



BMJ Open Optimisation, validation and field applicability of a ^{13}C -sucrose breath test to assess intestinal function in environmental enteropathy among children in resource poor settings: study protocol for a prospective study in Bangladesh, India, Kenya, Jamaica, Peru and Zambia

Gwenyth O Lee ¹, Robert Schillinger,² Nirupama Shivakumar,³ Sherine Whyte,⁴ Sayeeda Huq,⁵ Silvenus Ochieng Konyole,⁶ Justin Chileshe,⁷ Maribel Paredes-Olortegui,⁸ Victor Owino,⁹ Roger Yazbeck,^{10,11} Margaret N Kosek,^{8,12} Paul Kelly ^{13,14} Douglas Morrison²

To cite: Lee GO, Schillinger R, Shivakumar N, *et al*. Optimisation, validation and field applicability of a ^{13}C -sucrose breath test to assess intestinal function in environmental enteropathy among children in resource poor settings: study protocol for a prospective study in Bangladesh, India, Kenya, Jamaica, Peru and Zambia. *BMJ Open* 2020;**10**:e035841. doi:10.1136/bmjopen-2019-035841

► Prepublication history and additional material for this paper is available online. To view these files, please visit the journal online (<http://dx.doi.org/10.1136/bmjopen-2019-035841>).

Received 20 November 2019

Revised 08 May 2020

Accepted 23 October 2020



© Author(s) (or their employer(s)) 2020. Re-use permitted under CC BY-NC. No commercial re-use. See rights and permissions. Published by BMJ.

For numbered affiliations see end of article.

Correspondence to

Dr Gwenyth O Lee; golee@umich.edu

ABSTRACT

Introduction Environmental enteropathy (EE) is suspected to be a cause of growth faltering in children with sustained exposure to enteric pathogens, typically in resource-limited settings. A major hindrance to EE research is the lack of sensitive, non-invasive biomarkers. Current biomarkers measure intestinal permeability and inflammation, but not the functional capacity of the gut. Australian researchers have demonstrated proof of concept for an EE breath test based on using naturally ^{13}C -enriched sucrose, derived from maize, to assay intestinal sucrase activity, a digestive enzyme that is impaired in villus blunting. Here, we describe a coordinated research project to optimise, validate and evaluate the usability of a breath test protocol based on highly enriched ^{13}C -sucrose to quantify physiological dysfunction in EE in relevant target populations.

Methods and analysis We use the ^{13}C -sucrose breath test (^{13}C -SBT) to evaluate intestinal sucrase activity in two phases. First, an optimisation and validation phase will (1) confirm that a ^{13}C -SBT using highly enriched sucrose tracers reports similar information to the naturally enriched ^{13}C -SBT; (2) examine the dose–response relationship of the test to an intestinal sucrase inhibitor; (3) validate the ^{13}C -SBT in paediatric coeliac disease (4) validate the highly enriched ^{13}C -SBT against EE defined by biopsy in adults and (5) validate the ^{13}C -SBT against EE defined by the urinary lactulose:rahanose ratio (LR) among children in Peru. Second, a cross-sectional study will be conducted in six resource-limited countries (Bangladesh, India, Jamaica, Kenya, Peru and Zambia) to test the usability of the optimised ^{13}C -SBT to assess EE among 600 children aged 12–15 months old.

Ethics and dissemination Ethical approval will be obtained from each participating study site. By working as a consortium, the test, if shown to be informative of

Strengths and limitations of this study

- A validated non-invasive ^{13}C -sucrose breath test (^{13}C -SBT) would overcome a major current limitation of environmental enteropathy (EE) research by providing an assay that explicitly measures the function of the intestinal epithelium to digest and absorb nutrients.
- The test is supported by early proof-of-concept data from a study that used a naturally enriched ^{13}C -SBT to characterise intestinal function in children with possible EE. However, this test is limited by low signal to noise in the breath $^{13}\text{CO}_2$ signal.
- This coordinated research project design includes sequential validation and feasibility studies in both adult and paediatric populations.
- The large network permits validation in multiple geographic sites, in Southeast Asia (India, Bangladesh), Africa (Zambia, Kenya), Latin America (Peru) and the Caribbean (Jamaica).
- If the ^{13}C -SBT provides a functional readout related to EE, it would facilitate the validation and use of test substrates using a similar approach for the assessment of EE, with broad field applicability.

EE, will demonstrate strong evidence for utility across diverse, low-income and middle-income country paediatric populations.

Trial registration number NCT04109352; Pre-results.

INTRODUCTION

Retarded linear growth, resulting in stunting (length-for-age or height-for-age below 2 Z-scores of WHO growth standards), affects



23% of children under 5 years, most of whom live in low-income and middle-income countries.¹ Stunting is associated with increased child morbidity and mortality,² poorer cognitive development,³ school performance⁴⁻⁶ and lower adult wages,⁷ and, for girls, poorer maternal health outcomes.⁸

The aetiology of stunting is multifactorial, including generational and prenatal factors.^{9,10} Postnatal exposures also play a major role. Most postnatal growth faltering occurs around 6–18 months of age,¹¹ an age when the energy and nutrient requirements for rapid growth are high. For children living in communities without access to improved water and sanitation, this period is also associated with intense exposure to enteric pathogens, while protection from breast feeding and maternal antibodies begin to wane. Although driven by underlying environmental, social and familial factors, inadequate diet and exposure to pathogens are the major, proximal causes of infant growth faltering.¹⁰ However, nutritional interventions lead to only small to moderate improvements in growth,¹² and two recent, large studies to reduce enteric infection found no impact of water and sanitation interventions on child growth, or evidence of synergy between water, sanitation and hygiene (WASH) interventions and nutritional interventions.¹³⁻¹⁵ These findings reinforce the need to better understand the biological basis of stunting.

Since the 1960s, it has been noted that adults and children living without access to improved water and sanitation have altered small intestinal morphology,¹⁶ including diffuse, upper small bowel villous atrophy accompanied by evidence of barrier disruption and inflammation.^{17,18} This condition is linked to chronic bacterial translocation leading to systemic inflammation, and T-cell-mediated hyperstimulation of the mucosal immune system, negatively impacting nutrient absorption and utilisation.^{19,20} This increases the risk of nutrient deficiency, which may in turn further impair gut immune responses.¹⁰ It is theorised that this condition, successively termed, ‘tropical enteropathy’, ‘environmental enteropathy (EE)’ and most recently, ‘environmental enteric dysfunction’,²¹ is a major contributing cause of the failure of both nutritional, and water and sanitation-oriented interventions, to significantly improve child growth.

