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 13C-enriched sucrose, derived from maize, to assay intestinal sucrose 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 13C-sucrose to quantify physiological dysfunction in EE in relevant target populations.

Methods and analysis We use the 13C-sucrose breath test (13C-SBT) to evaluate intestinal sucrose activity in two phases. First, an optimisation and validation phase will (1) confirm that a 13C-SBT using highly enriched sucrose tracers reports similar information to the naturally enriched 13C-SBT; (2) examine the dose–response relationship of the test to an intestinal sucrose inhibitor; (3) validate the 13C-SBT in paediatric coeliac disease (4) validate the highly enriched 13C-SBT against EE defined by biopsy in adults and (5) validate the 13C-SBT against EE defined by the urinary lactulose:rahamose 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 13C-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.

Strengths and limitations of this study

► A validated non-invasive 13C-sucrose breath test (13C-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 13C-SBT to characterise intestinal function in children with possible EE. However, this test is limited by low signal to noise in the breath 13CO₂ 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 13C-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. Stunting is associated with increased child morbidity and mortality, poorer cognitive development, school performance, and lower adult wages, and, for girls, poorer maternal health outcomes.

The aetiology of stunting is multifactorial, including generational and prenatal factors. Postnatal exposures also play a major role. Most postnatal growth faltering occurs around 6–18 months of age, 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. However, nutritional interventions lead to only small to moderate improvements in growth, 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. 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, including diffuse, upper small bowel villous atrophy accompanied by evidence of barrier disruption and inflammation. 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. This increases the risk of nutrient deficiency, which may in turn further impair gut immune responses. It is theorised that this condition, successively termed, ‘tropical enteropathy’, ‘environmental enteropathy (EE)’ and most recently, ‘environmental enteric dysfunction’, 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. 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, or composite scores based on multiple biomarkers intended to capture multiple domains of gut dysfunction. 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. The relationship of alternative biomarkers to EE, and to each other, is also not well understood. 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. 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. Despite these limitations, the shift in nomenclature from ‘EE’ to ‘environmental enteric dysfunction’, first proposed by Keusch et al 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 macro- 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. Zinc metabolism can be measured though carefully performed, dual stable isotope studies. 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. 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. 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), inherited deficiencies are uncommon. SI activity is diminished in villus blunting, thus representing a possible surrogate marker of small intestinal function and integrity.
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. A recent transcriptomic study also described SI activity as potentially altered by EE.

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. 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. 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).

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)                                             | Adelaide, Australia |
| Study 4 Validation          | Case–control  | Villous atrophy Intestinal sucrase activity                                            | Adults from an EE setting (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)                                        | 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 (\( ^{13}C\) fructose; \( ^{13}C\) glucose and \( ^{13}C\) 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.\(^4\). To assess gut permeability, participants will also receive coadministration of 5g lactulose, 1g rhamnose, 0.5g xylose, 0.2g 3-O-methyl-D-glucose and 5g 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 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 re-measure 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 +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
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).\textsuperscript{32}  

Our primary outcome measures are  
1. Comparison of the $^{13}$C-SBT to the LR ratio, to the percent 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.\textsuperscript{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.\textsuperscript{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 $^{13}$C-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).\textsuperscript{53}  

The minimal detectable correlation within each site, would be 0.31.\textsuperscript{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\textsuperscript{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 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,\textsuperscript{37} 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
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, 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 $^{13}$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. 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.

Our staged validation approach and multisite design are intended to overcome these limitations and may serve as a template for future EE biomarker studies.

### Table 2
| 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                                                                         |

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

$^{13}$C-SBT, $^{13}$C-sucrose breath test.
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, $^{15}$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 peptides 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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Appendix 1: Unified Phase 2 Study Protocol

Phase 2 study protocol: The $^{13}$C-SBT, LR test, and D$_2$O dilution to assess body composition will be completed during a single visit with each participant. The study team will screen for recent diarrhea, antibiotic usage, and anti-inflammatory usage in the past month (anti-inflammatories are known to induce transient intestinal permeability$^1$ as well as enterocyte injury). If any of these are reported by the parents, the appointment will be scheduled one month from the date of the diarrheal episode or antibiotic/anti-inflammatory use.

