STUDY PROTOCOL

REVISED Novel gastrointestinal tools (GI Tools) for evaluating gut functional capacity in adults with environmental enteropathy in Zambia and Zimbabwe: A cross-sectional study protocol

[version 2; peer review: 2 approved]

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Abstract

Background

Environmental enteropathy (EE) is a highly prevalent subclinical inflammatory intestinal disorder associated with growth failure, impaired neurocognitive development, poor response to oral vaccines, and micronutrient deficiencies. However, EE research and clinical trials are hampered by the lack of non-invasive tools for measuring intestinal function in detail. This study aims to develop new tools for the measurement of multiple domains of gut functional capacity.

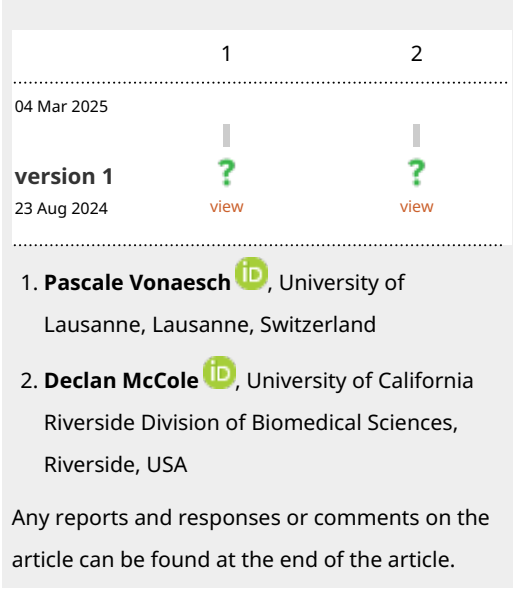
Methods

The GI TOOLS project is a cross-sectional study that will recruit adults aged 18-65 years with EE in Lusaka, Zambia. Each participant will undergo assessment of gut functional capacity using novel near-point-of-care tools and provide multiple samples for detailed laboratory analyses. Participants will also undergo endoscopy for collection of duodenal biopsies. Novel techniques include stable isotopes approaches to measuring digestion, absorption, and bidirectional transmucosal amino acid flux, a non-invasive fluorescence tool for real-time evaluation of gut permeability, and assessment of reverse permeation of intravenous antibiotics to be carried out separately in Zimbabwe. Stool and duodenal microbiome sequencing using MinION sequencing, metabolome analysis applied to plasma and intestinal fluids, blood immune cell phenotyping, *in vitro* epithelial barrier models, and duodenal immunohistochemistry will also be used to explore EE in depth. These will all be integrated with gold standard histology and mucosal morphometry, alongside lactulose permeation data, and stool and plasma biomarker analysis. The protocol has been approved by ethics committees and regulators in Zambia, Zimbabwe, and the UK. Participants will give informed consent before they can participate

Anticipated outcomes

Based on this extensive phenotyping, tests will be developed which can be simplified and refined for use in adults and children with EE, and for clinical trials. Findings from this project will be disseminated through in-person meetings with caregivers and regulatory bodies, presentations at conferences and in peer-reviewed journals.

Keywords
Environmental Enteric Dysfunction, Intestinal Absorption, Intestinal Permeability, Microbiome, Stable Isotopes



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REVISED **Amendments from Version 1**

The authors greatly appreciated the comments from the reviewers and the manuscript was significantly improved by incorporation of all of their comments. The first major revision was to the introduction, and reference list, to acknowledge the important contributions of work from Bangladesh, Pakistan, and USA, and from the Afribiota studies. The addition of other relevant literature was also included on the characterisation of EE and malnutrition in children. Additionally, the inclusion of 20 control participants from high socioeconomic status (SES) residential areas in Lusaka was incorporated into the protocol. Further detail was also provided in the sample collection and clinical assessments, particularly in the permeability, microbiome, and cell culture analyses. The discussion was therefore also redrafted accordingly to reflect the changes made throughout. These additions greatly improve the manuscript and provide a more balanced and wider view of current methods for biomarker and microbiome research in EED.

Any further responses from the reviewers can be found at the end of the article

Introduction

The gastrointestinal tract is a critical organ, simultaneously orchestrating digestion, absorption, and excretion of gut contents whilst providing a physical barrier between the external environment in the gut lumen and body tissues, creating conditions to tolerate dietary antigens and the gut microbiome.[1] However, measurement of these intestinal functions, which we refer to hereafter as gut functional capacity, is currently crude and imprecise. There is an urgent need to develop improved tools for the evaluation of a broader range of intestinal functions in studies of pathophysiology and clinical trials in both adults and children.[1] Following a golden age of research on human intestinal digestion and absorption in the 1970s and 1980s, work on gut physiology has slowed. Current gold-standard approaches to assess gut functional capacity, such as the lactulose-mannitol (or lactulose-rhamnose) test or endoscopy, are time- and labour-intensive or measure only one aspect of intestinal dysfunction, such as its permeability to molecules which normally are excluded from uptake in the healthy state.

Environmental enteropathy (EE) is a highly prevalent subclinical inflammatory intestinal disorder that is associated with exposure to unsanitary conditions and poor nutrition. In children, it is associated with growth failure, impaired neurocognitive development, and poor response to oral vaccines. EE is frequently associated with undernutrition. In moderate undernutrition, limited nutrient bioavailability, pathogen pressure, and gut barrier dysfunction can lead to impaired linear growth (stunting), impaired vaccine response and cognitive deficits in children.[2] In acute undernutrition, mortality remains unacceptably high, particularly those hospitalised with severe acute malnutrition (SAM).[3,4] Both stunting and incomplete recovery from SAM in childhood embed life-long and intergenerational health consequences which detrimentally impact population and economic health in low- and middle-income countries (LMICs).[5–7]

EE probably represents a chronic adaptation of the proximal small intestine to marginal diets and exposure to environmental enteropathogens.[8] In LMICs, this adaptation develops in early life and is characterised by reduced absorptive surface area, goblet and Paneth cell depletion and intraepithelial lymphocyte infiltration.[9] Our work indicates that SAM is associated with a more severe enteropathy[10]; a global disturbance of intestinal architecture and function, including maldigestion, malabsorption and impaired gut barrier function.[13] Impaired gut barrier function allows translocation of microbes and their products, which may explain systemic endotoxemia and, in some cases, sepsis and septic shock, which are major drivers of mortality.[11–13] Several non-invasive biomarkers of inflammation and microbial translocation have been proposed as biomarkers of severity of EE. Endotoxin core antibody (EndoCab), lipopolysaccharide (LPS) and myeloperoxidase (MPO) may be predictive of malnutrition status in children.[14] α-1-antitrypsin (AAT), MPO and neopterin, are elevated in malnourished children when compared to healthy children.[15] However, other studies do not report associations with malnutrition or lactulose-rhamnose tests.[16] These inconsistent associations hinder their broader application. Recent research in children with stunting has revealed significantly reduced HLA-DR expression on memory CD4+ and CD8+ T cells, along with a higher proportion of regulatory T cells and classical monocytes compared to non-stunted children.[17] Similarly, children with SAM exhibited lower HLA-DR upregulation on monocytes and neutrophils but demonstrated higher binding capacity for Escherichia coli than children without SAM.[18] These findings suggest an altered immune cell phenotype in children with SAM. Increased microbial metabolites, and acute phase proteins (CRP and calprotectin) and inflammatory markers were associated mortality in children with SAM.[19] These findings are further supported by another study which showed that markers associated with higher gut and systemic inflammation may be associated with higher mortality or hospital re-admission.[13] Additionally, biomarkers of environmental enteropathy (EE) may correlate with antigen-specific immune responses in SAM children at 18 months of age.[20] In the same study, maternal CRP and stool neopterin concentrations during pregnancy were associated with stronger heat-killed *Salmonella typhimurium*-specific IL-8 responses in the children at 18 months. Furthermore, children who received water, sanitation, and hygiene (WASH) interventions demonstrated higher inducible LPS-specific myeloperoxidase (MPO) levels compared to those who did not receive such interventions. These findings underscore the complex interplay

between the immune system, SAM, and EE. Further research is needed to better understand and differentiate the immune responses in SAM and EE, across both children and adults.

Both SAM and the antibiotic treatments recommended for children admitted to hospital with SAM also affect the gut microbiome, the composition of which is associated with lower bacterial diversity and maturity, which fail to persistently recover with standard nutritional therapies.[21] Characterization of the small intestinal microbiome in children is challenging due to the invasiveness of endoscopy procedures. Some studies showed through culture methods that small intestinal bacterial overgrowth (SIBO) is associated with stunting in children from Bangladesh,[22] Central African Republic and Madagascar. These studies also provided the first evidence, using 16s sequencing, of decompartmentalization of the microbiome along the GI tract in stunted growth and EE[23,24] and demonstrated a loss of carbohydrate-metabolising microbes in the small intestine.[25] These findings suggest that small intestinal microbes contribute to the pathophysiology of stunting however, these findings are relatively novel and need validation with different cohorts. There is a need to take a holistic approach to understanding the impact of undernutrition and EE on gut physiology across the whole gastrointestinal tract, which means developing tools for assessing multiple domains[26] of gut function (Figure 1). This necessitates simultaneous assessment of the structure of the intestine (e.g. villus height, and therefore surface area), barrier function and microbial translocation, digestive and absorptive capacity (e.g., enzyme activity for protein, fat, and carbohydrate digestion, transporter expression for absorption), systemic, and intestinal immune responses to pathogens, expression of epithelial pattern recognition receptors (PRRs), pathogen-associated molecular patterns (PAMP; an indicator of microbial translocation), and the composition, function and metabolic activity of the gut microbiota. The latter will be reflected in the measured host metabolome. Such measurements will allow the selection of an optimised 'toolkit' for assessing gut functional capacity and ultimately enable the design of novel therapeutic feeds, which actively promote improved gut function, support sustained rehabilitation, reduce mortality, and feed the superorganism (i.e., the host and microbiome), and not just the host. Recent evidence has demonstrated that designing a diet to enhance normal maturation of the colonic microbiota results in improved growth[21,27,28] versus standard therapies in children recovering

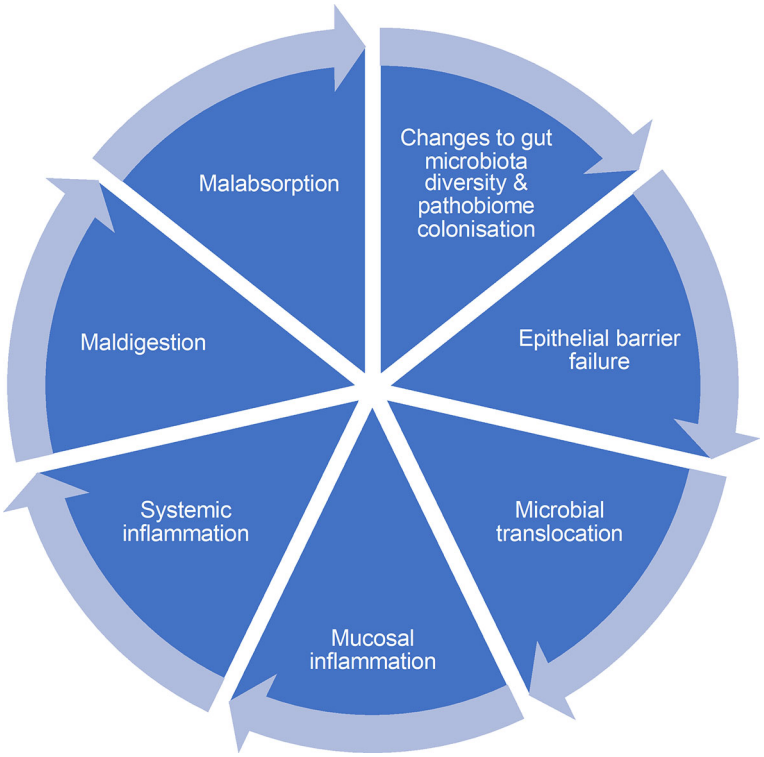

Figure 1. Domains of intestinal dysfunction leading to cycle of inflammation and persistence. Illustration of prominent elements of inflammation cycle, that includes (i) changes to gut microbiota diversity and pathobiome colonisation leading to decompartmentalization, which promotes (ii) epithelial barrier failure leading to increased permeability and (iii) microbial translocation of cell wall antigens and bacteria, which stimulates (iv) mucosal inflammation leading to (v) systemic inflammation which leads to increased demands for essential nutrients (e.g. amino acids), whose bioavailability may be limited by (vi) maldigestion caused by alterations to brush border architecture and enzyme activity and (vii) malabsorption caused by changes to the expression of nutrient transport proteins in epithelial cells. At the present time, it is not possible to determine how this network of functions is interrelated, and which functions are critical to resolve without new tools to assess each of these domains.

from acute malnutrition. Probiotic interventions have the potential to improve recovery and growth in various malnutrition disorders,[29] providing the proof-of-principle that rational design of feeds or biotherapeutics targeted to support gut function could be an achievable and effective therapeutic approach at scale.