Despite the potential significance of EE to child nutrition and health, there is a lack of simple diagnostic techniques to identify or classify the condition.²² A primary challenge has been that morphological confirmation of EE requires intestinal biopsy. In the absence of this, EE is studied using non-invasive biomarkers of gut function. The most common approaches to determining EE use the dual-sugar lactulose:mannitol or LR ratio,²³ or composite scores based on multiple biomarkers intended to capture multiple domains of gut dysfunction.^{24,25} However, dual-sugar tests, although non-invasive, are time-consuming to administer, vary greatly in protocol details, and concerns have been raised about interplatform consistency.²⁶ The relationship of alternative biomarkers to EE, and to each

other, is also not well understood.²⁷ Candidate biomarkers to define EE have frequently been ‘borrowed’ from the field of paediatric gastroenterology, and specifically, from the study of coeliac disease, which produces an enteropathy regarded as having histopathological and immune similarities to EE.²⁸ However, in many cases, there is a lack of descriptive data bridging biomarker performance between children with severe paediatric gastrointestinal disease, well-child controls in high-income countries and children from high-risk EE settings. This diminishes the interpretability of these tests. For example, faecal biomarkers of intestinal inflammation, myeloperoxidase and alpha-1-antitrypsin, have been proposed for EE, but reference values for healthy, well-growing children remain limited.²⁵ Despite these limitations, the shift in nomenclature from ‘EE’ to ‘environmental enteric dysfunction’, first proposed by Keusch *et al*²¹ and now adopted by a significant proportion of the research community, emerged from the viewpoint that, given the infeasibility of biopsy-confirmed ‘enteropathy’, diagnosis should be based instead on ‘functional’ biomarkers, as well as on functional consequences for child growth and development.

Most existing EE biomarkers reflect processes of intestinal or systemic inflammation, such as bacterial translocation and intestinal repair, and do not directly characterise functional deficits in the gut, such as deficits in macronutrient or micronutrient absorption. The lactulose:mannitol ratio is regarded as indirect marker of dysfunction, but this is based on reported associations with the D-xylose test, a measure of carbohydrate absorption that is challenging to administer and has been reported only infrequently in the EE literature.^{29,30} Zinc metabolism can be measured though carefully performed, dual stable isotope studies.¹⁹ Few other such tests are currently available.

Stable isotope-based tests have the potential to overcome this limitation by assessing host function across multiple domains of EE. Labelled substrate tracers can be designed to target specific domains of intestinal epithelial activity.³¹ Substrate tracers labelled with ¹³C are particularly promising for this purpose, as ¹³CO₂ expired in the breath can be non-invasively sampled to obtain a quantitative measure of substrate hydrolysis and absorption.

Intestinal sucrase-isomaltase (SI) activity has been identified as a potentially useful domain to target in a functional test of EE. SI is a small intestinal brush border enzyme that catalyses the hydrolysis of carbohydrates including starch, isomaltase and sucrose.³² It has a gradient expression along the small intestine, with highest expression in the duodenum and jejunum, and shows higher expression in the villi compared with the crypts. SI levels are reduced with mucosal injury but are relatively stable by race and age. Outside of a few higher-risk populations (eg, indigenous Greenlanders,³³ inherited deficiencies are uncommon.^{34,35} SI activity is diminished in villus blunting,³⁶ thus representing a possible surrogate marker of small intestinal function and integrity.³⁷ Inflammatory

pathologies of the gut, namely autoimmune disorders, inflammatory bowel disease and intestinal inflammation caused by HIV and giardiasis, have also been shown to cause SI deficiency.³⁸ A recent transcriptomic study also described SI activity as potentially altered by EE.³⁹

Breath tests that characterise intestinal SI activity through the ingestion of ¹³C labelled sucrose have been employed in animal models and in childhood cancer chemotherapy as a biomarker of enteropathy.^{37 40 41} A ¹³C-labelled sucrose breath test (¹³C-SBT) based on naturally ¹³C enriched sucrose from maize, was also used by Ritchie *et al* to assess aboriginal Australian children from enteropathy settings.³⁴ Threefold differences were observed between aboriginal and higher-socioeconomic status (SES) Australians children, and twofold differences among children with and without diarrhoea. However, this test was limited by the necessity for a large dose to produce an exhaled breath ¹³CO₂ signal above basal ¹³C abundance, making it unsuitable for routine use in very young children. Furthermore, between-population differences in the consumption of naturally ¹³C-enriched C₄ crops, such as maize and sugar cane, reduce the utility of this test as a diagnostic for EE or to compare the prevalence of EE between populations.

Inspired by this naturally enriched test, we recently developed a ¹³C-SBT based on highly enriched sucrose tracers. This test overcomes limitations of the previous test by dramatically reducing the quantity of substrate

required, as well elevating exhaled ¹³CO₂ substantially above the baseline value that might be expected due to diet, thereby improving the signal to noise ratio. To assess whether enriched ¹³C-SBT performance is altered among individuals with EE, we will conduct six integrated, complementary studies in two phases.

METHODS AND ANALYSIS

Our coordinated study design is summarised in [table 1](#).

Phase 1

In the first phase of the study, our overall objective is to optimise and validate a protocol for a non-invasive stable isotope test based on an enriched sucrose substrate (¹³C-SBT), among adults and children. To accomplish this, we will complete five coordinated studies. We will first optimise the ¹³C-SBT protocol and second, validate the ¹³C-SBT in successive adult and paediatric populations. We will then establish analytical validity (technical test performance), clinical validity (the test's ability to accurately and reliably identify a disorder of interest) and field usability (assessment of the test in the actual context where it would be used).^{42 43}

The objective of the first study is to establish that the highly enriched sucrose tracers for an ¹³C-SBT report similar information in comparison to the original naturally enriched ¹³C-SBT. We will conduct a cross-over study

Table 1 Features of the coordinated research projects that make up the study protocol