Guardians will be asked to fast their child for one hour prior to the appointment. Time will be given for the child to settle and adjust to their surroundings, including time for the child to play with, and become familiar with, the breath sampling equipment. Two baseline breath collection measurements will be completed, using a cannula apparatus for breath collection, consisting of a piece of PVC tubing taped just underneath the child’s nostril and controlled with a 3-way tap, or, if the field team prefers it, a face mask and breath collection bag. Exhaled breath will then be transferred to Labco Exetainers (evacuated tubes Labco Order No. 428W or 439W, Burlington, North Carolina, USA) either by positive displacement (syringe) or using evacuated extainer (bag). A baseline urine sample will be collected, and, for sites using deuterium dilution technique with analysis of saliva samples to assess body composition$^2$ (Bangladesh, India, Kenya, and Zambia), a baseline saliva sample will be collected.

To avoid $^{13}$C$_{12}$-sucrose loss, the $^{13}$C$_{12}$-sucrose solution and the LR solution will be administered separately. First, the 40 mg/mL $^{13}$C$_{12}$-sucrose solution will be administered at a dosage of 10µL per kg body weight (spiked in 1mL of drinking water). For example, for a child weighing 10kg, the dosage will be 100µL spiked in 1mL of drinking water. This will be followed a 2mL chaser of drinking water to rinse the vial.

Secondly, a 9mL of drinking water spiked with 1g lactulose and 0.2g rhamnose will be administered, and again a 2 mL chaser of drinking water will be used to rinse the vial. The total volume (1mL sucrose solution + 2mL chaser + 9mL LR solution + 2mL chaser) is 14 mL.

If the child spits out, vomits, or fails to swallow all the sugar solution, the test will be canceled and re-scheduled. After the child has consumed the sugars, a standard dose of deuterium oxide (D$_2$O) based on IAEA protocols will also be administered to the child. As soon as both the sugar solutions and the D$_2$O have been administered the child will be encouraged to drink water. After 90 minutes, the child will be given a standardized meal. The choice of standardized meal will be site-specific but may include egg, legumes, or rice and will not include any sugary foods or dairy products.

Breath sample collection: Using a stopwatch, breath samples will be collected every 15 minutes for the first 90 minutes following the administration of the sugar solution, and then every 30 minutes until 240 minutes (4 hours) have passed.

Urine sample collection: All urine passed in the 30-120 minutes of the test will be collected and combined for analysis, and any urine passed in the 120-300 minutes of the test will be collected and combined separately. The volume of all voids will be recorded. Both 2- and 5-hour LR tests are common in the literature$^3$–$^5$, with some evidence that 2-hour urine collections better capture small intestinal, rather than colonic, absorption$^5$. Here, both measures will be collected to enhance comparability with prior reports. 5-hour recoveries of lactulose and rhamnose will be calculated as the weighted average of the 30-120 and 120-300-minute samples. Samples will either be
stored with chlorhexidine or will be collected without preservative and stored immediately at -80. We will examine whether chlorhexidine results in interference during LC-MS/MS analysis.

**Saliva sample collection:** For sites measuring D\(_2\)O via saliva, the sample will be collected between 180 and 210 minutes of the test.

**Plasma sample collection:** After the breath/urine collection is complete, a second visit will be scheduled with study families to collect a plasma sample. Families will be asked to fast the child for 6 hours prior to the blood draw, and 2mL of blood will be collected in K2 EDTA. Following collection, the sample will be centrifuged and stored at -80C pending analysis.

**Anthropometry and questionnaire data:** The length, weight, and head circumference of each child will be measured, as will the height and weight of their mothers. Standard questionnaires to capture key socio-economic\(^6\) and demographic information, child dietary diversity (including recent consumption of C4 foods (e.g. maize, sugar cane, and sorghum or millet), and household food insecurity\(^7\), will be administered. Three months following the initial test, a third appointment will be made with the family to re-measure anthropometry, and to administer a questionnaire asking about morbidity over the past three months. Key data are summarized in Appendix 2.

**Additional site activities:** In addition to coordinated study activities, some activities will only be undertaken by one, or a sub-group, of study sites. In one site (Peru), the \(^{13}\)C-SBT test will be repeated after 2 days on 40 children, to assess test reproducibility. Several secondary EE biomarker assays of interest were also identified, to be assayed by sites resource permitting. These include, in order of priority, plasma fatty acid binding protein (FABP); plasma LPS binding protein (LPS-BP); and stool alpha-1-antitrypsin (AAT) and stool myeloperoxidase (MPO).