The urgent problem which we aim to address in the GI Tools study is the dearth of measurement tools to assess the gut functional capacity of communities affected by EE. These tools will 1) be essential for evaluating gut functional capacity in adults (across many disease states) 2) have the potential to assess gut functional capacity in children because of their largely non-invasive nature, and 3) direct the development of new therapeutic interventions to support optimal gut function, for example, recovery of children hospitalised with SAM.

Aims and objectives

The overarching objective of the GI Tools project is to develop a novel portfolio of tools for evaluating gut functional capacity in EE and malnutrition disorders. This protocol will allow for the determination of safety, acceptability, dosing, and timing for novel tools to evaluate gut functional capacity in adults with EE. This will be translated to work in malnourished children in further phases of this work.

Study aims

Aim 1: Evaluate a novel portfolio of stable isotope-based tools for evaluating carbohydrate and protein digestion and amino acid absorption.

Aim 2: Test a novel non-invasive fluorescence sensor-based tool for real-time evaluation of gut permeability.

Aim 3: Characterise the microbiome and metabolome across well characterised and novel physiological sites (duodenal aspirate, duodenal mucus, duodenal tissue, stool, plasma, urine).

Aim 4: Quantify systemic and intestinal inflammation using established biomarkers of EE, immune cell phenotyping, epithelial PRR expression, and in vitro epithelial barrier models.

Aim 5: Determine if antibiotics, given intravenously, permeate into the gut lumen in sufficient quantities to modulate the microbiota.

All of these will be correlated with histological mucosal morphometry, lactulose permeation, and established biomarkers of EE.

Study strengths and limitations

- A study of 80 adults with EE and 20 controls will develop novel analytical tools to demonstrate their viability and effectiveness for the assessment of gut functional capacity compared with current ‘gold standards’.
- This study will inform future work on EE disease assessment and in treating children with severe acute malnutrition (SAM) and/or stunting.
- These novel tools will have practical applications in clinical assessments not only in EE but other conditions that affect gut functional capacity allowing better understanding of the underlying mechanisms of different diseases.
- A limitation of this study is the lack of country-specific controls in whom the same assays would be performed for comparison.
- Including adults only in this study limits the applicability of some of the analytical methods when designing a child-based study due to smaller sample volumes.
- The data will be generated over the course of a single 3-day protocol; analysis of longitudinal changes will require additional studies.

Methods

Study outcomes

The expected outcomes for each aim are shown in Table 1.

Table 1. Summary of outcomes with reference to study aims.

	Measured outcome	Detail
Aim 1	*Protein digestion and absorption using stable isotope tracers*	Assessment of ^{13}C spirulina digestion (global index by breath $^{13}CO_2$) and absorption using ^{13}C Phe (from spirulina) and ^{2}H labelled free Phe administered orally.
	Bidirectional transmucosal amino acid flux (BTAAF)	A novel tool involving dual infusion of differentially labelled amino acid tracers (Phe/Leu) orally and intravenously for the assessment of EE severity.
Aim 2	*Fluorescein*	A novel non-invasive measure of intestinal permeability using orally dosed fluorescein and a finger probe (transcutaneous fluorescence spectroscopy) for detection of systemically circulating fluorescein.
Aim 3	*Microbiome analysis*	I. Faecal species abundance and diversity indexes by 16s rRNA sequencing. II. In country deep metagenomic sequencing by long read (Nanopore) sequencing compared with other short-read platforms (Illumina). III. Evaluation of microbial communities in EE and their ability to uptake intestinal amino acids, including conversion of L-amino acids to D-forms.
	Metabolic phenotyping	I. Untargeted metabolomics (^{1}H-NMR) of plasma, urine and faecal water. II. Targeted quantification (MS) of intestinal, plasma and urine amino acids (unlabelled + labelled) before and after administration of oral and intravenous stable isotopes.
	Metaproteomic analysis	1) Proteomic analysis of intestinal biopsies for expression of key mucosal proteins in biopsies (mucins and tight junction proteins including Zonulin, claudins and occludins).* 2) Dynamic proteomic analysis of key target proteins to assess mucosal and tight junction protein synthesis rates in biopsies by high-resolution MS and D2O labelling.
Aim 4	*Biomarker analysis*	Quantification of known markers of systemic inflammation, microbial translocation, and intestinal epithelial damage in plasma and stool.
	Duodenal expression of PRR	Profile the expression of bacterial, fungal, and viral PRR in the duodenal epithelium using immunohistochemistry.
	Systemic immune cell analysis	Characterisation of innate and adaptive systemic immune cell phenotypes using differential cell counting and flow cytometry.
	Plasma and faecal cell cultures	Evaluation of the immunogenic effect of PAMPs present in plasma and stool on epithelial barrier integrity and function using cell culture models.
Aim 5	*Antibiotic reverse permeation*	A novel technique for the assessment of reverse permeation of intravenously administered benzylpenicillin into the gut.
Aim 6	Compare all of the above with histological assessments of mucosal biopsies collected at endoscopy, lactulose/rhamnose testing, and established biomarkers of EE.	

*Does not give information on protein localisation.

Materials and analysis

Study design

This cross-sectional study will be conducted in adults in Zambia using a range of stable isotope labelled amino acids and fluorescent tracers,[1,30] systemic immune assessments, EE biomarker analysis, microbiome and metabolome analysis, enzyme functional capacity, proteomics and metaproteomic analysis, barrier integrity and function capacity analysis using cell lines, antibiotic reverse permeation, duodenal morphometry, and duodenal expression of PRR to identify potential new tools for measuring gut functional capacity in Zambian adults with EE.

In a sub-study in Zimbabwean adults, we will explore the extent to which antibiotics leak from systemic circulation into the gut, where they may modulate the microbiota.[31] The study will be carried out in Harare, where sampling of caecal luminal fluid collected at colonoscopy will be used to quantify reverse permeation of intravenously administered

benzylpenicillin from the systemic circulation into the gut lumen. Future work will focus on refinement and simplification of these techniques to make them suitable for use in children.

Study population

The initial study will be conducted in 80 adults from a population in Lusaka, Zambia, where our previous studies have demonstrated that EE is virtually ubiquitous in adults and children.[32] Additionally, 20 controls living in high socioeconomic status (SES) residential areas (Rhodes Park, Longacres, Kabulonga, Ibex Hill, Chelston, Avondale, Sunningdale, Roma, Chamba Valley, Lilayi, New Kasama and others) within Lusaka will be included. A further 20 patients attending for routine colonoscopy (for diagnostic purposes) in Harare, Zimbabwe will be enrolled for the antibiotic permeation study. As these participants will already be booked for colonoscopy they will be drawn from higher socio-economic groups.

Sample size estimation

As there are no, or very limited, data on these measurements in this population, the study was powered based on previous studies using stable isotope breath tests and permeability studies. Previous data from an enteropathy vs. healthy child cohort and ^{13}C-sucrose breath tests[33] has suggested that calculating the cumulative $^{13}CO_2$ excretion, % relative to the dose, at 90 minutes post-intake best represents the villus integrity and the absorption capacity of only the small intestine. Preliminary data have shown that in a group of adults from Misisi with demonstrable sucrase-isomaltase (SI) expression in biopsies,[34] the mean cumulative % of dose excreted as $^{13}CO_2$ at 90 minutes is 20.29% compared to 16.02% in the group without SI expression, with SD of 5.29% – resulting in an effect size (ES) of 0.81.[34] With $\alpha = 0.05$, power = $(1-\beta) = 0.80$ we estimate we need 20 samples per group for achieving 80% power with a one-tailed test. We will recruit 30 participants into the definitive studies, allowing for potential dropouts and incomplete sample sets. To estimate sample size for permeability studies we used data on circulating LPS concentrations in stunted children which were mean 481 EU/ml (SD 408) in Lusaka, compared to controls (mean 192, SD 113). This results in an effect size of 0.96, with $\alpha = 0.05$ and power = 0.80 and we would require 18 in each group for a two-tailed test. The overall sample size was set at 80 adults to allow for sufficient power to cover a range of studies in adults and children.

Participant recruitment

For adults from Misisi compound, consent will begin with an invitation delivered door-to-door, while social media approaches, like Facebook and/or Instagram will be used for the controls. All household members eligible to participate will be invited to group discussions. The study group discussion meetings will include the following:

- Introduction to the study team.
- Introduction to the study rationale and protocol in full; explanation of the study.
- Reading of participant information sheet in Nyanja or Shona; literacy rates in Misisi and Harare are variable, and this is therefore the most appropriate method for conveying this information.
- Benefits and risks of being involved in the research.
- Previous participants' descriptions of being involved in research which includes endoscopy.
- Questions to previous participants.
- Questions to the study team regarding the study.
- Invitation to attend a guided tour of the research and endoscopy facilities.

Potential participants who are unable to make any of the focus group meetings will be invited to the field clinic to discuss the information sheet and study procedures with a member of the study team. All potential participants will be invited to provide written informed consent in individual interviews with a member of the study team afterwards. They will be invited to ask further questions or express concerns. If happy to proceed, they will be asked to sign or thumbprint the consent form, which will be read out to them in the language of their choice if they are unable to read. Consenting and enrolment will occur at least 48 hours after they have attended a focus group meeting or study explanation visit. Only participants who give consent will undergo screening and enrolment into the study. However, if for any reason during the study, the participant decides to withdraw, they will be allowed to exit the study but will not be replaced. In the event of withdrawal, participants will have a choice whether their samples can be retained or must be destroyed.

Table 2. Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
• In Lusaka, only residents of Misisi compound and high SES residential areas, while in Harare, only patients who have been referred for a colonoscopy.	• This study will not include any individuals taking medication for type II diabetes.
• Only adults from high socio-economic status (i.e., living in detached houses with indoor flush toilets, tiled floors, separate kitchens with running water and indoor stoves, and own at least one vehicle) will be included in Harare and in the Zambian control group.	• Any contra-indication to study procedures including endoscopy, such as likely to cause problems with sedation (e.g., oropharyngeal anomaly, previous adverse reaction) or biopsy (e.g., bleeding diathesis).
• Participants of either sex will be included in the study.	• Pregnant or lactating.
• Participants aged between 18 and 65 years old.	• Currently taking any anticoagulants.
• Only participants who are able and willing to undergo HIV testing.	• Having renal failure or liver failure (any major organ system).
• Able and willing to give written, informed consent will be included.	• Any underlying condition, other than HIV, which in the opinion of the investigator would put the subject at undue risk of failing study completion or would interfere with analysis of study results.
	• Participants will be temporarily excluded from study procedures if they have had diarrhoea (by self-report) in the preceding month, and have taken antibiotics, non-steroidal anti-inflammatory drugs, and/or proton pump inhibitors in the preceding month until disqualifying condition has elapsed.

Inclusion and exclusion criteria

This study will include and exclude participants following the criteria shown in Table 2.

Protocol

Screening and initial assessment

Participants will be consented for HIV testing as part of the consent process, before enrolment. Consenting for HIV testing and communication of results will be performed by an experienced and fully trained team of research staff and counsellors. HIV counselling and testing have been performed by this team both as part of standard medical care in the Misisi clinic and as part of the routine research process in our recent studies for over 15 years.[10,35] Likewise, HIV counselling and testing are standard of care in Zimbabwean health care facilities. HIV test results will be given in person and confidence in the clinic of recruitment. Participants found to be HIV seropositive will be allowed to discuss their results and management in more detail. With their consent, they will be referred to HIV specialists for further investigation and management including anti-retroviral therapy per Ministry of Health guidelines and procedures in both Zambia and Zimbabwe. Test results remain confidential and will not be shared with anyone outside of the study team without the participant's explicit consent.