	Study goal	Study design	Primary study outcome	Population	Site
Phase 1					
Study 1	Optimisation	Cross-over	n/a	Healthy adults (N=20)	Glasgow, UK
Study 2	Validation	Cross-over	Intestinal sucrose inhibition (acarbose dose-response)	Healthy adults (N=20)	
Study 3	Validation	Case-control	Coeliac disease	Children with coeliac (N=20) Children with non-coeliac GI disorders (N=20) Healthy child controls (N=20)	Adelaide, Australia
Study 4	Validation	Case-control	Villous atrophy Intestinal sucrose activity	Adults from an EE setting (N=20) Healthy adult controls (n=20)	Lusaka, Zambia
Study 5	Validation	Cross-sectional	Urinary lactulose: rhamnose ratio Plasma kynurenine: tryptophan ratio	Infants from an EE setting (N=30)	Iquitos, Peru
Phase 2					
Study 6	Field utility and validation	Cross-sectional	Urinary lactulose: rhamnose ratio Length-for-age Z-score	Infants from EE settings (N=540) Infants from higher-SES settings (N=60)	Dhaka, Bangladesh Bangalore, India Kingston, Jamaica Kakamega, Kenya Iquitos, Peru Ndola, Zambia

EE, environmental enteropathy; n/a, not available; SES, socioeconomic status.

of 20 adults using three commercially available sucrose tracers ($^{13}\text{C}_6$ fructose; $^{13}\text{C}_6$ glucose and $^{13}\text{C}_{12}$ sucrose). We will also determine whether the addition of unlabelled carrier sucrose is necessary to replicate the original 'flooding dose' approach reported by Ritchie *et al.*³⁴ To assess gut permeability, participants will also receive coadministration of 5 g lactulose, 1 g rhamnose, 0.5 g xylose, 0.2 g 3-O-methyl-D-glucose and 5 g sucralose dissolved in water.

The objective of the second study is to characterise the dose response of the ^{13}C -SBT in response to three different doses of the intestinal sucrase inhibitor acarbose. A randomised cross-over trial of 20 adults will be conducted. Breath $^{13}\text{CO}_2$ will be collected serially for 4–6 hours and $^{13}\text{CO}_2$ recovery compared across treatments. Both studies 1 and 2 will be conducted in Glasgow, UK (University of Glasgow).

While the ideal 'gold standard' would be to breath test and biopsy children who are identified as having EE, from a logistical perspective, this study design is infeasible in the resource constrained environments where EE is prevalent. Therefore, the objective of the third study is to examine whether the ^{13}C -SBT, optimised according to the protocol established in study 1, varies between children with clinically diagnosed coeliac disease presenting with gastrointestinal symptoms to outpatient clinics (n=20) versus healthy coeliac controls (n=20) and healthy non-coeliac controls (n=20) (Adelaide, Australia, Flinders University). In this study, active disease is defined as positive serology for specific IgA antibodies in patients on a gluten-containing diet, or in patients undertaking a gluten challenge, followed by endoscopic evaluation and histological examination of duodenal biopsy for characteristic features of coeliac disease (villus atrophy, crypt hyperplasia and mucosal inflammation), with a Marsh III classification considered coeliac positive. The degree of villous atrophy will be correlated to the patients ^{13}C -SBT result. Additionally, tissue biopsies, collected from inflamed and normal sections of small bowel, will be assayed *ex vivo* for SI activity and correlated with the ^{13}C -SBT. The non-coeliac control group includes children presenting for endoscopy for non-coeliac disease, including abdominal pain and reflux disease, and do not present with any small intestinal pathology.

The objective of the fourth study is to validate the ^{13}C -SBT against intestinal sucrase activity and villus atrophy among adults with and without EE. Both villus atrophy and intestinal sucrase activity are measured using biopsy. A case-control study design (n=20 cases from a high-risk enteropathy setting and n=20 controls) will be used. (Lusaka, Zambia, University of Zambia)

The objective of the fifth study is to correlate the ^{13}C -SBT with an established, non-invasive biomarkers of EE (urinary LR ratio) and one secondary biomarker of EE (plasma kyrenine:tryptophan ratio) among children under 2 years of age from a high-risk enteropathy setting (n=30). (Iquitos, Peru, Asociación Benefica Proyectos de Informática, Salud, Medicina, y Agricultura (A.B.

PRISMA) and the University of Virginia). A substudy will remeasure the ^{13}C -SBT at 7 days to document test reproducibility.

Phase 2

In the second phase, we will conduct a multisite study in six resource-limited countries to determine the field usability of the optimised and validated ^{13}C -SBT in diagnosing EE in children aged 12–15 months. The specific primary objectives are to assess the relationship between the ^{13}C -SBT and the LR ratio among children 12–15 months of age, and to assess the relationship between the ^{13}C -SBT and child stunting. Our secondary objectives are to assess the relationship between the ^{13}C -SBT and secondary biomarkers of EE. We will also conduct exploratory analyses to characterise the relationship between the ^{13}C -SBT and child sex, SES, dietary diversity and household food security.

Study sites

This six study sites are: Dhaka, Bangladesh (the International Centre for Diarrhoeal Disease Research); Bangalore, India (St. John's Research Institute, St. John's National Academy of Health Sciences); Kingston, Jamaica (The Tropical Metabolism Research Unit of the Caribbean Institute for Health Research, University of West Indies); Kakamega, Kenya (Masinde Muliro University of Science and Technology); Iquitos, Peru⁴⁴ (Asociación Benefica PRISMA and the University of Virginia) and Ndola, Zambia (Tropical Disease Research Centre). These sites represent a range of epidemiological contexts which enhances the cross-context applicability of study results.

Coordinated study design

Each site will enrol 100 infants between 12 and 15 months of age. This range was selected because it is within the window of infant growth faltering that implies clinical or public health relevance but is also old enough to reduce the influence of breastfeeding on LR performance and to permit a several hour fast during initial assessment of the test.^{45 46} At each site, 90 children will be recruited from areas deemed high risks for EE, due to a lack of improved water and sanitation infrastructure or because the known prevalence of stunting is relatively elevated. Ten relatively high SES infants from a nearby community will also be enrolled. All children will be recruited and enrolled through convenience sampling, either at the community level (if the study site has previously censused the community) or through child clinic visits. Exclusion criteria include the presence of Severe Acute Malnutrition (weight for height z-score ≤ -3 SD), HIV positive status, any chronic illness medical or surgical contributing to growth retardation, or weight-for-height z-score more than +2SD.