**Laboratory analyses:** Breath samples from four sites (Peru, Jamaica, Kenya, and Zambia) will be sent to Dr. Roger Yazbek at the South Australian Breath Analysis Research Laboratories (SABAR Lab) where they will be analyzed via ABCA Isotope Ratio Mass Spectrometry. To minimize of inter-platform variability in dual-sugar testing\(^8\), urine samples for lactulose and rhamnose will be performed either on a single platform, on two or more standardized platforms (to be determined). Breath sample analysis for Bangladesh and India will be analyzed at Saint John’s Research Institute, Bangalore.

**Deuterium dilution testing:** Based on available instrumentation, one site (India) will assess body composition analysis through D\(_2\)O analysis of urine, and four sites (Peru, Jamaica, Bangladesh, Kenya, and Zambia) will use saliva. Deuterium equilibrates faster in saliva compared to urine\(^9\) and deuterium dilution analysis in saliva requires a higher dose and different instrumentation (Fourier Infrared Spectroscopy versus Isotope Ratio Mass Spectrometer) compared to urine sampling. However, the collection of urine or saliva for total body water analysis has been standardized and validated, with comparable performance demonstrated in infants younger than those we propose to measure in this study\(^10\). Deuterium enrichment of urine will be assessed using a Delta V advantage Isotope Ratio Mass Spectrometer (Thermo Fisher scientific Inc)\(^11\). Saliva samples will be measured for their deuterium enrichment in duplicate by Fourier Transformed Infrared Spectrophotometry (4500t FTIR, Agilent Technologies, CA, USA).
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Appendix 2: Key Study Data

| Key variable          | Detail                                      |
|-----------------------|---------------------------------------------|
| Child age             | Months                                      |
| Child sex             | Male; female                                |

**Anthropometry:**

| Detail                  |                              |
|-------------------------|------------------------------|
| Birthweight             | kg                            |
| Baseline weight         | kg                            |
| Baseline length         | cm                            |
| FM                      | Fat mass, measured by deuterium dilution |
| FFM                     | Fat free mass, measured by deuterium dilution |
| Weight at 3-month follow-up | kg                       |
| Length at 3-month follow up | cm                      |
| Maternal weight         | kg                            |
| Maternal height         | cm                            |

**Questionnaire data:**

| Detail                                      |
|---------------------------------------------|
| Household food security                     |
| Household socio-economic status             |
| Child dietary diversity score               |
| Recent child morbidity                      |
| EE Biomarkers:                              |

| Detail                  |
|-------------------------|
| LR                      |
| FABP                    |
| KT                      |
| MPO                     |
| AAT                     |

Urinary lactulose: rhamnose ratio
Plasma fatty-acid binding protein
Plasma kynurenine: tryptophan ratio
Fecal myeloperoxidase
Fecal alpha-1-antitrypsin
# Urine and Breath Collection Form Version 1.3

| Question                                                                 | Code | Response |
|--------------------------------------------------------------------------|------|----------|
| Study researcher/Nurse/Fieldworker ID                                   |      |          |
| Today's date (DD/MMM/YY)                                                |      |          |
| Time of arrival to Study Clinic                                         | Time (24 h Scale; HH:MM) |
| Screening questions                                                      |      |          |
| Diarrhea in past month                                                  | Yes = 01 | 00 |
| Antibiotics in past month                                               | Yes = 01 | 00 |
| Anti-inflammatories in past month                                       | Yes = 01 | 00 |
| If any of these questions (03, 04, 05) was yes, please re-schedule the test one month from the date of the diarrhea or antibiotic/NSAID use |
| Standard meal given (rice: legume mix, egg) or breastmilk              | Tick | Idly /Kichadi/ Hard-boiled egg |
| Time of completion of standard meal                                     | Time (24 h Scale; HH:MM) |

## BASELINE BREATH COLLECTION

| Breath sample tube number | Time of completion | Comments (note crying, difficult sample collection, etc.) |
|---------------------------|--------------------|----------------------------------------------------------|
| Baseline                  | 🟢 🟢 : 🟢 🟢       |                                                          |

## BASELINE URINE COLLECTION (LR and D₂O)

| Urinary collection Time* | Collection Volume (mL) | Time of Collection | Comments (please note any spillage) |
|--------------------------|------------------------|-------------------|-------------------------------------|
| Baseline                 | 🟢 🟢 : 🟢 🟢       |                  |                                     |