Anthropometry

All anthropometry measures will be taken by qualified research nurses trained in anthropometry. Weight will be measured using a calibrated scale, standing height using a height scale, and mid-upper arm circumference (MUAC) using a MUAC tape. Grip strength will be measured using a Takei Grip-D dynamometer, waist and hip circumferences using a measuring tape, and lean and fat mass using a BodyStat® 1500 Impedance instrument (BodyStat®, Douglas, Isle of Man).

Dietary assessment

A multi-pass 24-hour dietary recall will be used to assess participants on day 2. All participants will be asked about the food they may have had in the last 24 hours which will be documented (Described in detail elsewhere.[36] While this 24-hour period may not be representative of the participants' habitual intake, it was intended primarily to help interpretation of the metabolic profiling and thus would be most useful prior to the collection of samples for metabolic analysis.

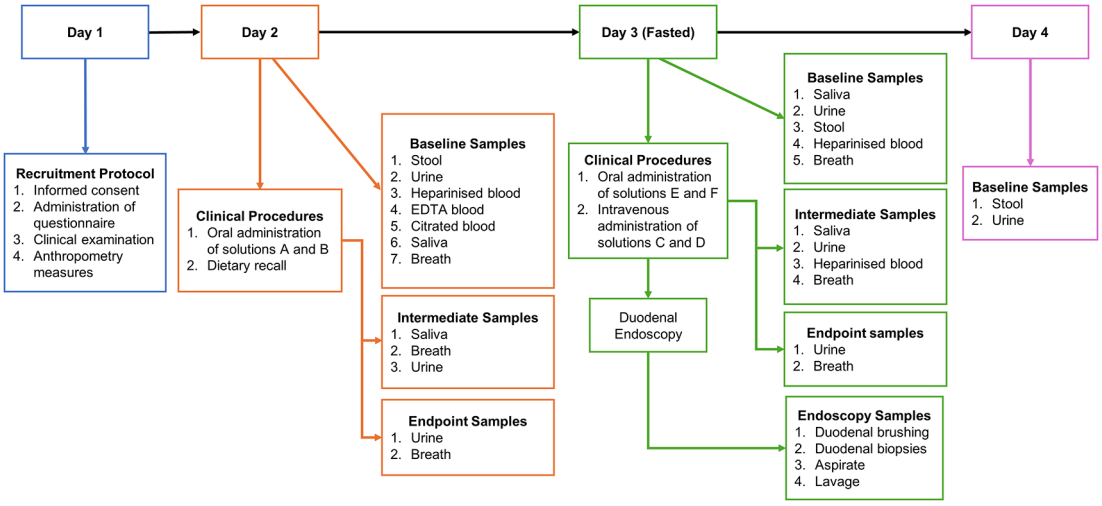

Figure 2. Study schema illustrating sample collection across the 4-day study period. Schema showing each sample collection time point throughout study, Day 1; No samples will be collected; Day 2; 9 mL heparinised blood; 2 mL EDTA blood; 5 mL citrated blood; 11 mL Urine at 00 and 180 mins; 4-8 g of stool; 0.5-2 mL of saliva at 00 and 180 mins; and breath samples every 20 mins. Day 3: 9 mL of heparinised blood; intermediate 2 mL heparinised blood samples every 20 mins for 1 h and every 30 mins for the last 2 h; breath samples every 20 mins; 0.5-2mL of saliva at 00, 60, 120 and 180 mins; 7 mL of urine at 00 and 180 mins; 4-8 g stool; 8 duodenal biopsies and 1 duodenal brushing; Day 4; 2-4 g of stool and 11 mL of urine.

Clinical investigations: Zambia

Investigations will be carried out over a four-day period (shown in Figure 2).

Day 1

This will include the final consent interview, administration of a questionnaire by the study nurse, clinical examination, and anthropometry (including weight, height, impedance, and deuterium approaches to body composition). Urine and stool collection devices will also be given.

Day 2

Participants will bring urine and stool samples with them. Before administration of the solutions, a saliva sample, 9 mL of blood will be collected in lithium heparin, 2 mL in EDTA and 4.5 mL in citrated blood collection tubes, respectively, by venepuncture. A drink (prepared as shown in Table 3) of $^{2}H_2O$ (deuterated water, 1 g $^{2}H_2O$ per kg body weight) containing ^{13}C-spirulina protein (1.25 mg kg^{-1}) and ($^{2}H_8$)-Phe (0.035 mg kg^{-1} in 200 mL clean water) will then be given at, or close to, 08:00 followed by breath sample collection using a straw and Exetainer tube (Labco, UK) every 20 mins for the first 1 h and every 30 mins for the remaining 3 h. A saliva sample will be collected at 180 mins, and a urine sample collection at 180 mins (3 h). A second drink of $^{2}H_2O$ (1 g $^{2}H_2O$ per kg body weight) will be given at 180 mins (3 h). During these procedures, a multi-pass 24-hour dietary recall will be administered by the study nurse. At the end of the procedures,

Table 3. Composition of test solutions.

Solution	Composition	Administration
Solution A	• Deuterated water (D_2O) (1 mL per kg body weight), • ^{13}C-spirulina protein (1.25 mg kg^{-1}), and • ($^{2}H_8$)-Phe (200 mg) in 200 mL water	Oral, given at the beginning of day 2 procedures
Solution B	• Deuterated water (D_2O) (1 mL per kg body weight)	Oral, given at the end of day 2 procedures
Solution C	• $^{2}H_5$-Phe (0.6 μmol kg^{-1}), • 5,5,5-$^{2}H_3$-Leu (1.2 μmol kg^{-1}) made up to 10 mL with sterile filtered normal saline	Day 3, given intravenously as slow bolus at beginning of procedures

Table 3. *Continued*

Solution	Composition	Administration
Solution D	• $^{2}H_5$-Phe (0.15 μmol kg^{-1}) • 5,5,5-$^{2}H_3$-leucine (0.3 μmol kg^{-1}) made up to 20 mL in sterile filtered normal saline	Day 3, given intravenously as continuous infusion over 3 hours
Solution E	• $^{13}C_6$-Phe (0.6 μmol kg^{-1}), • $^{13}C_6$-Leu (1.2 μmol kg^{-1}), • 5 g lactulose, • 1 g rhamnose, • 0.5 g D-xylose, • 0.2 g 3-O-methyl D-glucose, • 200 mg sodium fluorescein, • 1.667 mg L-arginine-(guanidineimino-$^{15}N_2$) HCl, • 1.0 g glycyl sarcosine in 200 mL water	Day 3, given orally as bolus at beginning of procedures
Solution F	• $^{13}C_6$-Phe (0.15 μmol kg^{-1}) • $^{13}C_6$-Leu (0.3 μmol kg^{-1}) made up to 600 mL in clean water	Day 3, given orally as sips every 20 mins

the participant will be asked to fast after midnight and given stool and urine collection devices in readiness for day 3 procedures.

Day 3

Following an overnight fast, an intravenous cannula will be introduced into both arms and 5 mL of blood will be collected into a lithium heparin tube via cannula. The fingertip probe will be clamped gently onto a fingertip, and continuous fluorescence recordings will begin. An intravenous bolus of $^{2}H_5$-phenylalanine (0.6 mmol kg^{-1}) and 5,5,5-$^{2}H_3$-leucine (1.2 mmol kg^{-1}) in 10 mL normal saline filtered through a 0.2 mm syringe filter (solution C) will be given over 2 mins, and an oral bolus of $^{13}C_6$-leucine (1.2 mmol kg^{-1}), $^{13}C_6$-phenylalanine (0.6 mmol kg^{-1}), 5 g lactulose, 1 g rhamnose, 0.5 g D-xylose, 0.2 g 3-O methyl-D-glucose, 200 mg fluorescein, 1.667 mg L-arginine-(guanidineimino-$^{15}N_2$) HCl and 1.0 g glycyl sarcosine (solution E) given. A continuous intravenous infusion of $^{2}H_5$-phenylalanine (0.15 mmol kg^{-1}) and $^{2}H_3$-leucine (0.3 mmol kg^{-1}) (solution D) will be commenced, at 5 mL h^{-1}, and sips of $^{13}C_6$-phenylalanine (0.15 mmol kg^{-1}) and $^{13}C_6$-leucine (0.3 mmol kg^{-1}) (solution F) administered as sips every 20 mins (prepared as shown in Table 3). Breath samples will be collected every 20 mins for the first 1 h and every 30 mins for the remaining 3 h. Blood samples will be collected into lithium heparin collection tubes via the intravenous cannula: 2 mL every 20 mins for the first 1 h and every 30 mins for the remaining 3 h, and 5mL for the last sample. Urine will be collected up to 180 mins (3 h) while saliva collected every 60 mins for 3 h. After 240 mins (4 h) the participant will be transferred to the endoscopy room where upper gastrointestinal endoscopy will be performed using doses of sedation with midazolam and pethidine selected by the endoscopist. Oximetry will be used throughout the procedure. During endoscopy, up to 10 mL of duodenal aspirate will be collected with clean tubing. Using the same tubing, 20 mL 0.9% of sterile saline will be used to collect duodenal lavage. A brushing will be collected using an endoscopic cytology brush, and then the brush will be cut off into 1000 μL PBS. For histology, 3 biopsies will be collected into normal saline using 2.8 mm biopsy forceps, orientated on cellulose acetate paper (Sartorius, Gottingen, Germany) in the endoscopy room and placed in formalin thereafter. An additional 5 biopsies will be collected into 3 cryovials (2+2+1). All samples collected during endoscopy, except for the 3 histology biopsies, will be snap-frozen in liquid nitrogen immediately after collection. At the end of day 3, the participant will be given urine and stool collection devices for the next day.

Day 4

Only stool and urine samples will be collected.

Colonoscopy: Zimbabwe

An intravenous injection of a single dose of 600 mg benzylpenicillin will be given in patients presenting for colonoscopy, 10 – 15 minutes before the procedure. Luminal fluid from the right side of the colon will be aspirated between 20 and 40 mins after benzylpenicillin administration. This fluid will be centrifuged, stored, and shipped to Imperial College where the assays for the presence of benzylpenicillin, which will indicate reverse permeation, will be performed. This sub-study is directed at understanding whether the degree of barrier loss in environmental enteropathy is sufficient to permit leakage of antibiotics into the right colon. Penicillin was selected as it is a narrow-spectrum antibiotic which would

not be expected to cause significant microbiome disruption in these healthy volunteers, which might be an ethical concern. A recent study[37] showed that antibiotic secretion into bile can modulate the microbiota during cardiac surgery, but this is a situation where gut hypoperfusion is well known to drive barrier failure. Such effects have not, to our knowledge, been shown in EE.

Sample handling

Samples for safety analysis (differential blood count using the Sysmex XP300™ automated haematology analyser (Sysmex UK Ltd., Milton Keynes, UK) and prothrombin time using the HumaClot Junior (HUMAN Diagnostics Worldwide, Wiesbaden, Germany)) will be processed immediately; excess samples will be retained as backup, but destroyed once the study is complete. Samples for research assays (Table 4) will be analysed immediately or stored at appropriate temperatures (usually -80°C). Blood samples from Day 2 (9 mL) will be centrifuged (2,300 *g* for 5 minutes

Table 4. Summary of laboratory analytical methods.

