Epidemiology of Environmental Enteric Dysfunction, malnutrition and intestinal microbiota amongst children in Kilifi, Kenya

Thesis for Doctor of Philosophy in Medical Sciences

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Abstract

Thesis title: **Epidemiology of Environmental Enteric Dysfunction, malnutrition and intestinal microbiota amongst children in Kilifi, Kenya** | Name: Rosie Crane | College: Kellogg College Degree: Doctor of Philosophy (D.Phil.) | Term and year of submission: Trinity 2019

Environmental Enteric Dysfunction (EED) is a chronic intestinal inflammatory disorder that is highly prevalent amongst children in tropical low-income settings and associated with stunting (low height-for-age). I undertook two studies in coastal Kenya that aimed to explore the aetiology of EED. Firstly, in a cross-sectional survey across 12 villages, 509 under-fives from 229 randomly selected homesteads underwent anthropometry and provided a stool sample. Participant sociodemographic and health characteristics were determined by questionnaire. 419 stool samples underwent enzyme-linked immunosorbent assay (ELISA) for three markers of EED: alpha-1-antitrypsin, myeloperoxidase and neopterin. EED risk factors identified included older age, geophagy and recent acute illness. Secondly, in a birth cohort of 99 participants aged zero to nine months, 839 dual sugar absorption tests and 934 breath hydrogen tests were carried out at monthly intervals to measure EED and small intestinal bacterial overgrowth (SIBO) respectively. Stool samples were collected bi-weekly and 434 underwent ELISA for alpha-1-antitrypsin, myeloperoxidase and neopterin. Acute illness and antibiotic episodes were captured by active and passive surveillance. Breastfeeding status was recorded weekly. EED increased with age and was associated with stunting. In a multivariable model, EED was not associated with SIBO, acute illnesses, household water/sanitation/hygiene or antibiotics. In the same model, adjusted for age, EED was worse amongst those who had ceased exclusive breastfeeding, and for one EED biomarker this association was only seen during the rainy season.

Chapter 1: Background and introduction*

Environmental Enteric Dysfunction (EED) is a term used to describe chronic inflammation and villous atrophy at the small intestinal epithelium that affects most children in tropical low-income settings. EED is a recently recognised and as yet poorly understood condition that may have far-reaching impacts on child growth, health and development in tropical low-income settings. Often associated with stunting (low height-for-age) and wasting (low weight-for-height), EED is now the subject of significant research interest as investigators seek to define its causes, pathogenesis, consequences and possible preventive or curative approaches. Stunting affects 149 million children worldwide and is associated with increased childhood morbidity and mortality from infectious disease, as well as poorer neurodevelopmental outcomes and even risk of noncommunicable disease in adulthood.(1, 2) However, efforts to treat or prevent stunting in childhood have achieved limited success to date. Better understanding of EED may aid prevention and treatment of stunting and improve understanding of the development of immunity at the gut-environment interface.

Early recognition of EED and case definition

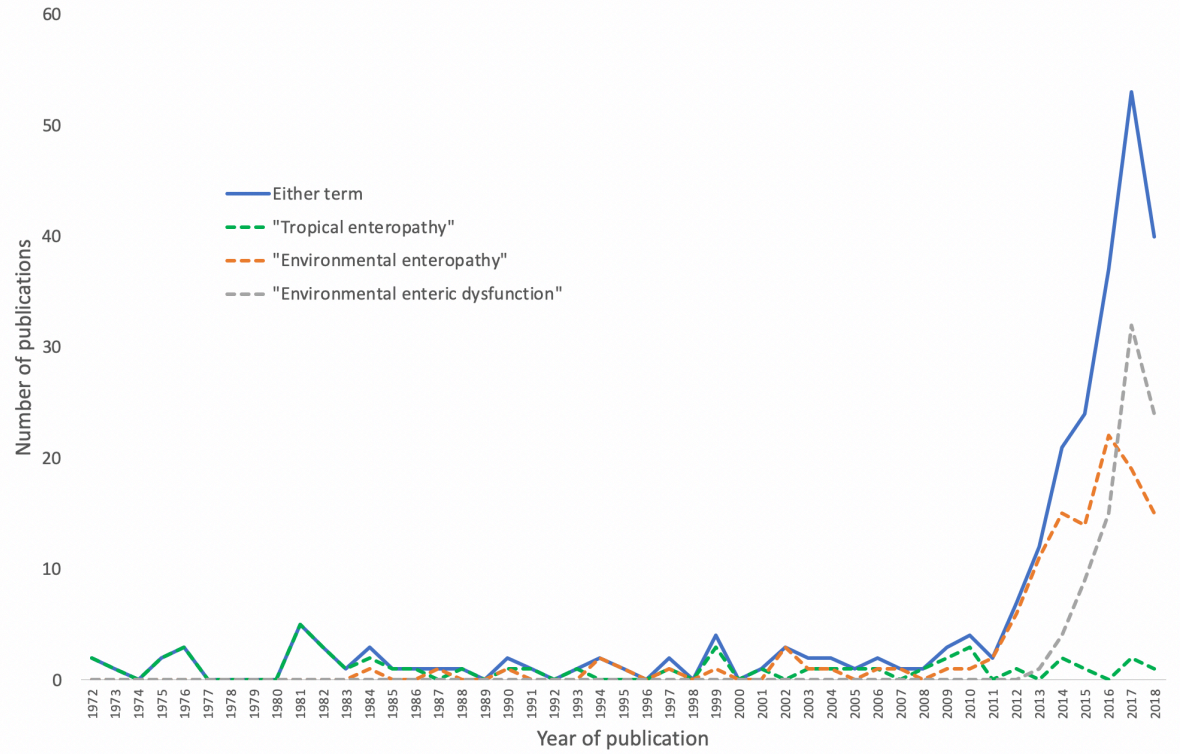
EED was first recognised in adults. The earliest descriptions, then labelled 'Tropical Enteropathy', occurred during the 1960s when an abnormal microscopic appearance of the

* N.B. The text for this chapter is an extended and significantly updated adaptation of a review that I wrote with colleagues: Crane RJ, Jones KD, Berkley JA. Environmental enteric dysfunction: an overview. *Food and Nutrition Bulletin* 2015; 36(1 Suppl): S76-87. See Appendix 4 for full text of this review.

small bowel was observed in adults returning from tropical low-income settings.(3) Villi were blunted and shortened, leading to decreased surface area for nutrient absorption. A 1971 study of USA Peace Corps volunteers is frequently referred to as the first to demonstrate that intestinal damage can be acquired and lost according to environment.(4) USA-born participants were assessed during and after placements in India and Pakistan. Gut absorptive function tests and biopsies confirmed abnormalities during residence abroad and recovery within two years of return to the USA. A later study of Zambian adults demonstrated the same histological abnormalities, which varied with season.(5) Histology studies of stillborn fetuses in India in the 1960s demonstrated normal gut mucosal appearance, suggesting that the changes seen in EED do not occur until after birth.(6) From the early 1990s, community-wide studies revealed that EED was a widespread and pervasive problem in infants and children. In these studies, dual sugar absorption tests characterised gut absorptive capacity and permeability. In a dual sugar absorption test, a mixture of a small sugar such as mannitol and a large sugar such as lactulose is given by mouth followed by either timed venesection or urine collection to measure absorption of each sugar across the gut wall. Mannitol is absorbed in proportion to gut absorptive capacity whilst lactulose is only found in blood or urine when the intestinal wall is abnormally permeable. Often the percentage excretion of lactulose and mannitol are combined to form an 'L:M ratio' where higher ratios indicate higher permeability and poorer absorptive capacity. The LM test is discussed in full later in this chapter. Abnormally high LM ratios were almost universally acquired by the end of infancy in The Gambia(7-9) and associated with stunting amongst infants and children in sub Saharan Africa, south Asia and South America.(7-30) In one Gambian study, the LM ratio was estimated to account for 43% of the variability observed in linear (length) growth amongst a cohort of 2-15-month-olds.(7) In 1999, a asymptomatic adults underwent LM testing in 14

countries across the world.(31) Abnormalities of both intestinal absorption and permeability were, in general, found in tropical rather than temperate countries. However, average intestinal absorptive capacity by country also correlated very closely with national Gross Domestic Product per capita, independent of climate, suggesting that poverty and its associated environments may play a more significant role. The term 'Environmental enteropathy' was first used in publication in 1984 and eventually overtook 'Tropical enteropathy' in recognition that quality of environment is more important than climate or latitude (Figure 1). Since 2013, some have begun using the term 'Environmental Enteric Dysfunction'.

Figure 1: Publications using the terms 'Tropical enteropathy', 'Environmental enteropathy' and 'Environmental enteric dysfunction' in publication between 1950 and the end of 2018.
Source: PubMed.gov (US National Library of Medicine, National Institutes of Health).



In 2017, Denno et al proposed a case definition for EED for intervention trials comprising: age 2-36 months; presence of linear growth failure; negative coeliac disease testing; two or

more biomarkers indicative of abnormal gut function; and supportive criteria including markers of systemic inflammation and/or intestinal dysbiosis.(32) This case definition has not yet been applied in any published EED studies, and allows for flexibility as understanding of EED expands. One problem is that the key demonstrable histological features at the intestinal epithelium (villous flattening, crypt hyperplasia and lymphocytic infiltration of lamina propria(5, 12, 33, 34)) are not unique to EED.

Tropical sprue (a syndrome of chronic diarrhoea, malabsorption and malnutrition seen in residents of tropical countries) and EED were initially thought to be the same process, with symptomatic tropical sprue the most severe pathology but representing the 'tip of the iceberg' of unrecognised EED, although this distinction remains unclear.(35) There are also similarities with gut inflammatory conditions common in high-income settings such as coeliac disease and inflammatory bowel disease (Crohn's disease and ulcerative colitis).(36) Enteropathies seen in human immunodeficiency virus (HIV), acute malnutrition (AM) and acute intestinal infection also overlap in both geographical and demographic distribution and in pathological features. These enteropathies are discussed in more detail later in this chapter.

EED is associated with wasting and impaired ponderal and linear growth

Undernutrition is quantified by, amongst other measures, weight-for-height, weight-for-age or height-for-age. These three measures are often expressed in comparison to median weight/age/height for age based on the 2006 World Health Organisation (WHO) Growth

Standards.(37) A 'Z-score' indicates how many standard deviations below or above the median any given measurement is. For example, a child with a height-for-age Z-score (HAZ) of -2.5 is 2.5 standard deviations below median height for their age. Cut-offs at <-1, <-2 and <-3 indicate 'at risk', 'moderate' and 'severe' categories respectively. Distinction is made between one-off measurement ('wast*ed*', 'underweight', 'stunt*ed*') and a dynamic process of worsening of Z-scores over time ('wast*ing*', 'becoming underweight', 'stunt*ing*'). Confusingly, the terms 'wasting' and 'stunting' also function as abstract nouns describing children found to be wasted (weight-for-height Z-score (WHZ) <-2) or stunted (HAZ<-2) in cross-sectional surveys. Moderate and severe wasting are also known as moderate and severe acute malnutrition (MAM and SAM). An alternative diagnostic criterion for SAM is a clinical diagnosis of kwashiorkor: nutritional oedema, thinning and reddening hair and skin peeling and ulceration. Where oedema is present for any reason, weight-for-age Z-score (WAZ) and WHZ are invalid since weight is inflated by interstitial fluid.

Being wasted, underweight and stunted all have exponential associations with mortality. Black et al used cohort data detailing nutritional status and disease-specific mortality for under-fives from eight low-income countries and undertook generalised linear modelling to adjust for confounders such as socio-economic position (SEP) and access to health care. They found that odds of death from all four infections studied (diarrhoea, pneumonia, measles and malaria) increased exponentially with worsening WHZ, WAZ and HAZ.(1)

Although less dangerous than wasting in the short-term, stunting is much more common and has long-term consequences for child health and neurodevelopment. Some children are born stunted but more become so, predominantly within infancy.(38) The time period between

conception and the second birthday (known as the 'first thousand days') is an important window for establishment of stunting, although belief that stunting is difficult to reverse after this window has been challenged.(39) The timing of onset of stunting, along with its heterogeneity within and between populations, suggests that stunting is at least partly determined by environmental exposures such as diet and pathogens. Very strong associations have repeatedly been demonstrated between maternal stunting and stunting at birth.(40) These are likely due to a combination of intrauterine environment, genetics and epigenetics. Maternal gastrointestinal health may contribute to the intrauterine environment. For example, markers of translocation of bacteria across the gut wall in the mother (maternal serum antibody towards the bacterial components flagellin and lipopolysaccharide (anti-flagellin and anti-LPS)) are associated with shorter gestation and shorter length at birth amongst Ugandan mother-newborn pairs.(41) Epigenetic changes occur where methylation of mother's deoxyribonucleic acid (DNA) occurs in response to her environment and is passed on to the newborn.(42) Lastly, the WHO Conceptual Framework on Stunting identifies distal factors such as political instability and urbanisation as additional important risk factors for stunting.(43)

Interventions against stunting have had limited success. A systematic review in 2008 estimated the effect of all evidence-based interventions against stunting in the 36 countries that together at that time bore 90% of the global burden of childhood stunting. These interventions included breastfeeding support, complementary and supplementary feeding, handwashing, prevention of malaria in pregnancy and family and community nutrition support. Together, even when implemented at 99% coverage, they were predicted to reduce stunting by only a third.(44) EED may be a key factor limiting effectiveness.

Not all studies have replicated the early Gambian findings of strong associations of EED with stunting. A 2014 systematic review of LM test studies found that, of eight studies identified, only five found significant association between EED (higher LM ratio) and poorer growth (defined as either one-off HAZ, WAZ or WHZ or change in HAZ, WAZ or WHZ).(45) Since publication of this review, 1,980 participants aged 0-2 years at eight south Asian, South American and sub Saharan African sites underwent 6,602 LM tests between age two and 15 months as part of the 'Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development' (MAL-ED) study. Initially, inverse association was found between LM-ratio-for-age Z-score (LMZ) and contemporaneous LAZ, i.e. poorer gut function was associated with poorer length-for-age in cross-section.(46) On extended follow-up of 1,253 MAL-ED participants to two years of age and this time assessing association between change in LAZ or WAZ over time rather than a single concurrent LAZ or WAZ, evidence was found for association between linear and ponderal growth and several faecal EED biomarkers (see page 20) but not with LMZ.(47) Finally, mean LMZ during the first two years of life was negatively associated with both HAZ and WAZ at five years of age amongst the 1,017 participants followed up this far.(48)

EED is unlikely to cause undernutrition solely via malabsorption. If this were the case, undernourished children would experience steatorrhoea and high stool volume as seen in short gut syndrome and other malabsorptive conditions. However, one of the hallmark features of EED is its lack of clinical symptoms aside from linear growth failure. There are several ways in which chronic intestinal inflammation could impact on growth. One proposed mechanism is appetite suppression, since this – rather than food availability – is often a key

factor restricting children's food intake.(49) Pro-inflammatory cytokines such as interleukin 1 (IL-1) and interleukin 6 (IL-6) may be raised in EED and act directly on appetite centres in the brain.(50) Another proposed mechanism is via negative regulation of systemic inflammatory activation on signalling through the growth hormone – insulin-like growth factor 1 (IGF-1) axis such as seen in paediatric Crohn's disease and also in other chronic extra-gastrointestinal illnesses such as juvenile rheumatoid arthritis.(50-52) A similar situation may occur in EED: indeed lower levels of IGF-1 were associated with higher systemic inflammatory markers and stunting amongst both Zimbabwean infants and severely malnourished children aged 12-60 months in urban Kenya.(53, 54) EED has also been demonstrated to inhibit ossification which results in poorer bone growth and loss of height potential.(55)

EED and stunting are associated with poor neurodevelopment

A meta-analysis in 2007 suggested that each year 200 million children do not reach their developmental potential due to stunting.(56) Stunted adults earn on average 22% less per year than their non-stunted counterparts.(56) The hypothesised pathway from EED to impaired neurocognition is via chronic sub-clinical neuroinflammation, anaemia, impaired liver function and abnormal gut metabolome early in life.(57) Circulating bacterial products may trigger the blood-brain-barrier endothelium to release pro-inflammatory cytokines such as IL-1 and IL-6 which in turn activate brain microglial cells, leading to chronic inflammation of the brain tissue itself. In the MAL-ED study, burden of subclinical enteropathogen carriage and number of days with acute illness were associated with anaemia, which in turn was associated with poorer neurodevelopment at 24 months of age.(58) Possibly detracting from

this hypothesis, amongst Tanzanian children, multiple markers of EED at age six weeks and six months were in fact associated with higher cognitive scores at age 15 months.(59) Also, in a large factorial cluster randomised trial of water, sanitation and hygiene (WASH) and nutritional interventions (Sanitation Hygiene Infant Nutrition Efficacy (SHINE): see page 38 for details) there was no effect of either intervention or a combination of the two on neurodevelopment at two years of age.(60)

Stunting is associated with higher risk of noncommunicable disease, and EED may mediate this association

Diabetes and cardiovascular disease in adulthood are associated with stunting in childhood.(61, 62) Possible mechanisms include epigenetic regulation within the individual or chronic inflammation such as EED.(63) Amongst Peruvian 3-5-year-olds in the MAL-ED study, decreased urinary mannitol excretion was associated with multiple biomarkers of later cardiometabolic risk (lower apolipoprotein A, lower high density lipoprotein, higher blood pressure).(64) Also, amongst Tanzanian children in a different study, serum anti-flagellin at six weeks was associated with increased blood pressure at 4-9 years of age.(65)

EED is characterised by poor mucosal and systemic immune development

The gut is a sophisticated immune organ, able to detect and destroy potential pathogens and maintain tissue integrity whilst avoiding inappropriate responses to foods and non-

pathogenic microorganisms. Undernutrition entails breakdown of this protective system and indeed is considered a form of immunosuppression.(66) Multiple human adult, human infant and animal studies support a theory that undernutrition during the first few years of life can confer life-long immunodeficiency.(66) A 2014 systematic review identified defects in both innate and adaptive immune systems in undernutrition.(67) Maternal undernutrition can also have intergenerational effects on immune function via epigenetic changes. A randomised placebo-controlled trial of multiple micronutrient supplementation of Gambian mothers resulted in methylation of several genes relevant to innate and adaptive immunity detectable in their infants at up to age nine months.(68)

Compared to UK controls, small intestine biopsies from Gambian children with and without diarrhoea and undernutrition all showed evidence of chronic T cell mediated inflammation with crypt hyperplasia, villous stunting and intraepithelial lymphocytosis.(12) It is not yet known whether these changes are an adaptive or maladaptive response to a challenging environment. Supportive of a maladaptive response theory, in a randomised controlled trial, the intestine-specific anti-inflammatory drug mesalazine was given to Kenyan 12-60-month-olds with SAM and evidence of EED (here defined as HAZ<-2 plus blood erythrocyte sedimentation rate >20mm/hour). Amongst the intervention group, neither increase in adverse events nor impairment in nutritional recovery were seen despite reductions in multiple inflammatory markers.(54)

In EED, impaired gut barrier function through breakdown of tight junctions and loss of the protective mucus layer results in luminal bacteria and their components translocating across the gut wall and into the bloodstream. For example, children in urban Zambia with SAM and

chronic diarrhoea had decreased intestinal mucosal expression of the tight junction protein claudin-4 and increased serum anti-LPS in comparison to healthy controls.(69) In the same study, detection of fragments of bacterial DNA in the bloodstream via 16S polymerase chain reaction (PCR) showed association with circulating anti-LPS and increased bloodstream bacterial in undernourished compared to healthy children and adults.

Chronic systemic inflammation may be triggered by this translocation. With worsening WAZ in Gambian children, mucosal cytokine expression became more biased towards a pro-inflammatory response with less transforming growth factor β (TGF-β: a cytokine produced by regulatory T cells). However, other evidence suggests that chronic bacterial translocation results in an acquired 'endotoxin tolerance' or 'immune desensitisation' in EED. Monocytes from adult patients recovering from septic shock have impaired response to stimulation in vitro.(70) Dendritic cell anergy has been demonstrated amongst Zambian children with SAM and endotoxaemia.(71) The enzyme indoleamine 2,3-dioxygenase 1 (IDO-1) plays a key role in immune tolerance by catalysing a rate-limiting step in the kynurenine pathway that converts tryptophan to kynurenine. Depletion of tryptophan inhibits T cell activity. A higher plasma kynurenine:tryptophan (K:T) ratio is therefore indicative of immune tolerance. At the MAL-ED Peru and Tanzania sites, lower plasma tryptophan was associated with poorer linear growth.(72) The ongoing Early Life Interventions for Childhood Growth and development in Tanzania (ELICIT) trial aims to replete the tryptophan-kynurenine-niacin pathway using nicotinamide supplementation.(73)

EED may impair response to oral vaccines

Oral polio and rotavirus vaccine immunogenicity and clinical efficacy are significantly reduced in low-income countries compared to high-income. For example, in a randomised double-blind placebo-controlled trial of oral rotavirus vaccine in Ghana, Kenya and Mali, efficacy against severe rotavirus gastroenteritis was estimated at 39% whilst similar figures in Europe and the USA are 85-100%.(74, 75) The same difference is not seen for parenteral (injected) vaccines. The emerging evidence described above of a possible impairment in mucosal immunity in EED and the geographical overlap seen between EED and low oral vaccine efficacy raises the question of a causative association between EED and poor oral vaccine response. However, evidence supporting this hypothesis is conflicting to date (Appendix 5).

Characterisation of EED

Since EED lacks a universally used case definition, it is misleading to suggest it can be 'diagnosed'. Instead, investigators aim to characterise its features using a variety of tests. As with many other pathologies, gold standard for characterisation of EED is histology. This can only be done via endoscopy and small intestinal biopsy. Endoscopy is time-consuming, requires skilled practitioners in a clean monitored environment and carries risks including gastrointestinal perforation, infection and adverse reaction to sedation or anaesthesia. The logistical and ethical challenges of carrying out endoscopies in EED-endemic settings, and the fact that the vast majority of children with EED are not acutely unwell and do not require endoscopy for a therapeutic reason, have led investigators to seek less invasive biomarkers

for EED. Keusch et al proposed four domains of EED amongst which biomarkers can be divided: 1) intestinal absorption and mucosal permeability, 2) enterocyte mass and function, 3) intestinal inflammation and 4) microbial translocation and systemic immune activation.(76) Others have since suggested the term 'regeneration/damage and repair' instead of 'enterocyte mass and function' and also the addition of a fifth domain related to intestinal dysbiosis.(32, 77)

Markers of intestinal absorption and mucosal permeability

The dual sugar absorption tests already described measure two key elements of gut function: permeability and absorption. The most commonly implemented of these at present is the LM test, although other sugars such as D-xylose and rhamnose can be used. The LM test requires cooperation from the participant and carer which may present a challenge when testing young children. Measurement of lactulose and mannitol in urine can be performed by Enzyme Linked Immunosorbent Assay (ELISA), anion exchange chromatography or mass spectrometry. All of these methods require centralised laboratory equipment and expertise, and results are not always comparable between laboratories.(78) Further technical background to the LM test is presented in Chapter 4.