*Use baseline urine for LR and Body composition (D₂O)

**DO NOT ADMINISTER SUGAR SOLUTION UNTIL THE CHILD HAS FULLY VOIDED THEIR BLADDER AND THE TIME IS 1 HOUR FROM THE LAST MEAL**

| Vial label of ¹³C₁₂-sucrose solution | Time the ¹³C₁₂-sucrose was first consumed | Total volume (mL) of sucrose solution consumed |
|--------------------------------------|------------------------------------------|-----------------------------------------------|
|                                      | 🟢 🟢 : 🟢 🟢                             |                                               |

| Total volume (mL) of chaser consumed | Time the LR solution was consumed: | Start time: _ : _ | End time: _ : _ | Total volume (mL) of LR solution consumed | Total volume (mL) of chaser consumed |
|--------------------------------------|------------------------------------|-----------------|----------------|------------------------------------------|-----------------------------------|
Child ID Code: S B T -

| How many minutes did it take the child to consume the LR solution in full? | 01-20 minutes |
|---------------------------------------------------------------|--------------|
| Was the LR solution consumed in full?                         | Yes = 01     |
| Weight of pre-weighed tissue =                                | No = 00      |
| Weight of soaked tissue =                                     |              |

If the child spits out, vomits, or fails to swallow all the sugar solution, the test cannot be completed. Please stop and re-schedule the test for another day.

| Total volume (mL) of deuterium water consumed                  |              |
|---------------------------------------------------------------|--------------|
| Total volume (mL) of chaser consumed                          |              |
| Was the deuterium dose consumed in full?                      | Yes = 01     |
| Weight of pre-weighed tissue =                                | No = 00      |
| Weight of soaked tissue =                                     |              |

A pre-weighed napkin should be placed under the neck to collect any spill from the dose. This should be weighed immediately after or placed in a small airtight container to avoid evaporation if immediate weighing is not possible.

Start collection: Encourage the child to drink water throughout the test.

Breath sample tube time

| Breath sample tube time | Exact collection time (use a stopwatch in minutes) | Comments (child was crying Y/N) | Urinary collection Time | Collection Volume (mL) | Exact collection time (using a stopwatch in minutes) | Comments (please note any spillage) |
|-------------------------|---------------------------------------------------|---------------------------------|-------------------------|------------------------|---------------------------------------------------|---------------------------------|
| 15 minutes              |                                                   |                                 |                         |                        | 30 to 90 minutes                                  |                                 |
| 30 minutes              |                                                   |                                 |                         |                        |                                                   |                                 |
| 45 minutes              |                                                   |                                 |                         |                        |                                                   |                                 |
| 60 minutes              |                                                   |                                 |                         |                        |                                                   |                                 |
| 75 minutes              |                                                   |                                 |                         |                        |                                                   |                                 |
| 90 minutes              |                                                   |                                 |                         |                        |                                                   |                                 |

At this time, please give the child the standardized meal

| Standard meal given (rice: legume mix, egg) or breastmilk | Tick | Idly /Kichadi/ Hard-boiled egg/ milk without sugar/ milk + beet sugar |
|-----------------------------------------------------------|------|---------------------------------------------------------------------|
| Time of consumption of standard meal                      |      |                                                                     |
| 120 minutes                                               |      |                                                                     |
| 150 minutes                                               |      |                                                                     |
| 180 minutes                                               |      |                                                                     |
| Urine                                                     |      |                                                                     |
| 210 minutes                                               |      |                                                                     |
| 240 minutes                                               |      |                                                                     |

Total:
Child ID Code: S B T -

| Question                                                                 | Range         | Yes | No |
|--------------------------------------------------------------------------|---------------|-----|----|
| How many loose stools were passed during the breath/urine collection?   | 00-15, NA     |     |    |
| Did any breastfeeding occur in the first 90 minutes of the test?        | Yes = 01      |     |    |
| (If the mother needed to comfort the child)                             | No = 00       |     |    |
| Did the child consume any non-breastmilk liquids or foods during the    | Yes = 01      |     |    |
| first 90 minutes of the test? (excluding water, which should be        | No = 00       |     |    |
| encouraged throughout the test)                                         |               |     |    |
| Weight of sipper with water at start of experiment (g)                  |               |     |    |
| Weight of sipper with water at end of experiment (g)                    |               |     |    |