Sample Type	Assays	Methods	Study participants	Time-points
Stool	Microbiome sequencing	Illumina HiSeq, MinION	All	Baseline
	Barrier integrity and function	Epithelial cell co-cultures		Baseline or Day 2
	Biomarkers	ELISA		Baseline
	Immune signalling assays	Reporter cell line co-culture		Baseline or Day 2
	Dynamic proteomics	LC-MS/MS		Day 3, Day 4
	Metabolic phenotyping	NMR spectroscopy		Day 2, Day 3
Urine	Metabolic phenotyping	NMR spectroscopy	All	Baseline
	Targeted isotope analysis	LC-MS/MS		Day 1, Day 2
Saliva	Body Composition	FTIR	All	Day 1, Day 2
Biopsies	Histology and morphometry	Microscopy	All	Endoscopy samples
	Enzymes and transporters	Immunofluorescence		Endoscopy samples
	Pattern recognition receptor expression	Immunofluorescence, Immunohistochemistry		Endoscopy samples
	Dynamic proteomics	LC-MS/MS		Day 3 samples
Duodenal aspirates	Microbiome sequencing	Illumina HiSeq, MinION	All	Endoscopy samples
	Metabolic phenotyping	NMR spectroscopy		Endoscopy samples
	Dynamic proteomics	LC-MS/MS		Endoscopy samples
	Amino acid uptake in microbiota	LC-MS/MS		Endoscopy samples
	Stable isotope measurement	LC-MS/MS		Endoscopy samples
Plasma	Stable isotope measurement	LC-MS/MS	All	Multiple measurements
	Biomarkers	ELISA, Luminex		Baseline or Day 3
	Barrier integrity and function	Epithelial cell co-cultures		Baseline or Day 3
	Immune function assays	Reporter cell line co-culture		Baseline or Day 3
	Metabolic phenotyping	NMR spectroscopy		Day 2, Day 3
Buffy Coats	Innate and adaptive immune cells	Flow cytometry	All	Baseline

Table 4. *Continued*

Sample Type	Assays	Methods	Study participants	Time-points
Whole blood	Haematology	Differential cell counts, coagulation	All	Baseline or Day 3
Breath tests	Stable isotope measurement	Infra-red isotope analyser	All	Multiple measurements
Colonic aspirates	Antibiotic assays	LC-MS, HPLC	Aim 3 only	Single aspirates per procedure

ELISA - Enzyme Linked Immunosorbent Assay; LC-MS/MS - liquid chromatography tandem mass spectrometry.

at 4°C) to separate plasma from buffy coat cells (enriched for immune cells) and red blood cells; plasma will be aliquoted and stored at -80°C and buffy coat cells will be treated to lyse red blood cells, fixed, washed, re-suspended in cell preservation media, and cryopreserved gradually to -80°C using cell freezing containers to maintain cell integrity. On day 3, blood collected at different time intervals in lithium heparin (Table 4) will be centrifuged under the same conditions to separate plasma which will be aliquoted and stored at -80°C. Stool samples collected on days 2, 3 and 4 will be aliquoted and stored at -80°C. All saliva samples collected on days 2 and 3, all urine and stool samples collected on days 2, 3, and 4 will also be aliquoted and stored at -80°C. All breath samples that will be collected on days 2 and 3 will be stored at room temperature until analysis. All cryo-stored samples will be retained until batch analyses at either TROPGAN or may be shipped to the relevant external laboratories for analyses (shown in Table 4). For sample types being shipped for analyses at external laboratories, an aliquot of each will be retained at TROPGAN wherever possible; these aliquots will act as a backup in case of analytical failures, or storage failures during transport to another laboratory.

Clinical assessments

Permeability analysis

The permeability will be measured by the appearance in the blood of fluorescein ingested orally and through use of a multi-agent urinary recovery assay. Fluorescein will be purchased from Sigma-Aldrich (46960-100G-F) and has a similar molecular size to lactulose (molecular weight of fluorescein = 332 g/mol, molecular weight of lactulose = 342 g/mol) and similar molecular radius (lactulose, 0.42 nm; fluorescein 0.5 nm). We have previously demonstrated that the rate and degree of permeation of fluorescein vary under different permeability conditions[38] and in patients with intestinal disorders.[39] Importantly, following permeation into the blood, fluorescein fluorescence is quantifiable using a fingertip probe similar to a pulse oximeter probe.[40] Hence, transcutaneous measurement (performed using a fingertip probe) of fluorescence from orally ingested fluorescein will be used to provide rapid, non-invasive assessment of permeability.

To complement these novel, non-invasive, fluorescent measurements, we will also assess permeability using the urinary recovery of five orally ingested sugar and dipeptide molecules (lactulose, rhamnose, D-xylose, 3-O methyl-D-glucose and glycyl sarcosine). Urinary recovery of these molecules will be quantified using mass spectrometry thereby permitting assessment of more traditional permeability measures (e.g. urinary lactulose recovery and lactulose: rhamnose ratio) as well as quantification of the permeation of multiple agents with different molecular weights. Together, these two approaches allow a comprehensive, minimally invasive assessment of intestinal permeability.

Laboratory assessments

Stable isotopes analysis

The ***non-radioactive*** stable isotope labelled amino acids are used to trace the digestion and absorption of protein (day 2) and bidirectional transmucosal amino acid flux (day 3). The labelling strategy is chosen to minimise isotopic crosstalk in the analysis and maximise the metabolic pools that can be sampled ethically. These include breath ($^{13}CO_2$), plasma (^{13}C / ^{2}H amino acids), intestinal aspirates (^{13}C / ^{2}H amino acids), and urine (^{13}C / ^{2}H amino acids). Breath samples will be analysed using a Thermo Scientific™ Delta Ray™ Isotope Ratio Infrared Spectrometer (Thermo Fisher Scientific, Bremen, Germany) in the TROPGAN laboratory (breath $^{13}CO_2$) and ^{13}C & ^{2}H labelled amino acids analysed by a Agilent 1290 Infinity LC coupled to a 6560 triple quadrupole mass spectrometer (Agilent Technologies Inc., Cheadle, UK) in SUERC ($^{13}C/^{2}H$ amino acid isotopologues).

Metabolic phenotyping

Urine, stool, plasma, and duodenal aspirates will be analysed by Nuclear Magnetic Resonance spectroscopy (NMR). High-throughput ^{1}H NMR spectra will be acquired using a 600MHz Bruker Avance III™ HD NMR spectrometer equipped with a 5 mm BBI Z-gradient probe, high-order shims, and automated tuning and matching (Bruker Biospin, Rheinstetten, Germany). Samples will be analysed in automation using standard pulse sequences with water suppression.

For urine, stool, and duodenal aspirates, two experiments will be acquired: (1D) ^{1}H NMR (noesygppr1d, standard Bruker pulse program), and (2D) ^{1}H−^{1}H *J*-resolved (*J*-Res) experiment (jresgpprqf). (1D) ^{1}H CPMG (Carr–Purcell–Meiboom–Gill, cpmgpr1d) will also be acquired for plasma only. Each 1D spectrum will be automatically phased and baseline corrected, digitized, and imported into MATLAB for preprocessing and statistical modelling.

Microbiome analysis

Stool samples, gastric and duodenal aspirates and intestinal biopsies will undergo nucleic acid extraction, library construction and whole metagenome shotgun sequencing at Imperial College London to identify taxonomic and functional composition of multi-site gastrointestinal microbiomes comparing standard short-read sequencing approaches (Illumina HiSeq™ (Illumina, San Diego, CA, USA)) with novel long-read approaches (MinION (Oxford Nanopore Technologies Ltd., Oxford, UK) and PacBio Sequel® (Pacific Biosciences Inc., Menlo Park, CA, USA)). An additional qPCR step will be performed to quantify the absolute abundance of bacteria by determining the total number of 16S rRNA gene copies. These results will also be compared across sequencing platforms. These sequencing efforts will be duplicated in parallel work in Lusaka to determine the ease of application to an African laboratory. Additional characterisation of microbial uptake of amino acids and conversion of L to D amino acids will also be carried out. Raw sequencing data will be analysed via established bioinformatics pipelines including DADA2 (for 16S rRNA genes), EPI2ME and MetaPhlAn3 (compositional), HUMAnN3 (functional) as with other data, these results will be compared with duodenal biopsy histology, lactulose permeation data, and biomarkers.

To evaluate microbial uptake of amino acids, the ratio of ^{13}C & ^{2}H labelled amino acids in bacterial cells derived from duodenal aspirates will be measured using mass spectrometry. This ratio will serve as an indicator for microbial usage of amino acids pathological leaked into the gut lumen from systemic circulation. In addition, a quantitative ultra-high pressure liquid chromatography mass spectrometry (UPLC–MS/MS) assay will be used to measure the concentrations of L- and D-amino acids in duodenal aspirates. The concentration of labelled D-amino acids will serve as an indicator of microbial consumption and usage of total amino acids content in the duodenum.

Histology and immunohistochemistry analysis

Formalin fixed biopsies will then be embedded in wax. Sections (4 μm) will be stained using haematoxylin and eosin (H&E) and scanned on an Olympus VS-120 scanning microscope. Morphometry will be performed to generate measurements of villus height and crypt depth as has been our standard practice for many years and on which several publications are based.[8,41,42] Recently a histological scoring system for EE has been developed in which we were involved, and this will be applied to generate scores for villus architectural change, Brunner's gland penetration, Paneth cell depletion, Goblet cell depletion, intra-epithelial lymphocytosis, and epithelial abnormalities.[43] The expression of PRRs will be assessed in separate histological sections of antibody-labelled duodenal biopsies via immunofluorescence microscopy.

EE biomarker analysis

To characterise the sample sets fully, giving context to observed changes in the composition of the microbiota, biomarkers of EE (shown in Table 5) will be quantified in plasma and stool aliquots via ELISA.

Cell culture analysis

To evaluate the capacity of circulating microbial products in plasma and microbial PAMP content in stool to activate innate immune signalling (immunogenicity; an indicator of microbial translocation), plasma and faecal water aliquots

Table 5. Plasma and faecal biomarkers of EE.

Marker	Sample type	Measure
1. Endotoxin-core antibody (EndoCab) IgG, IgM, and IgA	Plasma	Microbial translocation
2. Soluble (s)CD14	Plasma	Microbial translocation
3. LPS binding protein (LBP)	Plasma	Microbial translocation
4. sCD163	Plasma	Marker of systemic inflammation
6. C-reactive protein (CRP)	Plasma	Microbial translocation
7. Acid glycoprotein (AGP)	Plasma	Marker of systemic inflammation
8. Myeloperoxidase (MPO)	Stool	Intestinal Inflammation
9. Intestinal fatty acid binding protein (iFABP)	Plasma	Marker of epithelial damage

from each participant will be co-cultured with THP-1 Dual reporter cells for NFkB and IRF3 (monocytic cell line; obtained from Invivogen), which provide a global indicator of PAMPs that can trigger immune cell activation via Toll like receptors, a major pathogen recognition receptors of the innate immune system. We will then screen samples for more specific PRR signalling activity using PRR-specific HEK293 reporter cells (obtained from Invivogen), focusing initially on TLR4 (the receptor for bacterial LPS/endotoxin) and TLR5 (receptor for bacterial flagellin), but expanding to and other PRRs if indicated by microbial metagenomics/microbiome analyses. For PRR-specific reporter HEK293 assays, we will conduct parallel cultures with HEK293 lacking the receptor of interest (HEK293-null cells) as a negative control.

To evaluate the functional effect of PAMPs and immune mediators present in plasma and stool on gut epithelial barrier integrity (transepithelial resistance, TEER), permeability, PRR and tight junction expression, and epithelial cell activation, we will add aliquots of plasma/faecal water separately to the basal/apical side of *in vitro* models of the gut epithelial barrier. For the model epithelium we will use Caco-2 (cell line derived from the human colonic epithelium; obtained from American Type Culture Collection, ATCC, cultured in transwell inserts so that they have an apical side (equivalent to the epithelial side facing the gut lumen) and a basal side (equivalent to the epithelial side facing the lamina propria),[44] to recapitulate the polarity of the gut epithelium. PRR-specific inhibitors or antagonists and/or antibodies and filtered faecal water will be used in parallel experiments to attempt to disaggregate the effects of the specific PAMPs present in the stool samples. We will use these data to explore the relationship between immunogenicity (from THP-1 and HEK293 reporter assays) and functional impact of the sample on the epithelial barrier *in vitro* (from Caco-2 transwell models). We hypothesise that samples from participants with more circulating immunogenic PAMPs will be associated with greater loss of barrier function *in vitro*.

Antibiotic analysis

In the 20 patients attending for routine colonoscopy (for diagnostic purposes) in Harare, using luminal fluid, antibiotic analysis will be carried out by NMR or SUERC by LC-MS.