Study procedures for each participating child are outlined in figure 1 and described in detail in online supplemental appendix 1. In brief, the ^{13}C -SBT will be

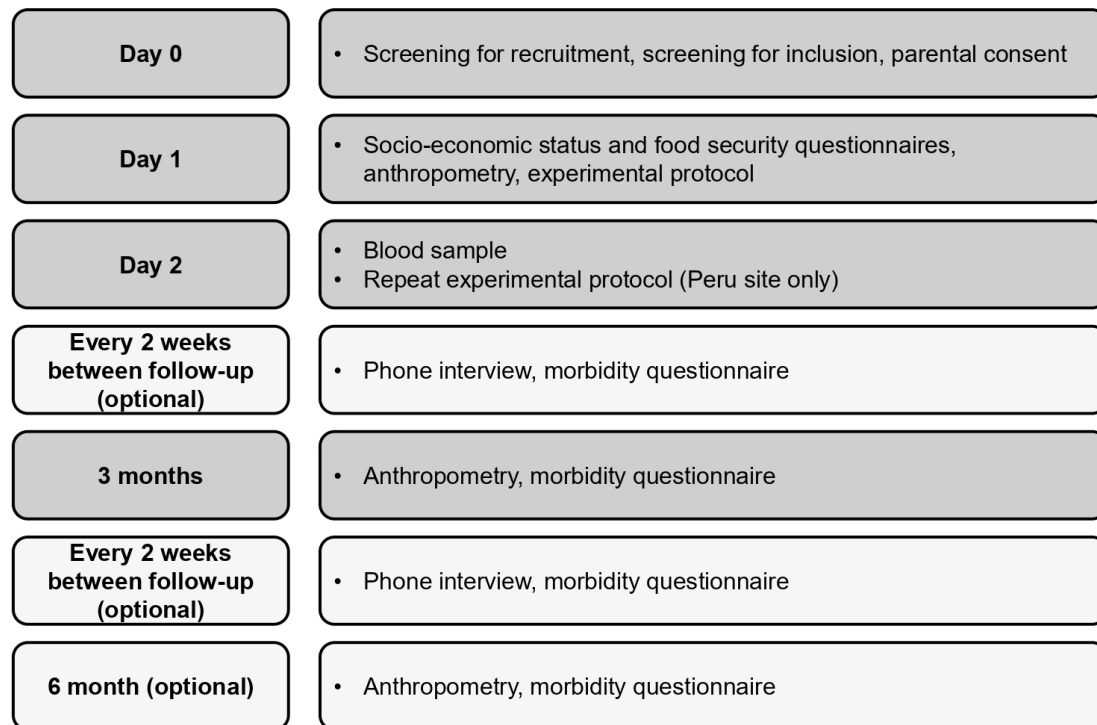


Figure 1 Flow diagram for phase 2 coordinated study protocol shown here is the timeline of participant activities. Darker grey boxes represent core study activities, while light grey boxes indicate activities that some, but not all, study sites will undertake. Primary and secondary study aims are based on core activities.

assessed in each child at one time point, as well as a 2-hour urinary LR test, an assessment of weight, length and body composition using the deuterium dilution technique (either saliva or urine), and a fasting plasma sample for the assessment of additional EE biomarkers. After 3 months, the height and weight measurement will be repeated. Each site will use the same harmonised study protocols for all data collection. Information about household SES, household food security (using the household food insecurity access scale), and child dietary diversity will also be asked of each caretaker using standardised instruments designed and validated for use across low-income and-middle income countries.^{47–49} Information about child morbidity and the consumption of C₄ foods will also be collected using standardised questions. In certain sites with high rates of cell phone coverage, ancillary morbidity data will also be collected by phone every 2 weeks, although this will not be used in the primary or secondary analysis. Key study data are summarised in online supplemental appendix 2, and study forms are provided in online supplemental appendix 3.

All data will be digitised by site via double-data entry and will be managed in accordance with institutional norms and local ethical committee approvals. Each site will confidentially maintain personal information about enrolled participants as necessary for study administration. Deidentified data will be shared with the University of Michigan where centralised data consistency checks will be performed, and inconsistencies will be communicated back to each site for resolution. After this process is complete, pooled analyses will be performed.

Outcomes and case definitions

¹³C breath tests can be summarised in several ways.⁵⁰ Based on expert opinion, cumulative per cent of dose recovered at 90 min post administration (cPDR90), and time to 50% recovery (T50), will be taken as our primary measures of the ¹³C-SBT. Other metrics for summarising the ¹³C-SBT test, at both specific time points and overall, will also be considered.

LR ratio: To assess the relationship between the ¹³C-SBT and EE, our case definition for EE, and primary outcome measure, will be based on the LR ratio, which is the most widely accepted, non-invasive test of EE. Because LR ratios vary by analytical platform²⁶ and may also be influenced by test administration procedures, cut-offs for ‘EE’ will be defined based on the empirical distribution of the data. To establish cut-offs for LR, the distribution of this variable among high-SES children (pooled across sites) will be examined (eg, LR ratios above the 90th percentile for upper SES children will be regarded as ‘elevated’). If insufficient numbers of higher-SES children are recruited, or if a subset of higher-SES children cannot be recruited across all sites, cut-off values for LR will be determined based on internal study percentiles (eg, below vs above the median) or on cut-offs from the literature.⁵¹ If the outcomes of the phase 1 studies support evidence for a LR cut-off based on the extent of villous atrophy, this will also be considered.

Anthropometry: We will use WHO growth standards to define length for age Z-score (LAZ), weight for age Z-score (WAZ) and weight for length Z-score. Stunting,

underweight and wasting will be defined based on WHO growth standards (≤ -2 Z-scores length for age, weight for age and weight for length).⁵²

Our primary outcome measures are

1. Comparison of the ¹³C-SBT to the LR ratio, to the per cent lactulose recovery, and to the per cent rhamnose recovery.
2. Characterise the relationship between the ¹³C-SBT and child anthropometry (LAZ and WAZ).
3. Characterise the relationship between ¹³C-SBT and childhood linear growth (change in LAZ) over 3 months and over 6 months.

Secondary outcome measures

1. Assess the relationship between the ¹³C-SBT and faecal myeloperoxidase concentration.
2. Assess the relationship between the ¹³C-SBT and serum fatty acid binding protein concentration.
3. Assess the relationship between the ¹³C-SBT and the kynurenine tryptophan ratio.
4. Assess the relationship between the ¹³C-SBT and faecal alpha-antitrypsin concentration.

Other prespecified outcome measures are

1. Reproducibility of the ¹³C-SBT.

We will assess the coefficient of variation and correlation coefficient between repeated cumulative percent of dose recovered at 90 min postadministration on separate SBT tests administered 1 week apart done on the same child (Peru site only).