**Anthropometry (take measurement in triplicates)**

| Measurement                 | Measurement 1 | Measurement 2 | Measurement 3 | Measurement 4 |
|-----------------------------|---------------|---------------|---------------|---------------|
| Height (cm)                 |               |               |               |               |
| Weight (kg)                 |               |               |               |               |
| Head circumference (cm)     |               |               |               |               |
| MUAC (cm)                   |               |               |               |               |
| Question                                                                 | Code | Response |
|------------------------------------------------------------------------|------|----------|
| 01 Study researcher/ Nurse/ Fieldworker ID                             |      |          |
| 02 Today’s date (DD/MMM/YY)                                            |      |          |
| 03 Sample ID:                                                          |      |          |
| 04 Time of arrival of sample at laboratory Time (24 Hr Scale; HH:MM)   |      |          |
| 05 Please confirm that each sample is complete:                        |      |          |
| 15-minute sample A                                                     |      | 15-minute sample B |
| 30-minute sample A                                                     |      | 30-minute sample B |
| 45-minute sample A                                                     |      | 45-minute sample B |
| 60-minute sample A                                                     |      | 60-minute sample B |
| 75-minute sample A                                                     |      | 75-minute sample B |
| 90-minute sample A                                                     |      | 90-minute sample B |
| 120-minute sample A                                                    |      | 120-minute sample B |
| 150-minute sample A                                                    |      | 150-minute sample B |
| 180-minute sample A                                                    |      | 180-minute sample B |
| 210-minute sample A                                                    |      | 210-minute sample B |
| 240-minute sample A                                                    |      | 240-minute sample B |

Breath samples should be labeled as follows: Study code + site code + participant code + timepoint + replicate identifier (A or B) e.g. SBT-ZAM1-P1-T60-A
Urine Reception in Laboratory  

| Question | Code | Response |
|----------|------|----------|
| 01 Study researcher/ Nurse/ Fieldworker ID | | |
| 02 Today’s date (DD/MMM/YY) | | / / |
| 03 Sample ID: | | |
| 04 Time of arrival of sample at laboratory | Time (24 Hr Scale; HH:MM) | : |
| 05 Number of aliquots of urine collected from 30 - 90 minutes (should be 4 aliquots of 250 µL) | 01-04 | |
| 06 Number of aliquots of urine collected from 90 minutes to 120 minutes (should be 2 aliquots of 250 µL) | 01-02 | |
| 07 Number of aliquots of urine collected from 120 minutes to 300 minutes (should be 2 aliquots of 250 µL) If applicable | 01-02 | |
| 08 Notes: | | |

_Urine samples should be labeled as follows:_ Study code + site code + participant code + timepoint (in minutes, from start time) + replicate identifier (A, B, C, D)

e.g. 30-90-minute urine: SBT-ZAM1-P001-T30-A

90-120-minute urine: SBT-ZAM1-P001-T90-A

120-300-minute urine: SBT-ZAM1-P001-T120-A
**Blood Sample Collection Version 1.0**  
(6 hours postprandial)

| Question                                                                 | Code       | Response |
|-------------------------------------------------------------------------|------------|----------|
| Study researcher/ Nurse/ Fieldworker ID                                 | 01         |          |
| Today's date (DD/MMM/YY)                                                | 02         |          |
| Before starting the test, when was the last time the child ate (either breastmilk or solid foods) (post prandial for how many hours) | 03         | Time (24 Hr Scale; HH:MM) : 00 |

*If the child has not fasted overnight, please reschedule the blood draw:*

| Question                                                                 | Code       | Response |
|-------------------------------------------------------------------------|------------|----------|
| Time of blood collection:                                               | 04         | Time (24 Hr Scale; HH:MM) : 00 |
| Tube with up to 2mL blood collected?                                     | 05         | Yes=01 No=00 |
| Notes:                                                                  | 06         |          |

