for Paediatric Cardiac Surgery, where 40 open cardiac surgeries are performed on infants annually. We therefore anticipate active recruitment will ensure the proposed target sample of 50 participants is achievable. No additional invasive procedures will be required for sample collection, enhancing the acceptability of the research for consenting parents or carers. This project will investigate the subdivisions of the gut microbiota of infants with CHD, and environmental factors such as the influence of mode of delivery, pre-operative fasting states and mode of feeding, and use of pre-operative antibiotics. Understanding the status of the intestinal microbiome of infants with CHD and the effects of undergoing surgery with CPB is vital in informing best care practices to enhance patient outcomes.

Objectives and Outcomes

The primary study objectives and outcomes are:

- To characterise the gut microbiota composition of infants with specified CHD undergoing surgery with CPB at specific time points perioperatively.
- To compare any differences in gut microbiota composition of infants who take part in this study at defined time points pre and post-operatively and compare with the microbiota of healthy babies from the INFANTMET study at matching time points.[19]
- To characterise the urine metabolite profile of infants with specified CHD undergoing surgery with CPB and compare with healthy infants from the INFANTMET study.[19]

Secondary objectives and outcomes:
To explore the influence of maternal and environmental factors on gut microbiome composition.

Methods

Study design

This study is a prospective cohort study of infants with CHD undergoing CPB at the National Centre for Paediatric Cardiac Surgery at Children's Health Ireland (CHI) at Crumlin, Dublin, Ireland. This single-site study will investigate the differences in the gut microbiome, metabolomics readouts, and stress levels between infants with CHD undergoing CPB and healthy age matched controls.

Participant selection

This study will involve the collection of demographic and clinical maternal and infant data from infants diagnosed with CHD scheduled for surgery involving CPB. CHD is typically diagnosed antenatally with fetal echocardiography performed routinely at 20-24 weeks gestation. Any child presenting with a murmur or features of cardiac conditions, or symptoms of CHD will be diagnosed using clinical examination, including palpation, auscultation, electrocardiogram (ECG) and echocardiography. Cardiac diagnoses are classified according to cardiovascular physiology, i.e., left to right shunt, cyanosis with biventricular circulation, univentricular circulation and outflow tract obstruction, as presented in table 1.

Table 1. Classification of CHD Subtypes

Group	CHD Group	CHD Subtypes
1	Left to right shunt	AVSD, VSD, large ASD
2	Cyanotic CHD with biventricular circulation	D-TGA/IVS, D-TGA/VSD
3	Cyanotic CHD with univentricular circulation	HLHS, HRHS (Tricuspid Atresia) Pulmonary Atresia
4	RVOT Obstruction	Tetralogy of Fallot, dysplastic PS
5	LVOT Obstruction	Shones Syndrome, Coarctation of Aorta, Interrupted Aortic Arch
6	Others	Ebstein Anomaly, Truncus Arteriosus

To be eligible for the study, the participants must meet the terms of the inclusion and exclusion criteria as presented in Table 2.

Table 2. Inclusion and Exclusion Criteria.

Inclusion criteria	Exclusion criteria
Infants born full term (≥37 weeks gestation)	Stillbirth or live birth where the baby is born alive but dies shortly after
Infants diagnosed with *CHD and scheduled for surgery involving CPB	Infants who are born healthy with no underlying illness, syndrome, or chronic disease
Infants born in Ireland to allow sample follow up	Participation in another study

Ability of the participant's parent/carers (in the investigator's opinion) to comprehend the full nature and purpose of the study	Infants not undergoing surgery involving cardiopulmonary bypass
Consent to participate in the study and willingness to comply with the protocol and study restrictions by the participant's parent/carers	Infants where parents/carers do not give consent to participate in the study
	Gastrointestinal pathology or intestinal surgery, excluding gastrostomy tube

*CHD diagnoses and subtypes are presented in Table 1.

Public and Patient Involvement (PPI) Statement

The mother of a child who had cardiac surgery as a baby was involved in reviewing the research questions, outcome measures and study literature at the study design phase. The PPI representative did not participate in the recruitment or the conduct of the study due to competing demands and availability. The PPI representative has offered to support dissemination of the study results through their involvement in charitable foundations and child and parent support fora.

Recruitment

Participants meeting all inclusion criteria will be selected after admission to the hospital, outpatient clinic or cardiac day unit. Study-related information will be given in written form as well as explained by a member of the project team. No study-related activities will begin before the potentially eligible participants' parents/carers have signed the Informed Consent Form (ICF). Participants parents/carers will be asked to refer to the Privacy Notice on the hospital website or they can receive a hardcopy if they wish. Signed ICFs will be stored safely in a locked cabinet in the research office.

Compensation

No compensation will be provided to the participants. There are no cost implications for the Health Service Executive (HSE) or to the participants. The management of patients and investigative tests will comply with current standards of care.

Study timeline

After completing recruitment procedures, i.e., determining whether the patient meets the study inclusion criteria, discussing the study with the parents/caregivers and obtaining informed consent, clinical and demographic data will start to be collected. The study will be undertaken for a period of 2 years after the infant is initially recruited.

Demographic Data

The infants' diagnosis including whether antenatal or postnatal diagnosis, co-morbidities, date of birth, gestational age, sex, mode of delivery, Appearance, Pulse, Activity, Grimace, Respiration (APGAR) scores, birth weight, head circumference, mode and type of feeding pre and post operatively, antibiotics administered post-delivery, complications and antenatal events will be recorded (case record form located in Appendix 1).

Maternal Data

Maternal history including age, antibiotics received, smoking status use of probiotics and significant antenatal events will be recorded.

Surgical course

The type of surgery performed, duration of CPB and cross-clamp time will be recorded. Antibiotic use and any intraoperative events will be recorded as well as clinical information including arterial blood gas data.

Post-operative Data

Paediatric Index of Mortality (PIM) score, duration of mechanical ventilation, haematology variables including haemoglobin and haematocrit, renal data e.g. blood urea nitrogen (BUN) and creatinine, fluid balance, and cardiovascular support including vasoactive inotrope score, as well as duration of stay in paediatric intensive care unit will be recorded.

Feeding Information Data

Feeding information including the type of feed and duration of the feed prior to and after surgery will be recorded. The date the patient is established on full feeds will be recorded. Full feeding is defined as when the patient no longer requires parenteral nutrition or intravenous fluids.

Discharge Information Data

Discharge information data: This will include the patient’s status on discharge from Paediatric Intensive Care Unit (PICU), as well as length of PICU and hospital stay.

Complications

The occurrence of complications will be recorded, for example the development of NEC. The timeline for recording NEC onset will be based on the initiation of triple antibiotic therapy, based on a full surgical review including clinical presentation, radiological and laboratory data. In addition, the occurrence of death, rehospitalisation for heart failure or cardiac problems will be included.

Subject withdrawal/exclusion

Under the Declaration of Helsinki, the research nurse will explain to the consenting adult that they have the right to withdraw from the study at any time and that this will in no way prejudice their future treatment. The reason for withdrawal will be recorded in the source documents and on the appropriate CRF. Consenting adults will be made aware that stored samples from individuals withdrawing from the study may have undergone processing and may be analysed in the study.

Regulatory procedures

The study is conducted following the version Fortaleza, Brazil, October 2013 of the Declaration of Helsinki 1964. The Protocol and the ICF have been approved by the Clinical Research Ethics Committee of Children's Health Ireland GEN/826/20. As biological samples will be procured in one institution and sent to another, a data sharing agreement is in place between The Cardiology Department, CHI at Crumlin Hospital, and APC Microbiome Ireland, in Cork. This research is fully compliant with the guidelines as set out in The General Data Protection Regulation (GDPR), the Irish Data Protection Acts 1988 to 2018 including Protection Act 2018 (Section 36(2)) (Health Research) Regulations 2018.

Data Statement

Once collected, the anonymised demographic, clinical and laboratory analysis data as well as statistical codes will be uploaded to the open access Research Repository University College Dublin.

Sample collection and analysis

Faecal samples

Stool samples will be collected at the following time points: within 24 hours after birth (timepoint (TP) 1), within 24 hrs pre surgery (TP 2), 1 week post-surgery (TP 3) 4 weeks post-surgery (TP 4), 24 weeks post-surgery (TP 5) at 52 weeks (TP 6) and 2 years of age (TP 7). Information about antibiotic therapy administered before or during the stool collection will be recorded.

As the study site is the National Centre for Paediatric Cardiology, all infants diagnosed with CHD antenatally are transferred to the study site from the Maternity Unit for management of CHD. The first study stool specimen is typically obtained after the infant is transferred to the study site and informed consent obtained, which is typically within 24 hours of delivery.