As well as lactulose excretion, intestinal permeability can also be measured by plasma zonulin and claudin-4 and faecal alpha-1-antitrypsin. Zonulin is a protein that modulates the permeability of tight junctions at the intestinal epithelium. Higher plasma zonulin indicates tighter junctions and less permeability. Claudin-4 is a component of the intestinal epithelium

tight junction. Histological studies of Zambian children with SAM demonstrated disruption of claudin-4 at tight junctions.(69) Alpha-1-antitrypsin is an abundant plasma protein not itself involved in inflammation but released into the gut lumen when gut permeability is high. Because of its resistance to degradation in stool, it is an established marker of protein-losing enteropathy in inflammatory bowel disease.

Markers of enterocyte mass and function / damage and repair

Citrulline is produced by enterocytes and passes into the bloodstream. Plasma citrulline is an established marker of total enterocyte mass in assessment of intestinal malabsorptive disease. Reg-1β is a gene that encodes proteins involved in intestinal epithelium repair, regeneration and growth. Reg-1β was measured in stool from Bangladeshi and Peruvian three-month-olds and found to independently predict growth shortfall at 24 months.(79) Intestinal fatty acid binding protein (I-FABP) is released into the bloodstream by the intestinal epithelium in response to damage. In Zimbabwean infants, I-FABP increased between three and 12 months of age, suggestive of damage taking place during this time.(53)

Markers of intestinal inflammation

Gut inflammation can be measured by, amongst others, host messenger ribonucleic acid (mRNA) transcripts, faecal calprotectin, myeloperoxidase and neopterin.

mRNA profiling provides insight into the proteins expressed by the intestinal epithelium. Amongst Malawian 2-5-year-olds, from 39 candidate mRNA transcripts identified mRNA encoding regeneration islet-derived protein 4 (REG4) as the most discriminatory between normal and abnormal LM ratio groups.(80) Also in Malawi, from a panel of seven candidate mRNA transcripts, CDX1 (a gene that encodes the protein homeobox protein CDX1, expressed by intestinal epithelium) and REG1A (a gene that encodes the protein islet cells regeneration factor) were positively associated with urinary lactulose excretion.(81)

Calprotectin is a zinc- and calcium-binding protein released by neutrophils into the bloodstream and intestinal lumen during inflammation. It remains stable in faeces where it can be detected by ELISA. It is used routinely in the diagnosis of inflammatory bowel disease. Faecal calprotectin is higher in infants compared to older children, in exclusively breastfed compared to non-exclusively-breastfed infants, and in Gambian compared to European infants.(24, 82, 83) In a study of inpatient Malawian children with complicated SAM, those who died had significantly higher faecal calprotectin.(84) Faecal calprotectin has also been shown to be associated with mouthing of soil-contaminated objects and caregiver visibly soiled hands amongst Bangladeshi 0-29-month-olds.(85, 86) On the other hand, a survey of 0-12-year-old healthy HIV-negative children in urban Uganda found no associations between faecal calprotectin and maternal education, enteropathogen infection, recent antibiotics or socioeconomic status.(83)

Appreciating that non-histologic diagnosis of EED probably requires more than one test, some investigators have started compiling composite disease activity scores from multiple biomarkers. Myeloperoxidase is a marker of mucosal neutrophil activity and, unlike other

markers such as calprotectin and lactoferrin, is not naturally elevated in stools of breastfed children. Neopterin is produced by dendritic cells, monocytes and macrophages in response to stimulation by interferon gamma, a pro-inflammatory inter-cell signalling hormone (cytokine). It has been used to assess disease severity in coeliac disease, which has many similarities with EED and in infections. Neopterin is also used as a marker of oxidative stress and as a prognostic marker in malignancy and HIV disease.(87) Neopterin and myeloperoxidase are also both resistant to degradation in stool. An EED severity score derived from myeloperoxidase, neopterin and the permeability marker alpha-1-antitrypsin was developed and validated against linear growth using data from a subset of 537 children from the MAL-ED study.(88) Also in the MAL-ED study, the same three markers were then measured in 22,846 non-diarrhoeal stool samples from all 1,253 participants aged zero to two years, along with a panel of 40 enteropathogens and the urine LM test.(47) Faecal concentrations of the three markers were found to decrease with age, to correlate poorly with each other, and to be highly unstable within individuals over time. Faecal myeloperoxidase and alpha-1-antitrypsin concentrations were 5-10 and two times higher than in high-income settings respectively. Directed Acyclic Graph analysis indicated support for association of higher enteropathogen burden with higher LM ratio and higher faecal alpha-1-antitrypsin, myeloperoxidase and neopterin, and in turn association with reduced linear and ponderal growth. For ponderal growth specifically, strongest evidence was seen for association with markers of intestinal inflammation; whereas for reduced linear growth strongest evidence was seen for association with the systemic inflammation marker plasma α-1-acid glycoprotein (AGP). Effect sizes of gut and systemic inflammation markers on growth were relatively small in general, with the largest being for plasma AGP followed by faecal myeloperoxidase. The study authors suggested that, given its demonstrated inverse

association with linear and ponderal growth, faecal alpha-1-antitrypsin may be a more accurate measure of intestinal permeability than the LMZ. However, the fact that the polar surface of alpha-1-antitrypsin is in fact much larger than that of lactulose raises questions about the exact nature of the gut permeability seen in EED.

Many subsequent EED studies have employed faecal alpha-1-antitrypsin, myeloperoxidase and/or neopterin. In the largest study, an analysis of 22,846 stool samples from 2,076 under-twos across the eight sites of the MAL-ED study and found all markers to decrease with age until two years, with myeloperoxidase approaching normal range values but alpha-1-antitrypsin and neopterin remaining high even by 24 months.(89) All three markers slightly increased during the rainy season and were associated with the burden of enteropathogen carriage as measured by multiplex PCR. Myeloperoxidase and alpha-1-antitrypsin (but not neopterin) were higher amongst those in lower socio-economic position groups. Myeloperoxidase and alpha-1-antitrypsin were also raised amongst children who had consumed breastmilk within the preceding seven days. This may be explained by the presence of α-lactalbumin in human milk which activates infant intestinal mucosal neutrophils, leading to the release of myeloperoxidase. Also, breastmilk contains alpha-1-antitrypsin. The study authors suggested this response is likely appropriate and therefore higher international normal reference ranges for these markers should be developed for breastfed children.

Many smaller studies have demonstrated association between the three markers and growth, poor WASH and enteropathogens. In Bangladesh, amongst multiple candidate enteropathogens tested, raised myeloperoxidase is associated with subclinical carriage of

Giardia duodenalis and Enteroaggregative *Escherichia coli* (EAEC), raised neopterin is associated with *Trichuris trichuria* carriage and raised alpha-1-antitrypsin with *Giardia*.(90) Neopterin is also associated with subclinical *Giardia* carriage in Gambian infants.(11) Higher neopterin is associated with poorer subsequent linear growth in Gambian children, and the same is true for myeloperoxidase in Bangladeshi and Pakistani children and for both myeloperoxidase and neopterin in Brazilian children.(11, 91-93) In Bangladesh, the combined severity score is associated with geophagy, sharing a sleeping space with animals and unsafe child faeces disposal methods.(85, 94, 95) In Peru, higher myeloperoxidase is associated with unreliable water supply and absent sanitation facilities.(96) Lastly, in Bangladesh, all three markers are independently associated with decreased immunogenicity of oral polio and rotavirus vaccines, and decreased clinical efficacy of oral rotavirus vaccine.(97)

Markers of microbial translocation and systemic immune activation

As already described, anti-LPS is produced when whole or fragmented bacteria translocate across a permeable bowel wall into the bloodstream. High plasma anti-LPS in early infancy represents maternally derived immunoglobulin G (IgG) so can be unreliable. The earliest EED anti-LPS studies were in 1996, when Gambian infants were followed until five years of age.(8) After a high start at eight weeks (likely maternal), titres dropped to levels similar to those seen in UK infants then from five months of age started to rise. In this same study, anti-LPS was also associated positively with LM ratio and negatively with linear growth. In a birth cohort in urban Bangladesh, serum anti-LPS at six months appeared predictive of stunting.(98) Amongst children in rural Pakistan, both serum anti-LPS and anti-flagellin at age

six and nine months were associated with subsequent linear growth failure over the following 18 months.(99) However, more recent studies have not been so conclusive. For example: also in rural Bangladesh, in a cross-sectional survey of children aged 0-4 years, slightly lower anti-LPS titres were seen in children from cleaner households, although statistical evidence for this difference was weak.(29) In the same study, unlike LM ratio, anti-LPS was not associated with stunting. Lastly, in a case-control study of Zimbabwean HIV-unexposed infants, an increase in anti-LPS titres was noted between three and six months of age, coinciding with cessation of exclusive breastfeeding.(53) However, in the same study, a summary measure of anti-LPS titres taken at six weeks and three, six and 12 months was not associated with increased likelihood of stunting by 18 months of age. Anti-LPS can now be measured reliably in dried blood spots, increasing utility in the field.(100)

Soluble CD14 (sCD14) is another marker of microbial translocation. Along with Toll-like receptor 4 (TLR-4), sCD14 is a co-receptor for the detection of LPS and expressed by macrophages, neutrophils and dendritic cells. It is present in blood and breastmilk. In Zimbabwean infants, sCD14 was found to rise steadily from birth to 12 months of age but was not found to independently predict risk of stunting by 18 months of age.(53)

Markers of general systemic immune activation include cytokine quantification, AGP and serum C reactive peptide (CRP). The latter is in regular use in clinical care worldwide. In Zimbabwean infants, raised CRP and AGP are associated with stunting.(53)

Evidence is emerging that the systemic inflammation component of EED may be its most consequential for child health and development. In a study of Malawian children with

complicated SAM, mortality was associated with the gut-specific marker faecal calprotectin but primarily via the more direct association between mortality and markers of systemic inflammation (here measured by a panel of 26 cytokines).(84) However, a cross-sectional study of Bangladeshi 18-month-olds did not demonstrate association between the LM ratio and markers of systemic inflammation (CRP and AGP).(101)

Biomarker combinations, and return to histology

Most recently, machine learning has been employed for a more objective approach to characterisation of EED. Tissue samples are also being obtained more frequently. Syed et al obtained duodenal biopsies from children with EED in Pakistan and Zambia and from children with coeliac disease and no disease from the USA.(102) Similar findings to the earlier Gambian studies were present: decreased villus-to-crypt ratios, intra-epithelial and lamina propria T lymphocytosis. Machine learning methods were then applied to automated image analysis and established certain differences in the histopathological appearances of the three clinical states. Using only the Pakistani data, the authors went on to discern which of 32 measured non-invasive EED biomarkers (from blood, stool and urine) were most predictive of histopathological features. 12 were identified as best predictors: plasma interleukins 1b, 6, 8, 9, and 12b, Reg-1β in stool and serum, myeloperoxidase in stool and platelet-derived growth factor subunit B (PDGFb) and tissue necrosis factor α (TNFα) in serum.(103) New biomarkers for EED are being investigated through comparison to duodenal histology in samples from children in Pakistan and the USA.(104)

In addition to the alpha-1-antitrypsin/myeloperoxidase/neopterin combined score, other scores are being proposed. Campbell et al developed scores for three domains (Gut Inflammation, Gut Permeability and Systemic Inflammation) from seven constituent biomarkers measured in Bangladeshi toddlers.(105) For all three domains, a worse score demonstrated some association with stunting but more strongly with becoming underweight.

Putative risk factors for EED

Early introduction of complementary foods; early cessation of breastfeeding; alternative first milks

The WHO recommends initiation of breastfeeding within one hour of birth then exclusive breastfeeding for the first six months of life followed by continued breastfeeding alongside complementary foods up to two years of age and beyond.(106) Adherence to these recommendations is poor globally.(107, 108) In tropical low-income settings, microbial contamination of complementary foods is likely to be high due to poor WASH provision and lack of refrigeration. Introduction of complementary foods earlier than six months of age is associated with morbidity and mortality from diarrhoea.(109) Recently, early circumstantial evidence has emerged for association between poor adherence to breastfeeding recommendations and EED. In a 2015 study of 24 six-month-old South African infants, exclusivity of breastfeeding was quantified using the deuterium dilution method and EED approximated by a panel of five faecal host mRNA markers of gut inflammation. Of the five, S100 calcium-binding protein A8 (S100A8) and interleukin-8 (IL-8) were found to correlate

significantly with the percentage of breastmilk intake.(110) In a cohort study of 590 Tanzanian infants aged six to 52 weeks, delay of breastfeeding initiation beyond one hour and prelacteal feeding were associated with elevated anti-LPS and anti-flagellin at six months of age.(111)

Human milk contains multiple substances that promote a healthy gut. In samples of breastmilk taken from 88 Malawian mothers, sialylated Human Milk Oligosaccharides (HMOs) were found to be much less abundant amongst mothers of severely stunted six-month-old infants.(112) Gnotobiotic (germ-free) mice and piglets colonised with gut microbiota from a six-month-old stunted Malawian infant and fed with bovine sialylated milk oligosaccharide were found to form higher bone and lean muscle mass than those with colonisation alone. No such difference was observed between germ-free mice and piglets receiving the same two diets. This suggests that sialylated milk oligosaccharide has beneficial effects on growth but that these are mediated via the gut microbiota. Human milk contains human epidermal growth factor which assists in gut epithelial regeneration and repair.(113) Lastly, two components of breastmilk – lactoferrin and lysozyme – given as supplements in a randomised controlled trial were associated with improved urinary lactulose excretion amongst Malawian one-year-olds.(114)

In the MAL-ED study, assessing 6,602 LM tests undertaken on 1,980 children across eight sites at age 3, 6, 9 and 15 months of age, with no evidence of interaction by age, higher LM ratio was surprisingly associated with consumption of breastmilk within the seven days preceding the LM test.(46) This is in contrast to previous studies amongst neonatal and premature babies in high-income settings that have demonstrated lower LM ratios with

breastmilk.(115, 116) Higher LM ratios amongst recently breastfed children were almost all accounted for by raised percent lactulose excretion, but breastmilk does not contain lactulose. The vast majority of participants at all sites were predominantly breastfed throughout follow-up, with frequent periods of reintroduction of exclusive breastfeeding, but that the majority of LM tests were preceded by both breastmilk and solids. The authors suggested further investigation into whether the LM test itself may be affected by breastfeeding.

Micronutrient and protein deficiencies

Zinc has known roles in enzyme catalysis, gene expression and cell stability. Zinc deficiency is common and known to be associated with poor growth and immune function.(117) Zinc is known to aid recovery of the intestinal mucosa following diarrhoea: indeed, zinc supplementation is recommended as part of standard therapy for diarrhoea by the WHO.(18) Exogenous zinc is first absorbed in the small intestine from food, then endogenous zinc is secreted then reabsorbed in the large intestine. This cycle of zinc homeostasis is affected by phytates present in plant foods, which impair absorption of exogenous zinc, and perhaps also by intestinal damage which may increase net loss of endogenous zinc. Zinc deficiency is associated with higher LM ratio in Malawian children.(23) Using stable isotope technology, zinc homeostasis has been characterised in Bangladeshi toddlers, allowing distinction between deficiency due to poor absorption versus increased faecal loss. Poor absorption rather than faecal loss was associated with higher urinary LM ratio.(118) In a mouse model, a zinc-deficient diet resulted in significant gut microbiome alteration – more so than a

protein-deficient diet.(119) It has been proposed that zinc deficiency and EED may promote each other, with EED destabilising zinc homeostasis and the resultant zinc deficiency causing intestinal damage.(117) However, results from intervention trials have so far not been conclusive. In two randomised controlled trials amongst Malawian toddlers, zinc supplementation was demonstrated to attenuate worsening of urinary LM ratios over time but resistant starch therapy – which was proposed to improve zinc absorption in the large intestine by promoting certain bacterial enzymes – did not impact on zinc homeostasis.(17, 120) In a randomised controlled trial amongst Tanzanian infants, neither zinc nor multivitamins had any effect on markers of EED, systemic inflammation or growth hormones.(121)

Vitamin A plays a known critical role in vision, neurodevelopment and intestinal integrity. In tropical low-income settings, vitamin A supplementation is routinely given every six months for the first five years of life. Vitamin A deficiency is associated with higher LM ratio and stunting in Brazilian children and with higher LM ratio in Bangladeshi 6-24-month-olds.(20, 122) In The Gambia, the least abnormal LM ratios are observed during the mango season, when vitamin A intake is highest.(19) In Indian infants, brief vitamin A supplementation yielded improvements in LM ratio within 30 days.(19) In Mexican infants, vitamin A supplementation improved linear growth.(123) In Brazilian children, although vitamin A supplementation did not improve nutritional status, it did reduce the number of new *Giardia* intestinal infections.(22) Proposed mechanisms through which vitamin A deficiency could lead to EED include via impaired regulation of intestinal immunity, impaired intestinal epithelium regeneration, and modulation of intestinal microbiota.(124) Similarly, vitamin D promotes intestinal health by regulating tight junctions, inhibiting intestinal epithelial cell

apoptosis, promoting anti-inflammatory immune regulatory responses and perhaps also altering gut microbiota.(125) Vitamin D deficiency is known to be associated with poorer outcomes in inflammatory bowel disease. The role of vitamin D in EED has not yet been explored.

Protein quality and quantity may be important. Comparison of community diets in Malawi shows some evidence that animal-source protein may be associated with higher HAZ amongst toddlers.(126) In Zambian adults, supplementation with the amino acids tryptophan, leucine and glutamine protected against a decline in intestinal villous height.(127) In Brazilian children, alanyl-glutamine – an two-amino-acid essential precursor for rapidly replicating cells such as those lining the small intestine – yielded improvements in urinary lactulose excretion, WHZ and WAZ but not HAZ.(21) Amongst Malawian 12-23-month-olds, replacing cereals with isoenergetic but higher-protein legumes was associated with improved intestinal permeability (lower percentage urinary lactulose excretion) but no improvements in linear growth.(128) The authors of this study suggested that common bean fibre, high in α-galactosides, may reduce intestinal inflammation via alteration of the intestinal microbiota. In the ongoing Modifying Intestinal Integrity and Microbiome in Severe Malnutrition with Legume-based Feeds (MIMBLE) study, Ugandan children with SAM will be randomised to standard treatment versus a lactose-free feed enriched with chickpea to provide fermentation substrate for gut microbiota to form short chain fatty acids for intestinal epithelium repair.(129) Study investigators will measure nutritional rehabilitation, new-onset (presumably lactose intolerance) diarrhoea and intestinal inflammation (faecal calprotectin).

Several other nutritional approaches have been proposed to tackle EED, for example: improving the digestibility of food through fermentation, hydrolysis or enzyme supplementation.(130) In Gambian infants, n-3 (omega-3) long chain polyunsaturated fatty acid (LC-PUFA – a dietary essential fatty acid thought to reduce intestinal inflammation) yielded improved mid upper arm circumference (MUAC) at nine and 12 months of age but no improvement in LM ratios.(24) In Ethiopian 6-24-month-olds, LC-PUFA supplementation did not result in improvements in linear growth or systemic inflammation.(131) In a factorial trial amongst Malawian 1-3-year-olds, multiple micronutrients were found to improve urinary LM ratio but not LC-PUFA.(132)

Toxins

Mycotoxins – aflatoxin, fumonisin and deoxynivaenol – are produced by certain species of fungi that are found on multiple crop species, primarily in tropical low-income settings. Mycotoxins damage enterocytes, interfere with zinc absorption and protein synthesis and nutrient metabolism in the gut and are associated with stunting in children.(133) In a cross-sectional study of Guatemalan children, higher aflatoxin exposure was associated with lower contemporaneous HAZ.(134) Other environmental toxins have been proposed to cause or exacerbate EED, within the context of an 'exposome' framework where multiple dietary, pathogen and chemical factors collide.(135) Proposed environmental contaminants include heavy metals, aerosols and pesticides.