Statistical analysis plan

With the complexity of the data set from the techniques described above, the biostatistical framework will be generated in a data-dependent manner. Data from novel tools will be integrated with mucosal morphometry, lactulose permeation, and biomarkers in blood and stool. All data will be tested for normality using a Shapiro-Wilk test and transformed accordingly (normalisation, scaling, etc.). Both parametric and non-parametric tests will be used and both univariate and multivariate analysis will be performed on all datasets generated, as appropriate. For univariate tests, to control for type-I errors, multiple testing will be corrected for by applying the Hommel correction (for Family-Wise Error Rate) or Benjamini-Hochberg (for False Discovery Rate) as appropriate for mildly (positively) correlated variables, or Benjamini-Yekutieli or Storey-Tibshirani (both for False Discovery Rate) methods for highly (positively and inversely) correlated variables. For multivariate tests, data will first be split into training (modelling) and test (evaluation) sets prior to any procedures on the data such as centering, scaling, or other transformations. The predictive classification models will be calculated only on the training portions and evaluated (accuracy and/or F1-score) on the test portions, with hyperparameter tuning using cross-validation on the training data. When small sample sizes do not allow setting aside a completely independent test set, repeated cross-validation will be used to ensure the robustness of the models by calculating a multitude of models (e.g. 1,000 individual models). The evaluation of performance is estimated across all models on the portion of the data that was set aside (test) in each partition. Where algorithms use random initializations of parameters, for each analysis the starting random number seed is saved to allow replication. All data generated will be compared with all duodenal morphometry data which is the most direct measure of EE severity; these comparisons will allow for validation of new real-team assessment tools and established laboratory techniques for sensitivity to differences in EE severity within the cohort.

Public involvement

Dissemination will include the participants and their families, together with Community Advisory Boards, the University of Zambia School of Medicine, and the University of Zimbabwe Faculty of Medicine and Health Sciences. We will also disseminate results to the Zambian National Health Research Authority and the Medical Research Council of Zimbabwe, and the relevant ethics committees in all the African partner countries, both in written form and by taking opportunities of local research dissemination meetings. This is established practice in all the research groups who will be collaborating on the work proposed. All publications will be open access.

Data management

Processed data and extracted variables from all sites will be stored on OneDrive (hosted at Imperial College London) with secure access for all researchers involved in the study. One copy of the raw data will be stored at the institution that generated it, and another copy will be stored in the same cloud storage as the processed data. All databases will be

archived with related questionnaires and any related dictionaries in soft copy on the same drive(s). All data records and materials will be archived appropriately (in accordance with ICH GCP Guidelines) and retained for 5 years according to the Sponsor's, QMUL's, requirements. Data with identifiers will not be stored, for example, personal identifiers on the front sheet of all clinical trial records will be detached prior to archiving and will not be entered on any database. These identifiers will be kept as hard-copy files in locked cabinets in study offices.

Confidentiality

Data security and confidentiality will be in line with General Data Protection Regulations, HRA Guidance, International Conference on Harmonisation, Guideline for Good Clinical Practice E6, Revision 2, and Queen Mary University of London Data Protection principles and policies. Only authorised study personnel will have access to study files and data. Any data that will be shared with other workers in the field will be anonymised. Identifiable personal data will not be used during analysis or when publishing results. The information that is collected will be treated confidentially. The information entered on separate forms only contains a participant number and the initials and only the data considered important for the study. Research staff are aware of confidentiality of personal data and meeting the requirements of the General Data Protection Regulation (GDPR). Personal information will only be used to contact the patient via letter or telephone after informed consent has been obtained. The only link between personalised data and anonymised data will be a register kept in hard copy in a locked cupboard by the compliance manager. Data will be maintained at an appropriate vigilance (RAID 1) level following current institutional advice. Data access will be restricted to only authorised personnel with individual passwords.

Ethical considerations

The protocol has been approved by the National Heath Research Agency (REF NHREB00001/02/2022, dated 2nd February 2022) and the University of Zambia Biomedical Research Ethics Committee (reference number 2291-2021, dated 20th December 2021). The Zimbabwean studies have been approved by the Medical Research Council of Zimbabwe (MRCZ/A/3065, dated 31 July 2023). The work will be carried out in full concordance with the principles of the Declaration of Helsinki, and all participants will give written consent. Queen Mary Ethics of Research Committee issued a letter of no objection (QMERC22.060, dated 22nd February 2020).

Discussion

Our project will take a holistic approach to understanding the impact of EE on gut functional capacity across the whole gastrointestinal tract, which means developing new tools for assessing multiple domains of physiology. This necessitates an assessment of the structure of the intestine (e.g., reduced villus height and therefore surface area, barrier function), digestive and absorptive capacity (e.g., enzyme activity for protein, fat and carbohydrate digestion, transporter expression for absorption), epithelial and immune responses to pathogens, PAMPs, and the microbiota, and the health of the gut microbiota and its metabolic activity. Through this study, we will be able to identify and select the combination of functional measures that are most sensitive to variations in gut physiology according to EE severity in this adult cohort. We will also be able to optimise protocols for communities, such as Misisi, where EE is the predominant gut phenotype. Such measurements will ultimately enable the design of novel therapeutic feeds which actively promote improved gut functional capacity, providing a novel approach to accelerate sustained rehabilitation, reduce mortality, and feed the superorganism (i.e., the host and microbiome), thereby improving growth in malnourished children.[21,28] The urgent problem which we aim to address in this proposal is the dearth of investigative tools available to measure these pathophysiological domains. These tools will be essential for the effective evaluation of new therapeutic feeds to be trialled in SAM and, critically, the selection of those that promote the healing of underlying defects as well as weight gain. Their application to childhood malnutrition will be highly original especially when linked to a clinical trial addressing patient-centred outcomes. Our research group will go on to conduct longitudinal studies and/or clinical trials to evaluate further the tools we will develop. This will provide a deeper understanding of the changing physiology and will generate essential data to support the findings of the trial. In the scenario where no benefit of a novel therapeutic intervention or feed is shown, our novel tools may provide essential for future refinements of the feeds to target specific domains of gut functional capacity and/or enhance effects on pathways showing positive sub-clinical modifications.

Stable (non-radioactive) isotope technology has much to offer in understanding intestinal physiology. Although some organisms can preferentially handle certain stable isotopes (e.g. maize can enrich for ^{13}C), in general terms these isotopes behave biochemically as faithful reporters of the molecule which is labelled. Such non-invasive approaches can be used in place of perfusion studies to study absorption, though interpretation of such data requires nuanced understanding of physiology alongside careful use of sophisticated spectrometry tools. There are many examples of such use to study micronutrient metabolism[45–47] and macronutrient metabolism.[48–51] Fluorescein use for measuring intestinal permeability has already been explored,[30] though here we propose to explore it as a tool in LMICs. There is considerable uncertainty, surprisingly, about the nature of the immunological defect in malnourished children and adults,[52] and our proposed work

will contribute to a better understanding of innate immunity in malnutrition. While there have been multiple studies of the microbiome in Africa, and some work on the metabolome, much more needs to be done. A unique feature of this proposed work is the intended application of these tools simultaneously to studies in vulnerable populations, ultimately in clinical trials in malnourished children.

In more general terms, these tools may find applications in patients with other enteropathies such as coeliac disease, or enteropathies consequent on cancer therapy. Objective measures of intestinal dysfunction may also help manage patients with intestinal failure. We believe that the strategy of using stable isotopes, fluorescence probes, and new sequencing tools to measure intestinal digestion and absorption, barrier function, and the function of the microbiome, will remain of great interest for years to come. Additionally, to date, there are few, if any, studies in adults or malnourished children with EE that have examined the expression of PRRs in the duodenal epithelium or measured the burden of PAMPs in plasma and stool, particularly in relation to their bidirectional impact on the epithelium. Therefore, the significance of this study lies not only in its potential to develop diagnostic tools but also in its ability to investigate various components of the immune system, microbiome, and metabolome within the same individuals.

Data availability

No data are associated with this article.

Anonymised data (accounting for potential small number disclosure) associated with each publication will be deposited in appropriate repositories and linked to using DOIs: European Nucleotide Archive (ENA) database for metagenomic sequencing data, MetaboLights for metabolomics data, and Zenodo and Mendeley Data for other data types.

Extended data

Mendeley Data: GI Tools SPIRIT Checklist, http://doi.org/10.17632/2536bhwx7g.1.[53]

The project contains the following underlying data:

- GITools_SPIRIT_Fillable-checklist-July2024.pdf

The remaining data in this article consists of bibliographic references, which are included in the References section.

References

1. Thompson AJ, Bourke CD, Robertson RC, *et al.*: **Understanding the role of the gut in undernutrition: what can technology tell us?** *Gut.* 2021 Jun 8; **70**(8): 1580–1594.
PubMed Abstract | Publisher Full Text | Free Full Text

2. Kosek M, Guerrant RL, Kang G, *et al.*: **Assessment of environmental enteropathy in the MAL-ED cohort study: theoretical and analytic framework.** *Clin Infect Dis.* 2014 Nov 1; **59** Suppl 4(Suppl 4): S239-47.
PubMed Abstract | Publisher Full Text | Free Full Text

3. Nabukeera-Barungi N, Grenov B, Lanyero B, *et al.*: **Predictors of mortality among hospitalized children with severe acute malnutrition: a prospective study from Uganda.** *Pediatr Res.* 2018 Jul; **84**(1): 92–98.
PubMed Abstract | Publisher Full Text

4. Talbert A, Thuo N, Karisa J, *et al.*: **Diarrhoea complicating severe acute malnutrition in Kenyan children: a prospective descriptive study of risk factors and outcome.** *PLoS One.* 2012; **7**(6): e38321.
PubMed Abstract | Publisher Full Text | Free Full Text

5. Bwakura-Dangarembizi M, Dumbura C, Amadi B, *et al.*: **Recovery of children following hospitalisation for complicated severe acute malnutrition.** *Matern Child Nutr.* 2022 Apr; **18**(2): e13302.
PubMed Abstract | Publisher Full Text | Free Full Text

6. Kerac M, Bunn J, Chagaluka G, *et al.*: **Follow-up of post-discharge growth and mortality after treatment for severe acute malnutrition (FuSAM study): a prospective cohort study.** *PLoS One.* 2014 Jun 3; **9**(6): e96030.
PubMed Abstract | Publisher Full Text | Free Full Text

7. Sow SO, Muhsen K, Nasrin D, *et al.*: **The Burden of Cryptosporidium Diarrheal Disease among Children < 24 Months of Age in Moderate/High Mortality Regions of Sub-Saharan Africa and South Asia, Utilizing Data from the Global Enteric Multicenter Study (GEMS).** *PLoS Negl Trop Dis.* 2016 May 24; **10**(5): e0004729.
PubMed Abstract | Publisher Full Text | Free Full Text

8. Amadi B, Zyambo K, Chandwe K, *et al.*: **Adaptation of the small intestine to microbial enteropathogens in Zambian children with stunting.** *Nat Microbiol.* 2021 Apr; **6**(4): 445–454.
PubMed Abstract | Publisher Full Text | Free Full Text

9. Kummerlowe C, Mwakamui S, Hughes TK, *et al.*: **Single-cell profiling of environmental enteropathy reveals signatures of epithelial remodeling and immune activation.** *Sci Transl Med.* 2022 Aug 31; **14**(660): eabi8633.
PubMed Abstract | Publisher Full Text | Free Full Text

10. Amadi B, Besa E, Zyambo K, *et al.*: **Impaired Barrier Function and Autoantibody Generation in Malnutrition Enteropathy in Zambia.** *EBioMedicine.* 2017 Aug; **22**: 191–199.
PubMed Abstract | Publisher Full Text | Free Full Text

11. Jones KD, Berkley JA: **Severe acute malnutrition and infection.** *Paediatr Int Child Health.* 2014 Dec; **34 Suppl 1**(Suppl 1): S1–S29.
PubMed Abstract | Publisher Full Text | Free Full Text

12. Sturgeon JP, Mufukari W, Tome J, *et al.*: **Risk factors for inpatient mortality among children with severe acute malnutrition in**

Zimbabwe and Zambia. *Eur J Clin Nutr.* 2023 Sep; **77**(9): 895–904.
PubMed Abstract | Publisher Full Text | Free Full Text

13. Sturgeon JP, Tome J, Dumbura C, *et al.*: **Inflammation and epithelial repair predict mortality, hospital readmission, and growth recovery in complicated severe acute malnutrition.** *Sci Transl Med.* 2024 Feb 28; **16**(736): eadh0673.
PubMed Abstract | Publisher Full Text | Free Full Text

14. Uddin MI, Hossain M, Islam S, *et al.*: **An assessment of potential biomarkers of environment enteropathy and its association with age and microbial infections among children in Bangladesh.** *PLoS One.* 2021 Apr 22; **16**(4): e0250446.
PubMed Abstract | Publisher Full Text | Free Full Text

