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Optimisation, validation and field applicability of a 13C-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

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

Trial registration number NCT04109352; Pre-results.

Strengths and limitations of this study

- A validated non-invasive 13C-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 $^{13}CO_2$ 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.

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 performance[4–6] 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 ^{13}C are particularly promising for this purpose, as $^{13}CO_2$ 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 ^{13}C labelled sucrose have been employed in animal models and in childhood cancer chemotherapy as a biomarker of enteropathy.[37 40 41] A ^{13}C-labelled sucrose breath test (^{13}C-SBT) based on naturally ^{13}C 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 $^{13}CO_2$ signal above basal ^{13}C abundance, making it unsuitable for routine use in very young children. Furthermore, between-population differences in the consumption of naturally ^{13}C- enriched C_4 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 ^{13}C-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 $^{13}CO_2$ substantially above the baseline value that might be expected due to diet, thereby improving the signal to noise ratio. To assess whether enriched ^{13}C-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 (^{13}C-SBT), among adults and children. To accomplish this, we will complete five coordinated studies. We will first optimise the ^{13}C-SBT protocol and second, validate the ^{13}C-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 ^{13}C-SBT report similar information in comparison to the original naturally enriched ^{13}C-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 sucrase 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 sucrase 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 ($^{13}C_6$ fructose; $^{13}C_6$ glucose and $^{13}C_{12}$ 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 $^{13}CO_2$ will be collected serially for 4–6 hours and $^{13}CO_2$ 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 kyurenine: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, Peru[44] (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 +2 SD.

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

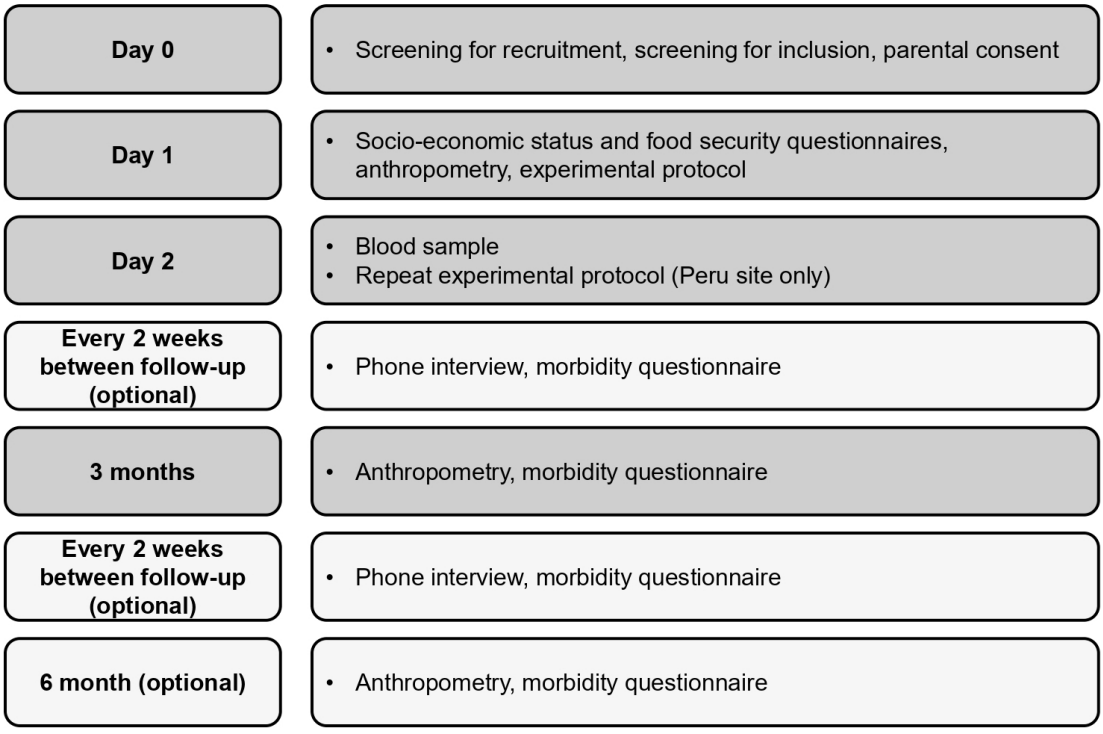

Figure 1 Flow diagram for phase 2 coordinated study protocol shown here is the timeline of participant activities. Darker grey boxes represent core study activities, while light grey boxes indicate activities that some, but not all, study sites will undertake. Primary and secondary study aims are based 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 C_4 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

^{13}C 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 ^{13}C-SBT. Other metrics for summarising the ^{13}C-SBT test, at both specific time points and overall, will also be considered.

LR ratio: To assess the relationship between the ^{13}C-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 platform[26] 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 ^{13}C-SBT to the LR ratio, to the per cent lactulose recovery, and to the percent rhamnose recovery.
2. Characterise the relationship between the ^{13}C-SBT and child anthropometry (LAZ and WAZ).
3. Characterise the relationship between ^{13}C-SBT and childhood linear growth (change in LAZ) over 3 months and over 6 months.

Secondary outcome measures

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

Other prespecified outcome measures are

1. Reproducibility of the ^{13}C-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 ^{13}C-SBT.
   We will determine if significant associations exist between ^{13}C-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 ^{13}C-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 ^{13}C-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 ^{13}C-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 ^{13}C-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 ^{13}C-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 Ritchie[34] 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 ^{13}C-SBT and lactulose and rhamnose excretion individually.

To characterise the relationship between the ^{13}C-SBT test and child anthropometry, we will compare be cPDR90 and T50 values for the ^{13}C-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 ^{13}C-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 ^{13}C-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 approachs,[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 ^{13}C-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 ^{13}C-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.
^{13}C-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 ^{13}C-SBT and subsequent change in WAZ, and LAZ,[25 56 57] which enhances the comparably 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 ^{13}C-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 ^{13}C-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 ^{13}C-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 ^{13}C-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 ^{13}C-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.

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