The sample will be collected by the bedside nurse or the parent/carers and transferred to the laboratory upon receipt of the sample during the weekdays or weekends. At night, the sample will be kept in the dedicated fridge and transferred to the laboratory within 4-5 hours for appropriate storage at -80°C until further analysis. A standard operating procedure (SOP) for sample collection when participant is no longer an in-patient is provided in Appendix 2.

Urine Samples

Testing the urinary metabolomics of study participants will allow the potential identification of altered metabolomic profiles, and explore the role of microbiota in such alterations. Mirroring the INFANTMET study, urine samples will be collected at 4-8 weeks post-surgery for metabolomic analysis using Sterisets Uricol Urine Collection Pack (MedGuard, Ireland). The urine sample will be collected from the urinary catheter if the participant is catheterised. Alternatively, a pad will be placed in the diaper and used to collect an unsoiled urine sample from the infant. The pad will then be placed in a biohazard bag and frozen immediately at -80 °C prior to processing. After all the sample collections are complete, they will be shipped to Teagasc Food Research, Moorepark, Ireland, using DHL overnight service for microbiome and metabolomics analyses. Styrofoam Saf-T-Pak STP-309 shipper box or equivalent will be used. DNA extraction will be performed on stool samples using the modification of the Repeated Bead Beating Plus Column (RBB+C) method.[20] LC-MS will be utilised for metabolomics analysis of urine.[19]

Sample collection for discharged participant

Parents/carers will receive a sample collection discharge pack and a parent diary/instruction served as reminder to collect due samples at different time-points prior to discharge home. They will receive a follow up text message or phone call to remind them on the due sample. The sample collection discharge pack consists of urine/stool collection containers with study code, sterile pad, syringe, zip-lock bag, gloves, biohazard bag and cooler bag. Parents/carers are asked to keep the collected sample at the dedicated section of their home freezer. They will transport the collected sample in the cooler bag provided when attending out-patient department for appointments. They will ask a member of the project team to transfer the collected sample to the laboratory upon arrival at the hospital. The study researcher is available at the dedicated contact number for any queries.

Adverse events and participant well-being

There are no expected safety concerns related to the study. All study participants will be under the care of the cardiology team at the hospital with access to psychological support, as well as nursing and medical professionals, social workers and chaplaincy.

Data collection and management

The study diaries, study dataset, and paper/digital CRF systems will be used for recording data from each study participant. All the data collected in this study is pseudonymised, as each of the participants will be assigned a specific study code and upon receipt, data will be referred to the study code. All study staff responsible for entering data into the CRF system received training in advance of the study commencement. This training included familiarity with the study diaries, study dataset, and paper/digital CRF system and have completed good clinical practice (GCP) in research training. They have individual access to the password protected study shared drive within the hospital. The study team will monitor the data/sample collection process. Any inconsistencies identified during the study will be presented as queries at the regular project team meeting.

Comparison Data

Data collected as part of the INFANTMET Study will serve as a healthy control comparison for this study. INFANTMET compared the gut microbiota development of breastfed infants born via C-section or vaginally at full –term or preterm at Cork University Maternity Hospital. Ethical approval for sample collection by Cork University Hospital Research Ethics Committee (reference number ECM(w) 07/02/2012). One hundred and ninety two infants were recruited to the INFANTMET study and stratified according to delivery mode and gestational age at birth. Faecal samples were collected from the infants at 1, 4, 8 and 24 weeks of age and year 1, 2 and 4 of life and stored under controlled conditions. Urine samples were collected at 4 weeks of age for metabolomic analysis and stored in a freezer at -80°C prior to processing. Samples were analysed in accordance with the analysis proposed for the GuMIBear study. Although INFANTMET study participants did not have CHD, they nonetheless serve as a valuable comparison group. The stool and urine samples collected as part of the GuMIBear study will be as closely time matched as possible to the INFANTMET study samples to capture the major developmental period of the early life gut microbiota.

Bioinformatics and Statistical analysis

Sample size justification

Published research in this area is lacking. However one case control study by Salomon et al. (2021) included 17 cases and 12 control participants and was sufficiently powered to determine a statistically significant difference in beta diversity in cases versus controls

(F=5.6, p<0.001). Our study proposes to include 50 patients with CHD undergoing surgery with CPB and age matched controls, almost three times the Solomon et al. (2021) study. We therefore anticipate that the proposed sample size is justified.

Demographic and Clinical Data

Demographic and clinical data and laboratory information will be tested for normality using the Shapiro-Wilk test. Descriptive statistics will be used to describe normally distributed data, and expressed as mean ± standard deviation (SD). Continuous data not normally distributed will be reported as median and interquartile ranges. Categorical variables will be expressed as counts and percentages. Groups will be compared using chi square tests for categorical variables and independent-samples student's *t*- tests for normally distributed continuous variables. For variables not normally distributed, the Mann-Whitney *U* test will be used. Comparison will include subgroup analysis of participants who experienced post-operative complications including NEC with those that did not. Comparisons will also include cyanotic versus acyanotic heart disease subgroup analysis, as well as mode and type of feeding pre and post-operatively.

Microbiome analysis

Metagenomic shotgun sequencing data will be analysed using bioBakery suite of tools (https://huttenhower.sph.harvard.edu/biobakery_workflows/). Trimmed and human reads filtered using KneadData (v0.7.2) with the default parameters. Quality controlled data will be taxonomically profiled at the species level with relative abundance by MetaPhlAn2. Functional profiling will be performed using HUMAnN3 and strain profiling using StrainPhlAn.

For alpha diversity analysis, samples will be rarefied to even depth and phyloseq::estimate richness will be used to calculate Chao1, Shannon and Simpson indices. Alpha diversity indices between groups will be univariately compared using the Wilcoxon rank sum test. A beta-diversity ordination will be generated using the Aitchison distance and visualised using Principal Component Analysis (PCA) plot. The Adonis function in the vegan package will be used to implement a permutational multivariate analysis of variance to test whether samples cluster beyond that expected by sampling variability. MaAsLin2 (Multivariate Associations with Linear Models) will be used to investigate multivariable associations between sequencing data and clinical metadata. MaAsLin2 performs boosted, additive, linear models to detect associations while adjusting for confounding factors. Sparse canonical correlation analysis (sCCA) will be used to calculate the overall correlation between metabolites and microbes, and to identify strongly associated biomarkers. Pairwise spearman rank correlation analysis will also be performed.

Discussion

Despite an increasing awareness of the early-life critical window of microbiome development on the health and wellness of infants, there remains much to learn about the interactions of the microbiome with the infant host with CHD undergoing surgery involving CPB. This study is designed to address this knowledge gap, and incorporates a sound methodology with particular strengths enhancing the value of its findings. The specimen collection strategy occurring at multiple time points over a 2 year period in the proposed study will deepen our understanding of the temporal dynamics of the colonising microbiota, and their interactions with host physiology. The study design will account for maternal and perioperative variables to determine changes to the microbiome. Access to existing microbiome data from healthy age matched infants provides a valuable opportunity to present high quality comparative information. A limitation of this study may include the failure to recruit infants with CHD not identified antenatally, despite active fetal screening services. While multi-centre trials capturing sufficient case numbers of NEC cases will offer robust conclusions, this study will offer valuable evidence to support the influence of CHD and CPB on the microbiome and intestinal epithelial barrier dysfunction (EBD). Future research can build on existing studies, and explore treatment strategies including recommendations for efficacious probiotic strain administration, including the supplements to promote a diverse gut microbiota to improve outcomes for this vulnerable population.

Ethics and Dissemination

This research study is ethically approved (REC REF No: GEN/826/20). Study results will be available to patients with CHD and their families, carers, support networks, paediatric cardiology and microbiome societies and other researchers. Study findings will provide a deeper understanding of the gut microbiota of infants with CHD and inform perioperative management options including strategies to prioritise the integrity of the gut microbiota.

Status of Study

The trial is ongoing and as of 5th February 2023 , 84 % of the participants have been recruited. Laboratory analysis has been carried out on 25% of study samples.

Funding Statement

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests

The authors declare no association with commercial entities, either financial or non-financial.

Author contributions

CM, CJMcM, CS, KM devised the project, and the main conceptual ideas. CJMcM, CM, CS, RPR, FK, DJ, MHT were involved in the study design and writing of the manuscript. MOT, JB, NM, SD, CJMcM are involved in consenting participants, collecting samples and acquiring data. DJ, CS, FK and RPR are responsible for analysing study samples. All authors read manuscript revisions, approved the final manuscript and accept accountability for the accuracy and integrity of the work.

Acknowledgements

All study participants, Paediatric Intensive Care Unit/ Children's Heart Centre nurses for assisting in sample collection. All laboratory staff including Ms Irene Regan who have facilitated tracking and secure storage of study samples. Dr Adam James, Dr Ross Foley and the Consultant Paediatric Consultants, CHI at Crumlin for their support with this study.