Enteropathogens

Microorganisms in the gut may be important in the establishment and/or maintenance of EED. Their relative importance may differ between geographical settings, season, age or feeding practices. Evidence supporting a role for enteropathogens in the pathogenesis of EED lies first in antibiotic intervention studies. Studies of chicks in the 1960s demonstrated that weight gain can be promoted by antibiotics alone, regardless of sanitation.(136) A recent systematic review concluded that good evidence exists in relation to moderate beneficial effects of antibiotics on growth in pre-pubertal children in tropical low income settings.(137) The 10 reviewed studies included empirical broad spectrum antibacterials for children with and without AM or diarrhoea, anthelminthics targeting *Giardia* infection and prophylactic cotrimoxazole for children with HIV infection. Since publication of this review, examination of the effects of periodic mass administration of azithromycin to children in rural Ethiopia – primarily aimed at trachoma – did not result in improved linear growth.(138) In Malawian toddlers, the anthelminthic albendazole prevented deterioration of urinary LM ratios(17) whilst in Indian infants azithromycin resulted in lower faecal calprotectin, myeloperoxidase and alpha-1-antitrypsin.(139) In certain arms of the ongoing factorial 'ELICIT' trial described above, the antimicrobials azithromycin and nitazoxanide aim to reduce subclinical enteropathogen carriage.(73)

Linear growth faltering has been linked to concurrent infection with multiple intestinal parasites (*Cryptosporidium*,(140) amoeba,(141) roundworm,(142) hookworm,(143)). The abnormal histological findings in EED have been linked to infection with hookworm and the bacteria *Citrobacter rodentium*.(5) The parasite *Giardia duodenalis* has been particularly

implicated in abnormal LM ratios in Nepal(27) and acute growth faltering in Gambian infants.(26) Acute rotavirus infection has been positively associated with concurrently high LM ratios in Bangladeshi and Peruvian children.(18, 144) In the MAL-ED study, which used non-diarrhoeal stool samples, the commonly-detected enteropathogens *Campylobacter* and EAEC were associated with increased faecal alpha-1-antitrypsin and myeloperoxidase whilst *Giardia* was associated with decreased alpha-1-antitrypsin, myeloperoxidase and neopterin and the rarer *Yersinia enterocolitica* was strongly associated with increased myeloperoxidase and decreased neopterin.(47) In a separate analysis, subclinical carriage of *Campylobacter* and *Giardia* was associated with higher LMZ.(47)

Helicobacter pylori is a bacterial infection best known as a cause of gastric ulcers. It has also been implicated in allowing the passage of live pathogens beyond the stomach through inhibition of stomach acid secretion.(145) In The Gambia, 15% of 0-20-month-olds and 46% of 40-60-month-olds had evidence of *H. pylori* infection, with higher prevalence amongst those acutely malnourished or with chronic diarrhoea.(146-148) Amongst urban Peruvian children, high frequency of *H. pylori* infection was noted and associated with subsequent higher risk of diarrhoea.(149)

H. pylori infection has been proposed as a trigger for Small Intestinal Bacterial Overgrowth (SIBO), a condition where the normally relatively sterile small intestine is colonised with large numbers of bacteria. In high-income settings, SIBO is best known to affect individuals with slowed gastric motility due to intrinsic neuromuscular conditions affecting intestinal peristalsis or external factors such as scoliosis and immobility. Also in these settings, SIBO is known to be associated with immune dysregulation, gut barrier dysfunction, bacterial

translocation and occasionally chronic diarrhoea.(150) Upper threshold normal values for concentration of bacteria in the intestinal tract are: 10^4 colony- forming units (CFU) per ml in the stomach, 10^5CFU/ml in the jejunum (upper small intestine), 10^6 CFU/ml in the ileum (lower small intestine) and 10^{10} CFU/ml in the large intestine.(151) SIBO is defined as greater than 10^5 CFU/ml of any bacterial species (or greater than 10^3CFU/ml of bacterial species of the types normally found in the large intestine) at any small intestinal site.(151)

There are several early examples of SIBO diagnosis via intestinal fluid sampling in low-income tropical settings. In the 1970s, 18 malnourished adults and 13 socioeconomic-position-matched non-malnourished controls in Calcutta, India, underwent endoscopy and sampling from the stomach and small intestine.(152) Malnourished participants had bacterial concentrations ranging from 10^2 to 10^8 CFU/ml. None of the non-malnourished adults had any bacteria isolated. Also in the 1970s, 25 Gambian children meeting a historical definition for acute malnutrition (weight <60% of Boston 50^{th} centile or clinical signs of kwashiorkor) underwent endoscopy and jejunal fluid sampling.(153) On culture, 22 of these 25 demonstrated bacterial concentrations greater than 10^5 CFU/ml. Bacterial species normally associated with both the respiratory tract (*Staphylococcus, Streptococcus, Pseudomonas*) and the large intestine (*Enterococci, E. coli, Candida, micrococci, Klebsiella*) were isolated. In the 1990s, 40 asymptomatic infants from a slum residential area in Sao Paulo, Brazil, underwent nasogastric intubation under fluoroscopy guidance followed by sampling of jejunal fluid.(154) Culture of this jejunal fluid showed concentrations of large-intestine-type bacteria ranging from 10^2 to 10^9 CFU/ml. No such bacteria were found in jejunal fluid from eight 'control' infants from a nearby richer neighbourhood.

Less invasive than small intestinal sampling is measurement of gases in exhaled breath. Intestinal bacteria metabolise carbohydrates in the gut lumen, producing hydrogen and methane gases that pass across the intestinal wall, and are carried dissolved in the bloodstream to the lungs where they excreted via exhalation. Technical background to breath testing for SIBO is discussed in Chapter 3. The earliest tropical low-income setting studies that used breath testing to measure SIBO took place in Myanmar in the early 1990s. In the first study, prevalence of SIBO was described at 47% amongst under-fives and associated with impaired linear growth within the 36-59 month age group subset.(155) In the second study, prevalence of SIBO was 27% and associated with male sex but not diarrhoea.(156) Amongst Brazilian children, SIBO measured by breath test was found to be 6% prevalent and associated with impaired response to oral cholera vaccine.(157) In the same area, prevalence of SIBO amongst children living in a slum settlement was found to be 38% compared to 2% amongst children from a nearby private school.(158) A subsequent study of the same comparison groups found 31% and 2% prevalence respectively.(159) Amongst Bangladeshi two-year-olds, SIBO measured by breath test was associated with stunting (less positive, or more negative, change in LAZ since birth), poor sanitation (open sewer outside house) and markers of EED (faecal Reg-1β and calprotectin) but not associated diarrhoea, wasting or several other EED markers.(160) In another study, amongst stunted and at-risk from stunting Bangladeshi 12-18-month-olds, SIBO prevalence was 15%. SIBO status did not affect the response of LAZ, WLZ or WAZ to a nutritional intervention.(161)

More recently, where samples of small intestinal fluid can be ethically obtained, next-generation sequencing techniques have been applied in an attempt to further characterise SIBO amongst children in tropical low-income settings. In 2018, Vonaesch et al published

findings from a study in which duodenal fluid samples were obtained via nasogastric tube from 46 stunted children aged 2-5 years from Madagascar and the Central African Republic.(162) 96% of these samples met the SIBO diagnostic criteria of $>10^5$ CFU/ml. 16S ribosomal ribonucleic acid (rRNA) sequencing of these samples revealed that the majority of overgrowth species were likely oro/naso-pharyngeal in origin. No particular species was over-represented amongst severely stunted compared to moderately stunted children. Following 16S sequencing of stool samples from 404 stunted and non-stunted children from the same two countries, oropharyngeal species were found to be over-represented in the large intestine also: dysbiosis was thus not just restricted to the small intestine. Amongst Brazilian children aged 5-11 years, SIBO as measured by breath test was 61% prevalent and associated with lower HAZ, lower blood haemoglobin and, as measured in stool by quantitative real-time PCR (qrtPCR), higher counts of the pathogenic *Salmonella* species and lower *Eubacteria* and *Firmicutes*.(163) In 2019, Donowitz et al found that, amongst the 17% of Bangladeshi two-year-olds diagnosed as SIBO positive by breath test, 16S rRNA sequencing of stool revealed the species *Lactobacillus* as over-represented amongst the SIBO positive children.(164) A mechanism suggested by the authors by which *Lactobacillus* overgrowth may lead to intestinal inflammation is via increased microbial lactic acid production which induces the host enzyme nicotinamide adenine dinucleotide phosphate oxidase which in turn produces damaging reactive oxygen species. Further discussion of the role of the gut microbiome in EED more generally is found below.

SIBO can be treated with antibiotics. Co-trimoxazole therapy was followed by normalisation of hydrogen breath testing in 19 of 20 previously SIBO positive urban Brazilian children.(165) However, there was no control group in this study and numbers were small. Metronidazole

targeted at SIBO (though likely impacting on specific bacterial or parasitic infections as well such as *Giardia*) aids recovery from malnutrition in Jamaican children.(166) Lastly, the non-absorbable oral antibiotic rifaximin, also targeted at SIBO, was found not to improve LM ratios in Malawian children, although SIBO was not measured.(167)

EED is unlikely, given its widespread distribution, to be caused by just one or a few species of enteropathogen. Chronic, sub-clinical multiple enteropathogen exposure may be more important. This frequently comes about through poor WASH. Observational study of young children in Zimbabwe suggests that a significant proportion of the burden of faeco-oral contamination is likely to come not only from well-characterised sources (food, water and hands) but also from direct eating of soil and animal faeces in the course of play and exploration.(168)

Observational evidence linking WASH, EED and stunting is weak. Bangladeshi children from cleaner households had lower risk of both abnormal LM ratio and stunting compared to those from less clean households.(29) In Malawian 3-5-year-olds, abnormal LM ratio was associated with lack of latrine access and low household water usage.(169) However, a recent systematic review of 14 trials of WASH interventions worldwide on anthropometric outcomes for children aged 0-18 years suggested only a modest impact of WASH on stunting (with no effect at all amongst 0-2-year-olds) and no impact on underweight or wasting.(170) In Uganda, although presence of *E. coli* in drinking water was not associated with type of water source, it was associated with higher LM ratios and lower HAZ and WAZ at 12-16 months of age.(171)

Two major cluster randomised trials of WASH and nutritional interventions have recently published results: 'WASH Benefits' and 'SHINE'. In the 'WASH Benefits' trial, 5,551 pregnant women in rural Bangladesh and 8,246 pregnant women in rural western Kenya were enrolled into a multi-site factorial cluster randomised trial of WASH and nutrition interventions with a primary endpoint of diarrhoea and linear growth. There were seven clusters: chlorinated drinking water ('water'); upgraded sanitation ('sanitation'); handwashing with soap ('handwashing'); combined water, sanitation, and handwashing; child nutrition counselling plus lipid-based nutrient supplements ('nutrition'); combined water, sanitation, handwashing, and nutrition; and control. Data on linear growth and diarrhoea were available for 4,584 children in Bangladesh and 6,583 in Kenya at age two years. At both sites, linear growth was improved amongst residents of clusters with nutritional interventions, but not for any of the WASH intervention arms.(172, 173) WASH interventions reduced caretaker recalled diarrhoea seven-day prevalence at the Bangladesh site but not Kenya. Examining the secondary endpoint of EED, a subgroup analysis of 1,512 trial participants from both sites aged 3-28 months found that at three months of age LM ratio was lower in the nutrition and WASH arms compared to control.(174) However, at 14 months no difference was seen and by 28 months LM ratio was in fact higher in nutrition and WASH arms compared to control (association of LM ratio with linear growth was not reported). The study authors suggested this unexpected result could have arisen from the earlier WASH and nutrition interventions delaying the peak gut insult, meaning worse EED amongst intervention groups when viewed at cross-section at 28 months.

In the SHINE trial, 5,280 pregnant women in rural Zimbabwe were randomised within 211 geographical clusters to four arms: combined WASH (ventilated improved pit latrine, two

handwashing stations, liquid soap, water chlorination, a clean play space for infants and hygiene counselling); nutrition (daily lipid-based nutritional supplement from six to 18 months, complementary feeding counselling); nutrition plus WASH; and control. As with the WASH Benefits study, at age 18 months, amongst the 3,686 children born to HIV-negative women, nutrition but not WASH improved linear growth.(175) The same results were true for the 668 HIV-exposed children.(176) In both groups, diarrhoea was not reduced by either intervention. In a further analysis of SHINE data, the WASH intervention was found to reduce the number of parasites detected in stool by multiplex PCR but no difference was found for bacteria or viruses, or overall enteropathogen prevalence or quantity.(177) The nutritional intervention had no effect on any enteropathogen group, and neither intervention had any effect on pathogen-attributable diarrhoea.

Lastly, in a randomised controlled trial of a WASH intervention in Timor-Leste, under-fives in the WASH intervention arm had lower faecal myeloperoxidase but this was not sustained over more than two years, and no change was seen in alpha-1-antitrypsin.(178)

Following these results, researchers in The Gambia measured SEP and nutritional status amongst 230 children aged 24 months living in one small geographical area but with wide-ranging family backgrounds from subsistence-farming to post-graduate education.(179) They found that WHZ demonstrated no social gradient, but that HAZ and WAZ demonstrated strong positive association with SEP. However, the majority of gains in HAZ and WAZ were seen only at the highest end of the SEP gradient, suggesting that major improvements in WASH ('transformative WASH') may be required for most low-income families: for example, piped water into the home. Noting that even in their WASH intervention arms high

enteropathogen carriage was seen at all three sites, the WASH Benefits and SHINE trial investigators echoed this call for development and testing of new transformative WASH interventions to radically reduce faeco-oral contamination in tropical low-income domestic settings.(180)

Abnormal gut microbiome

Newborns have sterile guts. The gut microbiome – the entire population of gut microorganisms including bacteria, viruses and parasites – subsequently diversifies with age, particularly during the first year of life and with introduction of complementary foods.(181) Next-generation sequencing techniques have recently allowed more comprehensive characterisation of the gut microbiota in larger-scale epidemiological studies. Across multiple settings worldwide, antibiotics, birth via caesarean section and diarrhoea are all associated with an immature gut microbiome – sometimes referred to as 'dysbiosis'.(182-188) The gut microbiome differs significantly between African and European infants,(189) between Amerindian/Malawian and USA children(113) and between Bangladeshi children with and without AM.(184, 190) Human breastmilk consumption is associated with age-appropriate gut microbiome diversification – specifically a dominance of the beneficial *Bifidobacterium* species – via multiple known mechanisms: free oligosaccharides serve as decoy receptors for epithelial binding sites, preventing epithelial colonisation by pathogen and also promoting *Bifidobacterium*; proteolysis of another human milk protein κ-casein produces glycomacropeptide which also serves as a decoy receptor; proteolysis of lactoferrin produces

lactoferricin, a potent antimicrobial; cytokines and secretory IgA facilitate pathogen killing in the gut lumen.(113)

The majority of gut microbiome studies in malnutrition to date have focussed on bacteria. Virus, parasite and even fungal species are likely important also.(191) Interaction between these groups occur, with certain species promoting or inhibiting others.(192) For example, amongst Zimbabwean children, dominant bacterial species were different before and after drug treatment for schistosomiasis.(193)

The earliest direct evidence that the microbiome affects nutritional status came from a study where faeces from 0-2-year-old nutritionally discordant Malawian twin pairs (one with kwashiorkor, one without) were transplanted into gnotobiotic mice that were fed a typical Malawian diet, resulting in only the mice that received faeces from the twin with kwashiorkor becoming malnourished.(194) Subsequently, machine learning algorithms were applied to 16S sequencing data from stool samples from healthy Malawian under-threes to identify age-discriminatory bacterial taxa.(195) A 'microbiota-for-age Z-score' was derived from this and used to describe other children with varying degrees of undernutrition as characterised by lean body mass, metabolic phenotype and bone maturation. Poorly nourished children had more immature microbiota-for-age. Again, transplantation of stool from these children to recipient gnotobiotic mice induced the same deteriorations in nutritional status in the mice. Efforts to improve gut microbiome maturity using lipid-based nutritional supplements in 6-12-month-old Malawian infants were not successful.(196)

Given the emerging evidence for the importance of the gut microbiome in AM, abnormal gut microbiome (sometimes referred to as dysbiosis) may also play a role in the pathogenesis of EED. Dysbiosis is seen in chronic inflammatory bowel disease and hypothesised to modulate and promote disease progression.(197) Changes in the upper and lower intestinal microbiome in SIBO have been discussed above. The first and to date only study of gut microbiome in EED suggests that differences are present. 81 stool samples from Malawian children with varying degrees of EED (defined here by LM test) underwent 16S rRNA gene sequencing.(198) The *Proteobacteria* phylum was reduced in severe EED. *Megasphaera*, *Mitsuokella* and *Sutterella* genera were higher and *Klebsiella* and *Clostridium_XI* were lower in EED. In high-income settings, probiotics are often given with the intention of improving the gut microbiome for varied desired outcomes. However, *Lactobacillus* probiotic supplementation in Malawian toddlers did not result in improvements in urinary LM ratio.(169) The study authors reasoned that the probiotic dose and/or duration may have been insufficient. The ongoing AFRIBIOTA project aims to describe dysbiosis in the context of EED and stunting via a case-control methodology amongst children in the Central African Republic and Madagascar.(199)

Extra-gastrointestinal and/or systemic illness

Pathways from gut to systemic inflammation have so far been discussed. In the reverse direction, gut inflammation has been observed in association with several acute systemic or non-gut illnesses: for example, respiratory illnesses and systemic infections such as malaria. Enteropathy is also commonly seen in HIV infection.

Malaria is an example of a systemic acute infection with possible effects on the gut. In Thai adults with acute slide-positive *Plasmodium falciparum* malaria, urinary LM ratios were abnormally high, reducing to normal during convalescence.(200) Certain gut microbiota profiles have been associated with protection against malaria transmission in humans and mice.(201) However, a small initial study has suggested that acute malaria infection does not result in appreciable differences in gut microbiota.(202) Globally, paediatric malaria morbidity and mortality is increased in undernutrition and in the specific nutrient deficiencies vitamin A, zinc, iron and folate.(203) Mendelian randomisation studies using sickle cell alleles have implicated malaria exposure as a risk factor for stunting.(204) The mechanism through which malaria may cause stunting is unclear. Neither mosquito net use nor recent treatment for malaria were associated with faecal calprotectin amongst urban Ugandan school-aged children.(83) However, this analysis was not adjusted for socio-economic position or other factors that may be co-linear with mosquito net usage. Malaria has not been included amongst measured putative risk factors for EED in studies to date.

Symptomatic enteropathy, with chronic diarrhoea and weight loss, is common in severely immune-suppressed people with HIV infection and those who have progressed to acquired

immune deficiency syndrome (AIDS).(205) The HIV virus itself may cause direct damage to intestinal epithelium, whilst also permitting injury by other gut pathogens by weakening host defences against them. More recently, it is understood that gut damage may in fact modulate progression of HIV disease itself.(205) The histopathological features of the gut in HIV infection are similar to those of EED, especially during advanced stage AIDS. In Zambian children with SAM and persistent diarrhoea, gut histological appearance was indistinguishable between HIV-positive and -negative children with the exception of deeper crypts and a 10% reduction in villous surface area to volume ratio amongst those HIV-positive.(69) Although CRP was raised amongst HIV-positive children, markers of intestinal bacterial translocation (serum anti-LPS, bacterial DNA in blood) did not differ. HIV enteropathy has been proposed as one of the overlapping subsets within the larger pool of EED, with other subsets being micronutrient deficiencies, persistent diarrhoea and SAM.(205)

Acute malnutrition

There is significant overlap between research into EED and into AM, and between the geographical and sociodemographic distributions of the two conditions.

EED has been proposed to arise from a combination of a moderately malnourished diet and chronic oral enteropathogen exposure. In a mouse model, expansion of certain bacterial species (*Bacteroidetes* and *Proteobacteria*) was seen in the small intestines of mice fed a malnourished diet compared to isocaloric controls.(206) Furthermore, mice exposed orally

to *Bacteroidales* and *E. coli* developed histopathological signs of EED only when this was combined with a malnourished diet. Bacterial exposure alone did not result in any EED-like changes. In Zambian children with SAM and persistent diarrhoea, mRNA transcripts from intestinal biopsies identified multiple abnormalities relating to mucus production and integrity, antimicrobial defence and nutrient absorption.(207) Many other studies demonstrate EED-like changes in children with AM.(208) In a study of Malawian children with complicated SAM already described above, as well as markers of systemic inflammation, faecal calprotectin concentrations were raised and multiple enteropathogen carriage common: both independent of diarrhoea.(84)

Summary

In summary, EED is a widespread pathological condition with good evidence for causative association with stunting and other forms of undernutrition amongst children in tropical low-income settings. Whilst there are many similarities with better known conditions common in high-income settings such as coeliac disease, Crohn's disease and ulcerative colitis, EED does appear to be distinct from these. EED has even more phenotypic overlap with HIV and AM enteropathies. Distinction from these is less clear, not least due to the high prevalence of these two underlying risk factors in EED-endemic settings. EED is characterised by intestinal and systemic inflammation, increased permeability, loss of absorptive surface area, microbial translocation and intestinal dysbiosis. Amongst these, inflammation seems most strongly associated with consequent undernutrition. Inflammation suppresses growth in EED via direct appetite suppression and growth hormone inhibition. There is conflicting evidence for

association of EED with poor neurodevelopment and impaired oral vaccine response, and early supportive evidence for EED as a mediator for the known association between childhood stunting and adult risk of non-communicable disease. As well as inflammation, immune tolerance is also seen in EED. It is not yet clear which of these is the predominant driver of detectable morbidity and mortality, or indeed whether a pro-inflammatory state is an adaptive or maladaptive response to a challenging environment. In recognition of its multi-faceted phenotype, multiple biomarkers are increasingly used and combined to characterise EED. EED is likely to be caused by a critical interplay between poor diet and enteropathogen exposure. Toxins may also play a role. No one enteropathogen is likely to be the main culprit, and chronic subclinical carriage rather than acute symptomatic diarrhoea is likely to be more important overall for growth. There is emerging evidence that the gut microbiome is abnormal in EED. Early evidence from Bangladesh supports association between SIBO, EED and stunting. The causative role of extra-gastrointestinal and systemic acute illnesses in EED is not yet clear. Evidence for a possible protective role for breastfeeding against EED is conflicting to date. Interventions against EED have so far met with limited success, with antibiotics, dietary changes, anti-inflammatory drugs and micronutrient supplements mostly effective and probiotics and WASH ineffective to date.

With the aim of addressing some of the knowledge gaps described above, the birth cohort study presented in Chapter 5 of this DPhil thesis was designed to investigate putative risk factors for EED during the first 10 months of life amongst infants in rural coastal Kenya. Particular emphasis was placed on appraising the possible roles of SIBO, acute illnesses, season, antibiotics and infant feeding method in the early development of EED. In Chapter 2, results from a cross-sectional survey of under-fives in the same study area as the birth

cohort are presented. Here, contextualising the subsequent birth cohort, distribution of EED and its putative risk factors by age and location of residence is explored, along with associations between stunting and EED and their putative risk factors.

Chapter 2: Social and environmental determinants of malnutrition and Environmental Enteric Dysfunction amongst under-fives in Junju, Kilifi: a cross-sectional survey

Background

Undernutrition – including stunting – is likely multifactorial. Individual studies have implicated risk factors for stunting at the level of the region, community, household, mother/caretaker and individual child. These include genetic and epigenetic factors and also environmental factors at both the intrauterine and post birth stages. Contributors to the intrauterine environment include specific intrauterine infections, maternal EED, air pollution and inadequate maternal diet. Postnatally, early introduction of complementary foods, poor nutritional and microbial quality of these foods, poor WASH, recurrent acute infections and environmental toxins have been implicated.(209) Distal factors such as political instability, urbanisation and climate act on stunting risk via these pre- and postnatal proximal mediators.

The most recent Demographic and Health Survey (DHS) in Kenya in 2014 recorded national prevalence of moderate stunting amongst under-fives as 26% and severe 8%.(210) Of the 47 counties in Kenya, Kilifi County had the third-highest overall prevalence of stunting at 39%. In the 2014 DHS, stunting in Kenya was most common amongst the lowest wealth quintile and amongst children whose mothers had no formal education. Stunting prevalence was also higher amongst children of male sex and in rural locations.

Evidence exists for spatial heterogeneity of malaria transmission intensity at the Junju study site.(211) Anecdotally, other putative stunting and EED risk factors such as socioeconomic status, housing density and water supply are known to vary across geographical space within the study area. The spatial epidemiology of stunting and EED has not yet been characterised in any setting.

This chapter describes a cross-sectional survey of under-fives that was undertaken during June, July and August 2015 in Junju, coastal Kenya. The survey was intended to contextualise and help plan for the later birth cohort study, as well as to explore associations in cross-section between stunting, EED and selected risk factors amongst under-fives in this low-income rural tropical setting.