*Blood samples should be labeled as follows:* Study code + site code + participant code + timepoint + replicate identifier (A or B) e.g. SBT-ZAM1-P1-T60-A
**Blood Sample Reception Version 1.0**

| Question                                                                 | Code | Response |
|-------------------------------------------------------------------------|------|----------|
| 01 Study researcher/ Nurse/ Fieldworker ID                              |      |          |
| 02 Date of blood collection (DD/MMM/YY)                                  |      |          |
| 03 Date of blood arrival in laboratory                                  |      |          |
| 04 Time of arrival                                                       |      |          |
| 05 Tube 1 with up to 2mL blood received?                                 | Yes=01 |          |
| 06 Evidence of hemolysis?                                                | No=00 |          |
|                                                                             | Slight=01 |          |
|                                                                             | Severe=02 |          |
| 07 Sample ID:                                                           |      |          |
| 08 Date of Centrifugation:                                               |      |          |
| 09 Time of Centrifugation:                                               |      |          |
| 10 Number of 500µL Plasma Aliquots:                                     | 00-03 |          |
| 06 Notes:                                                               |      |          |

*Blood samples should be labeled as follows:* Study code + site code + participant code + timepoint + replicate identifier (A or B) e.g. SBT-ZAM1-P1-T60-A
| Question                                                                 | Code          | Response |
|-------------------------------------------------------------------------|---------------|----------|
| 01 Study researcher/Nurse/Fieldworker ID                                |               |          |
| 02 Today’s date (DD/MMM/YY)                                             |               |          |
| **A. Child information and anthropometry**                              |               |          |
| 01 Date of birth (DD/MMM/YY)                                            |               |          |
| 02 Sex of child                                                         | Male=01       |          |
| 03 Birthweight (kg) * (from birth record, if available)                 |               |          |
| 04 Current weight (kg) *                                                |               |          |
| 05 Current length (cm)                                                  |               |          |
| 06 Current head circumference (cm)                                      |               |          |
| **B. Maternal anthropometry**                                           |               |          |
| 01 Mother’s date of birth                                               |               |          |
| 02 Is the mother currently pregnant?                                    | Yes = 01      |          |
| 03 Mother’s Weight (kg)                                                 |               |          |
| 04 Mother’s Height (cm)                                                 |               |          |
| **C. Socio-economic Information (WAMI)**                                 |               |          |
| Please explain to the mother that these questions are standardized     |               |          |
| questions used around the world, some questions may be more             |               |          |
| applicable to them than others.                                         |               |          |
| 01 Was this child chosen for participation in the study because they    | Low SES= 01   |          |
| come from a low-SES community, or a high SES community?                 | High SES = 02 |          |
| 02 What is the main source of drinking water for members of your        | Piped into     |          |
| household?                                                              | dwelling = 01 |          |
|                                                                         | Piped to yard/plot = 02 |    |
|                                                                         | Public tap/stand pipe = 03 |    |
|                                                                         | Tube well or borehole = 04 |    |
|                                                                         | Protected well = 05 |    |
|                                                                         | Unprotected well = 06 |    |
|                                                                         | Surface water (river/ dam/ lake/pond /stream/canal/irrigation canal) = 07 |    |
|                                                                         | Other = 08     |          |
| 02a If other, describe:                                                 |               |          |
| 03 What is the main source of water used by your household for other    | Piped into     |          |
| purposes such as cooking and hand-washing?                              | dwelling = 01 |          |
|                                                                         | Piped to yard/plot = 02 |    |
|                                                                         | Public tap/stand pipe = 03 |    |
|                                                                         | Tube well or borehole = 04 |    |
|                                                                         | Protected well = 05 |    |
|                                                                         | Unprotected well = 06 |    |
|                                                                         | Surface water (river/ dam/ lake/pond /stream/canal/irrigation canal) = 07 |    |
**Child ID Code:**