Appendix 1: Case Report Form PDF Version

Appendix 2: SOP for Obtaining a Stool Sample when Participant is no longer Inpatient

References

1. Skillington O MS, Gupta A, Mayer EA, Gill CIR, Del Rio D, O'Riordan KJ, Cryan JF, Ross RP and Stanton C The contrasting human gut microbiota in early and late life and implications for host health and disease. Nutrition and Healthy Ageing. 2012;6:157-78.
2. Cilieborg MS, Boye M, Sangild PT. Bacterial colonization and gut development in preterm neonates. Early human development. 2012;88 Suppl 1:S41-9.
3. Healy DB, Ryan CA, Ross RP, Stanton C, Dempsey EM. Clinical implications of preterm infant gut microbiome development. Nature microbiology. 2022;7(1):22-33.
4. Ohland CL, Jobin C. Microbial activities and intestinal homeostasis: A delicate balance between health and disease. Cellular and molecular gastroenterology and hepatology. 2015;1(1):28-40.
5. Cabrera-Perez J, Badovinac VP, Griffith TS. Enteric immunity, the gut microbiome, and sepsis: Rethinking the germ theory of disease. Experimental biology and medicine. 2017;242(2):127-39.
6. Halter J, Steinberg J, Fink G, Lutz C, Picone A, Maybury R, et al. Evidence of systemic cytokine release in patients undergoing cardiopulmonary bypass. The journal of extra-corporeal technology. 2005;37(3):272-7.
7. Centers for Disease Control and Prevention (CDC). Data and Statistics on Congenital Heart Defects. 2022 [updated January 24th 202226th July 2022]. Available from: https://www.cdc.gov/ncbddd/heartdefects/data.html Accessed 26th July 2022.
8. van der Linde D, Konings E, Slager M, Witsenburg M, Helbing W, Takkenburg J, Roos-Hesselink J. Birth Prevalence of Congenital Heart Disease Worldwide: A Systematic Review and Meta-Analysis. Journal of the American College of Cardiology. 2011;58(21):2241-2247.
9. Martin R, Makino H, Cetinyurek Yavuz A, Ben-Amor K, Roelofs M, Ishikawa E, et al. Early-Life Events, Including Mode of Delivery and Type of Feeding, Siblings and Gender, Shape the Developing Gut Microbiota. PloS one. 2016;11(6):e0158498.
10. Siano E, Lauriti G, Ceccanti S, Zani A. Cardiogenic Necrotizing Enterocolitis: A Clinically Distinct Entity from Classical Necrotizing Enterocolitis. European journal of pediatric surgery : official

journal of Austrian Association of Pediatric Surgery. 2019;29(1):14-22.
11. Kelleher ST, McMahon CJ, James A. Necrotizing Enterocolitis in Children with Congenital Heart Disease: A Literature Review. Pediatric cardiology. 2021;42(8):1688-99.
12. Murphy K, O'Donovan AN, Caplice NM, Ross RP, Stanton C. Exploring the Gut Microbiota and Cardiovascular Disease. Metabolites. 2021;11(8).
13. Carlo WF, Kimball TR, Michelfelder EC, Border WL. Persistent diastolic flow reversal in abdominal aortic Doppler-flow profiles is associated with an increased risk of necrotizing enterocolitis in term infants with congenital heart disease. Pediatrics. 2007;119(2):330-5.
14. McElhinney DB, Hedrick HL, Bush DM, Pereira GR, Stafford PW, Gaynor JW, et al. Necrotizing enterocolitis in neonates with congenital heart disease: risk factors and outcomes. Pediatrics. 2000;106(5):1080-7.
15. Miller TA, Minich LL, Lambert LM, Joss-Moore L, Puchalski MD. Abnormal abdominal aorta hemodynamics are associated with necrotizing enterocolitis in infants with hypoplastic left heart syndrome. Pediatric cardiology. 2014;35(4):616-21.
16. Nino DF, Sodhi CP, Hackam DJ. Necrotizing enterocolitis: new insights into pathogenesis and mechanisms. Nature reviews Gastroenterology & hepatology. 2016;13(10):590-600.
17. Typpo KV, Larmonier CB, Deschenes J, Redford D, Kiela PR, Ghishan FK. Clinical characteristics associated with postoperative intestinal epithelial barrier dysfunction in children with congenital heart disease. Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies. 2015;16(1):37-44.
18. Salomon J, Ericsson A, Price A, Manithody C, Murry DJ, Chhonker YS, et al. Dysbiosis and Intestinal Barrier Dysfunction in Pediatric Congenital Heart Disease Is Exacerbated Following Cardiopulmonary Bypass. JACC Basic to translational science. 2021;6(4):311-27.
19. Hill CJ, Lynch DB, Murphy K, Ulaszewska M, Jeffery IB, O'Shea CA, et al. Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. Microbiome. 2017;5(1):4.
20. Yu Z, Morrisson M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques. 2004;36(5):808-12.

GuMIBear Study Study ID: GMB: ______

^**999** – missing data; **0** – no data in medical notes

CASE RECORD FORM

Stool Sample Collections

Period/ Time Frame		Projected Date	Sample Date	Sample Collected	Comments
1.	Within 24h of Birth			☐ YES ☐NO	
2.	Pre-operatively			☐ YES ☐NO	
3.	Week 1 of life/Post-Op			☐ YES ☐NO	
4a.	Week 4 to 8 life/Post-op			☐ YES ☐NO	
4b.	**Urine** Sample Week 4 to 8			☐ YES ☐NO	
5.	Week 24 of life/Post-Op			☐ YES ☐NO	
6.	Week 52 of life/Post-Op			☐ YES ☐NO	
7.	Year 2 of life/Post-Op			☐ YES ☐NO	

PATIENT DEMOGRAPHIC

Date of Enrolment:	Date of Birth:	Cardiac Classification Group No:
Gender: ☐ Male ☐ Female ☐ Other/Ambiguous	Gestational Age: APGAR at ① ⑤ min	Multiple ☐ Yes ☐ No Birth Order:
Reason for admission:		
Comorbidities:		
Mode of Delivery: SVD: ☐Yes ☐No LSCS: ☐Yes ☐No, if Yes: ☐Elective/ ☐Emergency	Weight at Birth: ______ . ______ KGs Head Circumference: ______ cms	
Antibiotics to Infant post-delivery; ☐ Yes ☐ No If Yes, list:	Timing of cardiac diagnosis: Postnatal ☐ Antenatal ☐	
Significant Antenatal Events:		

MATERNAL INFORMATION

Maternal Age (years) at Birth:	Gestational Age at Booking Appt:
Antibiotics given Pre-Delivery: ☐ Yes ☐ No List:	Maternal Probiotics taken during pregnancy: ☐ Yes ☐ No List:
Maternal Smoking during Pregnancy: ☐ Yes ☐ No	Other Household Members Smoking during Pregnancy: ☐ Yes ☐ No

GuMIBear Study

Study ID: GMB: ______

^**999** – missing data; **0** – no data in medical notes

SURGERY INFORMATION

Surgery Date:	
Surgery Performed:	

Pre-Op Antibiotics[1]? List:		Post-Op Antibiotics[1]? List:	
1. Abs <48hrs pre sampling		1. Abs <48hrs pre sampling	
2. Abx <72hrs pre sampling		2. Abx <72hrs pre sampling	
3. Abx in last 7 days/during sample		3. Abx in last 7 days/during sample	

ABG	pH	PO^2
First Pre-Op ABG:		
First Post-Op ABG:		

Cardiopulmonary Bypass Duration:	
Aortic Cross Clamp Duration:	

Significant Intraoperative Events:

[1]Antibiotic treatment at time of stool sampling as below. Important not to include antibiotics which were started post the stool sampling

1) Abs < 48hrs pre sampling
2) Abx < 72 hrs pre sampling
3) Abx in previous 7 days/ during sample collection

POST-OPERATIVE INFORMATION

PIM3 Score:		No. of days in ICU (1st adm):	
Mechanical Vent:	☐ Yes ☐ No	No of Days on ECLS:	
No of Days Vent:		No of Days on RRT:	

Agent	Day 1	Day 2	Day 3
Milrinone			
Epinephrine			
Norepinephrine			
Vasopressin			
Midazolam			
Morphine			
Clonidine			
Others			
Fluid Balance:			
BUN:			
Creatinine:			
HCT:			
Hgb:			

FEEDING INFORMATION

Mode of Feeding (note ***date initiated*** and ***date d/c***): Breastmilk: Infant Formula: Other:
Prebiotics given to Infant: ☐ Yes ☐ No Type and Date Given:
Excessive Infantile Crying (cried for ≥3 Hrs for 3 Days in one week): ☐ Yes ☐ No

GuMIBear Study

Study ID: GMB: _______

^**999** – missing data; **0** – no data in medical notes

Date Trophic Feeds Commenced:	Type of Feed Used:
Time to Establishment of full feed[2]:	
Development of NEC[3]: Days post-op when developed NEC?	
~~Gut stasis:~~	Not applicable
Management Strategy:	

[2]Full feed – No longer requires parenteral nutrition or intravenous fluids supplement regardless the TFI.