15. Kosek M, Haque R, Lima A, *et al.*: **Fecal markers of intestinal inflammation and permeability associated with the subsequent acquisition of linear growth deficits in infants.** *Am J Trop Med Hyg.* 2013 Feb; **88**(2): 390–396.
PubMed Abstract | Publisher Full Text | Free Full Text

16. Harper KM, Mutasa M, Prendergast AJ, *et al.*: **Environmental enteric dysfunction pathways and child stunting: A systematic review.** *PLoS Negl Trop Dis.* 2018 Jan 19; **12**(1): e0006205.
PubMed Abstract | Publisher Full Text | Free Full Text

17. Andriamanantena Z, Randrianarisaona F, Rakotondrainipiana M, *et al.*: **Changes in Systemic Regulatory T Cells, Effector T Cells, and Monocyte Populations Associated With Early-Life Stunting.** *Front Immunol.* 2022 Jun 2; **13**: 864084.
PubMed Abstract | Publisher Full Text | Free Full Text

18. Phiri TN, Mutasa K, Rukobo S, *et al.*: **Severe acute malnutrition promotes bacterial binding over proinflammatory cytokine secretion by circulating innate immune cells.** *Sci Adv.* 2023 Nov 3; **9**(44): eadh2284.
PubMed Abstract | Publisher Full Text | Free Full Text

19. Wen B, Njunge JM, Bourdon C, *et al.*: **Systemic inflammation and metabolic disturbances underlie inpatient mortality among ill children with severe malnutrition.** *Sci Adv.* 2022 Feb 18; **8**(7): eabj6779.
PubMed Abstract | Publisher Full Text | Free Full Text

20. Mutasa K, Tome J, Rukobo S, *et al.*: **Stunting Status and Exposure to Infection and Inflammation in Early Life Shape Antibacterial Immune Cell Function Among Zimbabwean Children.** *Front Immunol.* 2022 Jun 13; **13**: 899296.
Publisher Full Text

21. Subramanian S, Huq S, Yatsunenko T, *et al.*: **Persistent gut microbiota immaturity in malnourished Bangladeshi children.** *Nature.* 2014 Jun 19; **510**(7505): 417–421.
PubMed Abstract | Publisher Full Text | Free Full Text

22. Donowitz JR, Haque R., Kirkpatrick BD, *et al.*: **Small Intestine Bacterial Overgrowth and Environmental Enteropathy in Bangladeshi Children.** *mBio.* 2016 Jan 12; **7**(1): e02102-15.
PubMed Abstract | Publisher Full Text | Free Full Text

23. Vonaesch P, Morien E, Andrianonimiadana L, *et al.*: **Stunted childhood growth is associated with decompartmentalization of the gastrointestinal tract and overgrowth of oropharyngeal taxa.** *Proc Natl Acad Sci U S A.* 2018 Sep 4; **115**(36): E8489–E8498.
PubMed Abstract | Publisher Full Text | Free Full Text

24. Iqbal NT, Chen RY, Griffin NW, *et al.*: **A shared group of bacterial taxa in the duodenal microbiota of undernourished Pakistani children with environmental enteric dysfunction.** *mSphere.* 2024 Jun 25; **9**(6): e0019624.
PubMed Abstract | Publisher Full Text | Free Full Text

25. Chen RY, Kung VL, Das S, *et al.*: **Duodenal Microbiota in Stunted Undernourished Children with Enteropathy.** *N Engl J Med.* 2020 Jul 23; **383**(4): 321–333.
PubMed Abstract | Publisher Full Text | Free Full Text

26. Owino V, Ahmed T, Freemark M, *et al.*: **Environmental Enteric Dysfunction and Growth Failure/Stunting in Global Child Health.** *Pediatrics.* 2016 Dec; **138**(6): e20160641.
PubMed Abstract | Publisher Full Text

27. Gehrig JL, Venkatesh S, Chang HW, *et al.*: **Effects of microbiota-directed foods in gnotobiotic animals and undernourished children.** *Science.* 2019 Jul 12; **365**(6449): eaau4732.
PubMed Abstract | Publisher Full Text | Free Full Text

28. Chen RY, Mostafa I, Hibberd MC, *et al.*: **A Microbiota-Directed Food Intervention for Undernourished Children.** *N Engl J Med.* 2021 Apr 22; **384**(16): 1517–1528.
PubMed Abstract | Publisher Full Text | Free Full Text

29. Barratt MJ, Nuzhat S, Ahsan K, *et al.*: **Bifidobacterium infantis treatment promotes weight gain in Bangladeshi infants with severe acute malnutrition.** *Sci Transl Med.* 2022 Apr 13; **14**(640): eabk1107.
PubMed Abstract | Publisher Full Text | Free Full Text

30. Dorshow RB, Johnson JR, Debreczeny MP, *et al.*: **Transdermal fluorescence detection of a dual fluorophore system for noninvasive point-of-care gastrointestinal permeability measurement.** *Biomed Opt Express.* 2019 Sep 13; **10**(10): 5103–5116.
PubMed Abstract | Publisher Full Text | Free Full Text

31. Kelly P, Besa E, Zyambo K, *et al.*: **Endomicroscopic and Transcriptomic Analysis of Impaired Barrier Function and Malabsorption in Environmental Enteropathy.** *PLoS Negl Trop Dis.* 2016 Apr 6; **10**(4): e0004600.
PubMed Abstract | Publisher Full Text | Free Full Text

32. Kelly P, Menzies I, Crane R, *et al.*: **Responses of small intestinal architecture and function over time to environmental factors in a tropical population.** *Am J Trop Med Hyg.* 2004 Apr; **70**(4): 412–419.
PubMed Abstract | Publisher Full Text

33. Ritchie BK, Brewster DR, Davidson GP, *et al.*: **13C-sucrose breath test: novel use of a noninvasive biomarker of environmental gut health.** *Pediatrics.* 2009 Aug; **124**(2): 620–626.
PubMed Abstract | Publisher Full Text

34. Schillinger RJ, Mwakamui S, Mulenga C, *et al.*: **13C-sucrose breath test for the non-invasive assessment of environmental enteropathy in Zambian adults.** *Front Med (Lausanne).* 2022 Jul 29; **9**: 904339.
PubMed Abstract | Publisher Full Text | Free Full Text

35. Louis-Auguste J, Kelly P: **Tropical Enteropathies.** *Curr Gastroenterol Rep.* 2017 Jul; **19**(7): 29.
PubMed Abstract | Publisher Full Text | Free Full Text

36. Jobarteh ML, McCrory MA, Lo B, *et al.*: **Development and Validation of an Objective, Passive Dietary Assessment Method for Estimating Food and Nutrient Intake in Households in Low- and Middle-Income Countries: A Study Protocol.** *Curr Dev Nutr.* 2020 Feb 7; **4**(2): nzaa020.
PubMed Abstract | Publisher Full Text | Free Full Text

37. Xue L, Ding Y, Qin Q, *et al.*: **Assessment of the impact of intravenous antibiotics treatment on gut microbiota in patients: Clinical data from pre-and post-cardiac surgery.** *Front Cell Infect Microbiol.* 2023 Jan 19; **12**(12): 1043971.
PubMed Abstract | Publisher Full Text | Free Full Text

38. Gan J, Monfort SE, Avery J, *et al.*: **Non-invasive assessment of intestinal permeability in healthy volunteers using transcutaneous fluorescence spectroscopy.** *Methods Appl Fluoresc.* 2022 Oct 10; **10**(4): 044014.
PubMed Abstract | Publisher Full Text

39. Mbuki R, Chileya S, Thompson AJ, *et al.*: **Rapid testing of gut permeability using oral fluorescein and confocal laser endomicroscopy in Zambian adults.** *Trans R Soc Trop Med Hyg.* 2021 Oct 1; **115**(10): 1226–1228.
PubMed Abstract | Publisher Full Text

40. Sanchez EM, Avery J, Gan J, *et al.*: **Transcutaneous fluorescence spectroscopy: development and characterization of a compact, portable, and fiber-optic sensor.** *J Biomed Opt.* 2024 Feb; **29**(2): 027003.
PubMed Abstract | Publisher Full Text | Free Full Text

41. Mulenga C, Sviben S, Chandwe K, *et al.*: **Epithelial Abnormalities in the Small Intestine of Zambian Children With Stunting.** *Front Med (Lausanne).* 2022 Mar 16; **9**: 849677.
PubMed Abstract | Publisher Full Text | Free Full Text

42. Louis-Auguste J, Greenwald S, Simuyandi M, *et al.*: **High dose multiple micronutrient supplementation improves villous morphology in environmental enteropathy without HIV enteropathy: results from a double-blind randomised placebo-controlled trial in Zambian adults.** *BMC Gastroenterol.* 2014 Jan 15; **14**: 15.
PubMed Abstract | Publisher Full Text | Free Full Text

43. Liu TC, VanBuskirk K, Ali SA, *et al.*: **A novel histological index for evaluation of environmental enteric dysfunction identifies geographic-specific features of enteropathy among children with suboptimal growth.** *PLoS Negl Trop Dis.* 2020 Jan 13; **14**(1): e0007975.
PubMed Abstract | Publisher Full Text | Free Full Text

44. Bourke CD, Gough EK, Pimundu G, *et al.*: **Cotrimoxazole reduces systemic inflammation in HIV infection by altering the gut microbiome and immune activation.** *Sci Transl Med.* 2019 Apr 3; **11**(486): eaav0537.
PubMed Abstract | Publisher Full Text | Free Full Text

45. Stoffel NU, Zeder C, Zimmermann MB: **Assessing Human Iron Kinetics Using Stable Iron Isotopic Techniques.** *Clin Pharmacokinet.* 2024 Oct; **63**(10): 1389–1405.
PubMed Abstract | Publisher Full Text | Free Full Text

46. Kurpad AV, Pasanna RM, Hegde SG, *et al.*: **Bioavailability and daily requirement of vitamin B12 in adult humans: an observational study of its colonic absorption and daily excretion as measured by [13C]-cyanocobalamin kinetics.** *Am J Clin Nutr.* 2023 Dec; **118**(6): 1214–1223.
PubMed Abstract | Publisher Full Text

47. Ariff S, Krebs NF, Westcott JE, *et al.*: **Exchangeable Zinc Pool Size at Birth in Pakistani Small for Gestational Age and Appropriate for**

Gestational Age Infants Do Not Differ But Are Lower Than in US Infants. *J Pediatr Gastroenterol Nutr.* 2018 Mar; **66**(3): 496–500.
PubMed Abstract | Publisher Full Text | Free Full Text

48. Guillin FM, Gaudichon C, Guérin-Deremaux L, *et al.*: **Values for the Digestibility of Pea Protein Isolate or Casein Amino Acids Determined using the Dual Isotope Method Are Not Similar to Those Derived with the Standard Ileal Balance Method in Healthy Volunteers.** *J Nutr.* 2024 Feb; **154**(2): 516–525.
PubMed Abstract | Publisher Full Text

49. Shivakumar N, Huq S, Paredes-Olortegui M, *et al.*: **A cross-sectional study of associations between the 13C-sucrose breath test, the lactulose rhamnose assay, and growth in children at high risk of environmental enteropathy.** *Am J Clin Nutr.* 2024 Dec; **120**(6): 1354–1363.
PubMed Abstract | Publisher Full Text | Free Full Text

50. Dale HF, Hagen M, Deb C, *et al.*: **Diagnosing sucrase-isomaltase deficiency: a comparison of a 13C-sucrose breath test and a duodenal enzyme assay.** *Scand J Clin Lab Invest.* 2024 Jul; **84**(4): 268–272.
PubMed Abstract | Publisher Full Text

51. Cox J, Wilkinson DJ, Atherton PJ, *et al.*: **Dietary protein splanchnic uptake and digestibility via stable isotope tracers.** *Curr Opin Clin Nutr Metab Care.* 2024 Sep 1; **27**(5): 451–456.
PubMed Abstract | Publisher Full Text | Free Full Text

52. Rytter MJ, Kolte L, Briend A, *et al.*: **The immune system in children with malnutrition–a systematic review.** *PLoS One.* 2014 Aug 25; **9**(8): e105017.
PubMed Abstract | Publisher Full Text | Free Full Text