Hypotheses

Amongst children aged 0-59 months, within the study area in Junju:

- HAZ is associated with WHZ;
- HAZ and EED both demonstrate spatial dependency;
- HAZ and EED are clustered by household, homestead and village;
- Markers of EED are associated with HAZ;
- HAZ and EED are associated with selected risk factors.

Aim and objectives

Aim

- Describe the prevalence, spatial dependency and associations of undernutrition, EED and putative endogenous, social, demographic, nutritional, biological and environmental risk factors amongst under-fives in Junju – both as a stand-alone study and also in order to A) help plan and B) help interpret results of the subsequent cohort study.

Objectives

Amongst children aged 0-59 months, in Junju:

- Describe distributions of HAZ, WHZ, WAZ, EED and risk factors by age;
- Test whether HAZ is associated with WHZ;
- Describe spatial dependency of HAZ and EED within the study area;
- Test whether HAZ and EED are clustered by household, homestead or village;
- Test whether markers of EED are associated with HAZ;
- Describe associations of HAZ and EED with selected risk factors.

Methods

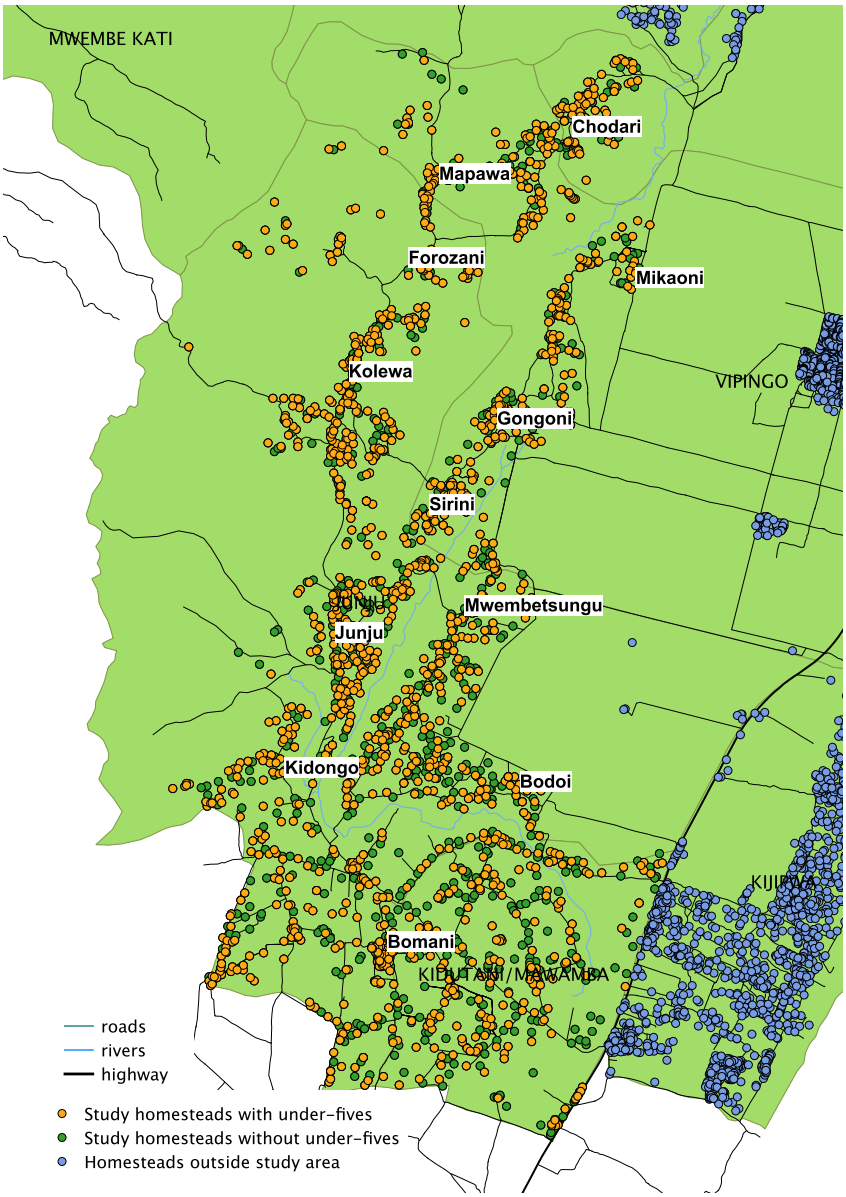
Overview

300 randomly selected homesteads within an area comprising 12 villages were approached for participation. In each participating homestead, a consent form was signed by the participant's primary caretaker (Appendix 6) and a questionnaire characterising exposures likely relevant to undernutrition and EED was administered (Appendix 7). For all resident under-fives, weight and height or length were measured, and stool collection attempted. Latitude and longitude of homesteads were recorded. The survey took place towards the end of the long rainy season during June to August 2015.

Study area and sampling frame

The study area for the cross-sectional survey was chosen to encompass the population within and surrounding the area already selected for the cohort (see page 180). This area comprised 12 villages in one contiguous zone measuring 26km by 13km. Three government primary healthcare facilities serve the area: Junju, Pingilikani and Bomani dispensaries. The area is located inside the Kilifi Health & Demographic Surveillance System (KHDSS). For participating homesteads, KHDSS entails quarterly enumeration rounds, where fieldworkers confirm identities of current residents as well as noting any recent births, deaths, in-migrations and out-migrations.(212) Using 'round 35' (April 2015) KHDSS data, 2,178 homesteads (homestead definition: Appendix 8) were identified as lying within the boundaries of the chosen area, of which 1,420 were known to contain at least one child aged less than five years: a total of 3,384 under-fives (Figure 2).

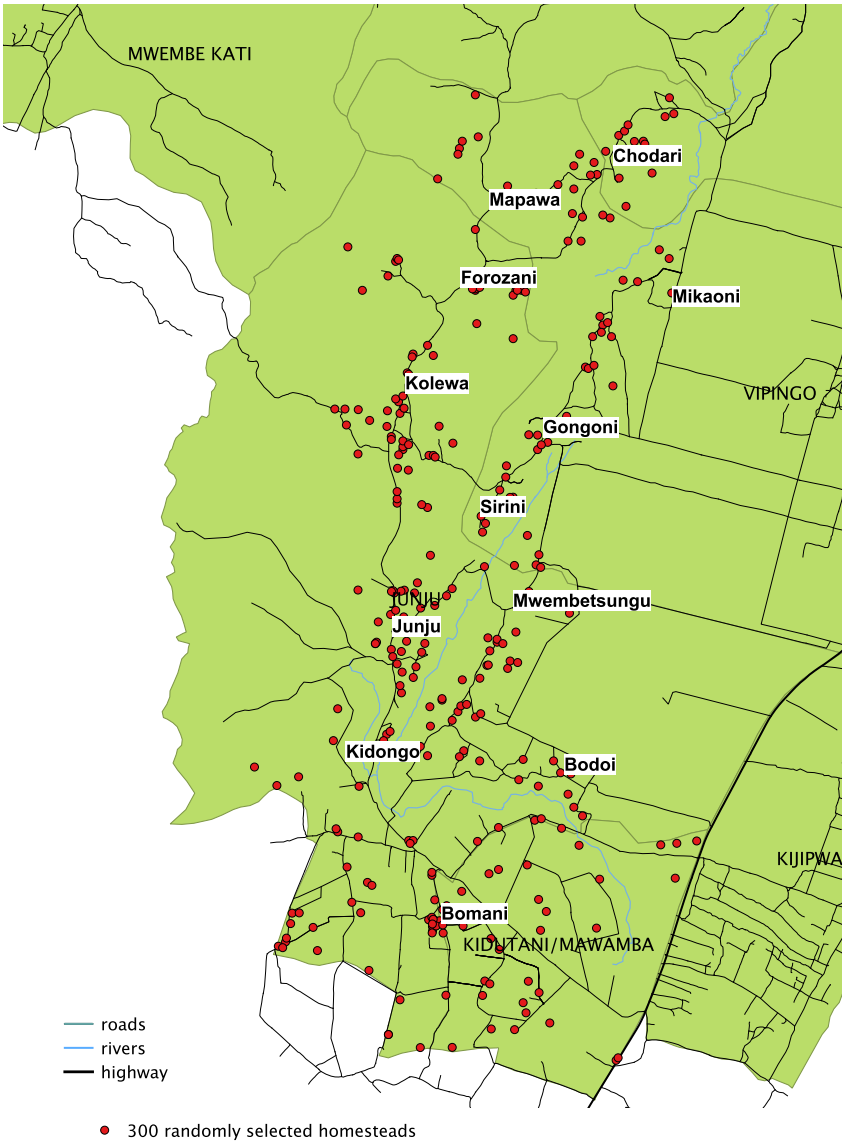
Figure 2: Map of the study area for the cross-sectional survey.
2,178 homesteads lay within the 13x26km study area boundary, of which 1,420 contained at least one under-five during the April 2015 35th KHDSS re-enumeration round. Green background indicates Kilifi County. White background indicates neighbouring Mombasa County (or, bottom right, the Indian Ocean). The two-lane highway connects Mombasa in the south to Kilifi in the north. The uninhabited expanse of land east of the study area is Vipingo sisal plantation. West of the study area is uninhabited forest and agricultural land. Bomani village in the south of the study area is close to sea level and bordering the more densely populated Mombasa suburb of Mtwapa. All roads are dirt except the highway. The nine villages north of Bodoi and Kidongo are arranged in an elevated hilltop ring around an uninhabited central valley and flood plain.



Sample size and sampling method

From these 1,420 homesteads, using a random number generator, a simple random sample of 300 homesteads was selected for approach (Figure 3). Sample size was chosen, using data from the area's most recent DHS,(213) assuming 50% prevalence of stunting (HAZ<-2) and standard error 3%. With alpha 0.05, a sample size of 300 participants was found to enable estimation of stunting prevalence with minimum precision +/-5%. Within each participating homestead, to increase sample size above 300 with minimum extra logistical effort, every under-five was included. With reference to the level of the participant (child), the sampling method was therefore two-stage (first stage: select homesteads from KHDSS; second stage: select all under-fives in homestead). Under-fives across the study area each had an equal chance of being selected regardless of their homestead size: i.e. the sampling method was self-weighting.

Figure 3: Map of homesteads selected for inclusion in the cross-sectional survey.
300 selected homesteads from the 26x13km study area sampling frame of 1,420 homesteads recorded as containing at least one under-five during the April 2015 35th KHDSS re-enumeration round.



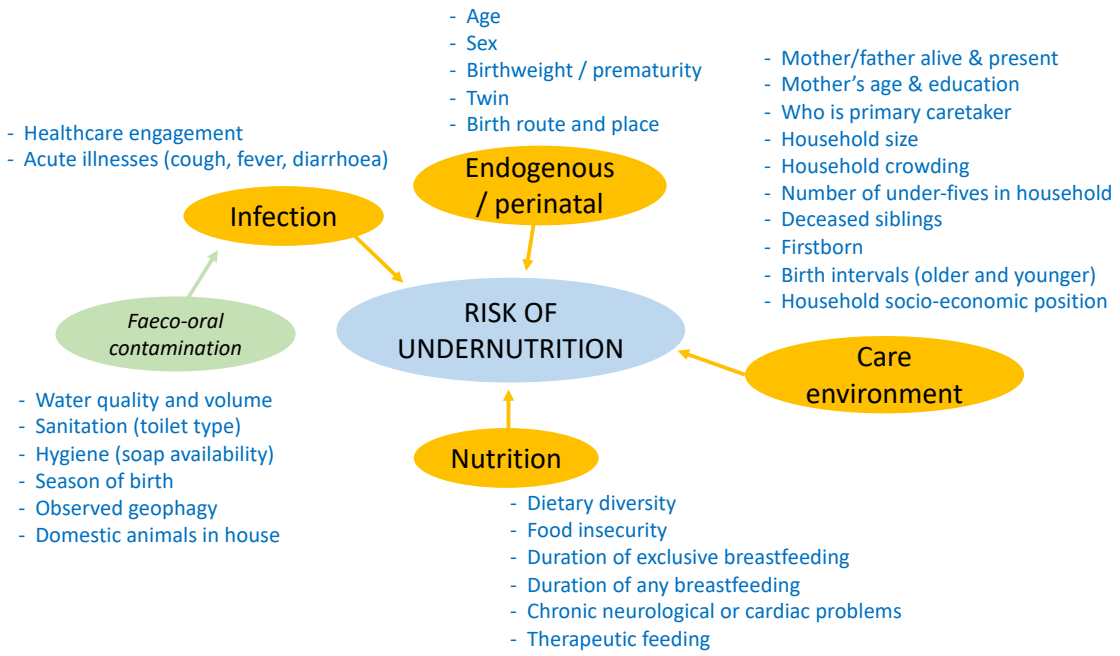
Questionnaire

A questionnaire was developed with the aim of measuring potential exposures and relevant confounders for undernutrition (Appendix 7). Factors were selected according to their hypothesised influence on risk of undernutrition and grouped into the proximal mediators 'infection', 'nutrition', 'endogenous/perinatal' and 'care environment' (Figure 4). These

proximal mediators were hypothesised to encompass the major biological pathways through which each factor gives rise to increased or decreased risk of undernutrition. In this schematic, EED is not shown but considered as a pathogenic process through which some or all of the proximal mediators exert their influence on the outcome of undernutrition.

Factors within each proximal mediator were selected following review of existing established nutritional and general surveys (United Nations Children's Fund (UNICEF) Standardised Monitoring and Assessment of Relief and Transition (214) and Multiple Indicators Cluster Survey(215)), from anecdotal experience of clinical care in Kilifi County Hospital malnutrition ward, and through discussion with fieldworkers as to what they deem important risk factors for undernutrition in their local area.

Figure 4: Factors, and their unifying mediators, that may influence risk of childhood undernutrition in Junju. Yellow = proximal mediator; green = distal mediator.



The questionnaire was piloted with local volunteer mothers and administered by trained fieldworkers on hand-held Android tablets using 'Open Data Kit' software(216) with inbuilt real-time data quality checks and user feedback. The questionnaire was written in English but administered verbally by 10 locally resident fieldworkers in the local first language, Giryama. A period of fieldworker training preceded fieldwork and covered research ethics, principles of informed consent, interviewing skills, subject context and technical background pertaining to each of the selected factors and their measurement. All fieldworkers obtained formal certification in Good Clinical Practice.

Residents of Junju typically live in nuclear family units (household) within an extended contiguous cluster of physical dwellings (homestead). The questionnaire was administered at the household level and administered in three stages: household, mother, and child. Household-stage questions were asked of the family member most familiar with day-to-day household activities. In most cases, this was the mother. Occasionally, more than one respondent was involved if a question needed family consensus or discussion. Mother-stage questions were asked of the mother only, in privacy. Child-stage questions were asked of the child's primary caretaker.

Where available, hand-held child health records, KHDSS records and national identification cards were cross-checked against participant responses to optimise accuracy of mother's age, child dates of birth and birthweight. Where a questionnaire response could be confirmed by fieldworker direct observation (e.g. housing materials, type of toilet, presence of domestic animals, water source), this was done with permission. However, fieldworkers did not request to see inside dwellings, nor did they request to see any documents beyond

those confirming mother's age, child date of birth or birthweight. Where they were not already familiar with a described communal household water source, fieldworkers would walk to that source to confirm its type and its distance from the homestead. Gestational age at birth was estimated by comparing documented Estimated Date of Delivery (EDD) against caretakers' recall, or, where one or more of these dates were missing or forgotten, by asking caretakers whether the child was born more than one month before their EDD. Prematurity was defined as birth more than one month before EDD. Household dietary diversity was defined using the 2006 FANTA (Food And Nutrition Technical Assistance) II Household Dietary Diversity Score.(217) Respondents were asked to list foods consumed by all household members during the past 24 hours. These foods were recorded in 12 categories (cereals, fish, roots and tubers, vegetables, fruits, meats and poultry, eggs, pulses and legumes, milk, fats and oils, sugars, miscellaneous). Following the FANTA II categorical criteria, a household was deemed to have high dietary diversity if residents had consumed food items from six or more categories during the past 24 hours; medium diversity four or five; low diversity three or fewer. Water sources and sanitation facilities were categorised by fieldworkers according to WHO definitions for 'improved' and 'unimproved'. Examples of each category are given in Appendix 9.

Where suitable respondents were absent from a homestead on the day of visit, up to three further visits would be made, at different times of day including weekends or evenings, each at least two days apart and spread over up to 14 days. After a third unsuccessful visit, fieldworkers would determine through local enquiry whether absence was due to short-term travel or permanent out-migration.

Anthropometry

Fieldworkers were trained and assessed according to WHO anthropometry guidelines(218) and followed a Standard Operating Procedure (SOP) (Appendix 10). Weight, MUAC and length or height were measured for every participating under-five. As recommended by WHO, length was measured for those aged less than two years whilst height was measured for those aged two years and above. Following the WHO guideline, where a child aged two years or older could not stand, length was measured and 0.7cm deducted from this reading to give an estimate of height. Where a child aged less than two years refused to lie down, height was measured and 0.7cm added to this reading to give an estimate of length.(218) Participants meeting MUAC-based WHO criteria for AM (clinical signs of kwashiorkor and/or MUAC less than 12.5cm amongst those aged six months or more) were referred to their nearest government dispensary for further screening and treatment. In addition, any participant who was acutely unwell for any reason on the day of visit was referred to their nearest government dispensary.

Focus group discussions

Nine focus group discussions were planned and carried out by Research Assistant John Muriuki with support from fieldworkers. They took place over two days during survey fieldwork in nine of the 12 villages. At each site, participants were 10-15 local mothers and caretakers intended to represent a cross-section of the community and selected by village

elders. Discussions were facilitated by a fieldworker, were held in Giryama and Swahili languages according to the participants' preferences, and grouped around six themes: food availability, food access, vulnerability and coping strategies during times of food insecurity, water and sanitation, childcare practices and perceptions of child health, infant and young child feeding practices. Predetermined specific questions were asked of the group (Appendix 11), but further issues and questions arose as each group's discussions took varying paths.

Stool sampling and quantification of EED biomarkers

After questionnaire and anthropometry were completed, participants' caretakers were instructed on stool collection technique using the sterile container and spatula provided and asked to collect their child's first stool of the following morning from a provided new plastic non-absorbable nappy cover (for children who used nappies) or sheet of clean paper (for older children). Caretakers were asked to place the filled specimen container in a cool dark place in the home, wrapped in the tin foil provided, whilst awaiting collection. The next morning, starting from 6am, fieldworkers would visit homesteads to collect stool samples. At the homestead, samples were placed in a coolbox at 2-8^{o}C and transported to KEMRI Wellcome Trust Research Programme Clinical Trials Laboratory (KWTRP CTL). Timing of stool passage, collection at homestead and freezing at KWTRP CTL were noted. When a participant had not yet passed stool by the fieldworker's visit, two repeat visits would be made. If stool was still not obtained after the third attempt, collection was abandoned. For participants experiencing diarrhoea (three or more loose stools in the past 24 hours), stool collection was delayed until at least seven diarrhoea-free days had passed.

On arrival at KWTRP CTL, stool was immediately stored in aliquots at -80^{o}C. Prior to freezing, stool was examined for ova, cysts and parasites (OCP) by formol/formalin ether concentration method. Participants with positive OCP result were notified by the study clinician within 2-3 days and referred to their nearest participating government dispensary for further assessment and treatment with mebendazole where indicated. The three participating dispensaries (Junju, Bomani and Pingilikani) were given weekly lists of participants with positive OCP results, and participants followed up at home by fieldworkers if they did not attend their local dispensary within a week.

Stool samples underwent ELISA for alpha-1-antitrypsin, myeloperoxidase and neopterin using commercial kits, and four-parameter logistic regression used to fit non-linear standard curves for conversion of optical densities to marker concentrations. See Appendices 12 and 13 for detailed methods and laboratory SOPs.

Statistical analysis

Statistical analysis was carried out using Stata 15.0.(219) Principal Component Analysis (PCA) was used to group households by water quality and socioeconomic status. Methods for these two PCAs followed the principles set out by Vyas et al.(220) Insights from focus group discussions were used to select variables that were deemed discriminatory within the local area.

HAZ, WHZ and WAZ were calculated using the Stata software add-on package 'zscore06' which compares to the WHO 2006 Growth Standards.(37, 221)

Global Moran's index was used to test for spatial dependency of mean HAZ, mean log faecal alpha-1-antitrypsin, mean log faecal myeloperoxidase and mean log faecal neopterin by homestead. Using the inbuilt Stata command 'spatwmat', a matrix was generated to cover all 229 homestead geolocations and the greatest Euclidian distance between homesteads calculated. Subsequently, using the inbuilt Stata command 'spatgsa', a Global Moran's index (and derived p-value; null hypothesis being no spatial autocorrelation) was generated for each of the four selected outcomes within the band defined by the greatest Euclidian distance.

Following initial descriptive analyses, testing a hypothesis that participants with lower HAZ also have lower WHZ, a linear regression model was run with outcome HAZ and fixed effects WHZ, age and sex. Following this, testing a hypothesis that HAZ and EED biomarkers are clustered by village, homestead or household, for each of four outcomes (HAZ, log faecal alpha-1-antitrypsin, log myeloperoxidase and log neopterin) a null mixed-effects linear regression model was compared using likelihood ratio testing with models including village, homestead or household as a random effect. To explore factors associated with concurrent low HAZ, exposure variables were then selected for inclusion in a multivariable mixed-effects linear regression model with the outcome of HAZ. Homestead was included in this model as a random effect. Variable selection was prospective and hypothesis driven. Variables were dropped prior to model building if deemed of insufficient quality, co-linear, or categorical or binary and too highly skewed. Following this, to assess performance of the three faecal EED

biomarkers against HAZ in a cross-sectional context, three multivariable linear regression models were developed, each with the outcome HAZ and adjusted for age and sex, with the additional fixed effect of alpha-1-antitrypsin, myeloperoxidase and neopterin respectively. Pearson's coefficient was used to assess correlation between each of the three faecal EED biomarkers. Lastly, testing a hypothesis that selected factors are associated with markers of EED, a smaller number of proximal, biological factors were selected for inclusion in three multivariable mixed-effects linear regression models with outcomes alpha-1-antitrypsin, myeloperoxidase and neopterin. These models aimed to characterise factors associated with biomarkers of EED and were adjusted for clustering by homestead using random-effects.