[3]NEC – Initiation of triple IV antibiotic therapy and nil by mouth for at least 5 days, based on a full surgical review including clinical presentation, radiological and laboratory data.

DISCHARGE INFORMATION

Date of Discharge		
Ward:	Home:	RIP:

READMISSION TO ICU

Total ICU Readmission days:

	Date of Admission	Date of Discharge	Total ICU stays	Reason for admission
1.				
2.				
3.				
4.				
5.				

DATA ENTRY BY (NAME)

Admission	**Date**	**Paper**	**Date**	**G-Drive**
First				
Second				
Third				

Participant Withdrawal from Study: ☐ Yes ☐ No

GCP Procedure Followed: ☐ Yes ☐ No

See Study Folder Appendix 4. Signed:_______________ Date: _____________________

The Gut Microbiota of Infants with Complex Congenital Heart Disease Undergoing Cardiopulmonary Bypass

(GuMiBear)

STANDARD OPERATING PROCEDURE FOR STOOL SAMPLE COLLECTION AS AN OUTPATIENT

Purpose: To collect infant stool samples while the infant is an out-patient from CHI at Crumlin

Objective: To collect infant stool samples for the study in a uniform manner and under a set of conditions, so that they can be processed by the laboratories to achieve optimal results.

Procedure:

1. Ensure that at least one legal guardian has provided written informed consent for their infant to participate in the study and that they are happy for their infant to remain in the study.
2. Ensure that in the hospital chart of the infant that it is noted that he/she is participating in the study and contact details of the study team.
3. Ensure the parent/guardian has been supplied with a study pack containing the requisites for the collection of the stool sample
    a. Completed labels
    b. Disposable Gloves
    c. Stool collection containers
    d. Bio-hazard bags
4. Explain to the legal guardian that a minimum of a teaspoon of stool has to be collected. Explain to the legal guardian when the samples have to be collected as close as possible to the next out-patient appointment.
5. Explain to parent/guardian how to collect the sample as follows:
    a. Have requisites for collection at the ready.(Gloves, sample container, red biohazard bag & labels)
    b. Place appropriate label on the sample bottle.
    c. Wear disposable gloves
    d. Unscrew cap of sample bottle

The Gut Microbiota of Infants with Complex Congenital Heart Disease Undergoing Cardiopulmonary Bypass

(GuMiBear)

e. Spoon in stool sample (1 teaspoon in volume)

f. Screw cap on tightly

g. Place in red hazard bag

h. Remove disposable gloves

i. Dispose with soiled nappy

j. Wash hands

k. Place stool sample in fridge

l. Text research nurse that sample is ready for collection.

6. Label the sample bottles with Subject Number, Date of Birth, Initials, Sample Number, Date of Collection

7. Ask the parent/guardian to attach the appropriate label to the sample container and immediately place container in freezer.

8. Text the parent/guardian the night before the sample is to be collected. Meet the parent/guardian in out-patients to collect the stool sample and store the sample in the study container in the designated study freezer.

9. Update the study spreadsheet to indicate sample has been obtained.

BMJ Open

A Protocol for a Prospective Cohort Study Exploring the Gut Microbiota of Infants with Congenital Heart Disease Undergoing Cardiopulmonary Bypass (the GuMiBear study)

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2022-067016.R3
Article Type:	Protocol
Date Submitted by the Author:	01-Mar-2023
Complete List of Authors:	Magner, Claire; University College Dublin, School of Nursing, Midwifery and Health Systems Jenkins, Dominic; Children's Health Ireland at Crumlin Koc, Fatma; University College Cork, School of Microbiology; Teagasc Food Research Centre, Food Biosciences Tan, Mong Hoi; Children's Health Ireland at Crumlin O’Toole, Molly; Children's Health Ireland at Crumlin Boyle, Jordan; Children's Health Ireland at Crumlin Maguire, Niamh; Children's Health Ireland at Crumlin Duignan, Sophie; Children's Health Ireland at Crumlin Murphy, Kiera; University College Cork APC Microbiome Institute; Teagasc Food Research Centre, Food Biosciences Ross, Paul; University College Cork College of Science Engineering and Food Science Stanton , Catherine ; University College Cork APC Microbiome Institute; Teagasc Food Research Centre McMahon, Colin J.; Children's Health Ireland at Crumlin, Department of Paediatric Cardiology; University College Dublin School of Medicine
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Paediatrics
Keywords:	Congenital heart disease < CARDIOLOGY, Cardiac surgery < SURGERY, Paediatric cardiology < CARDIOLOGY, Quality in health care < HEALTH SERVICES ADMINISTRATION & MANAGEMENT, Biochemistry < NATURAL SCIENCE DISCIPLINES

Title: A Protocol for a Prospective Cohort Study Exploring the Gut Microbiota of Infants with Congenital Heart Disease Undergoing Cardiopulmonary Bypass (the GuMiBear study)

Authors: Claire Magner[1], Dominic Jenkins[2], Fatma Koc[3,4,5], Mong Hoi Tan[2], Molly O'Toole[2], Jordan Boyle[2], Niamh Maguire[2], Sophie Duignan[2], Kiera Murphy[4,5], R. Paul Ross[4] , Catherine Stanton[4,5], Colin J. McMahon[6]

[1] School of Nursing, Midwifery & Health Systems, University College Dublin, Ireland.

[2] Children's Health Ireland at Crumlin, Dublin, Ireland

[3] School of Microbiology, University College Cork

[4] APC Microbiome Ireland, University College Cork, Co Cork, Ireland

[5] Food Biosciences, Teagasc Food Research Centre, Moorepark, Co Cork, Ireland

[6] Department Paediatric Cardiology, Children's Health Ireland at Crumlin, Dublin, Ireland

Please address all correspondence to Prof Colin McMahon, Department of Paediatric Cardiology, Children's Health Ireland at Crumlin, Dublin. D12 N512. Email: colin.mcmahon@olchc.ie

Abstract

Introduction

The gut microbiota develops from birth and matures significantly during the first 24 months of life, playing a major role in infant health and development. The composition of the gut microbiota is influenced by several factors including mode of delivery, gestational age, feed type and treatment with antibiotics. Alterations in the pattern of gut microbiota development and composition can be associated with illness and compromised health outcomes.

Infants diagnosed with 'Congenital Heart Disease' (CHD) often require surgery involving cardiopulmonary bypass (CPB) early in life. The impact of this type of surgery on the integrity of the gut microbiome is poorly understood. In addition, these infants are at significant risk of developing the potentially devastating intestinal condition Necrotising Enterocolitis (NEC).

Methods and Analysis

This study will employ a prospective cohort study methodology to investigate the gut microbiota and urine metabolome of infants with CHD undergoing surgery involving CPB. Stool and urine samples, demographic and clinical data will be collected from eligible infants based at the National Centre for Paediatric Cardiac Surgery in Ireland. Shotgun metagenome

sequencing will be performed on stool samples and urine metabolomic analysis will identify metabolic biomarkers. The impact of the underlying diagnosis, surgery involving CPB, and the influence of environmental factors will be explored. Data from healthy age matched infants from the INFANTMET study will serve as a control for this study.

Ethics and Dissemination

This study has received full ethical approval from the Clinical Research Ethics Committee of Children's Health Ireland GEN/826/20.

Abbreviations:

APGAR Score Newborn assessment score rating: Appearance, Pulse, Activity, Grimace, Respiration

ASD	Atrial Septal Defect
AVSD	Atrioventricular Septal Defect
BUN	Blood Urea Nitrogen
CHC	Children's Heart Centre
CHD	Congenital Heart Disease
CHI	Children's Health Ireland
CPB	Cardiopulmonary Bypass
CRF	Case Report Form
D-TGA	Dextro-Transposition of the Great Arteries
EBD	Epithelial Barrier Dysfunction
ECG	Electrocardiogram
GCP	Good Clinical Practice
GDPR	General Data Protection Regulation
HLHS	Hypoplastic Left Heart Syndrome

HRHS	Hypoplastic Right Heart Syndrome
HSE	Health Service Executive
ICF	Informed Consent Form
IV	Intravenous
IVS	Intra Ventricular Septum
L-TGA	Levo-Transposition of the Great Arteries
LVOT	Left Ventricular Outflow Tract
MaAsLin2	Multivariate Associations with Linear Models
NEC	Necrotising Enterocolitis
PCA	Principal Component Analysis
PICU	Paediatric Intensive Care Unit
PIM3	Paediatric Index of Mortality
PPI	Public and Patient Involvement
PS	Pulmonary Valve Stenosis
RBB+C	Repeated Beat Beating Plus Column (RBB+C) method
RVOT	Right Ventricular Outflow Tract
sCCA	Sparse canonical correlation analysis
SD	Standard Deviation
SOP	Standard Operating Procedure
TGA	Transposition of the Great Arteries
TP	Time Point
VSD	Ventricular Septal Defect