53. Weatherill J: **GI Tools SPIRIT Checklist.** *Mendeley Data.* **V1**.
Publisher Full Text

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Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 18 March 2025

https://doi.org/10.5256/f1000research.178271.r369467

✔ **Pascale Vonaesch**
University of Lausanne, Lausanne, Switzerland

The authors have clarified many of the unclear points. While the overview in the field is stills somehow limited, I do not have any further comments to make to this article. I wish the authors all the best for the actual study and look forward reading their results in the future.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: childhood undernutrition, environmental enteropathy, human microbiome, small intestinal microbiome, biomarker analysis, clinical microbiome research in Sub-Saharan Africa and South-East Asia

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 11 March 2025

https://doi.org/10.5256/f1000research.178271.r369466

✔ **Declan McCole**
University of California Riverside Division of Biomedical Sciences, Riverside, California, USA

The authors have addressed my prior concerns.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Intestinal permeability, epithelial tight junctions, epithelial transport, intestinal inflammation.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 14 November 2024

https://doi.org/10.5256/f1000research.169506.r317023

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Declan McCole
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The GI TOOLS project is a cross-sectional study that will recruit adults aged 18-65 years with environmental enteropathy (EE) in Lusaka, Zambia that aims to develop new tools for the measurement of gut functional that can be utilized in future clinical trials. The methodology of this project requires each participant to undergo an assessment of gut functional capacity using novel nearpoint-of-care tools and to provide multiple samples for detailed laboratory analyses. Novel techniques include stable isotope approaches to measuring digestion, absorption, and bidirectional transmucosal amino acid flux, a non-invasive fluorescence tool for real-time evaluation of gut permeability, and assessment of gut luminal access of intravenous antibiotics. Stool and duodenal microbiome sequencing, metabolome analysis of plasma and intestinal fluids, blood immune cell phenotyping, *in vitro* epithelial barrier models, and duodenal immunohistochemistry will also be used to explore EE in depth. These will be integrated with histology and mucosal morphometry using an established histological scoring system for EE, alongside stool and plasma biomarker analysis. The proposal builds on prior foundational work from this consortium that has tested several of the experimental approaches listed in the current proposal. An important feature of the proposal is that local dissemination of research data in Zambia and Zimbabwe will also be undertaken in written form and in presentations at local meetings. This will be an important means of engaging with local research and public health communities. Overall, this represents a very comprehensive set of approaches to functionally characterize the impact of EE on multiple aspects of gut physiology from permeability to digestion of nutrients. The study has high clinical relevance and if it meets its goals it could not only create a new paradigm for functional verification of EE, but also provide robust readouts that could be tested for efficacy of different dietary and therapeutic interventions to rescue subjects from the damaging effects of EE.

There are some concerns with the inclusion/exclusion criteria. The preference for inclusion of

subjects only from higher socioeconomic backgrounds in the Harare (Zimbabwe) study on i.v. antibiotic pharmaco-distribution requires some explanation and justification.

What considerations will be made if there is a high rate of avoidance of HIV testing?

What size (kDa and diameter) of fluorescein will be used as the permeability probe? This should be specifically stated and the source/catalog number of the actual fluorescein to be used should also be provided.

Since proteomic analysis of intestinal biopsies will be performed, it should be clarified if tight junction protein expression will be examined as altered expression could correlate with functional changes in permeability – with the caveat that this approach will not identify changes in localization of TJ proteins that occur without any change in expression levels.

In Aim 5, Benzylpenicillin is the iv antibiotic that will be used in this study to identify if i.v. administered antibiotics can access the gut lumen. It is unclear what the goal of this aspect of the study is meant to determine. Do the authors consider antibiotic administration to exacerbate symptoms of EE? With respect to the question of whether i.v. antibiotics can enter the gut lumen, this has already been demonstrated that antibiotics can pass into the gut lumen via biliary secretions, and alter bacterial populations (Xue et al, 2023. [Ref 1]). In addition, no explanation of why this particular antibiotic is being used has been included. It is hard to justify this particular aim (#5) as it will not generate any novel findings and could be considered an unnecessary human subjects intervention.

A limitation of the study is that samples will not be collected longitudinally to identify if readings are sustained or transient. However, longitudinal assessment is proposed in the Discussion section describing future studies.

Clarification of the “reporter” characteristics of HEK293, THP-1 cell lines should be provided. Will the group be using modified cells lines expressing actual reporter constructs (i.e. to quantify enzymatic activity) or are these cells just being used as ‘model’ cell lines. Given that changes in gut epithelial physiology are integral to the symptoms of EE, what is the rationale for choosing a kidney epithelial cell line (HEK293) as a model epithelium?

In vitro experiments assessing the effect of PAMPs in fecal samples on permeability of intestinal epithelial cell cultures, will likely exhibit responses to bacterial LPS and it is unclear how the authors will be able to screen for effects of other PAMPs (in feces) in this system with any degree of specificity.

References

1. Xue L, Ding Y, Qin Q, Liu L, et al.: Assessment of the impact of intravenous antibiotics treatment on gut microbiota in patients: Clinical data from pre-and post-cardiac surgery.*Front Cell Infect Microbiol*. 2022; **12**: 1043971 PubMed Abstract | Publisher Full Text

Is the rationale for, and objectives of, the study clearly described?

Yes

Is the study design appropriate for the research question?

Yes

Are sufficient details of the methods provided to allow replication by others?
No

Are the datasets clearly presented in a useable and accessible format?
Not applicable

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Intestinal permeability, epithelial tight junctions, epithelial transport, intestinal inflammation.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 13 Feb 2025

James Weatherill

We are grateful for affording us the opportunity to improve this manuscript.
Please find the following specific responses to your comments:

Comments:

- *There are some concerns with the inclusion/exclusion criteria. The preference for inclusion of subjects only from higher socioeconomic backgrounds in the Harare (Zimbabwe) study on i.v. antibiotic pharmaco-distribution requires some explanation and justification.*

Response: This point was also made by Reviewer 1 and has been addressed (specific point 2 above).

- *What considerations will be made if there is a high rate of avoidance of HIV testing?*

Response: We consider that HIV testing is required to allow interpretation of data and so those potential participants who decline HIV testing cannot be included. Recruitment will continue until the pre-specified sample size is reached.

- *What size (kDa and diameter) of fluorescein will be used as the permeability probe? This should be specifically stated and the source/catalogue number of the actual fluorescein to be used should also be provided.*

Response: We use fluorescein sodium as the non-invasive permeability probe, not fluorescein conjugated to other molecules (e.g. FITC-dextran, FITC-PEG, etc). Fluorescein sodium was purchased from Sigma-Aldrich (catalogue number: 46960-100G-F). Fluorescein sodium has a molecular weight of 376 Da. However, the sodium ions dissociate in solution to leave free fluorescein, which has a molecular weight of 332 Da. This means that the molecular weight of the molecule probing the gut barrier in this assay is 332 Da, which is very close to that of lactulose (342 Da), a molecule that has been used to assess gut barrier integrity for decades. Importantly, most previous studies investigating gut barrier integrity in animals and cells using fluorescence-based techniques have used FITC-dextran's as fluorescent probe molecules. These have much higher molecular weights than fluorescein

(e.g. 4 kDa and above). However, as stated above, the molecular weight of free fluorescein is better suited to study of the gut barrier in humans in vivo (due to its similarity to lactulose). Furthermore, our previous results have demonstrated that fluorescein passes the human gut barrier in vivo to differing degrees under different states of intestinal permeability [http://doi.org/10.1088/2050-6120/ac9513, https://doi.org/10.1093/trstmh/trab083] and that orally ingested fluorescein is detectable using non-invasive, transcutaneous spectroscopy while FITC-dextran (4kDa) is not [https://doi.org/10.1038/s41598-020-73149-2]. Thus, taken together, this demonstrates that fluorescein is a suitable fluorescent probe molecule for non-invasive assessment of human gut barrier function.

Finally, we note that we also use a multi-agent urinary recovery assay for permeability analysis. Using this approach, urinary recovery of five orally ingested molecules (lactulose, rhamnose, D-xylose, 3-O methyl-D-glucose and glycyl sarcosine) is quantified using mass spectrometry. This permits more traditional permeability assessment, for example through measurement of urinary recovery of lactulose and calculation of the lactulose: rhamnose ratio. We have amended the manuscript on pages 13&14 (under Clinical assessments, Permeability analysis) to clarify these points.

- *Since proteomic analysis of intestinal biopsies will be performed, it should be clarified if tight junction protein expression will be examined as altered expression could correlate with functional changes in permeability – with the caveat that this approach will not identify changes in localization of TJ proteins that occur without any change in expression levels.*

Response: The proteomic profiling is intended mainly to inform us about synthesis rates. We anticipate that tight junction proteins will be included in the data, and we aim to be able to estimate synthesis rates of these and other proteins which are central to barrier function (including mucins). Additionally, we aim to quantify tight junction proteins in intestinal biopsies (Zonulin, Claudin, Occluding proteins) and for targeted proteins (e.g. ZO-1, Claudin-4) measure 2H incorporation to determine synthesis rates. This clarification has been made in table 1 on page 28.

- *In Aim 5, Benzylpenicillin is the iv antibiotic that will be used in this study to identify if i.v. administered antibiotics can access the gut lumen. It is unclear what the goal of this aspect of the study is meant to determine. Do the authors consider antibiotic administration to exacerbate symptoms of EE? With respect to the question of whether i.v. antibiotics can enter the gut lumen, this has already been demonstrated that antibiotics can pass into the gut lumen via biliary secretions, and alter bacterial populations (Xue et al, 2023. [Ref 1]). In addition, no explanation of why this particular antibiotic is being used has been included. It is hard to justify this particular aim (#5) as it will not generate any novel findings and could be considered an unnecessary human subjects' intervention.*

Response: Thank you for this interesting point. Although it is now known that antibiotics can enter the gut via biliary secretions (penicillin G included), this paper was published (2023) since the protocol was approved. However, we believe that this question still needs to be answered as the Xue study evaluated the impact of antibiotics in patients undergoing cardiac surgery, which is a very different group to the patients we propose to study, notably because hypoperfusion during surgery is well known to drive bowel ischaemia which causes profound barrier loss. Our work is directed at understanding whether the degree of barrier loss in environmental enteropathy is sufficient to permit leakage of antibiotics into the right colon. Penicillin was selected as it is a narrow-spectrum antibiotic which would not be

expected to cause significant microbiome disruption in these healthy volunteers, an ethical concern. We have added this to the paper (page 12).

- *A limitation of the study is that samples will not be collected longitudinally to identify if readings are sustained or transient. However, longitudinal assessment is proposed in the Discussion section describing future studies.*

Response: This is an important point. In the last stage of the proposed work, we will generate some short term follow up data. Further funding will be sought to explore longitudinal changes in future studies.

- *Clarification of the "reporter" characteristics of HEK293, THP-1 cell lines should be provided. Will the group be using modified cells lines expressing actual reporter constructs (i.e. to quantify enzymatic activity) or are these cells just being used as 'model' cell lines. Given that changes in gut epithelial physiology are integral to the symptoms of EE, what is the rationale for choosing a kidney epithelial cell line (HEK293) as a model epithelium?*

Response: We agree that we could have been clearer about the specific use of each type of cell line; the reporter lines (THP-1 and HEK293 sub-sets) will be used to evaluate immunogenicity (PAMP content) of the samples in the first instance with THP-1 Dual reporters for NFkB and IRFs used initially and PRR-specific HEK293 used to explore specific PAMP-PRR signalling pathways. The Caco-2 cells, grown in transwell cultures, will be used to assess how the samples impact epithelial barrier function. We have updated this section with more specific details on the separate assays on page 15-16.

- In vitro experiments assessing the effect of PAMPs in fecal samples on permeability of intestinal epithelial cell cultures, will likely exhibit responses to bacterial LPS and it is unclear how the authors will be able to screen for effects of other PAMPs (in faeces) in this system with any degree of specificity.

Response: To disaggregate the effects of the different PAMPs in the stool sample, filtered faecal water, PRR-specific inhibitors, antagonists, and antibodies will be used. This information has been added on page 16. We will also use data from PRR-specific HEK293 reporter assays of the samples to identify both LPS/TLR4-driven and non-LPS/TLR4-mediated immunogenicity.

Competing Interests: No competing interests were disclosed.