| Question                                                                 | Yes | No |
|--------------------------------------------------------------------------|-----|----|
| **03a** If other, describe: **Child ID Code:** Other = 08 |     |    |
| **04** What kind of toilet facility do members of your household usually use? |     |    |
| No facility/bush/field or bucket toilet = 01                            |     |    |
| Pit latrine with flush = 02                                             |     |    |
| Flush to piped sewer system = 03                                       |     |    |
| Flush to septic tank = 04                                               |     |    |
| Flush to pit latrine = 05                                               |     |    |
| Flush to somewhere else = 06                                            |     |    |
| Other = 07                                                              |     |    |
| **04a** If other, describe:                                              |     |    |
| **05** Do you have a separate room which is used as a kitchen?          | Yes = 01 | No = 00 |
| **06** Does any member of your household have a bank account?           | Yes = 01 | No = 00 |
| **07** Does your household have a mattress?                             | Yes = 01 | No = 00 |
| **08** Does your household have a refrigerator?                         | Yes = 01 | No = 00 |
| **09** Does your household have a television?                           | Yes = 01 | No = 00 |
| **10** Does your household have a table?                                | Yes = 01 | No = 00 |
| **11** Does your household have a chair or bench?                       | Yes = 01 | No = 00 |
| **12** How many rooms are there in your house?                          | Yes = 01 | No = 00 |
| **13** How many people usually sleep in this household? 01-30 (people) |     |    |
| **14** Have you (the mother of the study child) ever attended school?   | Yes = 01 | No = 00 |
| *If no, skip to question 18.*                                            |     |    |
| **15** How many years of schooling have you completed? 00-20             |     |    |
| **16** *If younger than 25 years old:* Are you currently attending school or college? | Yes = 01; No = 00 |    |
| **17** What is the average monthly income for the entire household?     |     |    |
| **18** Currency                                                         |     |    |
| I = Indian rupees; J = Jamaica dollars; K = Zambian kwacha; P = Peruvian soles; S= Kenyan Shilling; T= taka |     |    |
| **D. Child’s dietary diversity**                                        |     |    |
| **01** Are you breastfeeding <CHILD>? If NO, then skip to Q.6            | Yes = 01 | No = 00 |
| **01a** Last night, how many times did you breastfeed <CHILD> from sunset to sunrise? |     |    |
| **01b** Yesterday, during the day, how many times did you breastfeed <CHILD>? |     |    |
| **02** Do you give <CHILD> infant formula? If NO, then skip to Q.9       | Yes = 01 | No = 00 |
| Question                                                                 | Answer Options |
|-------------------------------------------------------------------------|----------------|
| Last night, how many times did you feed <CHILD> formula from sunset to sunrise? | 00-99          |
| Yesterday, during the day, how many times did you feed <CHILD> formula? | 00-99          |
| Do you give <CHILD> other milks, such as tinned, powdered or fresh animal milk? If NO, then skip to Q.12 | Yes = 01  
No = 00       |
| Last night, how many times did you feed <CHILD> animal milks from sunset to sunrise? | 00-99          |
| Yesterday, during the day, how many times did you feed <CHILD> animal milk? | 00-99          |
| Yesterday, during the day or last night, did <CHILD> have:              |                |
| Plain water                                                             | Yes = 01  
No = 00       |
| Tea, coffee <local examples>?                                           | Yes = 01  
No = 00       |
| Fruit or vegetable juices?                                              | Yes = 01  
No = 00       |
| Any other liquids, such as sugar water, thin soup or broth, carbonated drinks <local examples> | Yes = 01  
No = 00       |
| Is <CHILD> eating any semi-solid, mashed or solid foods? If NO, go to Q24 | Yes = 01  
No = 00       |
| Thinking about yesterday, during the day and at night, did <CHILD> have any of these foods, even if they were in combination with other foods? |               |
| Maize?                                                                  | Yes = 01  
No = 00       |
| Sorghum?                                                                | Yes = 01  
No = 00       |
| Millet (any kind)?                                                      | Yes = 01  
No = 00       |
| Sugar cane or cane-derived sugar                                        | Yes = 01  
No = 00       |
| Rice, porridge, bread, noodles or other foods made from grains? (do not include foods made from maize, sorghum, or millet) Mention <local examples> | Yes = 01  
No = 00       |
| White potatoes, white yams, manioc, or other foods made from roots? Mention <local examples> | Yes = 01  
No = 00       |
| Question                                                                 | Yes | No |
|-------------------------------------------------------------------------|-----|----|
| 15 Carrots, squash, or sweet potatoes that are yellow or orange inside? Mention <local examples> | Yes = 01  
No = 00 |    |
| 16 Any dark green leafy vegetables such as spinach? Mention <local examples>  | Yes = 01  
No = 00 |    |
| 17 Foods made with beans, lentils, peas, corn, ground nuts? Mention <local examples> | Yes = 01  
No = 00 |    |
| 18 Ripe mangoes, papayas, or other sweet yellow/orange or red fruit? Mention <local examples> | Yes = 01  
No = 00 |    |
| 19 Any other fruits or vegetables such as banana, apple, oranges, tomatoes, avocado? Mention <local examples> (not including sugar cane) | Yes = 01  
No = 00 |    |
| 20 Liver, kidney, heart or other organ meats? Mention <local examples>  | Yes = 01  
No = 00 |    |
| 21 Any meat, such as chicken, beef, lamb, goat, duck (others)? Mention <local examples> | Yes = 01  
No = 00 |    |
| 22 Eggs? Mention <local examples> | Yes = 01  
No = 00 |    |
| 23 Fresh or dried fish or shellfish? Mention <local examples> | Yes = 01  
No = 00 |    |
| 24 Cheese, yogurt or other dairy products? Mention <local examples> | Yes = 01  
No = 00 |    |
| 25 Any sugary foods such as pastries, cakes or biscuits? Mention <local examples> | Yes = 01  
No = 00 |    |
| 26 Any commercially available foods for infants or young children? Mention <local examples> | Yes = 01  
No = 00 |    |
| 27 Yesterday, counting meals and snacks, how many times did you feed <CHILD>? | 00-99 |    |
| 28 How would you describe your child’s appetite? Would you say it is: | 01=poor,  
02=fair,  
03=good,  
04=very good |    |