Keywords: Congenital Heart disease, Microbiota, Infant, Antibiotic, Development, Cardiac Surgery, Cardiopulmonary Bypass

Word Count: 3,887

Article Summary

Strengths and Limitations of this Study

- The GuMIBear study will collect microbiome data from children with CHD at 7 time points over a 2 year timeframe
- The study design incorporates comparison with age matched healthy controls, allowing insights into clinically relevant microbiome alterations throughout and beyond the surgical course
- The study involves urine metabolomic analysis providing novel insights into the metabolite profile of study participants compared with healthy age matched infants
- The primary limitation of GuMIBear is that it is a single centre study limiting the generalisability of the findings

Introduction

What is currently known

The establishment of gut microbiota begins at birth and continues over the first years of life. Continued evolution of the gut microbiome after birth is governed by host factors such as both the adaptive and innate immune system, as well as external factors such as diet, medication and toxin exposure, and illness.[1] Understanding the role of the gut microbiome in metabolism, immune function and nutrition is gaining increasing recognition, as it is accepted that an altered colonisation has been associated with a higher risk of disease later in life.[2] In the critical first weeks and months of life, perturbations to the infant gut microbiome have implications for growth development and health.[3]

The Microbiome and Systemic Inflammation

It is evident that under certain conditions, disruption of the normal microbiota that colonise the intestinal tract can occur.[4] These conditions include systemic inflammatory processes, which can result in intestinal inflammation, where proinflammatory bacteria can flourish, interacting with the intestinal epithelium to cause cytokine release, activating key inflammatory pathways increasing morbidity and prolonging critical illness.[5] The pattern of cytokine release in patients undergoing CPB is described as comparable to those released in systemic inflammation such as trauma and sepsis.[6] However, the nature of gut microbiota compositional changes in infants undergoing surgery with CPB remains understudied. This research aims to address this knowledge gap to enhance our understanding and inform care practices.

CHD and Necrotising Enterocolitis (NEC)

CHD affects approximately 1 in every 100 babies born throughout the US every year.[7] It is the most common congenital defect worldwide.[8] A diagnosis of 'Complex Congenital Heart Disease' can include conditions such as Hypoplastic Left Heart Syndrome (HLHS); Hypoplastic Right Heart Syndrome (HRHS), Transposition of the Great Arteries (TGA) requiring intervention in the first week of life, while CHD such as Atrioventricular Septal Defect (AVSD), Tetralogy of Fallot may require corrective surgery in the first few months of life. CHD requiring surgery involving cardiopulmonary bypass (CPB) present a greater risk to patients. This increased risk is not limited directly to the surgery, compromised ventricular function or low cardiac output state, but includes the risk of developing NEC.

There is a well-established connection between CHD and NEC, a potentially devastating intestinal condition of infancy. [9] NEC carries a reported incidence of between 3 and 9% in infants with CHD with all-cause mortality rates as high as 38% in children with 'cardiogenic' NEC.[10] While CHD remains one of the most common risk factors for NEC, the underlying pathophysiology of this association is complex. [11] Growing evidence suggests that perturbations in the early-life gut microbiota composition increase the risk for NEC. [3,12] A significant association between episodes of low cardiac output and shock in the development of NEC is recognised. [13, 14] It is reported that infants with certain types of CHD, mainly HLHS, may possess abnormal systemic vasculature contributing to the increased risk for NEC. [15] Whether it is those, or other causes of impaired perfusion to the gut, the resulting damage to the mucosal barrier can provide an entry point to bacteria provoking an inflammatory cascade, and the devastating consequences that can ensue.[16] The vulnerability of infants with CHD is enhanced during the course of surgical intervention involving CPB (11), and the role of the gut microbiome has received little research focus in this context.

Surgery involving CPB

Infants diagnosed with CHD are at risk of alterations to their intestinal homeostasis, a further threat is presented in the context of surgery involving CPB. [11] There is evidence to suggest intestinal ischaemia reperfusion injury occurs after CPB and contributes to epithelial barrier dysfunction (EBD) potentially exposing the bloodstream to bacteria or bacterial products.[17] Although alterations in gut barrier integrity and resident microbiota have been demonstrated.[3] it is not fully understood what changes to the microbiome occur following CPB, and the nature and severity of EBD. While the gut microbiota in infants with CHD following CPB remains understudied, a small single centre case control study recently identified significant gut microbiota perturbations in patients with CHD.[18] This case-control study highlighted that children with CHD had a disrupted gut microbiome at baseline with an over-representation of pro-inflammatory bacteria, this was further exacerbated by CPB. Samples were collected pre-operatively and in a limited 24 and 48 hour time frame postoperatively. The significance of intraoperative variables including aortic cross clamp time and duration of CPB was not determined.

Our study proposes to address the knowledge gap and advance existing research by examining the gut microbiome of infants with CHD pre-operatively, and at defined time points up to 2 years of life/ postoperatively. This timeline will account for the recovery phase post cardiac surgery, including time to re-establish full feeds, wean from mechanical ventilation and circulatory support, and allow for surveillance of outcome measures including NEC, repeat surgery, and mortality postoperatively. Comparisons will be made with healthy age-matched infants recruited as part of the INFANTMET study.[19] As well as collecting intraoperative variables such as duration of CPB and aortic cross clamp time, a novel aspect of this research will be to profile the metabolites in urine to assess potential metabolic biomarkers and pathway changes. Our research will recruit patients in a National Centre for Paediatric Cardiac Surgery, where 40 open cardiac surgeries are performed on infants annually. We therefore anticipate active recruitment will ensure the proposed target sample of 50 participants is achievable. No additional invasive procedures will be required for sample collection, enhancing the acceptability of the research for consenting parents or carers. This project will investigate the subdivisions of the gut microbiota of infants with CHD, and environmental factors such as the influence of mode of delivery, pre-operative fasting states and mode of feeding, and use of pre-operative antibiotics. Understanding the status of the intestinal microbiome of infants with CHD and the effects of undergoing surgery with CPB is vital in informing best care practices to enhance patient outcomes.

Objectives and Outcomes

The primary study objectives and outcomes are:

- To characterise the gut microbiota composition of infants with specified CHD undergoing surgery with CPB at specific time points perioperatively.
- To compare any differences in gut microbiota composition of infants who take part in this study at defined time points pre and post-operatively and compare with the microbiota of healthy babies from the INFANTMET study at matching time points.[19]
- To characterise the urine metabolite profile of infants with specified CHD undergoing surgery with CPB and compare with healthy infants from the INFANTMET study.[19]

Secondary objectives and outcomes:
To explore the influence of maternal and environmental factors on gut microbiome composition.

Methods

Study design

This study is a prospective cohort study of infants with CHD undergoing CPB at the National Centre for Paediatric Cardiac Surgery at Children's Health Ireland (CHI) at Crumlin, Dublin, Ireland. This single-site study will investigate the differences in the gut microbiome, metabolomics readouts, and stress levels between infants with CHD undergoing CPB and healthy age matched controls.

Participant selection

This study will involve the collection of demographic and clinical maternal and infant data from infants diagnosed with CHD scheduled for surgery involving CPB. CHD is typically diagnosed antenatally with fetal echocardiography performed routinely at 20-24 weeks gestation. Any child presenting with a murmur or features of cardiac conditions, or symptoms of CHD will be diagnosed using clinical examination, including palpation, auscultation, electrocardiogram (ECG) and echocardiography. Cardiac diagnoses are classified according to cardiovascular physiology, i.e., left to right shunt, cyanosis with biventricular circulation, univentricular circulation and outflow tract obstruction, as presented in table 1.

Table 1. Classification of CHD Subtypes

Group	CHD Group	CHD Subtypes
1	Left to right shunt	AVSD, VSD, large ASD
2	Cyanotic CHD with biventricular circulation	D-TGA/IVS, D-TGA/VSD
3	Cyanotic CHD with univentricular circulation	HLHS, HRHS (Tricuspid Atresia) Pulmonary Atresia
4	RVOT Obstruction	Tetralogy of Fallot, dysplastic PS
5	LVOT Obstruction	Shones Syndrome, Coarctation of Aorta, Interrupted Aortic Arch
6	Others	Ebstein Anomaly, Truncus Arteriosus

To be eligible for the study, the participants must meet the terms of the inclusion and exclusion criteria as presented in Table 2.

Table 2. Inclusion and Exclusion Criteria.