2. Assess the relationship between epidemiological factors and the ¹³C-SBT.

We will determine if significant associations exist between ¹³C-SBT measured as cPDR90/T50 and the Water Assets Maternal Education and Income (WAMI index). The WAMI index is a previously validated composite index of environmental variables to create an index that expresses the socioeconomic and physical environment in diverse geographical contexts.⁴⁷

Sample size

The sample size for the phase 2 study was predetermined (N=10 upper SES and 90 lower SES children per site), power calculations were conducted. Calculations were based on previously reported Pearson's correlations of 0.67 (95% CI 0.42 to 0.82) between the naturally enriched ¹³C-SBT and LR.³⁴ The current test, using highly enriched sucrose, is expected to be more sensitive than the original test, so sample size estimates are regarded as conservative. Assuming the Pearson's correlation between the enriched ¹³C-SBT and LR is similar, 90 children per site is sufficient to estimate the correlation between the two tests with an SD of 0.58 (95% CI 0.55 to 0.79).⁵³ The minimal detectable correlation within each site, would be 0.31.⁵⁴

Between sites, we estimate statistical power to detect meaningful differences between children with and without EE, based on a cut-off of the LR ratio. The proportion of children who will be classified as having EE relative to this these cut-offs is unknown, so the estimated detectable difference was calculated across a range

of values (10%–50%). We estimate that differences in the ¹³C-SBT on the order of 0.60 standard deviations (50% prevalence of EE) to 0.99 SD (10% prevalence of EE) will be detectable with 80% power. For comparison, Ritchie observed differences in the ¹³C-SBT cumulative percentage of dose recovered at 90 min between healthy Aboriginal and non-Aboriginal children on the order of ~0.84 SD, and differences between Aboriginal children with and without acute diarrhoea on the order of ~0.92 SD.

Power calculations were also performed to assess the relationship between the ¹³C-SBT and child stunting based on the known prevalence of stunting in each site (table 2).

Data analysis plan

All analyses will be stratified by site and then pooled. Analysis stratified by site will be limited to bivariable comparisons, and pooled data will be used to construct multivariable models, using either fixed or random effects to account for site.

We will examine the relationship between the ¹³C-SBT and LR both continuously, and as dichotomous variables. Continuous analyses will include calculation of both site-specific and pooled correlation coefficients (to provide direct comparison to Ritchie³⁴ and regression models will be developed where the dependent variables will be log-transformed LR, and the independent variable will be cPDR90 and T50. For dichotomous analyses, Receiver Operating Characteristic (ROC) curves will be used to calculate the sensitivity and specificity of be cPDR90 and T50 cut-offs to predict relatively elevated LR test results. We will also examine the association between the ¹³C-SBT and lactulose and rhamnose excretion individually.

To characterise the relationship between the ¹³C-SBT test and child anthropometry, we will compare be cPDR90 and T50 values for the ¹³C-SBT and concurrently measured LAZ and WAZ. We will also consider fat mass and fat-free mass. Nutritional status will be analysed both continuously and will dichotomised (ie, into stunted and non-stunted). T-tests will be used to compare be cPDR90 and T50 between the stunted and non-stunted groups within sites, and simple and multivariable linear regression models including random effects for country membership, where the dependent variables will be LAZ, WAZ and the independent variable will be ¹³C-SBT. In addition, we will consider adjustment for factors such as the age, sex, breastfeeding status and recent illness history of the child.

Our first secondary objective is to assess the relationship between the ¹³C-SBT and secondary biomarkers of EE. This activity will be contingent on the availability of these biomarker results from a sufficient number of infants across the study sites. Following the prior approaches,^{27 55} the relationship between EE biomarkers will be explored and EE scores will be generated via principal components analysis, partial least squares regression, or other variable reduction techniques, and comparisons between

Table 2 Power calculations are based on the primary comparison of stunted to non-stunted children

Country	Estimated prevalence of stunting, %	Sample size	Detectable difference in ¹³ C-SBT between stunted and non-stunted (per SD)
Bangladesh	36	90	0.62 SDs
India	27	90	0.67 SDs
Jamaica	20	90	0.74 SDs
Kenya	26	90	0.67 SDs
Peru	38	30	0.61 SDs
Zambia	40	90	0.61 SDs
Pooled- no design effect	30.4	480	0.28 SDs
Pooled – design effect=0.9	30.4	432*	0.30 SDs
Pooled- design effect=0.7	30.4	336*	0.34 SDs
Pooled- design effect=0.5	30.4	240*	0.40 SDs

Table 2 shown here are estimated detectable differences in the ¹³C-SBT between stunted and non-stunted children, based on the estimated prevalence of stunting in specifically proposed study communities (estimates of stunting prevalence provided by study community). The percentage of variability in the ¹³C-SBT based on site is unknown, so a range of design effects (1.0–0.5) are provided.

*Asterisks refer to the overall sample size adjusted for the design effect.

¹³C-SBT, ¹³C-sucrose breath test .

be cPDR90 and T50 and these scores will be examined similarly to LR.

Following previous approaches, we will examine the association between the ¹³C-SBT and subsequent change in WAZ, and LAZ,^{25 56 57} which enhances the comparability of our results to those of other studies. We will again consider adjustment for factors that may influence child growth trajectory such as the age, gender, breastfeeding status, and recent illness history of the children in pooled models only.

Finally, we will conduct exploratory analyses. To assess the reproducibility of the ¹³C-SBT we will examine the coefficient of variation and correlation coefficient between repeated tests from the same child (Peru site only). We will also examine the relationship between the ¹³C-SBT and child sex, SES, dietary diversity and household food security. Regression models will be developed where the dependent variable will be the result of the ¹³C-SBT test (transformed if necessary) and the independent variables will include factors that may be associated with the infant gut function, including breastfeeding, dietary diversity, age, sex, food security, history of recent illness and SES scores.⁴⁷ If between-site differences in ¹³C-SBT are observed, the models will include site-level random intercepts.

Patient and public involvement

No patient involvement.

Ethics and dissemination

Each study protocol has been approved (Zambia, Australia, Peru, Bangladesh, India, UK, Zambia, Kenya) or is pending approval (Jamaica) by the institutional review board or boards relevant to that study site. Written

informed consent will be obtained from the participant themselves, and/or the legal guardian of each participant, by members of each local study team. In both the phase 1 and the coordinated phase 2 studies, each study site will use a unique consent form, reflective of both of core study activities and any site-specific activities also being performed.