Reviewer Report 17 October 2024

https://doi.org/10.5256/f1000research.169506.r326299

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Pascale Vonaesch
[1] University of Lausanne, Lausanne, Switzerland
[2] University of Lausanne, Lausanne, Switzerland

In this article, the authors describe a study to take place in Zambia and Zimbabwe, aiming at

assessing for possible biomarkers of environmental enteric disorder (EED). They propose to study this in a group of adults which undergo collection of different biospecimens, including blood and stool samples as well as endoscopy and duodenal sampling, allowing to correlate the potential biomarkers with the gold standard, based on histology of the small intestinal mucosa.

The study is interesting, well designed and addresses a pressing problem, as to date, there are still no widely accepted, easy to measure biomarkers for EED that clearly delineate the disease and distinguish it from other syndromes/infections affecting the GI tract. The study is very interesting as it assesses for macronutrient digestion and absorption as well as intestinal permeability using advanced techniques *in homo*.
These analyses are combined with a description of the duodenal microbiota using 16S and shotgun metagenomic sequencing, performed in parallel in Africa as well as in the UK using Nanopore technology.

The study protocol reads very nicely and is complete in its approach and description.

Major points:
My main concern for the study is the introduction and reference list, which is currently biased towards work from the consortium, leaving out previous work performed on biomarkers in studies performed on children by consortia located in Bangladesh, Pakistan, the Central African Republic and Madagascar. In my opinion, even if the target group is children, adding this previous work would greatly strengthen the point of the study performed and situate it better in the overall research performed so far. It would furthermore also give credit to previous work from other groups on the same/similar subject.

Furthermore, the description on previous work on the (duodenal) microbiota in undernutrition and EED is very scarce and misses to cite previous work in children in the Bangladeshi cohort (Chen R, et al., 2020 [Ref 1]), the Pakistan cohort (Iqbal et al., mSphere 2024 [Ref 3]) and the Afribiota project (Vonaesch et al., PNAS 2018 & 2022 [Ref 2]). Indeed, these studies were the first to show decompartmentalization of the GI tract (first mentioned in Vonaesch et al., PNAS 2018) in stunted growth and EED and could show first hints that the duodenal microbiota could be causally linked to the EED pathophysiology (Chen R, et al., 2020 [Ref 1], Vonaesch et al., 2022 [Ref 2]). This link is currently missing in the manuscript.
Furthermore, the authors also do not mention previous work linking EED with decreased lipid absorption (Vonaesch et al., PNAS 2022) and metabolization (Habermann et al., Gastroenterology 2021 [Ref 4]).

Last, there is also only limited references provided for the characterization of the immune system associated with undernutrition and EED (see. i.e. Andrianamantena et al., 2022 [Ref 5] among others). While I acknowledge this previous work is performed in children and not in adults like in he proposed study, I think that the information should still be given, even the more as this work was likely motivating the analyses proposed in the current study protocol and as the authors stress the fact that the biomarkers might be used in children thereafter.
In conclusion, I strongly encourage the authors to have a more balanced overview of the current state of work on biomarker discovery, the microbiome and EED beyond work performed by the study team in Zambia and in adults.

Specific points:

In the section on study size estimation, it was not clear to me what the prevalence of sucrase-isomaltase expression is. This information would ease the reader to better understand the overall sample size estimations.

Regarding the inclusion criteria, it was not clear to me why only patients of high economic status are recruited. Will this not lead to a potential bias? Please explain a bit more in detail, so the rationale behind this choice is more evident to the reader.

Regarding the consenting and consent withdrawal in the study, it was not clear to me what happens to the data and samples if a patient retracks. Are the data kept and anonymized? Destroyed? This should be clarified.

Regarding the microbiota analysis, it is not clear to me how the characterization of microbial uptake of AA and the subsequent L to D conversion will be measured. Can this be specified?

In addition, seen the importance attributed to the absolute abundance of the microbiome in the small intestinal tract (demonstrated i.e. in Chen et al, 2020), I encourage the study team to also assess for bacterial absolute abundance in Aim3 of their proposed study aims.

In the discussion section, it would have been nice to see a bit better how this research is situated in previous work, what it will bring in addition to what has been done before and what the main novelties of this project are compared to previous studies performed on children and/or adults in other countries around the globe. This would allow the reader to better understand the uniqueness and novelty of this study (which is evident to me, but it would be nice to also more clearly showcase this to readers less familiar with the topic).

Minor points:
Page 10, under the heading Day 4: Should this not read: “Colonoscopy: Zimbabwe” (with a capital Z)?

Data management: who has access to the study data? Will the Zambian study team have access to all data also if generated in the UK? How will the data been made openly accessible after publication (future data availability statement)? Please specify this in the study protocol.

Ethical considerations: is there a Nagoya approval needed to export samples from Zambia to the UK? If yes, has this approval been applied for/ been granted? Please provide some information on if and how the Nagoya approval applies to this study.

References
1. Chen R, Kung V, Das S, Hossain M, et al.: Duodenal Microbiota in Stunted Undernourished Children with Enteropathy. *New England Journal of Medicine*. 2020; **383** (4): 321-333 Publisher Full Text
2. Vonaesch P, Araújo J, Gody J, Mbecko J, et al.: Stunted children display ectopic small intestinal colonization by oral bacteria, which cause lipid malabsorption in experimental models. *Proceedings of the National Academy of Sciences*. 2022; **119** (41). Publisher Full Text
3. Iqbal NT, Chen RY, Griffin NW, Hibberd MC, et al.: A shared group of bacterial taxa in the duodenal microbiota of undernourished Pakistani children with environmental enteric dysfunction.*mSphere*. 2024; **9** (6): e0019624 PubMed Abstract | Publisher Full Text

4. Haberman Y, Iqbal NT, Ghandikota S, Mallawaarachchi I, et al.: Mucosal Genomics Implicate Lymphocyte Activation and Lipid Metabolism in Refractory Environmental Enteric Dysfunction. *Gastroenterology*. 2021; **160** (6): 2055-2071.e0 PubMed Abstract | Publisher Full Text
5. Andriamanantena Z, Randrianarisaona F, Rakotondrainipiana M, Andriantsalama P, et al.: Changes in Systemic Regulatory T Cells, Effector T Cells, and Monocyte Populations Associated With Early-Life Stunting.*Front Immunol*. 2022; **13**: 864084 PubMed Abstract | Publisher Full Text

Is the rationale for, and objectives of, the study clearly described?
Yes

Is the study design appropriate for the research question?
Yes

Are sufficient details of the methods provided to allow replication by others?
Partly

Are the datasets clearly presented in a useable and accessible format?
Not applicable

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: childhood undernutrition, environmental enteropathy, human microbiome, small intestinal microbiome, biomarker analysis, clinical microbiome research in Sub-Saharan Africa and South-East Asia

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 13 Feb 2025

James Weatherill

We are grateful for affording us the opportunity to improve this manuscript.
Please find the following specific responses to your comments:

Major points:

- *1) My main concern for the study is the introduction and reference list, which is currently biased towards work from the consortium, leaving out previous work performed on biomarkers in studies performed on children by consortia located in Bangladesh, Pakistan, the Central African Republic, and Madagascar. In my opinion, even if the target group is children, adding this previous work would greatly strengthen the point of the study performed and situate it better in the overall research performed so far. It would furthermore also give credit to previous work from other groups on the same/similar subject.*
- *2) Furthermore, the description on previous work on the (duodenal) microbiota in undernutrition and EED is very scarce and misses to cite previous work in children in the*

Bangladeshi cohort (Chen R, et al., 2020 [Ref 1]), the Pakistan cohort (Iqbal et al., mSphere 2024 [Ref 3]) and the Afribiota project (Vonaesch et al., PNAS 2018 & 2022 [Ref 2]). Indeed, these studies were the first to show decompartmentalization of the GI tract (first mentioned in Vonaesch et al., PNAS 2018) in stunted growth and EED and could show first hints that the duodenal microbiota could be causally linked to the EED pathophysiology (Chen R, et al., 2020 [Ref 1], Vonaesch et al., 2022 [Ref 2]). This link is currently missing in the manuscript.

Response: We fully accept these points and have revised the Introduction (page 5) substantially to embrace the important contributions of seminal work from Bangladesh, Pakistan, and USA, and from the Afribiota studies.

- *3) Furthermore, the authors also do not mention previous work linking EED with decreased lipid absorption (Vonaesch et al., PNAS 2022) and metabolization (Habermann et al., Gastroenterology 2021 [Ref 4]).Response:* Again, we should have included this. These references have now been included (page 5).
- *4) Last, there is also only limited references provided for the characterization of the immune system associated with undernutrition and EED (see. i.e. Andrianamantena et al., 2022 [Ref 5] among others). While I acknowledge this previous work is performed in children and not in adults like in he proposed study, I think that the information should still be given, even the more as this work was likely motivating the analyses proposed in the current study protocol and as the authors stress the fact that the biomarkers might be used in children thereafter.*

Response: This point is acknowledged, and the introduction now cites more references characterising EE and malnutrition in children where most studies have been done (page 5).

- *5) In conclusion, I strongly encourage the authors to have a more balanced overview of the current state of work on biomarker discovery, the microbiome and EED beyond work performed by the study team in Zambia and in adults.*

Response: We have addressed this and hope these points are satisfactory.

Specific comments:

- *In the section on study size estimation, it was not clear to me what the prevalence of sucrase-isomaltase expression is. This information would ease the reader to better understand the overall sample size estimations.*

Response: These estimates are based on a recent publication which was erroneously omitted. We have now included it (Schillinger *et al*).

- *Regarding the inclusion criteria, it was not clear to me why only patients of high economic status are recruited. Will this not lead to a potential bias? Please explain a bit more in detail, so the rationale behind this choice is more evident to the reader.*

Response: This choice is partly based on the demographic of the clinic where this work will be done, but also because we reasoned that if antibiotic leakage into the gut lumen is not detectable with higher SES participants, who have milder EE histologically, then it will not be of significance in people with lower SES. This has been added into the paper (page 8).

- *Regarding the consenting and consent withdrawal in the study, it was not clear to me what happens to the data and samples if a patient retracts. Are the data kept and anonymized? Destroyed? This should be clarified.*

Response: In the event of withdrawal, the participant is given the choice whether or not to allow retention of samples and data, or if they wish them to be destroyed. This is now included (page 10).

- *Regarding the microbiota analysis, it is not clear to me how the characterization of microbial uptake of AA and the subsequent L to D conversion will be measured. Can this be specified?*

Response: A fuller description has now been included (page 15).

- *In addition, seen the importance attributed to the absolute abundance of the microbiome in the small intestinal tract (demonstrated i.e. in Chen et al, 2020), I encourage the study team to also assess for bacterial absolute abundance in Aim3 of their proposed study aims.*

Response: This helpful suggestion has been added (pages 14&15).

- *In the discussion section, it would have been nice to see a bit better how this research is situated in previous work, what it will bring in addition to what has been done before and what the main novelties of this project are compared to previous studies performed on children and/or adults in other countries around the globe. This would allow the reader to better understand the uniqueness and novelty of this study (which is evident to me, but it would be nice to also more clearly showcase this to readers less familiar with the topic).*

Response: This point was well made. We have expanded the Discussion to include this (pages 19&20).

Minor points:

- *Page 10, under the heading Day 4: Should this not read: "Colonoscopy: Zimbabwe" (with a capital Z)?*

Response: Yes, now corrected (now page 12).

- *Data management: who has access to the study data? Will the Zambian study team have access to all data also if generated in the UK? How will the data been made openly accessible after publication (future data availability statement)? Please specify this in the study protocol.*

Response: In compliance with the policies of UKRI, the funder, all data will be open access in a fully anonymised form so that it is fully compliant also with GCP and the requirements of the University of Zambia Biomedical Research Ethics Committee and the Joint Research Council of the University of Zimbabwe. We have described this on pages 17,18&26.

- *Ethical considerations: is there a Nagoya approval needed to export samples from Zambia to the UK? If yes, has this approval been applied for/ been granted? Please provide some information on if and how the Nagoya approval applies to this study.*

Response: The research work is fully collaborative, governed by transfer agreements which protect all collaborating researchers and ensure that all data and intellectual property are retained by the institutions in the countries of origin. We have taken advice and been informed that our practises are compliant with the Nagoya protocol.

Competing Interests: No competing interests were disclosed.