Inclusion criteria	Exclusion criteria
Infants born full term (≥37 weeks gestation)	Stillbirth or live birth where the baby is born alive but dies shortly after

Infants diagnosed with *CHD and scheduled for surgery involving CPB	Infants who are born healthy with no underlying illness, syndrome, or chronic disease
Infants born in Ireland to allow sample follow up	Participation in another study
Ability of the participant's parent/carers (in the investigator's opinion) to comprehend the full nature and purpose of the study	Infants not undergoing surgery involving cardiopulmonary bypass
Consent to participate in the study and willingness to comply with the protocol and study restrictions by the participant's parent/carers	Infants where parents/carers do not give consent to participate in the study
	Gastrointestinal pathology or intestinal surgery, excluding gastrostomy tube

*CHD diagnoses and subtypes are presented in Table 1.

Public and Patient Involvement (PPI) Statement

The mother of a child who had cardiac surgery as a baby was involved in reviewing the research questions, outcome measures and study literature at the study design phase. The PPI representative did not participate in the recruitment or the conduct of the study due to competing demands and availability. The PPI representative has offered to support dissemination of the study results through their involvement in charitable foundations and child and parent support fora.

Recruitment

Participants meeting all inclusion criteria will be selected after admission to the hospital, outpatient clinic or cardiac day unit. Study-related information will be given in written form as well as explained by a member of the project team. No study-related activities will begin before the potentially eligible participants' parents/carers have signed the Informed Consent Form (ICF). Participants parents/carers will be asked to refer to the Privacy Notice on the hospital website or they can receive a hardcopy if they wish. Signed ICFs will be stored safely in a locked cabinet in the research office.

Compensation

No compensation will be provided to the participants. There are no cost implications for the Health Service Executive (HSE) or to the participants. The management of patients and investigative tests will comply with current standards of care.

Study timeline

After completing recruitment procedures, i.e., determining whether the patient meets the study inclusion criteria, discussing the study with the parents/caregivers and obtaining informed consent, clinical and demographic data will start to be collected. The study will be undertaken for a period of 2 years after the infant is initially recruited.

Demographic Data

The infants' diagnosis including whether antenatal or postnatal diagnosis, co-morbidities, date of birth, gestational age, sex, mode of delivery, Appearance, Pulse, Activity, Grimace, Respiration (APGAR) scores, birth weight, head circumference, mode and type of feeding pre and post operatively, antibiotics administered post-delivery, complications and antenatal events will be recorded (case record form located in Appendix 1).

Maternal Data

Maternal history including age, antibiotics received, smoking status use of probiotics and significant antenatal events will be recorded.

Surgical course

The type of surgery performed, duration of CPB and cross-clamp time will be recorded. Antibiotic use and any intraoperative events will be recorded as well as clinical information including arterial blood gas data.

Post-operative Data

Paediatric Index of Mortality (PIM) score, duration of mechanical ventilation, haematology variables including haemoglobin and haematocrit, renal data e.g. blood urea nitrogen (BUN) and creatinine, fluid balance, and cardiovascular support including vasoactive inotrope score, as well as duration of stay in paediatric intensive care unit will be recorded.

Feeding Information Data

Feeding information including the type of feed and duration of the feed prior to and after surgery will be recorded. The date the patient is established on full feeds will be recorded. Full feeding is defined as when the patient no longer requires parenteral nutrition or intravenous fluids.

Discharge Information Data

Discharge information data: This will include the patient's status on discharge from Paediatric Intensive Care Unit (PICU), as well as length of PICU and hospital stay.

Complications

The occurrence of complications will be recorded, for example the development of NEC. The timeline for recording NEC onset will be based on the initiation of triple antibiotic therapy, based on a full surgical review including clinical presentation, radiological and laboratory data. In addition, the occurrence of death, rehospitalisation for heart failure or cardiac problems will be included.

Subject withdrawal/exclusion

Under the Declaration of Helsinki, the research nurse will explain to the consenting adult that they have the right to withdraw from the study at any time and that this will in no way prejudice their future treatment. The reason for withdrawal will be recorded in the source documents and on the appropriate CRF. Consenting adults will be made aware that stored samples from individuals withdrawing from the study may have undergone processing and may be analysed in the study.

Regulatory procedures

The study is conducted following the version Fortaleza, Brazil, October 2013 of the Declaration of Helsinki 1964. The Protocol and the ICF have been approved by the Clinical Research Ethics Committee of Children's Health Ireland GEN/826/20. As biological samples will be procured in one institution and sent to another, a data sharing agreement is in place between The Cardiology Department, CHI at Crumlin Hospital, and APC Microbiome Ireland, in Cork. This research is fully compliant with the guidelines as set out in The General Data Protection Regulation (GDPR), the Irish Data Protection Acts 1988 to 2018 including Protection Act 2018 (Section 36(2)) (Health Research) Regulations 2018.

Data Statement

Once collected, the anonymised demographic, clinical and laboratory analysis data as well as statistical codes will be uploaded to the open access Research Repository University College Dublin.

Sample collection and analysis

Faecal samples

Stool samples will be collected at the following time points: within 24 hours after birth (timepoint (TP) 1), within 24 hrs pre surgery (TP 2), 1 week post-surgery (TP 3) 4 weeks post-surgery (TP 4), 24 weeks post-surgery (TP 5) at 52 weeks (TP 6) and 2 years of age (TP 7). Information about antibiotic therapy administered before or during the stool collection will be recorded.

As the study site is the National Centre for Paediatric Cardiology, all infants diagnosed with CHD antenatally are transferred to the study site from the Maternity Unit for management of CHD. The first study stool specimen is typically obtained after the infant is transferred to the study site and informed consent obtained, which is typically within 24 hours of delivery.

The sample will be collected by the bedside nurse or the parent/carers and transferred to the laboratory upon receipt of the sample during the weekdays or weekends. At night, the sample will be kept in the dedicated fridge and transferred to the laboratory within 4-5 hours for appropriate storage at -80°C until further analysis. A standard operating procedure (SOP) for sample collection when participant is no longer an in-patient is provided in Appendix 2.

Urine Samples

Testing the urinary metabolomics of study participants will allow the potential identification of altered metabolomic profiles, and explore the role of microbiota in such alterations. Mirroring the INFANTMET study, urine samples will be collected at 4-8 weeks post-surgery for metabolomic analysis using Sterisets Uricol Urine Collection Pack (MedGuard, Ireland). The urine sample will be collected from the urinary catheter if the participant is catheterised. Alternatively, a pad will be placed in the diaper and used to collect an unsoiled urine sample from the infant. The pad will then be placed in a biohazard bag and frozen immediately at -80 °C prior to processing. After all the sample collections are complete, they will be shipped to Teagasc Food Research, Moorepark, Ireland, using DHL overnight service for microbiome and metabolomics analyses. Styrofoam Saf-T-Pak STP-309 shipper box or equivalent will be used. DNA extraction will be performed on stool samples using the modification of the Repeated Bead Beating Plus Column (RBB+C) method.[20] LC-MS will be utilised for metabolomics analysis of urine.[19]

Sample collection for discharged participant

Parents/carers will receive a sample collection discharge pack and a parent diary/instruction served as reminder to collect due samples at different time-points prior to discharge home. They will receive a follow up text message or phone call to remind them on the due sample. The sample collection discharge pack consists of urine/stool collection containers with study code, sterile pad, syringe, zip-lock bag, gloves, biohazard bag and cooler bag. Parents/carers are asked to keep the collected sample at the dedicated section of their home freezer. They will transport the collected sample in the cooler bag provided when attending out-patient department for appointments. They will ask a member of the project team to transfer the collected sample to the laboratory upon arrival at the hospital. The study researcher is available at the dedicated contact number for any queries.

Adverse events and participant well-being

There are no expected safety concerns related to the study. All study participants will be under the care of the cardiology team at the hospital with access to psychological support, as well as nursing and medical professionals, social workers and chaplaincy.

Data collection and management

The study diaries, study dataset, and paper/digital CRF systems will be used for recording data from each study participant. All the data collected in this study is pseudonymised, as each of the participants will be assigned a specific study code and upon receipt, data will be referred to the study code. All study staff responsible for entering data into the CRF system received training in advance of the study commencement. This training included familiarity with the study diaries, study dataset, and paper/digital CRF system and have completed good clinical practice (GCP) in research training. They have individual access to the password protected study shared drive within the hospital. The study team will monitor the data/sample collection process. Any inconsistencies identified during the study will be presented as queries at the regular project team meeting.

Comparison Data

Data collected as part of the INFANTMET Study will serve as a healthy control comparison for this study. INFANTMET compared the gut microbiota development of breastfed infants born via C-section or vaginally at full –term or preterm at Cork University Maternity Hospital. Ethical approval for sample collection by Cork University Hospital Research Ethics Committee (reference number ECM(w) 07/02/2012). One hundred and ninety two infants were recruited to the INFANTMET study and stratified according to delivery mode and gestational age at birth. Faecal samples were collected from the infants at 1, 4, 8 and 24 weeks of age and year 1, 2 and 4 of life and stored under controlled conditions. Urine samples were collected at 4 weeks of age for metabolomic analysis and stored in a freezer at -80°C prior to processing. Samples were analysed in accordance with the analysis proposed for the GuMIBear study. Although INFANTMET study participants did not have CHD, they nonetheless serve as a valuable comparison group. The stool and urine samples collected as part of the GuMIBear study will be as closely time matched as possible to the INFANTMET study samples to capture the major developmental period of the early life gut microbiota.