We will publish and disseminate our results once the project is complete. By conducting the field usability phase of our study across six countries, our test, if shown to be informative of EE, will demonstrate strong evidence for utility across diverse, low-income and middle-income country paediatric populations.

DISCUSSION

We propose to optimise, validate and assess the field usability of a ¹³C-SBT to evaluate EE. The research team brings together experts in stable isotopes, biochemistry, gastroenterology, human nutrition and epidemiology, as well as field teams with extensive expertise conducting human research in resource-limited settings. This breadth of expertise is necessary to overcome previous limitations of EE research. Proposed EE biomarkers have often been carried over from studies of severe gastrointestinal disorders, such as coeliac disease, into enteropathy settings, with unclear biological interpretation and without validation against the ‘gold-standard’ definition of EE. In other instances, analytical variability has hindered cross-study or cross-context comparisons of test performance.²⁶ Our staged validation approach and multisite design are intended to overcome these limitations and may serve as a template for future EE biomarker studies.

The protocol is relatively intensive, with the phase 2 study requiring 4-hour breath collections and 2-hour urine collections from 600 infants. This decision to emphasise the comprehensive testing of a relatively smaller number of children was deliberate. If the study results support the utility of the ^{13}C -SBT, future work would aim to shorten and streamline our protocol for future clinical and epidemiological research.

At present, there is a true deficiency in the number of non-invasive tests to measure intestinal function. The critical evaluation of this test will add to collective knowledge if findings affirm, refute or partially affirm the utility of the test. This study will ascertain whether a stable isotope breath test can report information on the functional activity of an important small intestinal enzyme, or not. This will serve as an important paradigm on the potential utility of other functional breath tests which could be used to characterise EE. Looking ahead, ^{13}C breath tests have potential utility for better understanding multiple other enzymes and factors of nutrient uptake in addition to SI activity. The digestion of proteins and uptake of one or more amino acids and/or peptides through the development of probes for peptidases would be a logical next step, as would the integration of zinc assays to probe multiple nutrient uptake capacities concurrently. The overarching vision of the coordinated research project is the eventual development of a breath test that, like the urea breath test for *Helicobacter pylori*, can be deployed to rapidly identify children with potential EE. Although the ^{13}C -SBT we describe here is non-invasive and feasible in low-resource settings, the analysis of these samples currently cannot be performed at the point of care. However, with advancing breath analysis technology, these tests are becoming less expensive and more field-deployable, opening these approaches to a wider user base and especially to researchers and clinicians in low-income and middle-income countries.

Author affiliations

¹Department of Epidemiology, University of Michigan, Ann Arbor, Michigan, USA

²Scottish Universities Environmental Research Centre, University of Glasgow, Glasgow, UK

³Division of Nutrition, Saint John's Research Institute, Bangalore, Karnataka, India

⁴Caribbean Institute for Health Research (formerly, Tropical Medicine Research Institute), University of the West Indies at Mona, Mona, Saint Andrew, Jamaica

⁵Nutrition and Clinical Services Division, International Centre for Diarrhoeal Disease Research, Bangladesh (icDDR), Dhaka, Bangladesh

⁶Biomedical Sciences Department, Tropical Diseases Research Centre, Ndola, Copperbelt, Zambia

⁷Department of Nutritional Sciences, Masinde Muliro University of Science and Technology, Kakamega, Kenya

⁸Research and Development Area, Asociación Benéfica Proyectos de Informática, Salud, Medicina, y Agricultura (A.B. PRISMA), Iquitos, Loreto, Peru

⁹Nutritional and Health Related Environmental Studies Section, Division of Human Health, International Atomic Energy Agency, Vienna, Austria

¹⁰Department of Surgery, College of Medicine and Public Health, Flinders University, Adelaide, South Australia, Australia

¹¹Flinders Centre for Innovation in Cancer, Flinders University, Adelaide, South Australia, Australia

¹²Division of Infectious Diseases & International Health, University of Virginia, Charlottesville, Virginia, USA

¹³Blizard Institute, Barts and The London School of Medicine, London, UK

¹⁴Tropical Gastroenterology and Nutrition group, University of Zambia School of Medicine, Lusaka, Lusaka, Zambia

Acknowledgements We sincerely thank all institutions for administrative support of the project to date. We also thank Dr Mamane Zeilani from Nutriset for constructive discussions and for providing funding, in part, for this work.

Contributors GOL: contributed to the development of the combined research protocol, drafted the manuscript and will contribute to the analysis and interpretation of data. She will support analysis and interpretation of data for all sites. RS: contributed to the development of the site-specific protocol for the first and second studies (Glasgow, UK), the development of the combined research protocol, and critically reviewed the manuscript for accuracy and intellectual content. He will support collection of data in the UK, and analysis and interpretation of data for all sites. NS: contributed to the development of the site-specific protocol for the sixth study (Bangalore, India), the development of the combined research protocol, and critically reviewed the manuscript. She will support collection of data in India, and analysis and interpretation of data for all sites. SW: contributed to the development of the site-specific protocol for the sixth study (Kingston, Jamaica), the development of the combined research protocol and critically reviewed the manuscript. She will support collection of data in Jamaica, and analysis and interpretation of data for all sites. SH: contributed to the development of the site-specific protocol for the sixth study (Dhaka, Bangladesh), the development of the combined research protocol, and critically reviewed the manuscript. She will support collection of data in Bangladesh and analysis and interpretation of data for all sites. SOK: contributed to the development of the site-specific protocol for the sixth study (Kakamega, Kenya), the development of the combined research protocol and critically reviewed the manuscript. He will support collection of data in Kenya and analysis and interpretation of data for all sites. JC: contributed to the development of the site-specific protocol for the sixth study (Ndola, Zambia), the development of the combined research protocol, and critically reviewed the manuscript. He will support collection of data in Zambia and analysis and interpretation of data for all sites. MP-O: contributed to the development of the fifth study and the site-specific protocol for the sixth study (Iquitos, Peru), the development of the combined research protocol, and critically reviewed the manuscript. She will support collection of data in Peru and analysis and interpretation of data for all sites. VO: contributed to the development of the combined research protocol, contributed to the drafting the manuscript, and critically reviewed the manuscript. He will support analysis and interpretation of data for all sites. RY: contributed to the development of the site-specific protocol for the third study (Adelaide, Australia) and the development of the combined research protocol, contributed to the drafting the manuscript, and critically reviewed the manuscript. He will support laboratory analysis of breath test results and analysis and interpretation of data for all sites. MNK: contributed to the development of the fifth study and the site-specific protocol for the sixth study (Iquitos, Peru), the development of the combined research protocol, contributed to the drafting the manuscript, and critically reviewed the manuscript. She will support data collection in Peru, Ianalysis of EED biomarkers, and analysis and interpretation of data for all sites. DM: contributed to the development of the site-specific protocol for the first and second studies (Glasgow, UK) and the development of the combined research protocol, contributed to the drafting the manuscript, and critically reviewed the manuscript. He will support laboratory analysis of breath test results and analysis and interpretation of data for all sites. All authors approve the final version to be published and agree to be accountable for all aspects of the work.