**E. Food Security (HFIAS)**

| Question                                                                 | Yes | No |
|-------------------------------------------------------------------------|-----|----|
| 01 In the past four weeks, did you worry that your household would not have enough food? | No = 00  
Rarely = 01  
Sometimes = 02  
Often = 03 |    |
| 02 In the past four weeks, were you or any household member not able to eat the kinds of foods you preferred because of a lack of resources? | No = 00  
Rarely = 01  
Sometimes = 02  
Often = 03 |    |
| 03 In the past four weeks, did you or any household member have to | No = 00  
Rarely = 01  
Sometimes = 02  
Often = 03 |    |
## F. Child Morbidity

| Question                                                                 | Response Options                                      |
|--------------------------------------------------------------------------|-------------------------------------------------------|
| Does the child have diarrhea today?                                      | Yes = 01, No = 00, Doesn't know = 88                  |
| Over the past 1 week (including today), has your child had diarrhea?     | Yes = 01, No = 00, Doesn't know = 88                  |
| If yes to 02, for how many days?                                         | 01-07                                                 |
| Over the past 4 weeks (including today), has your child had diarrhea?    | Yes = 01, No = 00, Doesn't know = 88                  |
| If yes to 04, how many separate episodes?                                | 01-20, Doesn't know = 88                              |
| How many days per episode?                                               |                                                       |

**Note:** Episodes must be separated by at least 2 days without diarrhea

| Epidemiologic Cutpoints | Days |
|-------------------------|------|
| a. First episode        |      |
| b. Second episode       |      |
| c. Third episode        |      |

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**Epidemiologic Cutpoints:**

- **Diarrhea:** 3 days per episode.
- **Vomiting:** 10 days per episode.
- **Weight for Age:** Underweight if Z score <-2.0.
- **Weight for Height:** Stunting if Z score <-2.0.
| Morbidities       | Does child have symptom today? | If yes, how many days in past week including today? (1-7) | Has child had symptom in past 1 month? | If yes, how many episodes in the past month? (1-28) | Has child had symptom in past 3 months, if yes how many episodes? |
|-------------------|--------------------------------|--------------------------------------------------------|-------------------------------------|--------------------------------------------------|--------------------------------------------------------|
| 14 Common cold    | □ Yes □ No                    | □                                                   | □ Yes □ No                          | □                                                | □                                                      |
| 15 Cough          | □ Yes □ No                    | □                                                   | □ Yes □ No                          | □                                                | □                                                      |
| No. | Condition                          | Yes | No | Yes | No |
|-----|-----------------------------------|-----|----|-----|----|
| 16  | Difficulty in breathing           |     |    |     |    |
| 17  | Fever                             |     |    |     |    |
| 18  | Pus draining from ears            |     |    |     |    |
| 19  | Vomiting                          |     |    |     |    |
| 20  | Rashes                            |     |    |     |    |
| 21  | Convulsions                       |     |    |     |    |
| Question                                                                 | Code | Response |
|-------------------------------------------------------------------------|------|----------|
| 01 Study researcher/Nurse/Fieldworker ID                                |      |          |
| 02 Today’s date (DD/MMM/YY)                                             |      |          |
| 03 Current weight (kg)                                                  |      |          |
| 04 Current length (cm)                                                  |      |          |
| 05 Current head circumference (cm)                                       |      |          |