Bioinformatics and Statistical analysis

Sample size justification

Published research in this area is lacking. However one case control study by Salomon et al. (2021) included 17 cases and 12 control participants and was sufficiently powered to determine a statistically significant difference in beta diversity in cases versus controls ($F=5.6$, $p<0.001$). Our study proposes to include 50 patients with CHD undergoing surgery with CPB and age matched controls, almost three times the Solomon et al. (2021) study. We therefore anticipate that the proposed sample size is justified.

Demographic and Clinical Data

Demographic and clinical data and laboratory information will be tested for normality using the Shapiro-Wilk test. Descriptive statistics will be used to describe normally distributed data, and expressed as mean $\pm$ standard deviation (SD). Continuous data not normally distributed will be reported as median and interquartile ranges. Categorical variables will be expressed as counts and percentages. Groups will be compared using chi square tests for categorical variables and independent-samples student's *t*- tests for normally distributed continuous variables. For variables not normally distributed, the Mann-Whitney *U* test will be used. Comparison will include subgroup analysis of participants who experienced post-operative complications including NEC with those that did not. Comparisons will also include cyanotic versus acyanotic heart disease subgroup analysis, as well as mode and type of feeding pre and post-operatively.

Microbiome analysis

Metagenomic shotgun sequencing data will be analysed using bioBakery suite of tools (https://huttenhower.sph.harvard.edu/biobakery_workflows/). Trimmed and human reads filtered using KneadData (v0.7.2) with the default parameters. Quality controlled data will be taxonomically profiled at the species level with relative abundance by MetaPhlAn2. Functional profiling will be performed using HUMAnN3 and strain profiling using StrainPhlAn.

For alpha diversity analysis, samples will be rarefied to even depth and phyloseq::estimate richness will be used to calculate Chao1, Shannon and Simpson indices. Alpha diversity indices between groups will be univariately compared using the Wilcoxon rank sum test. A beta-diversity ordination will be generated using the Aitchison distance and visualised using Principal Component Analysis (PCA) plot. The Adonis function in the vegan package will be used to implement a permutational multivariate analysis of variance to test whether samples cluster beyond that expected by sampling variability. MaAsLin2 (Multivariate Associations with Linear Models) will be used to investigate multivariable associations between sequencing data and clinical metadata. MaAsLin2 performs boosted, additive, linear models to detect associations while adjusting for confounding factors. Sparse canonical correlation analysis (sCCA) will be used to calculate the overall correlation between metabolites and microbes, and to identify strongly associated biomarkers. Pairwise spearman rank correlation analysis will also be performed.

Discussion

Despite an increasing awareness of the early-life critical window of microbiome development on the health and wellness of infants, there remains much to learn about the interactions of the microbiome with the infant host with CHD undergoing surgery involving CPB. This study is designed to address this knowledge gap, and incorporates a sound methodology with particular strengths enhancing the value of its findings. The specimen collection strategy occurring at multiple time points over a 2 year period in the proposed study will deepen our understanding of the temporal dynamics of the colonising microbiota, and their interactions with host physiology. The study design will account for maternal and perioperative variables to determine changes to the microbiome. Access to existing microbiome data from healthy age matched infants provides a valuable opportunity to present high quality comparative information. A limitation of this study may include the failure to recruit infants with CHD not identified antenatally, despite active fetal screening services. While multi-centre trials capturing sufficient case numbers of NEC cases will offer robust conclusions, this study will offer valuable evidence to support the influence of CHD and CPB on the microbiome and intestinal epithelial barrier dysfunction (EBD). Future research can build on existing studies, and explore treatment strategies including recommendations for efficacious probiotic strain administration, including the supplements to promote a diverse gut microbiota to improve outcomes for this vulnerable population.

Ethics and Dissemination

This research study is ethically approved by the Clinical Research Ethics Committee of Children's Health Ireland (REC REF No: GEN/826/20). Study results will be available to patients with CHD and their families, carers, support networks, paediatric cardiology and microbiome societies and other researchers. Study findings will provide a deeper understanding of the gut microbiota of infants with CHD and inform perioperative management options including strategies to prioritise the integrity of the gut microbiota.

Status of Study

The trial is ongoing and as of 5th February 2023 , 84 % of the participants have been recruited. Laboratory analysis has been carried out on 25% of study samples.

Funding Statement

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests

The authors declare no association with commercial entities, either financial or non-financial.

Author contributions

CM, CJMcM, CS, KM devised the project, and the main conceptual ideas. CJMcM, CM, CS, RPR, FK, DJ, MHT were involved in the study design and writing of the manuscript. MOT, JB, NM, SD, CJMcM are involved in consenting participants, collecting samples and acquiring data. DJ, CS, FK and RPR are responsible for analysing study samples. All authors read manuscript revisions, approved the final manuscript and accept accountability for the accuracy and integrity of the work.

Acknowledgements

All study participants, Paediatric Intensive Care Unit/ Children's Heart Centre nurses for assisting in sample collection. All laboratory staff including Ms Irene Regan who have facilitated tracking and secure storage of study samples. Dr Adam James, Dr Ross Foley and the Consultant Paediatric Consultants, CHI at Crumlin for their support with this study.

Appendix 1: Case Report Form PDF Version

Appendix 2: SOP for Obtaining a Stool Sample when Participant is no longer Inpatient

References

1. Skillington O MS, Gupta A, Mayer EA, Gill CIR, Del Rio D, O'Riordan KJ, Cryan JF, Ross RP and Stanton C The contrasting human gut microbiota in early and late life and implications for host health and disease. Nutrition and Healthy Ageing. 2012;6:157-78.
2. Cilieborg MS, Boye M, Sangild PT. Bacterial colonization and gut development in preterm neonates. Early human development. 2012;88 Suppl 1:S41-9.
3. Healy DB, Ryan CA, Ross RP, Stanton C, Dempsey EM. Clinical implications of preterm infant gut microbiome development. Nature microbiology. 2022;7(1):22-33.
4. Ohland CL, Jobin C. Microbial activities and intestinal homeostasis: A delicate balance between health and disease. Cellular and molecular gastroenterology and hepatology. 2015;1(1):28-40.
5. Cabrera-Perez J, Badovinac VP, Griffith TS. Enteric immunity, the gut microbiome, and sepsis: Rethinking the germ theory of disease. Experimental biology and medicine. 2017;242(2):127-39.
6. Halter J, Steinberg J, Fink G, Lutz C, Picone A, Maybury R, et al. Evidence of systemic cytokine release in patients undergoing cardiopulmonary bypass. The journal of extra-corporeal technology. 2005;37(3):272-7.

7. Centers for Disease Control and Prevention (CDC). Data and Statistics on Congenital Heart Defects. 2022 [updated January 24th 202226th July 2022]. Available from: https://www.cdc.gov/ncbddd/heartdefects/data.html Accessed 26th July 2022.
8. van der Linde D, Konings E, Slager M, Witsenburg M, Helbing W, Takkenburg J, Roos-Hesselink J. Birth Prevalence of Congenital Heart Disease Worldwide: A Systematic Review and Meta-Analysis. Journal of the American College of Cardiology. 2011;58(21):2241-2247.
9. Martin R, Makino H, Cetinyurek Yavuz A, Ben-Amor K, Roelofs M, Ishikawa E, et al. Early-Life Events, Including Mode of Delivery and Type of Feeding, Siblings and Gender, Shape the Developing Gut Microbiota. PloS one. 2016;11(6):e0158498.
10. Siano E, Lauriti G, Ceccanti S, Zani A. Cardiogenic Necrotizing Enterocolitis: A Clinically Distinct Entity from Classical Necrotizing Enterocolitis. European journal of pediatric surgery : official journal of Austrian Association of Pediatric Surgery. 2019;29(1):14-22.
11. Kelleher ST, McMahon CJ, James A. Necrotizing Enterocolitis in Children with Congenital Heart Disease: A Literature Review. Pediatric cardiology. 2021;42(8):1688-99.
12. Murphy K, O'Donovan AN, Caplice NM, Ross RP, Stanton C. Exploring the Gut Microbiota and Cardiovascular Disease. Metabolites. 2021;11(8).
13. Carlo WF, Kimball TR, Michelfelder EC, Border WL. Persistent diastolic flow reversal in abdominal aortic Doppler-flow profiles is associated with an increased risk of necrotizing enterocolitis in term infants with congenital heart disease. Pediatrics. 2007;119(2):330-5.
14. McElhinney DB, Hedrick HL, Bush DM, Pereira GR, Stafford PW, Gaynor JW, et al. Necrotizing enterocolitis in neonates with congenital heart disease: risk factors and outcomes. Pediatrics. 2000;106(5):1080-7.
15. Miller TA, Minich LL, Lambert LM, Joss-Moore L, Puchalski MD. Abnormal abdominal aorta hemodynamics are associated with necrotizing enterocolitis in infants with hypoplastic left heart syndrome. Pediatric cardiology. 2014;35(4):616-21.
16. Nino DF, Sodhi CP, Hackam DJ. Necrotizing enterocolitis: new insights into pathogenesis and mechanisms. Nature reviews Gastroenterology & hepatology. 2016;13(10):590-600.
17. Typpo KV, Larmonier CB, Deschenes J, Redford D, Kiela PR, Ghishan FK. Clinical characteristics associated with postoperative intestinal epithelial barrier dysfunction in children with congenital heart disease. Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies. 2015;16(1):37-44.
18. Salomon J, Ericsson A, Price A, Manithody C, Murry DJ, Chhonker YS, et al. Dysbiosis and Intestinal Barrier Dysfunction in Pediatric Congenital Heart Disease Is Exacerbated Following Cardiopulmonary Bypass. JACC Basic to translational science. 2021;6(4):311-27.
19. Hill CJ, Lynch DB, Murphy K, Ulaszewska M, Jeffery IB, O'Shea CA, et al. Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. Microbiome. 2017;5(1):4.
20. Yu Z, Morrisson M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques. 2004;36(5):808-12.