Results

Survey participation

Of the 300 homesteads approached, 24 were absent due to short-term travel, 24 had permanently out-migrated, nine no longer contained any under-fives and 14 declined to participate. The remaining 229 homesteads participated in the study. These 229 homesteads comprised 336 households and contained 509 under-fives. These 509 represent 15% of the total 3,384 under-fives recorded as resident in the study area during KHDSS round 35.

Anthropometry findings

Frequency distribution for HAZ was significantly shifted towards negative values, with a peak between -1 and -2 Z-scores, a significant minority of values below -3 Z-scores and a tail of outlying negative values as low as -6 Z-scores (Figure 5). WAZ distribution followed a similar pattern, with a peak around -1 Z-scores, less dispersed, and with a lesser outlying tail extending to around -5 Z-scores. WHZ distribution peaked symmetrically around zero and was even less dispersed.

Figure 5: Frequency distribution of HAZ, WAZ and WHZ for 509 under-fives.
Red lines indicate thresholds for at-risk (-1), moderate (-2) and severe (-3) stunted, underweight and wasted status.

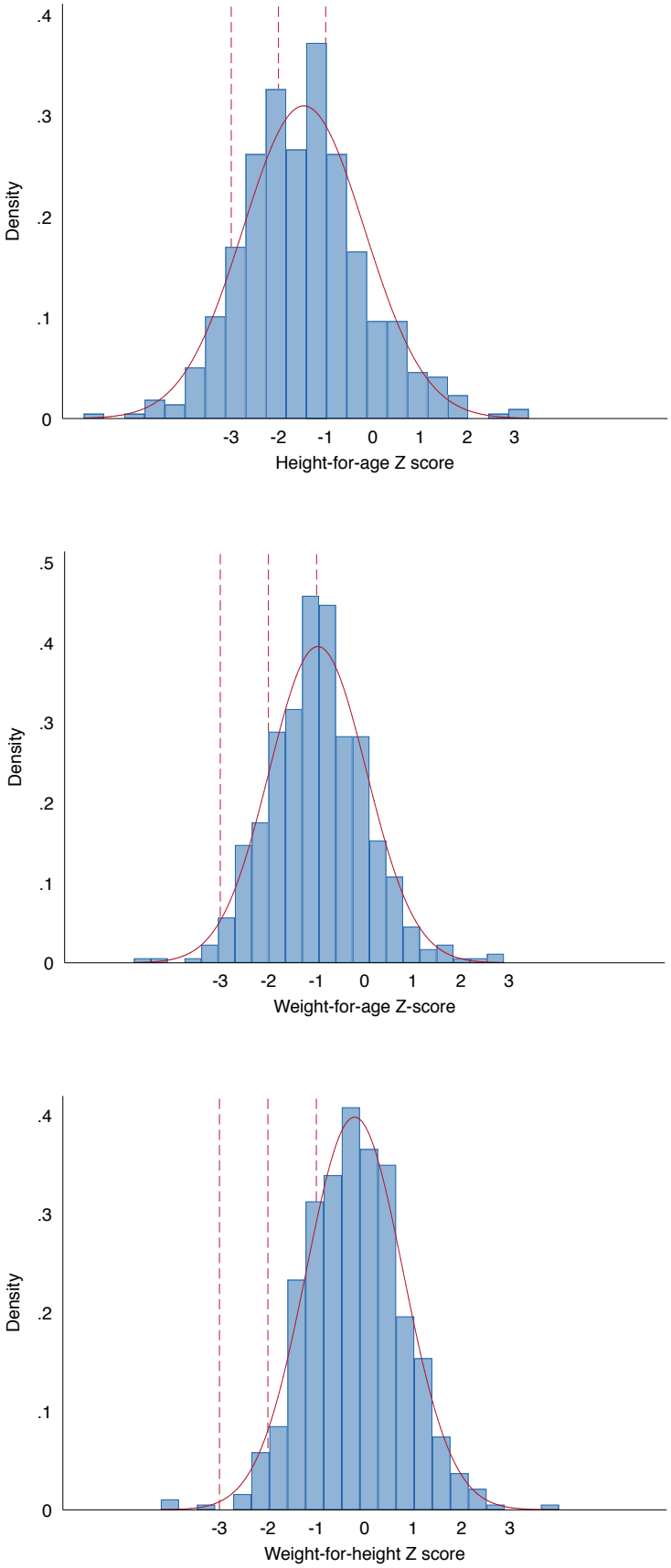


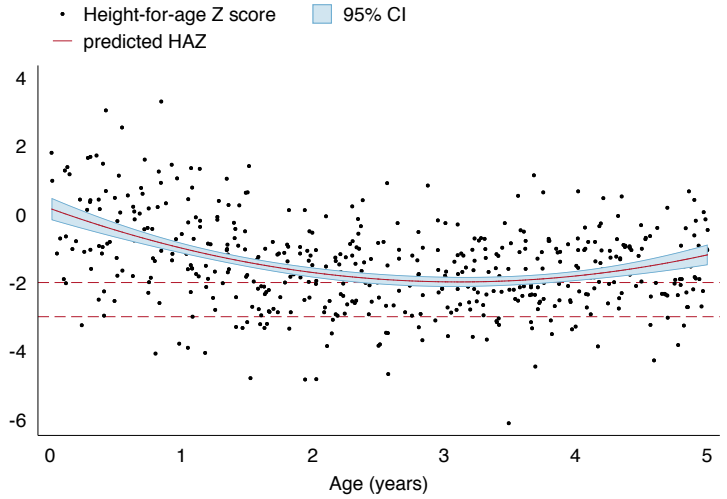
Table 1 details categorised anthropometry findings for the 509 participants. For three (0.6%) participants, weight was measured but WAZ and WHZ were not calculated because of the presence of oedema. These three participants were confirmed as meeting clinical criteria for kwashiorkor. For one participant, length could not be reliably measured due to fixed flexion contractures of both legs. HAZ and WHZ were therefore not calculated for this individual. Prevalence of moderate or severe wasted status (WHZ<-2) was low, at 3% (95% CI 1 to 4%). Prevalence of stunted status was relatively higher with at-risk (HAZ<-1) at 33% (95% CI 29 to 38), moderate (HAZ<-2) 25% (22 to 29) and severe (HAZ<-3) 9% (7-12).

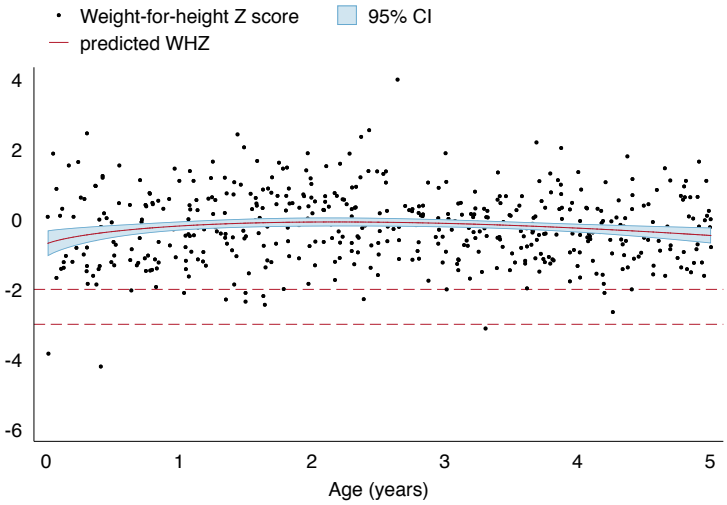
Table 1: Frequency distribution of indicators of nutritional status amongst 509 under-fives in Junju, Kilifi.

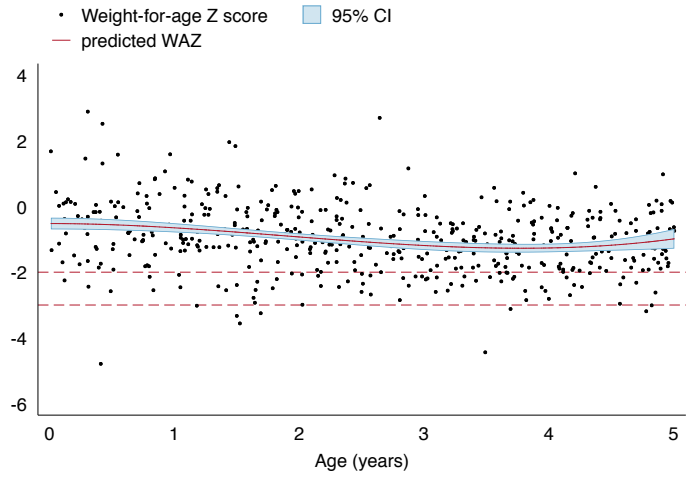
Factor	Category	Distribution (%)	Missing data (%)
Stunting	Not at risk (HAZ≥-1)	164 (32)	1 (<1)
	At risk (-2≤HAZ<-1)	169 (33)	
	Moderate (-3≤HAZ<-3)	129 (25)	
	Severe (HAZ<-3)	47 (9)	
Wasting	Not at risk (WHZ≥-1)	392 (77)	4 (<1)
	At risk (-2≤WHZ<-1)	101 (20)	
	Moderate (-3≤WHZ<-3)	10 (2)	
	Severe (WHZ<-3)	3 (<1)	
Underweight	Not at risk (WAZ≥-1)	251 (50)	3 (<1)
	At risk (-2≤WAZ<-1)	182 (36)	
	Moderate (-3≤WAZ<-3)	64 (13)	
	Severe (WAZ<-3)	9 (2)	
Overweight	No (WHZ<2)	497 (98)	3 (<1)
	Yes (WHZ>=2)	9 (2)	
Kwashiorkor	No	506 (99)	none
	Yes	3 (1)	
Moderate or severe acute malnutrition	Neither	493 (97)	none
	MAM	10 (2)	
	SAM	6 (1)	
MUAC (cm; amongst age ≥6 months)	≥12.5	460 (99)	none
	11.5-12.4	6 (1)	
	<11.5	0	

HAZ declined with age (although this decline was not linear: a fractional polynomial was selected by Stata as a better fit than a straight line) most steeply during the first 24 months then appeared to increase again from around 36 months onwards. (Figure 6; top). WHZ showed little variability with age (Figure 6; middle). WAZ was lowest at around four years of age (Figure 6; bottom).

Figure 6: Observed and predicted HAZ, WHZ and WAZ by age for 509 under-fives in Junju.
Blue lines indicate 95% confidence intervals for values predicted by fractional polynomials. Red dotted lines indicate y axis values at -2 (threshold for moderately stunted, wasted or underweight) and -3 (threshold for severely stunted, wasted or underweight).

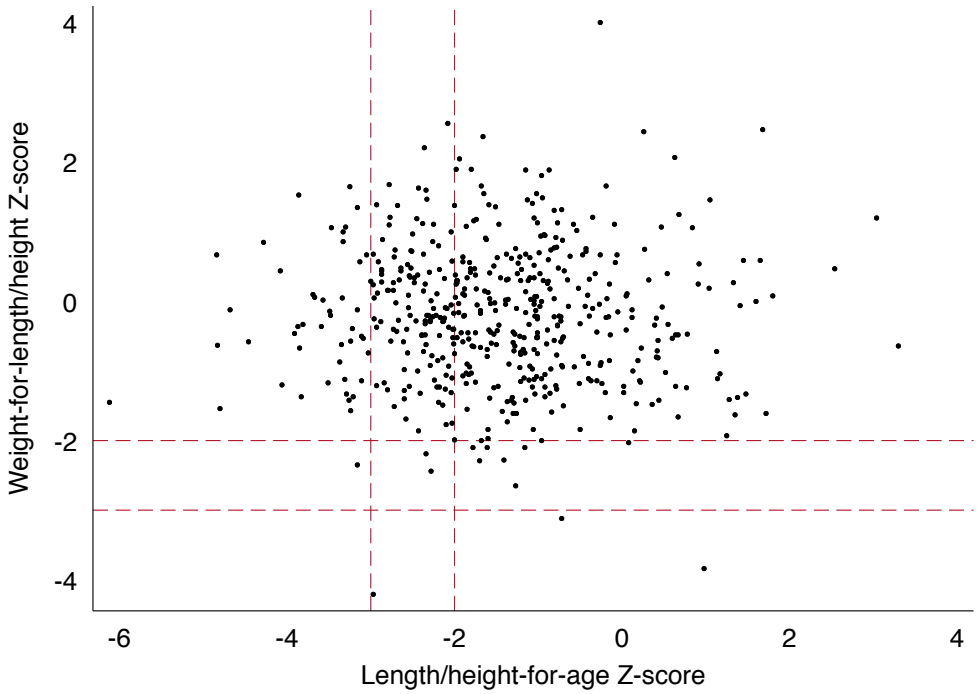






Testing a hypothesis that more stunted participants (lower HAZ) were more likely to also be more wasted (lower WHZ), linear regression modelling of the association between HAZ and WHZ adjusted for age and sex yielded no evidence to support such an association (Figure 7).

Figure 7: Scatter plot showing HAZ and WHZ by individual survey participants.
n=505 where data for both Z-scores are available. Red dotted lines indicate threshold values for severely and moderately stunted and wasted. Linear regression of WHZ with HAZ, age and sex as fixed effects: coefficient for WHZ -0.04, 95% CI -0.15 to 0.07, p=0.49.



Perceived risk factors for undernutrition: endogenous/perinatal

Focus group discussions yielded the following paraphrased[†] insights into putative local endogenous/perinatal risk factors for undernutrition:

[†] Focus group discussion output is paraphrased here and in subsequent sections by Research Assistant John Muriuki

- "A healthy mother during pregnancy will not always guarantee a healthy newborn. However, eating well during pregnancy mostly leads to healthy children. Poor nutrition during pregnancy can lead to premature birth."
- "Minimum birth interval spacing recommendations are unknown. Family planning is not carried out. A woman is usually pregnant again by around nine months after delivery."

Questionnaire responses indicated that 4% of participants were a twin and 10% were born premature (Table 2; defined here as having been born more than one month before estimated date of delivery). 62% of participants had unknown birthweight: this was almost always because they had been born at home.

Table 2: Frequency distribution of putative 'endogenous/perinatal' risk factors for undernutrition amongst 509 participating under-fives.

Factor	Category	Distribution (n(%))	Missing (%)
Sex	Male	285 (56)	none
	Female	224 (44)	
Age (months)	0-5	43 (8)	none
	6-11	45 (9)	
	12-23	114 (22)	
	24-35	93 (18)	
	36-59	214 (42)	
Twin	Not twin	489 (96)	none
	Twin	20 (4)	
Delivery place	Home	266 (52)	none
	Healthcare facility	243 (48)	
Birth route	Vaginal	500 (98)	none
	Caesarean	9 (2)	
Premature*	Not premature	459 (90)	none
	Premature	50 (10)	
Birthweight	Macrosomic (≥4kg)	15 (8)	316 (62)
	Normal (2.5 to <4kg)	164 (84)	
	Low (1.5 to <2.5kg)	15 (8)	
	Very low (1 to <1.5kg)	1 (1)	
	Extremely low (<1kg)	0 (0)	

** Prematurity defined as having been born more than one month before estimated date of delivery*

Perceived risk factors for undernutrition: care environment

Following insights from the focus group discussions, six factors were selected for PCA for household SEP (Appendix 14). These were: floor, roof and wall material, household land and livestock ownership and household per capita income.

16% of participants had birth intervals to both younger and older siblings of fewer than 24 months (Table 3). 21% of participants had a sibling who had died. Here, this was defined via the mother – i.e. 21% of participants were born to a mother who herself had previously lost a child. Mother's age at first baby ranged from 13 to 38 years.

Table 3: Frequency distribution of putative undernutrition risk factors related to the proximal mediator 'care environment' amongst 509 participating under-fives.

Factor	Category	Distribution[1]	Missing (%)
Firstborn to mother	No	392 (77)	none
	Yes	117 (23)	
Birth interval to older sibling (months)	<24	83 (16)	none
	≥24 or firstborn	426 (84)	
Birth interval to younger sibling (months)	<24	50 (16)	202 (40)[2]
	≥24	257 (84)	
Mother alive	No	11 (2)	none
	Yes	498 (98)	
Mother's age when child was born (years)		26 (13-46)	56 (11)
Primary caretaker present daily	No	30 (6)	none
	Yes	474 (94)	
Father alive	No	20 (4)	none
	Yes	489 (96)	
Sibling has died	No	400 (79)	none
	Yes	109 (21)	
Mother's education	None	131 (26)	none
	Some primary	258 (51)	
	Completed primary or higher	120 (24)	
Mother's marital status	Single/divorced/widowed	15 (3)	none
	Married	494 (97)	
Primary caretaker	Mother	471 (93)	none
	Not mother	38 (7)	
Mother's age at first baby (years)		19 (13-38)	46 (9)
Number of residents		6 (2-12)	none
Number of under-fives		2 (1-4)	none
Number of residents per sleeping room		3 (0.75-10)	none
Sex of household head	Male	463 (91)	none
	Female	46 (9)	
Socio-economic position[3]	Poorest	144 (28)	none
	Poorer	121 (24)	
	Richer	121 (24)	
	Richest	123 (24)	

1. For categorical variables: n (% of total households). For continuous variables: median (range).
2. Missing because inappropriate to report due to age (≥2 years).
3. Derived from principal components analysis of six household attributes (see above).

Perceived risk factors for undernutrition: faeco-oral contamination

Focus group discussions yielded the following observations concerning local WASH practices:

- Residents generally wash hands only before eating a main meal and on no other occasions.
- When a child defecates, most caretakers bury the stool or throw it into a nearby bush. Very few will throw the faeces into a latrine, even if one is available nearby. The child is then wiped using leaves which are thrown into small open landfill sites within the homesteads (these are generated by digging for earth to construct mud walls).
- Uncooked food (fruits) are never washed before eating.

Also arising from focus group discussions, the following local beliefs were paraphrased:

- "Children are most vulnerable to illness during the rainy season (March to August) and mango season (May and June)."
- "Two-, three- and four-year-olds are particularly at risk of illness because they explore their environment and eat food without consideration for hygiene. Their mothers are usually preoccupied caring for a younger sibling."

A PCA-derived score for household sanitation and hygiene was explored using sanitation type (WHO improved versus unimproved), disposal method for children's faeces (throw in bush or bury versus throw in latrine or other sanitation facility), availability of soap in the household and presence of domestic animals inside the house. However, no collinearity was seen for these variables. Notably, households with improved sanitation were in fact less likely

to dispose of children's faeces in a latrine. No composite score was therefore developed for this domain. Instead, PCA was carried out for household water provision using six factors: drinking water source, time to fetch water and return, whether water is paid for, treatment of water, storage of water and volume used per person per day (Table 4; Appendix 15).

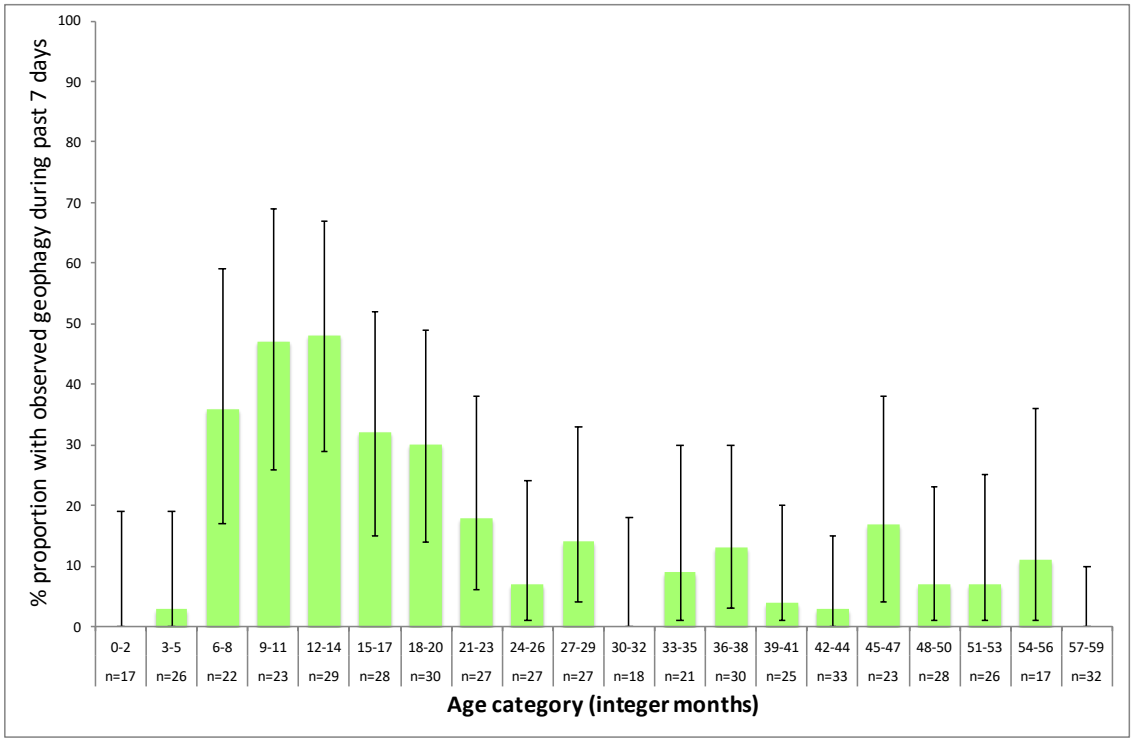
Reported geophagy (eating soil) was most common between ages nine and 14 months (Figure 8).

Table 4: Frequency distribution of putative undernutrition risk factors related to the proximal mediator 'faeco-oral contamination' amongst 509 participating under-fives.

Factor	Category	Distribution (n(%))	Missing (%)
Season of birth[1]	Dry	295 (58)	none
	Rainy	214 (42)	
Geophagy noted during past seven days	No	428 (84)	none
	Yes	81 (16)	
Water position[2]	Lowest	139 (27)	None
	Lower	132 (26)	
	Higher	151 (30)	
	Highest	87 (17)	
Sanitation type[3]	WHO unimproved	425 (84)	None
	WHO improved	84 (17)	
Method of disposal of children's faeces	Throw in bush / bury	89 (17)	None
	Latrine	420 (83)	
Soap availability in household	No	294 (58)	None
	Yes	215 (42)	
Domestic animals allowed in house	Yes	484 (95)	None
	No	25 (5)	

1. Rainy: calendar months of April, May, June, October and November. Dry: all other calendar months.
2. Derived from principal components analysis of 10 household attributes (see above).
3. See Appendix 9 for examples of WHO improved and unimproved sanitation facility.