Funding This work was supported by the International Atomic Energy Agency (IAEA) coordinated research project (E4.10.16.) and a grant to DM from Nutriset in support of developing the SBT.

Competing interests None declared.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

Supplemental material This content has been supplied by the author(s). It has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content

includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>.

ORCID iDs

Gwenyth O Lee <http://orcid.org/0000-0002-7889-3852>

Paul Kelly <http://orcid.org/0000-0003-0844-6448>

REFERENCES

- United Nations' Children's Fund, World Health Organization WB. *Joint child malnutrition Estimates—Levels and trends*. Geneva, Switzerland, 2017.
- United Nations' Children's Fund. *Malnutrition: current status and progress, 2017*. Available: <https://data.unicef.org/topic/nutrition/malnutrition/#>
- Sudfeld CR, McCoy DC, Danaei G, et al. Linear growth and child development in low- and middle-income countries: a meta-analysis. *Pediatrics* 2015;135:e1266–75.
- Gandhi M, Ashorn P, Maleta K, et al. Height gain during early childhood is an important predictor of schooling and mathematics ability outcomes. *Acta Paediatr* 2011;100:1113–8.
- Fink G, Rockers PC. Childhood growth, schooling, and cognitive development: further evidence from the young lives study. *Am J Clin Nutr* 2014;100:182–8.
- Adair LS, Fall CHD, Osmond C, et al. Associations of linear growth and relative weight gain during early life with adult health and human capital in countries of low and middle income: findings from five birth cohort studies. *Lancet* 2013;382:525–34.
- McGovern ME, Krishna A, Aguayo VM, et al. A review of the evidence linking child stunting to economic outcomes. *Int J Epidemiol* 2017;46:1171–91.
- Black RE, Allen LH, Bhutta ZA, et al. Maternal and child undernutrition: global and regional exposures and health consequences. *Lancet* 2008;371:243–60.
- Dewey KG. Reducing stunting by improving maternal, infant and young child nutrition in regions such as South Asia: evidence, challenges and opportunities. *Matern Child Nutr* 2016;12 Suppl 1:27–38.
- Budge S, Parker AH, Hutchings PT, et al. Environmental enteric dysfunction and child stunting. *Nutr Rev* 2019;77:240–53.
- Shrimpton R, Victora CG, de Onis M, Hallal PC, et al. Worldwide timing of growth faltering: implications for nutritional interventions. *Pediatrics* 2001;107:e75.
- Dewey KG, Adu-Afarwah S. Systematic review of the efficacy and effectiveness of complementary feeding interventions in developing countries. *Matern Child Nutr* 2008;4 Suppl 1:24–85.
- Luby SP, Rahman M, Arnold BF, et al. Effects of water quality, sanitation, handwashing, and nutritional interventions on diarrhoea and child growth in rural Bangladesh: a cluster randomised controlled trial. *Lancet Glob Health* 2018;6:e302–15.
- Null C, Stewart CP, Pickering AJ, et al. Effects of water quality, sanitation, handwashing, and nutritional interventions on diarrhoea and child growth in rural Kenya: a cluster-randomised controlled trial. *Lancet Glob Health* 2018;6:e316–29.
- Humphrey JH, Mbuya MNN, Ntozini R, et al. Independent and combined effects of improved water, sanitation, and hygiene, and improved complementary feeding, on child stunting and anaemia in rural Zimbabwe: a cluster-randomised trial. *Lancet Glob Health* 2019;7:e132–47.
- Prendergast A, Kelly P. Enteropathies in the developing world: neglected effects on global health. *Am J Trop Med Hyg* 2012;86:756–63.
- Veitch AM, Kelly P, Zulu IS, et al. Tropical enteropathy: a T-cell-mediated crypt hyperplastic enteropathy. *Eur J Gastroenterol Hepatol* 2001;13:1175–81.
- Kelly P, MWANSA J, Menzies IAN, et al. Responses of small intestinal architecture and function over time to environmental factors in a tropical population. *Am J Trop Med Hyg* 2004;70:412–9.
- Manary MJ, Abrams SA, Griffin IJ, et al. Perturbed zinc homeostasis in rural 3–5-y-Old Malawian children is associated with abnormalities in intestinal permeability attributed to tropical enteropathy. *Pediatr Res* 2010;67:671–5.
- Kelly P, Besa E, Lees J, et al. PTH-231 Pathways of epithelial leakage and zinc malabsorption in environmental enteropathy imaged by confocal endomicroscopy. *Gut* 2015;64:A511.3–2.
- Keusch GT, Denno DM, Black RE, et al. Environmental enteric dysfunction: pathogenesis, diagnosis, and clinical consequences. *Clin Infect Dis* 2014;59 Suppl 4:S207–12.
- Jimenez L, Duggan CP. Biomarkers of environmental enteric dysfunction: the good, the bad and the ugly. *J Pediatr Gastroenterol Nutr* 2018;65:4–5.
- Faubion WA, Camilleri M, Murray JA, et al. Improving the detection of environmental enteric dysfunction: a lactulose, rhamnose assay of intestinal permeability in children aged under 5 years exposed to poor sanitation and hygiene. *BMJ Glob Health* 2016;1:e000066.
- Denno DM, Tarr PI, Nataro JP. Perspective piece environmental enteric dysfunction : a case definition for intervention trials. *Am J Trop Med Hyg* 2017:1–9.
- 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;88:390–6.
- Lee GO, Kosek P, Lima AAM, et al. Lactulose: mannitol diagnostic test by HPLC and LC-MS/MS platforms: considerations for field studies of intestinal barrier function and environmental enteropathy. *J Pediatr Gastroenterol Nutr* 2014;59:544–50.
- Campbell RK, Schulze KJ, Shaikh S, et al. Biomarkers of environmental enteric dysfunction among children in rural Bangladesh. *J Pediatr Gastroenterol Nutr* 2017;65:40–6.
- Watanabe K, Petri WA. Environmental enteropathy: elusive but significant subclinical abnormalities in developing countries. *EBioMedicine* 2016;10:25–32.
- Fagundes-Neto U, Viaro T, Wehba J, et al. Tropical enteropathy (environmental enteropathy) in early childhood: a syndrome caused by contaminated environment. *J Trop Pediatr* 1984;30:204–9.
- Haghighi P, Wolf PL, Durie P. Tropical sprue and subclinical enteropathy: a vision for the nineties. *Crit Rev Clin Lab Sci* 1997;34:313–41.
- Owino V, Ahmed T, Freemark M, et al. Environmental enteric dysfunction and growth Failure/Stunting in global child health. *Pediatrics* 2016;138:e20160641.
- Gorvel JP, Ferrero A, Chambaud L, et al. Expression of sucrase-isomaltase and dipeptidylpeptidase IV in human small intestine and colon. *Gastroenterology* 1991;101:618–25.
- Fenger HJ, Madsen PR, Fenger HJ, et al. Sucrase Deficiency in Greenland : Incidence and Genetic Aspects Sucrase Deficiency in Greenland. *Scan J Gastroenterol* 2009;22:24–8.
- Ritchie BK, Brewster DR, Davidson GP, et al. 13C-sucrose breath test: novel use of a noninvasive biomarker of environmental gut health. *Pediatrics* 2009;124:620–6.
- Robayo-Torres CC, Diaz-Sotomayor M, Hamaker BR, et al. 13C-Labeled-Starch breath test in congenital sucrase-isomaltase deficiency. *J Pediatr Gastroenterol Nutr* 2018;66:S61–4.
- Gupta SK, Chong SK, Fitzgerald JF. Disaccharidase activities in children: normal values and comparison based on symptoms and histologic changes. *J Pediatr Gastroenterol Nutr* 1999;28:246–51.
- Tooley KL, Saxon BR, Webster J, et al. A novel non-invasive biomarker for assessment of small intestinal mucositis in children with cancer undergoing chemotherapy. *Cancer Biol Ther* 2006;5:1275–81.
- Gericke B, Amiri M, Naim HY. The multiple roles of sucrase-isomaltase in the intestinal physiology. *Mol Cell Pediatr* 2016;3:2.
- Yu J, Ordiz MI, Stauber J, et al. Environmental Enteric Dysfunction Includes a Broad Spectrum of Inflammatory Responses and Epithelial Repair Processes. *Cell Mol Gastroenterol Hepatol* 2016;2:158–74.
- Clarke JM, Pelton NC, Bajka BH, et al. Use of the ¹³C-sucrose breath test to assess chemotherapy-induced small intestinal mucositis in the rat. *Cancer Biol Ther* 2006;5:34–8.
- Mauger CA, Butler RN, Geier MS, et al. Probiotic effects on 5-fluorouracil-induced mucositis assessed by the sucrose breath test in rats. *Dig Dis Sci* 2007;52:612–9.
- Hayes DF. Biomarker validation and testing. *Mol Oncol* 2015;9:960–6.
- Teutsch SM, Bradley LA, Palomaki GE, et al. The evaluation of genomic applications in practice and prevention (EGAPP) initiative: methods of the EGAPP Working group. *Genet Med* 2009;11:3–14.
- Yori PP, Lee G, Olórtgui MP, et al. Santa Clara de Nanay: the MAL-ED cohort in Peru. *Clin Infect Dis* 2014;59 Suppl 4:S310–6.
- Lee GO, McCormick BJJ, Seidman JC, et al. Infant nutritional status, feeding practices, enteropathogen exposure, socioeconomic status,