GuMIBear Study

Study ID: GMB: ______

^**999** – missing data; **0** – no data in medical notes

CASE RECORD FORM

Stool Sample Collections

Period/ Time Frame		Projected Date	Sample Date	Sample Collected	Comments
1.	Within 24h of Birth			☐ YES ☐NO	
2.	Pre-operatively			☐ YES ☐NO	
3.	Week 1 of life/Post-Op			☐ YES ☐NO	
4a.	Week 4 to 8 life/Post-op			☐ YES ☐NO	
4b.	**Urine** Sample Week 4 to 8			☐ YES ☐NO	
5.	Week 24 of life/Post-Op			☐ YES ☐NO	
6.	Week 52 of life/Post-Op			☐ YES ☐NO	
7.	Year 2 of life/Post-Op			☐ YES ☐NO	

PATIENT DEMOGRAPHIC

Date of Enrolment:		Date of Birth:	Cardiac Classification Group No:
Gender:	☐ Male ☐ Female ☐ Other/Ambiguous	Gestational Age: APGAR at ① ⑤ min	Multiple ☐ Yes ☐ No Birth Order:
Reason for admission:			
Comorbidities:			
Mode of Delivery: SVD: ☐Yes ☐No LSCS: ☐Yes ☐No, if Yes: ☐Elective/ ☐Emergency		Weight at Birth: ______ . ______ KGs Head Circumference: ______ cms	
Antibiotics to Infant post-delivery; ☐ Yes ☐ No If Yes, list:		Timing of cardiac diagnosis: Postnatal ☐ Antenatal ☐	
Significant Antenatal Events:			

MATERNAL INFORMATION

Maternal Age (years) at Birth:	Gestational Age at Booking Appt:
Antibiotics given Pre-Delivery: ☐ Yes ☐ No List:	Maternal Probiotics taken during pregnancy: ☐ Yes ☐ No List:
Maternal Smoking during Pregnancy: ☐ Yes ☐ No	Other Household Members Smoking during Pregnancy: ☐ Yes ☐ No

GuMIBear Study

Study ID: GMB: ______

^**999** – missing data; **0** – no data in medical notes

SURGERY INFORMATION

Surgery Date:					
Surgery Performed:					
Pre-Op Antibiotics[1]? List:			Post-Op Antibiotics[1]? List:		
1. Abs <48hrs pre sampling			1. Abs <48hrs pre sampling		
2. Abx <72hrs pre sampling			2. Abx <72hrs pre sampling		
3. Abx in last 7 days/during sample			3. Abx in last 7 days/during sample		
ABG	pH	PO^2			
First Pre-Op ABG:			Cardiopulmonary Bypass Duration:		
First Post-Op ABG:			Aortic Cross Clamp Duration:		
Significant Intraoperative Events:					

[1]Antibiotic treatment at time of stool sampling as below. Important not to include antibiotics which were started post the stool sampling

1) Abs < 48hrs pre sampling
2) Abx < 72 hrs pre sampling
3) Abx in previous 7 days/ during sample collection

POST-OPERATIVE INFORMATION

PIM3 Score:		No. of days in ICU (1st adm):	
Mechanical Vent:	☐ Yes ☐ No	No of Days on ECLS:	
No of Days Vent:		No of Days on RRT:	
Agent	**Day 1**	**Day 2**	**Day 3**
Milrinone			
Epinephrine			
Norepinephrine			
Vasopressin			
Midazolam			
Morphine			
Clonidine			
Others			
Fluid Balance:			
BUN:			
Creatinine:			
HCT:			
Hgb:			

FEEDING INFORMATION

Mode of Feeding (note ***date initiated*** and ***date d/c***):		
Breastmilk:	Infant Formula:	Other:
Prebiotics given to Infant: ☐ Yes ☐ No	Type and Date Given:	
Excessive Infantile Crying (cried for ≥3 Hrs for 3 Days in one week): ☐ Yes ☐ No		

GuMIBear Study

Study ID: GMB: ______

^**999** – missing data; **0** – no data in medical notes

Date Trophic Feeds Commenced:	Type of Feed Used:
Time to Establishment of full feed[2]:	
Development of NEC[3]: Days post-op when developed NEC?	
~~Gut stasis:~~	Not applicable
Management Strategy:	

[2]Full feed – No longer requires parenteral nutrition or intravenous fluids supplement regardless the TFI.

[3]NEC – Initiation of triple IV antibiotic therapy and nil by mouth for at least 5 days, based on a full surgical review including clinical presentation, radiological and laboratory data.

DISCHARGE INFORMATION

Date of Discharge		
Ward:	Home:	RIP:

READMISSION TO ICU

Total ICU Readmission days:

	Date of Admission	Date of Discharge	Total ICU stays	Reason for admission
1.				
2.				
3.				
4.				
5.				

DATA ENTRY BY (NAME)

Admission	**Date**	**Paper**	**Date**	**G-Drive**
First				
Second				
Third				

Participant Withdrawal from Study: ☐ Yes ☐ No

GCP Procedure Followed: ☐ Yes ☐ No

See Study Folder Appendix 4. Signed:______________ Date: ____________________

The Gut Microbiota of Infants with Complex Congenital Heart Disease Undergoing Cardiopulmonary Bypass

(GuMiBear)

STANDARD OPERATING PROCEDURE FOR STOOL SAMPLE COLLECTION AS AN OUTPATIENT

Purpose: To collect infant stool samples while the infant is an out-patient from CHI at Crumlin

Objective: To collect infant stool samples for the study in a uniform manner and under a set of conditions, so that they can be processed by the laboratories to achieve optimal results.

Procedure:

1. Ensure that at least one legal guardian has provided written informed consent for their infant to participate in the study and that they are happy for their infant to remain in the study.
2. Ensure that in the hospital chart of the infant that it is noted that he/she is participating in the study and contact details of the study team.
3. Ensure the parent/guardian has been supplied with a study pack containing the requisites for the collection of the stool sample
   a. Completed labels
   b. Disposable Gloves
   c. Stool collection containers
   d. Bio-hazard bags
4. Explain to the legal guardian that a minimum of a teaspoon of stool has to be collected. Explain to the legal guardian when the samples have to be collected as close as possible to the next out-patient appointment.
5. Explain to parent/guardian how to collect the sample as follows:
   a. Have requisites for collection at the ready.(Gloves, sample container, red biohazard bag & labels)
   b. Place appropriate label on the sample bottle.
   c. Wear disposable gloves
   d. Unscrew cap of sample bottle

The Gut Microbiota of Infants with Complex Congenital Heart Disease Undergoing Cardiopulmonary Bypass

(GuMiBear)

e. Spoon in stool sample (1 teaspoon in volume)

f. Screw cap on tightly

g. Place in red hazard bag

h. Remove disposable gloves

i. Dispose with soiled nappy

j. Wash hands

k. Place stool sample in fridge

l. Text research nurse that sample is ready for collection.

6. Label the sample bottles with Subject Number, Date of Birth, Initials, Sample Number, Date of Collection

7. Ask the parent/guardian to attach the appropriate label to the sample container and immediately place container in freezer.

8. Text the parent/guardian the night before the sample is to be collected. Meet the parent/guardian in out-patients to collect the stool sample and store the sample in the study container in the designated study freezer.

9. Update the study spreadsheet to indicate sample has been obtained.