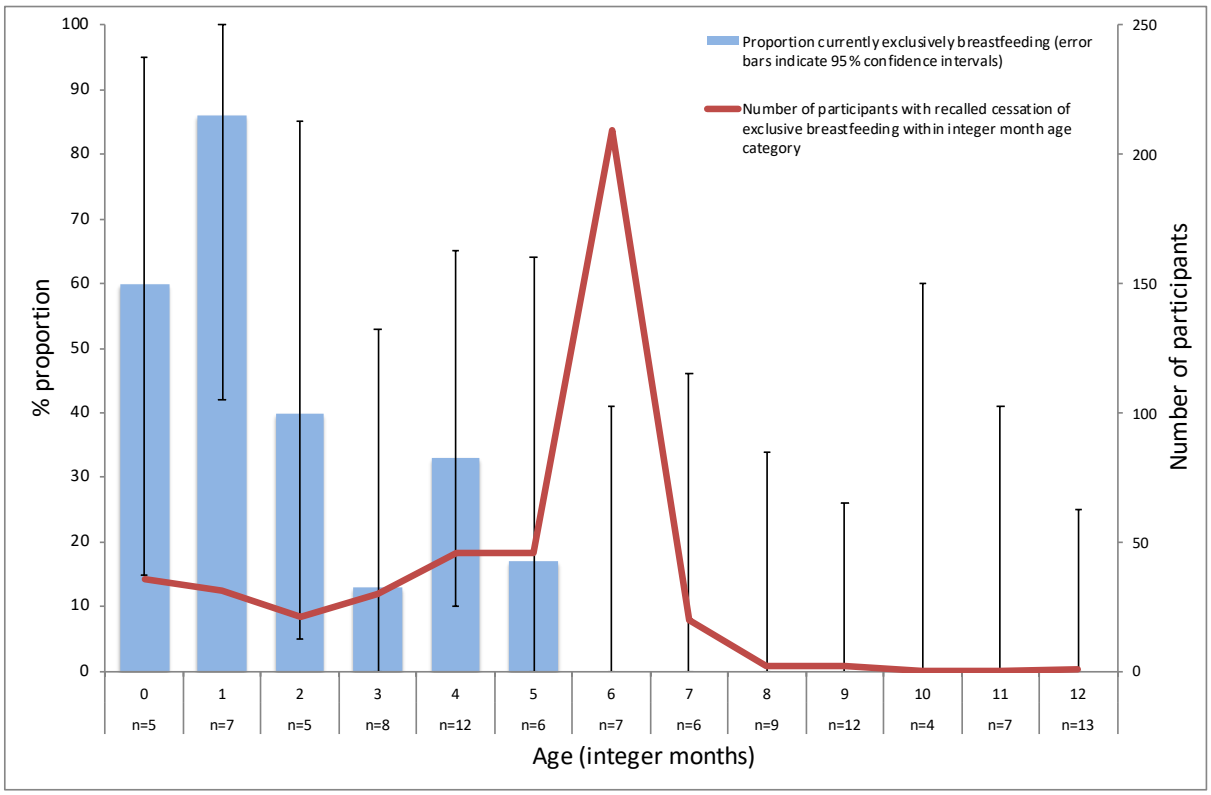
Figure 8: Proportion with observed geophagy during the seven days preceding interview, by age. Error bars indicate 95% confidence intervals.



Perceived risk factors for undernutrition: nutrition

Caretaker-recalled age at cessation of exclusive breastfeeding amongst those no longer exclusively breastfeeding demonstrated a very high peak at six months: the WHO recommendation (Figure 9). Overlaying the proportion of infants within each integer month age category with caretaker reported current exclusive breastfeeding status demonstrates introduction of complementary foods before six months of age in the majority. Discord between recalled and current statuses suggests that the recalled age may be unreliable.

Figure 9: Current and recalled exclusive breastfeeding status by age.
Percentage proportion reported to be currently exclusively breastfeeding since birth amongst 101 participants aged less than 13 months (left vertical axis; blue bars); recalled age at cessation of exclusive breastfeeding amongst 444 participants aged 6-59 months who have already started complementary feeding (right vertical axis; red line). The 'n=' notations below the horizontal axis indicate the denominator for each of the left vertical axis data points (blue bars). No participants were reported – either concurrently or via recall – to be exclusively breastfed beyond 13 months of age.

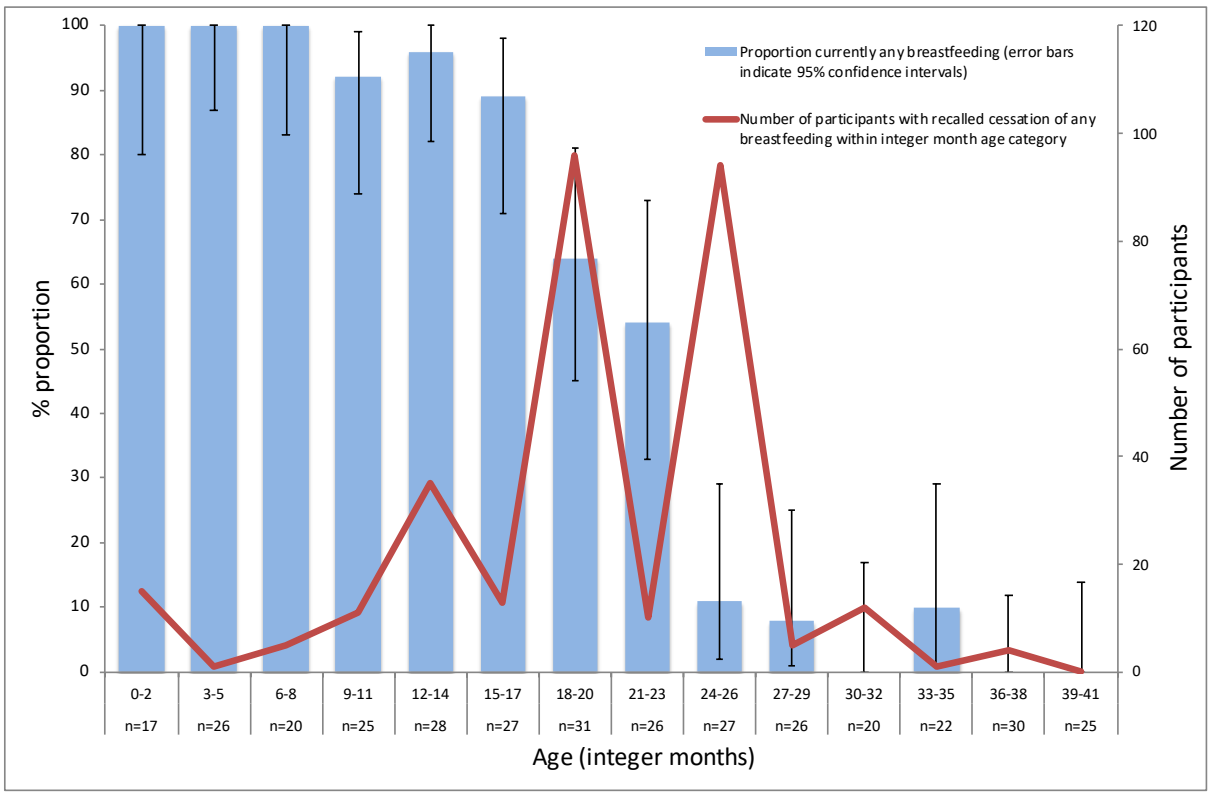


Focus group discussion participants reported that first complementary foods are introduced from anywhere between one week and six months of age. Respondents also stated that, within the first week of birth, and especially if there is a problem with supply of mother's milk, salt/sugar water, sugar water or coconut water are given "to aid digestion". They described that a thin porridge made from mixed cereals cooked in water (*uji*) is then introduced at one to two months, and that *uji* (cooked porridge made from maize flour and water) is believed to provide energy to a crying baby whose mother has insufficient breastmilk. Focus group discussion participants were in consensus that around six to eight

months of age, once the baby is perceived able to swallow solids, a soft paste made from maize flour cooked in water (*bokoboko*), bananas and thicker *uji* is introduced.

The focus group discussions revealed local beliefs that a child is at greater risk of illness if they did not breastfeed until at least two years of age. Accordingly, amongst the 307 participants aged 24-59 months, recalled duration of any breastfeeding demonstrated a high peak at 24 months: the WHO recommendation (Figure 10). However, a second equally high peak existed at 18-20 months. This latter peak is corroborated by overlaid reports of current breastfeeding by age category, which demonstrated sharpest decline between 18 and 23 months. Only around half of participants aged 21-23 months were still breastfeeding.

Figure 10: Current and recalled any breastfeeding status by age.
Percentage proportion reported to be <u>currently</u> breastfeeding amongst 350 participants aged less than 42 months (left vertical axis; blue bars); <u>recalled</u> age at cessation of exclusive breastfeeding amongst 307 children aged 24-59 months who have ceased breastfeeding (right vertical axis; red line). The 'n=' notations below the horizontal axis indicate the denominator for each of the left vertical axis data points (blue bars). No participants were reported – either concurrently or via recall – to be breastfed beyond 42 months of age.



Focus group discussions yielded the following insights into topics concerning household foods:

- *Food availability*
    - Food is currently available in adequate quantities in local shops.
    - Local residents feel they are currently eating enough to avert hunger but not a nutritionally balanced diet.
    - Between January and July, food (maize flour, beans and *omena* (dried small oily fish)) is mainly sourced from local shops.
    - Between August and December, food (maize, pigeon peas, sugar beans and *pojo* (mung beans)) is mainly sourced from residents' own subsistence farms.
    - Throughout the year, green leafy vegetables (amaranthus, *mrenda* (jute), okra and *mchunga* (bitter lettuce)) are sourced from the farm or foraged in nearby forest.
    - The commonest meal throughout the year is *ugali* (cooked stiff porridge prepared using maize flour and water) with foraged amaranthus (green leaf).
- *Food access*
    - When food stores are depleted, residents turn to income-generating activities to purchase food from local shops.
- *Vulnerability and coping strategies during food insecurity*
    - Main triggers for food insecurity are limited opportunities for casual labour and insufficient rainfall leading to poor maize harvest.

- The three months leading up to the cross-sectional survey fieldwork have been the toughest this year in terms of food security.
- When families have insufficient food, coping strategies include eating only once per day, borrowing from neighbours, and foraging for mangoes and coconuts. Children are given first priority when food is limited.
- Households at particular risk of food insecurity are those with low or no income, casual labourers, those who cannot afford fertilizers, those that do not own the land they farm, and those with no employment.

Even in this rural, agricultural area, 91% of participants were resident in households whose dominant source of food was purchase (Table 5). Only 8% were resident in households whose dominant source of food was own production. Only 52% of participants were resident in households that had demonstrated high dietary diversity. 13% of respondents came from a household where members had missed one or more meals during the preceding seven days due to food shortage.

Table 5: Frequency distribution of putative undernutrition risk factors related to the proximal mediator 'nutrition' amongst 509 participating under-fives.

Factor	Category	Distribution[1]	Missing (%)
Ever breastfed	No	13 (3)	none
	Yes	496 (97)	
Currently breastfeeding	No	332 (65)	none
	Yes	177 (35)	
Recalled duration of any breastfeeding (months)	<18	79 (26)	202 (40)[2]
	18-23	106 (35)	
	≥24	122 (40)	
Currently exclusively breastfeeding (never had complementary foods)	No	492 (97)	none
	Yes	17 (3)	
Recalled duration of exclusive breastfeeding (months)		6 (0-12)	43 (8)[3]
Chronic neurological problem	No	491 (96)	none
	Yes	18 (4)	
Chronic cardiac problem	No	509 (100)	None
	Yes	0 (0)	
Enrolled on a feeding programme	No	508 (>99)	none
	Yes	1 (<1)	
Food security (missed meal during past 7 days)	No	441 (87)	none
	Yes	68 (13)	
Dominant food source	Own production	43 (8)	none
	Purchase	465 (91)	
	Trade	1 (<1)	
	Food aid	0 (0)	
	Borrowed	0 (0)	
	Gathered/hunted	0 (0)	
Dietary diversity score	Low	47 (9)	none
	Medium	199 (39)	
	High	263 (52)	

1. For categorical variables: n (% of total). For continuous variables: median (range)
2. Missing because inappropriate to report due to age (≥2 years)
3. Missing because inappropriate to report due to age (<6 months)

Perceived risk factors for undernutrition: infection

Focus group discussion participants voiced the following paraphrased beliefs and observations regarding child health and healthcare engagement in their community:

- "Main illnesses amongst under-fives in this community are: malaria, pneumonia, diarrhoea, ringworm, asthma, 'yellow fever' and 'stomach ache'."

- "Malaria is caused by sleeping within a bednet or receiving mosquito bites before going to bed. Malaria is also caused by a dirty environment caused by livestock urine and faeces."
- "Pneumonia arises from becoming cold whilst motorbike riding."
- "Diarrhoea is caused by dirty food, water or cooking utensils."
- "Main approaches to the sick child include seeking Western healthcare, traditional herbs and prayers."

Amongst survey participants, diarrhoea was the least common acute illness with only 7% of respondents having experienced three or more loose stools within a 24-hour period during the last seven days (Table 6). 76% of respondents had slept under a bednet the night before the survey. Primary care had been sought for cough, fever or diarrhoea within the preceding seven days for 30% of respondents.

Table 6: Frequency distribution of putative undernutrition risk factors related to infection amongst 509 participating under-fives.

Factor	Category	Distribution[1]	Missing (%)
Bednet use last night	No	120 (24)	none
	Yes	389 (76)	
Cough during past 7 days	No	296 (58)	none
	Yes	213 (42)	
Fever during past 7 days	No	399 (78)	none
	Yes	110 (22)	
Diarrhoea during past 7 days	No	472 (93)	none
	Yes	37 (7)	
Primary care sought during past 7 days for cough, fever or diarrhoea	No	357 (70)	none
	Yes	152 (30)	

1. For categorical variables: n (% of total). For continuous variables: median (range).

Stool microscopy

Stool was obtained for 419 (82%) of the 509 participants. Cold chain and time to freezing information is presented in Appendix 16. Of the 419 stool samples, 45 (11%) yielded positive results for helminths upon microscopy. Helminth species were mainly *Ascaris lumbricoides* with the exception of three *Trichuris trichuria*, one hookworm species and one *Hymenolepis diminuta* (Table 7). One participant had co-infection with *Ascaris lumbricoides* and *T. trichuria*.

Table 7: Cross-sectional survey stool formol ether method microscopy findings.

Species identified	Number of samples (% of total)
None	374 (89)
Ascaris lumbricoides alone	40 (10)
Trichuris trichuria alone	2 (<1)
Ascaris lumbricoides and *Trichuris trichuria*	1 (<1)
Hymenolepis diminuta	1 (<1)
Hookworm species (undetermined)	1 (<1)
TOTAL	**419**

Markers of EED in stool by age

ELISA results for alpha-1-antitrypsin, myeloperoxidase and neopterin were successfully obtained for all 419 stool samples. Table 8 and Figure 11 show faecal alpha-1-antitrypsin, myeloperoxidase and neopterin concentrations by age category. Overall, 239 (57%; 95% CI 52-62%) samples had higher alpha-1-antitrypsin concentrations than consensus normal threshold of 0.27mg/g.(222) Equivalent figures for neopterin were 367 (88%; 95% CI 84-91%) above 70nmol/L(223) and for myeloperoxidase 333 (79%; 95% CI 75-83%) above 2000ng/g.(224) Faecal alpha-1-antitrypsin and neopterin concentrations declined with age, with neopterin most closely reflecting the sharp decline during the first 24 months of life that

is seen with HAZ (Table 8 and Figure 11). Myeloperoxidase was highest in the 6-11-month age category. All three markers showed poor correlation between each other (Figure 12).

Table 8: Summary statistics for faecal log alpha-1-antitrypsin, myeloperoxidase and neopterin by age in month categories amongst 419 cross-sectional survey participants.
Numbers are presented in different styles: alpha-1-antitrypsin to 2 decimal places, myeloperoxidase and neopterin to nearest integer.

Age (months)	n	Faecal concentration (median (interquartile range))		
		Alpha-1-antitrypsin (mg/g)	Myeloperoxidase (ng/g)*	Neopterin (nmol/L)*
0-5	25	0.76 (0.31 to 1.11)	26,168 (13.544 to 46,971)	2,166 (1,755 to 3,886)
6-11	34	0.51 (0.27 to 0.85)	32,862 (7,027 to 63.786)	2,112 (897 to 3,529)
12-23	98	0.49 (0.15 to 0.84)	12,379 (3,132 to 29,880)	612 (262 to 1,753)
24-35	79	0.27 (0.13 to 0.49)	6,350 (1,938 to 15,381)	305 (155 to 690)
36-59	183	0.26 (0.77 to 0.50)	4,057 (2,168 to 11,505)	231 (112 to 521)
TOTAL	419	0.33 (0.13 to 0.70)	6,950 (2,524 to 20,640)	426 (162 to 1,141)

Figure 11: Box plots for faecal alpha-1-antitrypsin, myeloperoxidase and neopterin by age in month categories amongst 419 cross-sectional survey participants.
Box midlines denote median, box edges denote 25th and 75th centiles, whiskers denote upper and lower adjacent values, hollow dots denote outlying values. Horizontal red dashed lines indicate proposed threshold normal values (for alpha-1-antitrypsin 0.27mg/g, for myeloperoxidase 2,000ng/g, for neopterin 70nmol/L). Note y axis scale is logarithmic.

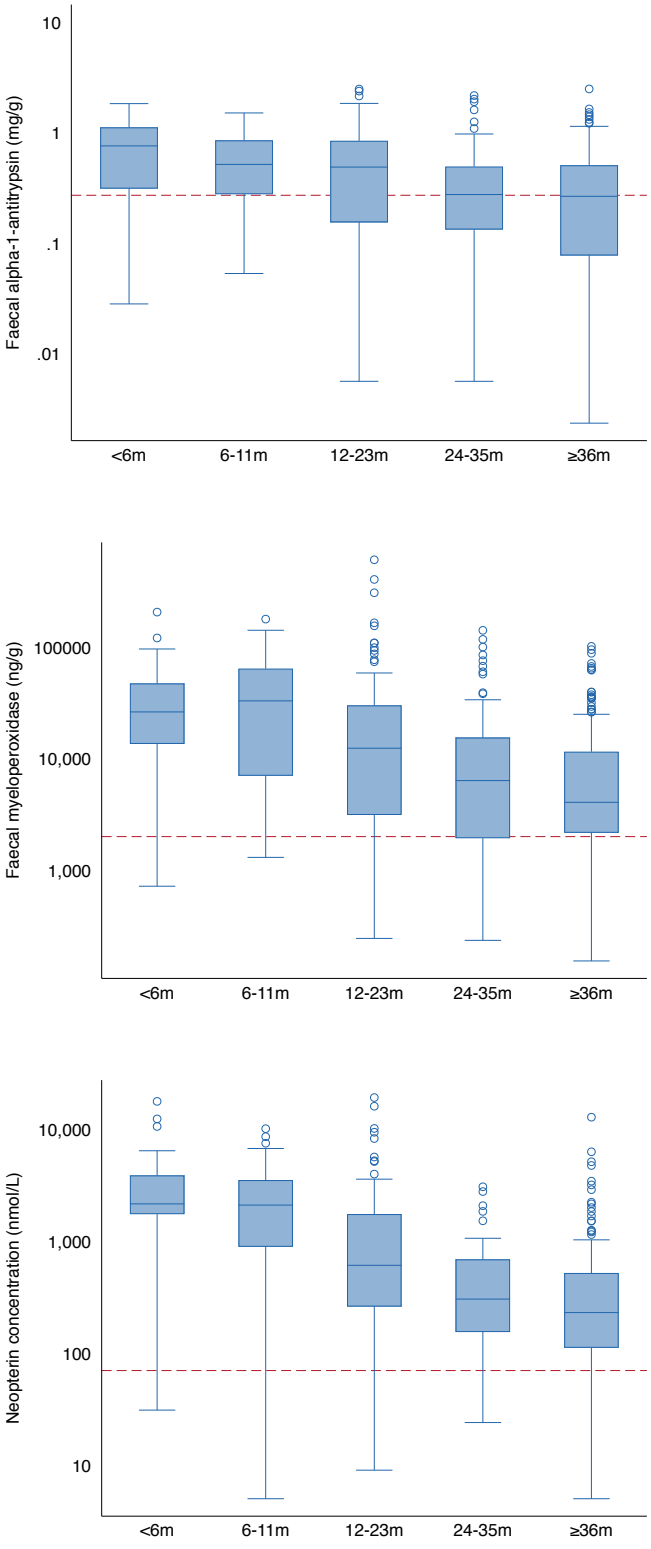
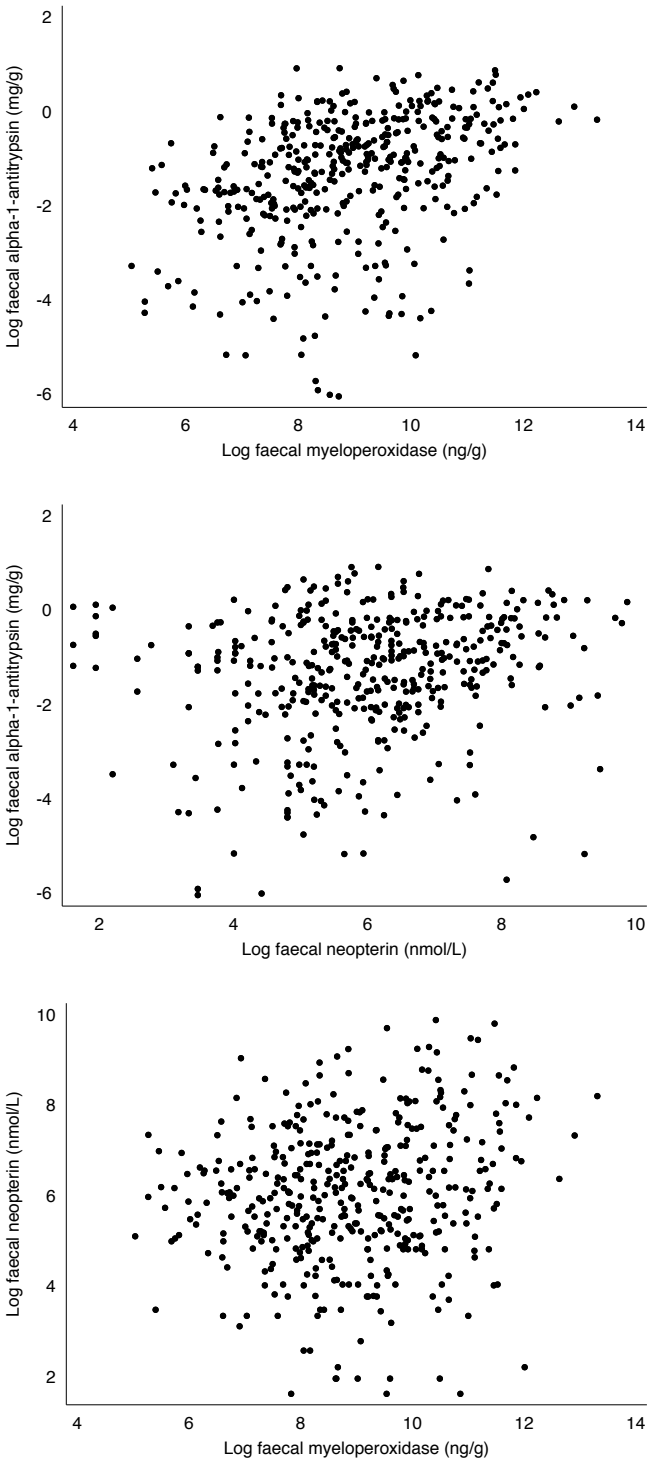