- and illness are associated with gut barrier function as assessed by the lactulose mannitol test in the MAL-ED birth cohort. *Am J Trop Med Hyg* 2017;97:281–90.
- 46 McCormick BJJ, Lee GO, Seidman JC, *et al.* Dynamics and trends in fecal biomarkers of gut function in children from 1–24 months in the MAL-ED study. *Am J Trop Med Hyg* 2017;96:465–72.
- 47 Psaki SR, Seidman JC, Miller M, *et al.* Measuring socioeconomic status in multicountry studies: results from the eight-country MAL-ED study. *Popul Health Metr* 2014;12:1–11.
- 48 Coates J, Swindale A, Bilinsky P. *Household food insecurity access scale (HFIAS) for measurement of food access: indicator guide.* Washington DC, 2007.
- 49 Ruel MT. *Operationalizing dietary diversity: a review of measurement issues.* 2003.
- 50 Braden B, Lembcke B, Kuker W, *et al.* 13C-breath tests: current state of the art and future directions. *Dig Liver Dis* 2007;39:795–805.
- 51 Harper KM, Mutasa M, Prendergast AJ, *et al.* Environmental enteric dysfunction pathways and child stunting: a systematic review. *PLoS Negl Trop Dis* 2018;12:e0006205:1–23.
- 52 WHO Multicentre Growth Reference Study Group. *Who child growth standards based on length/height, weight and age.* 2006.
- 53 Moinester M, Gottfried R. Sample size estimation for correlations with pre-specified confidence interval. *TQMP* 2014;10:124–30.
- 54 Hulley SB, Cummings SR, Browner WS, *et al.* *Designing clinical research.* Lippincott Williams & Wilkins, 2013.
- 55 Naylor C, Lu M, Haque R, *et al.* Environmental enteropathy, oral vaccine failure and growth faltering in infants in Bangladesh. *EBioMedicine* 2015;2:1759–66.
- 56 Kosek MN, Mduma E, Kosek PS, *et al.* Plasma tryptophan and the kynurenine tryptophan ratio are associated with the acquisition of linear growth deficits and oral vaccine underperformance. *Am J Trop Med Hyg* 2016;95:928–37.
- 57 Colston JM, Peñataro Yori P, Colantuoni E, *et al.* A methodologic framework for modeling and assessing biomarkers of environmental enteropathy as predictors of growth in infants: an example from a Peruvian birth cohort. *Am J Clin Nutr* 2017;106:245–55.