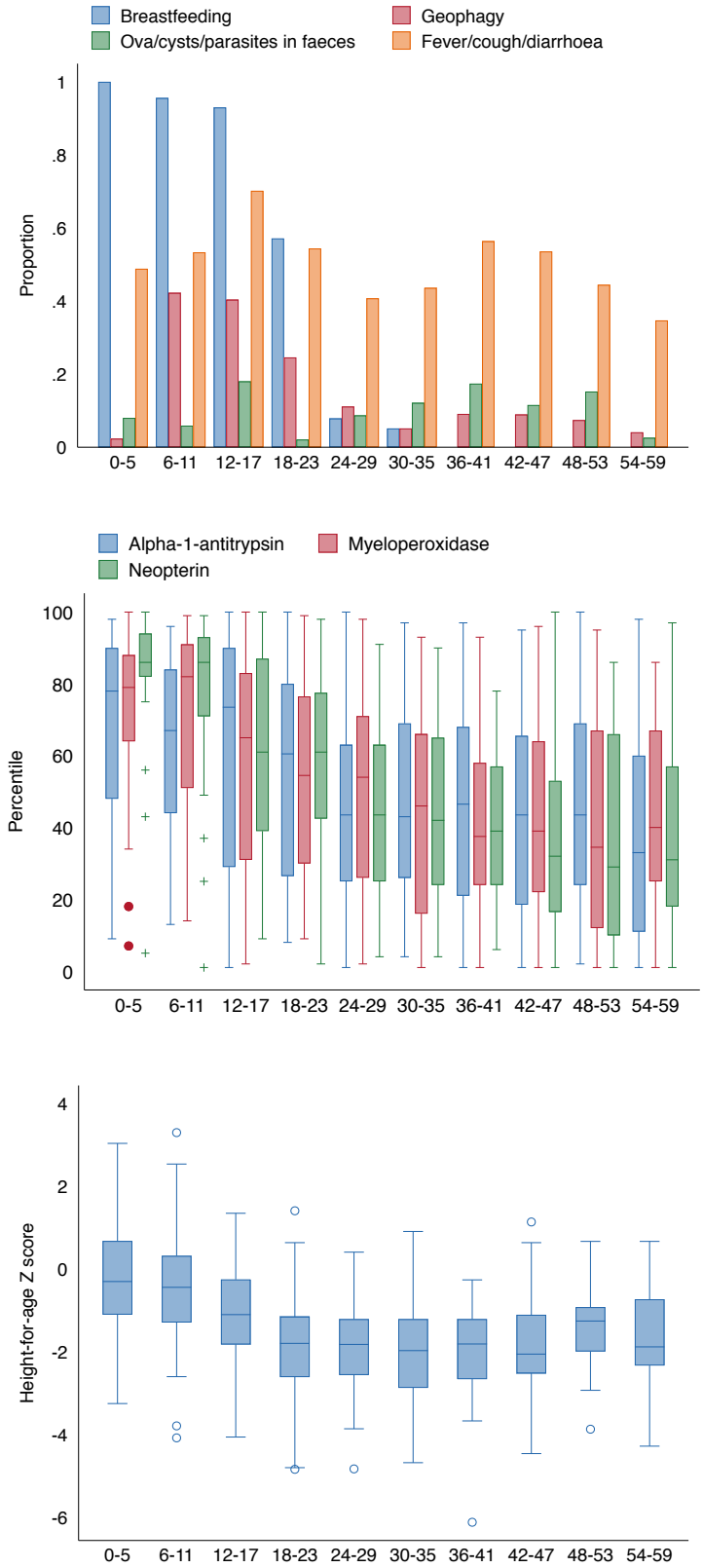
Figure 12: Scatter plots demonstrating association between log faecal biomarkers of EED measured from the same stool sample amongst 419 under-fives.
Pearson's correlation coefficients: alpha-1-antitrypsin and myeloperoxidase: 0.37; alpha-1-antitrypsin and neopterin: 0.18; neopterin and myeloperoxidase: 0.16.



Distribution of multiple risk factors by age

Of all the measured factors in this cross-sectional survey, most are likely to either be static over time and age (e.g. sex), change with season (e.g. household water source) or change infrequently and unpredictably (e.g. household socio-economic status). However, six are more likely to show common and shared patterns of age-related change. These are: breastfeeding (any and exclusive), OCP stool microscopy result, observed geophagy, acute illness (fever, cough or diarrhoea), EED biomarker ELISA results and anthropometry results (HAZ, WHZ and WAZ). Charting these cross-sectional survey-derived factors by age can, assuming there has been no cohort effect in the study area during the past five years, provide a suggestion as to the changes that the average individual child may be expected to undergo during their first five years of life. Here, contemporaneous peaks in geophagy and acute illness are seen amongst 6-23-month-olds and possibly also a smaller peak of intestinal helminthiasis at around 12-17 months (Figure 13; top). During the first half of this same time period (0-12 months of age) faecal concentrations of EED biomarkers are higher, especially neopterin (Figure 13: middle). Breastfeeding is ceased mainly between 12 and 23 months. Decline in HAZ occurs between six and 23 months (Figure 13; bottom). A later second peak in acute illnesses at around three years of age is not matched by peaks in any of the faecal EED biomarkers or further declines in HAZ.

Figure 13: Frequency distributions of putative risk factors for EED and stunting by age category in months. Proportion of participants currently breastfeeding, with ova, cysts or parasites detected in stool, or geophagy or fever, cough or diarrhoea noted during the past seven days (top); box plots of percentiles of faecal EED biomarkers (middle); box plots of HAZ (bottom). Box midlines denote median, box edges denote 25th and 75th centiles, whiskers denote upper and lower adjacent values, hollow dots denote outlying values.



Spatial dependency of HAZ and faecal markers of EED

Global Moran's index suggested no strong evidence for spatial dependency of either mean homestead HAZ or mean log homestead alpha-1-antitrypsin, myeloperoxidase or neopterin (Table 9). To assess risk of confounding by age, Moran's index analysis was also carried out for mean homestead age. No evidence was found for spatial dependency of this factor.

Table 9: Global Moran's index assessment of spatial autocorrelation of selected factors by participating homestead.

Factor	Number of homesteads	Moran's I	p
Mean age (months)	229	0.01	0.17
Mean HAZ	229	0.000	0.42
Mean log faecal alpha-1-antitrypsin (mg/g)	205	-0.009	0.43
Mean log faecal myeloperoxidase (ng/g)	205	-0.007	0.47
Mean log faecal neopterin (nmol/L)	205	0.03	0.05

Clustering of nutritional status and EED by village, homestead and household

With the continuous outcome of HAZ, mixed-effects linear regression models were run, each allowing for a random effect among the values of village, homestead and household respectively and with age as a fixed effect. Estimates from these models were each compared using likelihood ratio test against an equivalent age-adjusted model of HAZ that did not allow for clustering. Resultant p-values indicated strong evidence for clustering of HAZ by village, homestead and household (Table 10). The same age-adjusted analysis using log faecal alpha-1-antitrypsin, myeloperoxidase and neopterin as outcomes yielded strong evidence for clustering of only neopterin by village, homestead and household (Table 10). Given these

results, and the fact that it was the unit of the sampling framework, homestead was selected as the random effect for all subsequent models.

Table 10: P-values derived from likelihood ratio tests comparing null mixed-effects linear regression models with and without adjustment for clustering of outcomes by village, homestead or household. All models are adjusted for age in continuous months.

Level of clustering	Outcome: HAZ			Outcome: faecal EED biomarkers				
	n	Groups	p	n	Groups	p		
						AAT	MPO	NEO
Village	508	10	0.0003	419	10	0.35	0.15	0.002
Homestead	508	229	0.0003	419	205	0.17	0.24	0.03
Household	508	336	0.003	419	291	0.20	0.26	0.008

Data preparation for multivariable modelling

An objective, prospective, hypothesis-driven approach was used when selecting factors for multivariable regression modelling (see Appendix 17 for detailed methods). From a list of 46 potential exposure variables (Tables 2-6), 20 exposures were shortlisted for inclusion as fixed effects in multivariable models exploring risk factors for low HAZ and EED.

Factors associated with stunting

From this shortlist, for a multivariable model exploring factors associated with low HAZ, a further two exposure variables were removed as they were deemed too transient to have any likely meaningful association with HAZ in a cross-sectional survey (recent acute illness and recent observed geophagy). Presence of parasites in stool was removed as it was deemed on a causal pathway between water and sanitation variables and HAZ. Current breastfeeding was removed due to very strong collinearity with age. 15 exposure variables

were therefore inputted into a mixed-effects linear regression model with HAZ as the outcome and homestead as a random effect to adjust for possible clustering of associations at the homestead level. Amongst 508 participants for whom HAZ data were available, strongest evidence exists for association of lower HAZ with low birthweight, a sibling who has died and lower household socioeconomic position (Table 11).

A low p-value is also observed for the variable describing birth interval to younger sibling. However, this is an overall p-value derived by likelihood ratio testing comparing models with and without this entire variable, which itself includes a category for those in whom birth interval to younger sibling cannot yet be fairly evaluated since they have not yet attained 24 months of age. A positive coefficient is observed for this category, in keeping with known higher HAZ values, on average, for those aged 24 months or older compared to those aged less than 24 months. The coefficient for the category describing participants aged 24 months or older who have a birth interval to their younger sibling of less than 24 months is negative but it has a wide 95% confidence interval that spans zero. There is therefore insufficient evidence to support association of younger sibling birth interval spacing with HAZ.

Model outputs with and without adjustment for clustering by homestead were compared. There were no large increases in confidence interval size or shifting of coefficients when adjustment was introduced.

A sensitivity model was run excluding two factors (deceased sibling and household socio-economic position) that, due to their distal, over-arching nature, were at risk of masking associations for more proximal factors in the model. These sensitivity models excluding

deceased sibling and household socio-economic position in turn revealed borderline and strong evidence respectively for association of lack of bednet use with lower HAZ (Appendix 18). In both models, outputs for other variables were largely unchanged. Bednet use is indeed weakly co-linear with household socio-economic position (households using bednets had higher socio-economic position scores) but not with deceased sibling or any other of the model exposure variables. Bednet use was therefore removed from the main model due to collinearity.

Table 11: Factors associated with HAZ amongst 508 children aged 0-59 months in Junju, Kilifi.
A mixed-effects linear regression model. Analysis is adjusted for clustering by homestead (229 groups).

Factor	Category	Distribution[1]	Coefficient	95% CI	p
Age (months)		30 (16 to 45)	-0.01	-0.02 to 0.00	0.23
Sex	Male	284 (56)	-	-	0.72
	Female	224 (44)	-0.04	-0.02 to 0.17	
Birth weight (kg)	≥2.5kg	177 (35)	-	-	0.0002
	<2.5kg	15 (3)	-1.33	-1.95 to -0.71	
	Unknown	316 (62)	-0.09	-0.32 to 0.14	
Birth interval to younger sibling	≥24 months or no younger sibling	50 (10)	-	-	0.03
	<24 months	257(51)	-0.04	-0.39 to 0.32	
	Cannot evaluate (age <24 months)	201 (40)	0.51	0.12 to 0.91	
Birth interval to older sibling	≥24 months or firstborn	425 (84)	-	-	0.61
	<24 months	83 (16)	0.07	-0.21 to 0.35	
Season of birth	Dry	295 (58)	-	-	0.09
	Rainy	213 (42)	-0.18	-0.39 to 0.03	
Deceased sibling	No	399 (79)	-	-	0.02
	Yes	109 (21)	-0.33	-0.59 to -0.06	
Mother's education	Completed primary or higher	120 (24)	-	-	0.49
	None, or some primary	389 (76)	0.09	-0.35 to 0.17	
Primary caretaker	Mother	470 (93)	-	-	0.66
	Not mother	38 (7)	-0.09	-0.51 to 0.33	
Household socio-economic position PCA[2] first component score		-0.8 (-1.1 to 6.2)	0.13	0.05 to 0.21	0.001
Household water position PCA[2] first component score		0.1 (-0.6 to 0.9)	-0.02	-0.11 to 0.08	0.68
Household sanitation type	WHO improved	83 (16)	-	-	0.86
	WHO unimproved	425 (84)	0.03	-0.28 to 0.34	
Household dietary diversity	High	262 (52)	-	-	0.78
	Low or medium	246 (48)	-0.03	-0.26 to 0.20	
Household food security	No missed meal during past 7 days	440 (87)	-	-	0.63
	Missed meal during past 7 days	68 (13)	-0.08	-0.40 to 0.24	

1. For categorical variables: n (% of total). For continuous variables: median (interquartile range).
2. Principal Component Analysis

In separate linear regression models of HAZ amongst the 193 participants for whom birthweight was known, comparison of models – each with fixed effects of birthweight quintiles and age categories – treating birthweight as a linear versus a categorical exposure indicates a linear association between quintiles of birthweight and HAZ (comparison likelihood ratio p=0.93). Further comparison of models allowing and not allowing for modification by age of the association between birthweight and HAZ (with no other exposure variables) demonstrate no evidence for interaction (comparison likelihood ratio p=0.17), i.e. the unadjusted effect of birthweight on HAZ is linear and sustained throughout the first five years. There was strong evidence for modification of the association between HAZ and season of birth by age (likelihood ratio test p=0.0002). However, stratum-specific coefficients for age do not indicate a linear trend and all of their 95% confidence intervals cross zero, suggesting that there is insufficient evidence for association between season of birth and HAZ even within limited age categories.

Association between faecal EED biomarkers and concurrent HAZ

In three separate linear regression models adjusted for age and sex, none of the three EED faecal biomarkers demonstrate evidence for association with HAZ (Table 12). In sensitivity models unadjusted for age and sex, higher log faecal neopterin shows strong evidence for association with higher concurrent HAZ (Appendix 19). These models were not adjusted for clustering by homestead because none of the included variables had been measured at the homestead/household level: they were instead all child specific.

Table 12: Biomarkers associated with HAZ amongst 418 under-fives.
Three separate multivariable linear regression models, each with age and sex and one EED biomarker as fixed effects.

EED biomarker	n	Median (interquartile range)	Coefficient	95% CI	p
Log faecal alpa-1-antitrypsin (mg/g)	418	-1.1 (-2.0 to -0.4)	-0.06	-0.54 to 0.03	0.21
Log faecal myeloperoxidase (ng/g)	418	8.8 (7.8 to 9.9)	-0.02	-0.10 to 0.07	0.72
Log faecal neopterin (nmol/L)	418	6.0 (5.1 to 7.0)	0.06	-0.03 to 0.15	0.17

Factors associated with biomarkers of EED

Since stool ELISA results were not available for all 509 participants, fewer exposure variables were selected to preserve power in these smaller models. Proximal factors included in the above HAZ model were retained, whilst distal factors (birth interval to younger and older siblings, bednet use, season of birth, deceased sibling, mother as primary caretaker, mother's education, household SEP) were discarded. Stool microscopy results were added, as well as current breastfeeding status, recent acute illness and geophagy. These resulting 10 exposure variables were inputted into three mixed-effects linear regression models, each with alpha-1-antitrypsin, myeloperoxidase or neopterin as the outcome and homestead as a random effect. Amongst the 419 participants for whom all data were available, aside from younger age, strong evidence exists for association of higher faecal myeloperoxidase with male sex and association of higher faecal neopterin with current breastfeeding, higher household water score and unimproved sanitation (Table 13). To investigate possible associations for factors co-linear with age, in a sensitivity model excluding age, sex and current breastfeeding status (the latter excluded due to its strong collinearity with age), strong evidence exists for association of higher faecal alpha-1-antitrypsin and high faecal neopterin with geophagy and association of higher faecal myeloperoxidase with recent acute illness (Appendix 20).

Table 13: Factors associated with log faecal alpha-1-antitrypsin, myeloperoxidase and neopterin amongst 419 children aged 0-59 months in Junju, Kilifi. Three separate multivariable mixed-effects linear regression models, one for each faecal EED biomarker outcome. Analysis is adjusted for clustering via inclusion of homestead as a random effect (205 groups).

Factor	Category	Distribution[1]	Log faecal alpha-1-antitrypsin (mg/g)			Log faecal myeloperoxidase (ng/g)			Log faecal neopterin (nmol/L)		
			Coefficient	95% CI	p	Coefficient	95% CI	p	Coefficient	95% CI	p
Age (months)		30 (18 to 45)	-0.02	-0.03 to 0.00	0.007	-0.02	-0.03 to 0.01	0.004	-0.03	-0.04 to -0.02	<0.001
Sex	Male	237 (57)	-		0.55	-	-	0.04	-	-	0.56
	Female	182 (43)	-0.07	-0.32 to 0.18		-0.28	-0.56 to -0.01		-0.08	-0.33 to 0.18	
Current breastfeeding status	Exclusive	10 (2)	-	-	0.43	-	-	0.07	-	-	0.02
	Mixed	128 (31)	-0.39	-1.22 to 0.44		-0.01	-0.95 to 0.93		-0.63	-1.51 to 0.25	
	None	281 (67)	-0.58	-1.55 to 0.34		-0.55	-1.59 to 0.48		-1.06	-2.03 to -0.08	
Fever, cough or diarrhoea during past seven days	No	204 (49)	-	-	0.15	-	-	0.10	-	-	0.85
	Yes	215 (51)	0.18	-0.06 to 0.43		0.23	0.04 to 0.51		-0.02	-0.29 to 0.23	
Geophagy noted during past seven days	No	350 (84)	-	-	0.26	-	-	0.22	-	-	0.09
	Yes	69 (16)	0.20	-0.15 to 0.55		-0.25	-0.64 to 0.14		0.32	-0.05 to 0.70	
Faecal ova, cysts and parasites	Negative	375 (90)	-	-	0.07	-	-	0.41	-	-	0.57
	Positive	44 (11)	0.37	-0.03 to 0.77		0.19	-0.26 to 0.64		0.12	-0.30 to 0.55	
Water PCA[2] first component score		0.1 (-1.4 to 1.4)	0.09	0.02 to 0.19	0.11	-0.02	-0.14 to 0.10	0.76	0.13	0.01 to 0.24	0.03
Household sanitation WHO type	Improved	77 (18)	-	-	0.90	-	-	0.43	-	-	0.001
	Unimproved	342 (82)	-0.02	-0.35 to 0.30		-0.15	-52 to 0.22		0.60	0.24 to 0.96	
Household dietary diversity	High	220 (53)	-	-	0.11	-	-	0.65	-	-	0.91
	Low / medium	199 (47)	-0.21	-0.47 to 0.05		0.07	-0.22 to 0.36		-0.02	-0.30 to 0.26	
Household food security: missed meal during past seven days	No	363 (87)	-	-	0.52	-	-	0.06	-	-	0.43
	Yes	56 (13)	0.12	-0.24 to 0.48		0.40	-0.01 to 0.81		0.16	-0.24 to 0.55	

1. For categorical variables: n (% of total). For continuous variables: median (interquartile range).
2. Principal Component Analysis

Discussion and conclusions

In this study area, there is a high (35%) prevalence of moderate or severe stunting amongst under-fives. This figure is comparable to the latest Kenya DHS which took place in 2014 and reported 39% prevalence of moderate or severe stunting amongst under-fives for Kilifi County and 26% nationally.(210) Prevalence of wasting (moderate or severe) is much lower at 3%, and again comparable to the 2014 Kenya DHS which reported 4% prevalence of for both Kilifi County and Kenya overall.

Amongst study participants, HAZ declines evenly throughout the first 24 months of age. HAZ then stabilises before starting to slightly improve from 36 months onwards.

HAZ was not associated with WHZ. A caveat to this observation is that HAZ and WHZ were measured concurrently for each individual. If a time lag were introduced between one-off measurements of HAZ and WHZ, associations may be present. Low HAZ takes longer to develop than low WHZ: wasting occurs and resolves quickly. For example, a child who had been wasted and increasingly stunted for the first three years of their life may still be stunted by four years of age despite no longer being wasted. Another possible explanation for the lack of association is that few WHZ recordings were significantly abnormal. In a setting with higher prevalence of AM, association may be seen between WHZ and HAZ.

Multiple indicators of severe deprivation were present at higher than expected prevalence in this study area. For example, 21% of participants had a deceased sibling; only 24% of

participants' mothers had completed primary education; median monthly per capita income was only 1,500 Kenya shillings (US$15); the majority of dwellings were built using non-permanent materials; only 3% had improved sanitation. Lastly, although 54% of participants are from households with WHO improved water sources, none have this water piped directly into the house or compound. Despite these multiple indicators of severe deprivation, wasting was rare. One factor that protects against wasting in this and similar populations is stunting: a shorter child has a smaller body frame over which to distribute lean and fat body mass.

Based on caretaker recall, there appeared to be poor adherence to WHO breastfeeding duration recommendations. If discounting all responses that were equal to the WHO recommendations (six months for exclusive breastfeeding and 24 or more months for any breastfeeding), underlying – perhaps more accurate – responses appeared to demonstrate peaks for cessation of exclusive breastfeeding at age 4-5 months and cessation of any breastfeeding at around 18 months. This underlying pattern was supported by the focus group discussion insights. Further discussion of the reliability of these breastfeeding data is below.

Prevalence of helminthiasis measured by stool microscopy for ova, cysts and parasites was low at 11%. Previous studies in coastal Kenya have demonstrated high prevalence (median 33%) of *Schistosoma haematobium* infection (but absent *S. mansoni*) amongst school-aged children.(225) However, these studies mostly used the Kato-Katz method for examining stool which has higher sensitivity for diagnosis of schistosomiasis. General soil-transmitted helminth prevalence in previous coastal studies has ranged between 0.1% and 50%.(225) A study in a similar population comparing formol ether concentration method against a gold

standard of combination formol ether plus two other methods (Kato-Katz and wet mount) has demonstrated sensitivity of 78% and negative predictive value of 63% for formol ether.(226) Formol ether examination of a single stool sample has particularly low sensitivity in detection of *Giardia* species and *Strongyloides stercoralis* infections.(227) The observed prevalence of 11% may therefore be an underestimation.

In a multivariable model exploring risk factors for low HAZ, risk factors with strong evidence for association with lower HAZ were low birthweight, a deceased sibling and lower household socio-economic position. There was also weak evidence for association of lower HAZ with having been born in the rainy season. In contrast, in a similar cross-sectional survey of Tanzanian children aged 22-28 months, stunted children were more likely than non-stunted children to be from households with severe hunger, low dietary diversity and no soap.(228)

The 95% confidence interval for the coefficient for age in the multivariable HAZ model crossed zero. However, age was co-linear with the variable 'birth interval to younger sibling', where a coefficient whose 95% confidence interval does not span zero was observed amongst those aged 0-23 months (whose birth interval to younger sibling was not recorded) compared to those in the reference category (all aged 24-59 months). This collinearity is likely to explain the lack of observed association with age in the multivariable model.

Household SEP was not strongly co-linear with any of the other exposures in the model that might be expected to change systematically with household wealth (e.g. water source, dietary diversity, food insecurity). It was, however, weakly co-linear with bednet use, and a sensitivity model excluding household socio-economic position revealed strong evidence for

association of low HAZ with sleeping without a bednet. It is not possible to conclude from this model whether lack of bednet use has a direct causative association with low HAZ, or whether its association with HAZ observed in the sensitivity model is due to collinearity with lower socio-economic position which itself is a strong risk factor for low HAZ when included.

Having a deceased sibling was not co-linear with any of the measured exposures – both included and not included in the multivariable model. Biological pathways through which a deceased sibling may be associated with HAZ may include via shared specific characteristics with the deceased sibling (inherited disease, prematurity, twin or triplet), a shared more general environment or possibly maternal or other caretaker depression arising from bereavement leading to suboptimal care environment.

Interaction testing in a separate model including just HAZ, birthweight and age suggests that the unadjusted association between birthweight and HAZ is both linear and sustained with increasing age: i.e. during the first five years there appears to be no age beyond which low birthweight no longer has an adverse effect on HAZ. This is in contrast to results from the MAL-ED cohort where the effect of low birth WAZ on HAZ was highest at birth then declined by 12 months and further still by 24 months.(229)

A major limitation of this HAZ multivariable explanatory model is its omission of factors that may be strong risk factors for low HAZ but could not be included due to their skewed distribution (rarity) leading to risk of sparsity. These include prematurity, neurodisability and congenital cardiac disease. Maternal nutritional and HIV status were not measured and may be important. (However, prevalence of HIV infection was only 1% amongst mothers

attending ante-natal care at Junju dispensary during the study period). Other potentially important factors were not included in the model due to missing data: notably mother's age and recalled exclusive breastfeeding duration. Breastfeeding is a particularly complex exposure variable. Its association with HAZ or EED may be modified by age, and also by time that has elapsed since change in breastfeeding status. For example, exclusive breastfeeding may be beneficial for a three-month-old's growth but harmful for a 15-month-old who has different nutritional requirements. A child noted to be currently no longer breastfeeding may have had their last breastfeed yesterday or three years ago. For these reasons, any observed association of HAZ with current breastfeeding status, even when adjusted for age, is difficult to interpret. In addition, recalled duration of exclusive or any breastfeeding is vulnerable to bias. Even where recollection is accurate, breastfeeding duration can be problematically co-linear with general poor health. Anecdotal experience in the local area has led to our understanding that caretakers who perceive their child to be malnourished or chronically ill will breastfeed for longer (although duration of *exclusive* breastfeeding will be the same or even shorter as caretakers introduce complementary foods earlier in the belief that breastmilk is providing insufficient nutrition). Another problem with recording recalled breastfeeding duration is that any responses given for participants aged younger than the WHO-recommended minimum duration (24 months) must be recoded as missing to avoid unfair labelling as 'inadequate duration'. If only those who are still breastfeeding have duration recoded as missing, and not all participants aged below 24 months, this risks over-representation of those who stop earlier. The same principle applies to those aged less than six months when recording recalled duration of exclusive breastfeeding. Solutions to some of these problems of measuring breastfeeding status include adequately powered, age-restricted sampling (e.g. 1,000 12-month-olds), or longitudinal data with multiple

measurements of exposure and outcome per participant over time. Lastly, another possible limitation of this multivariable HAZ model is insufficient variability amongst participants. HAZ is normally distributed, albeit about an abnormally low median. This suggests that risk factors for low HAZ are population-wide and may not be easily detectable in a homogenous population.

Inclusion of HAZ in all models as a continuous outcome rather than dichotomised around a threshold Z-score value has served to maximise statistical power. It has also had the added benefit of reflecting the likely biological truth, which is that associations of lower HAZ with adverse events (and their causative risk factors) do not commence once a threshold has been reached but rather are linear or even exponential and start with HAZ values greater than -2 and perhaps even greater than -1. This hypothesis is supported by data already discussed on page nine concerning increased odds of death from infectious disease amongst children worldwide with HAZ, WHZ and WAZ between -1 and -2 compared to >-1.(1) Including HAZ as a continuous variable, assuming linear associations, could have been problematic if a large proportion of participants were tall for their age. However, very few participants had HAZ greater than +1. HAZ comparisons made here can therefore confidently be interpreted as spanning a 'short-to-normal' range of HAZ rather than also including a 'normal-to-tall' range wherein risk factors and their associations with HAZ may be very different.

Faecal alpha-1-antitrypsin concentrations are initially high at birth, declining to normal levels by 24 months of age onwards. Age-related frequency distribution of faecal myeloperoxidase shares some similarities, but with a peak amongst age 6-11 months and levels still slightly higher than normal by five years of age. For neopterin, concentrations are much higher than

normal at birth, decline more rapidly until 24 months, then stabilise but are still abnormal by five years of age. The observed age-related patterns for all three biomarkers – including the particularly high early neopterin concentrations – amongst participants aged 0-23 months are comparable to results obtained from the MAL-ED cohorts.(89) High alpha-1-antitrypsin at birth could partially be explained by presence of alpha-1-antitrypsin in breastmilk as discussed on page 21. Colostrum has particularly high concentration (0.3g/L compared to 0.1g/L in breastmilk from 30 days onwards(89)). These age-related declines during the first 24 months are mirrored by HAZ, especially for neopterin. It is during these 24 months that geophagy also peaks (specifically: 6-23 months) and a small peak is also seen in acute illnesses. Age-related change in intestinal helminthiasis is difficult to ascertain due to low prevalence but may peak around 12-17 months.

The three faecal biomarkers of EED show poor correlation between themselves and, adjusted for age, no associations with concurrent HAZ. In a sensitivity model without adjustment for age, neopterin shows strong positive association with HAZ. This unexpected observation may be due to lack of consideration for a possible time lag between putative exposure (an EED/stunting risk factor such as geophagy), marker of pathology (neopterin) and outcome (low HAZ). Since both neopterin and HAZ both demonstrate the same age-related change, from this cross-sectional data it is not possible rule out true independent positive (even causative) association between neopterin and HAZ. However, it is more likely that the IFNγ-stimulated release of neopterin into the gut by monocytes, macrophages and dendritic cells is part of a wider pathological process that occurs during the first 24 months of life and results – with minimal time lag – in HAZ decline. However, whilst the rate of decline in HAZ is

constant throughout the first 24 months, faecal neopterin concentration is not constant during this time: it falls.

Neither HAZ nor any of the three faecal EED biomarkers demonstrated spatial dependency within the study area. However, neopterin shows strong age-adjusted evidence for clustering by village, homestead and household, as does HAZ. The fact that neopterin varies more widely with age and also between cluster units suggest that, amongst this study population, faecal neopterin may be more discriminatory than myeloperoxidase or alpha-1-antitrypsin in measuring differences in intestinal health between individuals in this setting. There may have been insufficient variability in community and physical characteristics within the study area to give rise to spatial dependency of stunting or EED.

In multivariable models exploring risk factors for EED, after adjustment for age and sex, strong evidence exists for association of higher faecal neopterin with breastfeeding, higher household water score and unimproved sanitation. The unexpected association with water score may possibly be explained by its collinearity with sanitation type. In the study area, households with unimproved sanitation are more likely to also have an improved water source. Common combinations within households are various categories of piped water (WHO improved) alongside an uncovered hand-dug latrine or trench (WHO unimproved) (Appendix 21). The observed association of higher household water score with higher faecal neopterin may therefore be an artefact arising from a coalescence of this collinearity and neopterin's very strong association with unimproved sanitation. Neopterin's positive association with breastfeeding can possibly be explained by residual confounding by age. Age was included in the model as a continuous variable, assuming linearity of association with

HAZ. However, breastfeeding status followed a similar age-related change to neopterin concentration, which is not linear. The breastfeeding status category with a 95% confidence interval that does not span zero is those no longer breastfeeding in comparison to those exclusively breastfeeding (the coefficient for those mixed feeding is also negative, but its 95% confidence interval does span zero). Interquartile ranges for age within these two categories were 30.2 to 50.0 and 1.3 to 4.5 months respectively. Faecal neopterin was much higher within these youngest participants. In the sensitivity model that excludes age, sex and current breastfeeding status, strong evidence is also demonstrated for positive association of geophagy with both alpha-1-antitrypsin and neopterin, and positive association of recent acute illness with myeloperoxidase. A possible limitation of these three models is their multiple comparisons. A composite score derived from all three markers could have been used, for example the score developed by Kosek et al discussed on page 20. However, at this exploratory stage I chose to keep the three markers separate. Consideration was given to risk of sparsity during model building, but no statistical adjustment for multiple comparisons was made.

Faecal neopterin's observed spatial dependency, age-adjusted multi-level clustering and strong age-related change that is contemporaneous with HAZ decline all promote its potential usefulness as a discriminatory biomarker of EED in this study population. However, as discussed above, confounding of the association between neopterin and HAZ by other measured or unmeasured factors that also change with age cannot be ruled out with this cross-sectional study design. Normal age- and breastfeeding status-stratified reference ranges for alpha-1-antitrypsin, myeloperoxidase and neopterin do not exist. If upper normal

values were known, binary classification of markers as 'normal' or 'abnormally high' may be more useful than raw continuous data in detecting biologically meaningful associations.

There were several limitations to this study. Firstly, varying elements of the survey data were by their nature clustered at three levels: homestead (the unit of sample selection; extended family living in close proximity), household (the unit of survey administration for questions about the child's environment) and mother. There was insufficient power for multilevel modelling, so homestead was chosen as the random effect following discussion with fieldworkers concerning which of the three levels they perceived demonstrates the highest inter-unit attribute variability. This was hopefully an appropriate strategy given the limited sample size.

Other possible study limitations relate to selection bias and generalisability. Regarding selection bias, the sampling framework – homesteads that contain one or more under-fives – was generated in April 2015 (KHDSS enumeration round 35) but the survey did not take place until June 2015, two months later. Any baby born to a homestead that did not previously contain any under-fives during those three months would not have had a chance of selection. This may have led to minor under-representation of babies aged less than three months in the sample. Regarding generalisability, since the survey took place during the rainy season, results can only apply to this period. The long rainy season is locally described as the 'hungry season', as stored food from the previous year's harvest runs low. At Kilifi County Hospital, the main government secondary care facility for the study area, incidence of admission with complicated SAM is anecdotally highest during this time. Prevalence of certain putative risk factors for EED and low HAZ (e.g. food insecurity, recent illness) are likely

to be higher during the rainy season and strength of associations with HAZ and EED may differ.

Strengths of this study include its relatively large sampling proportion (15%) and well-characterised sampling frame (KHDSS). Significant efforts were made through careful questionnaire design and by fieldworkers during home visits to ensure high levels of participation and accurate questionnaire responses. Statistical analysis was prospective and hypothesis driven.

In summary, this cross-sectional survey of 509 under-fives, their mothers and households has demonstrated high prevalence of stunting and abnormally high concentrations of faecal biomarkers of intestinal inflammation and permeability amongst under-fives during the long rainy season at this rural coastal Kenyan site. Multiple indicators of socio-economic deprivation and faeco-oral contamination were present, as well as shorter exclusive breastfeeding duration, food insecurity and low dietary diversity. Low HAZ was associated in cross-section with low birthweight, a deceased sibling, low household socio-economic position and possibly also lack of bednet use. Markers of EED, in particular neopterin, were highest during the first 23 months of life (a time during which HAZ declines) and associated in cross-section with unimproved sanitation, male sex, geophagy and recent acute illness. Low HAZ showed clustering by village, homestead and household, as did neopterin. Of note, in all models, neither of the two diet variables (dietary diversity and food insecurity) demonstrated association with any of the EED biomarkers or HAZ. The effect of breastfeeding, both current and historical, on HAZ and EED was difficult to ascertain.

This survey measured stunted growth in cross-section: as a one-off snapshot of a child's HAZ. However, stunting is a dynamic and reversible process that fluctuates over weeks to months. A single length or height measurement episode will not distinguish the growth faltering child from the static or recovering child. Better understanding of risk factors for the stunting process can come from longitudinal studies where difference in HAZ over time can be calculated, lag times introduced, and risk factors measured on multiple occasions.

The observed associations of the faecal EED biomarkers with events that occurred within the past seven days (illness and geophagy) suggest that they may fluctuate quickly in this setting. This, plus the limitations of HAZ in a cross-sectional context as discussed above, and the inclusion of a wide range of participant ages when true associations may in fact only be present within a limited age category, may explain the lack of observed association between any of the faecal biomarkers and HAZ when adjusted for age. A case control study of 22-28-month-olds in Tanzania similarly found no differences in faecal alpha-1-antitrypsin, myeloperoxidase or neopterin between the stunted cases and non-stunted controls.(228) A longitudinal approach with more frequent sampling may address this issue.

This study demonstrates that, in this population, stunting occurs during the first 24 months of life and is associated with lower birthweight, a deceased sibling, lower socio-economic position, shorter younger sibling birth interval and possibly also birth in the rainy season and lack of bednet use. During this same period, breastfeeding ceases, acute illnesses, intestinal helminthiasis and geophagy peak and markers of intestinal inflammation (especially neopterin) are highest. A closer focus on this age group is therefore required, along with a longitudinal approach, to better understand EED and stunting within this population.

Chapter 3: Breath hydrogen test to measure Small Intestinal Bacterial Overgrowth amongst infants in Junju, Kilifi

Background

The gold standard for the diagnosis of SIBO is sampling and culture of fluid from the small intestinal lumen.(230) Early studies using these methods are described on page 33. However, obtaining small intestinal fluid usually requires endoscopy under sedation or general anaesthesia, and is therefore invasive and costly. A less invasive method for diagnosis of SIBO is breath testing. Intestinal bacteria metabolise carbohydrates in the gut lumen, producing hydrogen and methane that passes across the intestinal wall, into the bloodstream and is excreted via the lungs. Testing the hydrogen or methane content of exhaled air is a reasonable approximation of intestinal bacterial presence since the human body does not produce either gas except via metabolically active intestinal bacteria.(231)

The Breath Hydrogen Test (BHT) was employed as part of a prospective longitudinal birth cohort in Junju, Kilifi, Kenya, during June 2015 to January 2017. Detailed methods and results for enrolment and follow up of this cohort are described in Chapter 5. Background to the possible role of SIBO in EED and undernutrition is presented on pages 32-36. As a reminder, SIBO as defined by BHT has been found to be associated with stunting in Burmese 3-4-year-olds and Bangladeshi two-year-olds, with impaired response to oral cholera vaccine and lower SEP in Brazilian school-aged children, and with poor sanitation and some markers of

EED in Bangladeshi two-year-olds.(157-160) The focus of this Chapter is on methods for the measurement and diagnosis of SIBO.

Hydrogen and methane can be detected in breath by table-top (mains electricity powered) or hand-held (battery powered) machines. During breath tests, subjects undergo a period of fasting followed by oral ingestion of a substrate such as glucose or lactulose. Depending on the ability of the subject to comply with instructions, breath can be sampled actively (blowing into a tube) or passively (nasal prongs or face mask). Breath can be collected via one-way-valve into a sealed container, stored and later injected into the measurement device, or the device can be connected directly to the subject as they exhale. During a breath test, for 2-3 hours following substrate ingestion, serial measurements of exhaled air are taken. Where the substrate is absorbed along the length of the intestine, a large, late peak (beyond 60-120 minutes post ingestion) in the hydrogen or methane content of exhaled air over time depicts metabolism of the substrate by normally resident bacteria in the large intestine. An additional earlier, smaller peak indicates abnormal presence of bacteria in the small intestine. Where the substrate is fully absorbed by the small intestine (e.g. glucose), any peak is diagnostic of SIBO, and no later peak is expected either in presence or absence of SIBO. With hydrogen as the measured gas, glucose breath testing is more sensitive than and similarly specific to lactulose breath testing when compared against the gold standard of jejunal culture (Table 14). Lactulose breath testing has the additional disadvantage that slower oro-caecal transit time (OTT) may lead to confusion between ‘early’ and ‘late’ peaks.

Table 14: Performance of lactulose and glucose breath hydrogen tests against the gold standard of jejunal culture (>105 CFU/ml) in diagnosis of small intestinal bacterial overgrowth.
Table from Gasbarrini et al.(230) Median values from 11 studies involving adults and children using hydrogen breath test diagnostic thresholds ranging >10-12ppm above baseline fasting breath hydrogen.

Breath hydrogen test substrate	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Lactulose	52.4	85.7	61.5	53.6
Glucose	62.5	81.8	65.5	71.7

Breath testing is an established method for the diagnosis of SIBO in clinical practice amongst children in high-income settings. Using this method, SIBO has been found associated with abdominal surgery, obesity, proton pump inhibitor therapy, Irritable Bowel Syndrome, cirrhosis, cystic fibrosis and other functional gastrointestinal disorders.(232-236) Prevalence of positive BHT is around 4% amongst asymptomatic control children.(232) However, no studies of asymptomatic infants are available from high-income settings.

The earliest published example of breath testing for diagnosis of SIBO amongst children in tropical low-income settings is a 1991 study in rural Myanmar.(156) Here, in a cross-sectional survey of 340 children aged 1-59 months, lactulose was given orally followed by breath hydrogen measurement at intervals over three hours. 145 participants (43% of total) were excluded from further analysis after being labelled 'non-hydrogen-producers'. Non-hydrogen-producer status was defined by peak breath hydrogen <10ppm throughout, including after 60 minutes when a large intestine peak would normally be expected. These childrens' intestinal flora were assumed to not include bacteria that produce hydrogen, or to include these flora but also include species that metabolise hydrogen to methane, acetate or hydrogen sulphide. Amongst the 195 'hydrogen-producers', 27% fitted the authors'

definition of SIBO (an early peak, within the first hour, distinguishable from the later, larger, peak denoting hydrogen produced in the large intestine) and SIBO defined in this way was found to follow the same age-related pattern as incidence of diarrhoea. The same authors went on to conduct breath hydrogen testing on 256 'hydrogen-producer' children aged 1-59 months following an oral carbohydrate rice meal.(237) Early (within 60 minutes) peaks in breath hydrogen of 10ppm or more were defined as meeting SIBO criteria. Here, SIBO was detected among 21% of participants. There followed four further published examples of lactulose hydrogen breath testing amongst children in settings where EED may be common: all in Brazil, amongst school-aged children and using similar methods.(157, 158, 165)

In an effort to capture evidence of bacterial fermentation among non-hydrogen-producers, methane can be measured alongside or instead of hydrogen. In 2012, 85 slum-resident school-aged children and 43 age-matched children from a nearby private school in Sao Paulo, Brazil, underwent lactulose breath testing with detection of both hydrogen and methane.(159) Here, SIBO was defined as either an increase by ≥20ppm hydrogen or ≥10ppm methane during the first 60 minutes after lactulose ingestion compared to pre-ingestion baseline. Again, breath hydrogen was higher amongst the slum-resident children, with methane showing the same association. Supporting a theory that hydrogen non-production is in part due to metabolism of hydrogen to methane, the normal 'large intestine' (later, larger) hydrogen peak was smaller amongst those who produced methane.

There have to date been three studies published employing the glucose BHT for characterisation of SIBO amongst children in tropical low-income settings. The first occurred in 2007 and involved 50 children aged 5-11 years from the same slum area of Sao Paulo,

Brazil, and 50 similarly aged children from the same neighbouring private school.(158) Participants were asked to fast for eight hours, following which a baseline (fasting) breath sample was taken and then 50g glucose in 250ml water was given by mouth. Breath samples were then taken 15, 30, 45, 60, 90, 120 and 180 minutes after glucose ingestion. A lactulose BHT was undertaken on the next day. For both substrates, SIBO-positive status was allocated when any of the breath samples taken up to 60 minutes post ingestion showed hydrogen concentration ≥20ppm higher than the fasting (baseline) sample. Using these criteria, SIBO was detected by lactulose BHT amongst 38% of slum-resident participants and 2% of private school participants. For the glucose BHT, very few met the same SIBO criteria (4% in both groups). However, the authors concluded that a glucose substrate may be useful for subjects with quicker OTT where risk of confusion between small and large intestine peaks is higher. Shortly afterwards, the 2009 Rome Consensus Conference recommended glucose as the optimum substrate for diagnosis of SIBO by breath testing due to its complete absorption by the small intestine, with a smaller diagnostic threshold of ≥12ppm greater than fasting baseline, rather than ≥20ppm used for lactulose breath testing.(230) This threshold was decided upon following observation that higher cut-off values reduced BHT sensitivity below 60% compared to fluid sampling. The consensus also recommended specific dietary restrictions, fasting and mouthwashes to minimise baseline breath hydrogen prior to substrate administration but did not define an acceptable upper value or give direction on whether to proceed with testing if baseline breath hydrogen is high.

The second published example of using glucose BHT to measure SIBO amongst children in tropical low-income settings is from Bangladesh. In 2016, Donowitz et al published results of a cross-sectional survey of 103 two-year-olds from a low-income neighbourhood of Dhaka,

Bangladesh.(160) Following exclusions for SAM (fasting was deemed unsafe for these children), declining to participate and failure to complete testing, results were successfully obtained for 90 children aged 24-25 months. After three hours of fasting and a baseline breath sample, participants were given 1g/kg glucose in 5ml/kg water and breath samples were taken at 20-minute intervals between 20 and 180 minutes post ingestion. Alveolar breath sampling was ensured by concomitant measurement of exhaled carbon dioxide and repeating of samples until a minimum 5% CO_2 concentration was reached. Only water was allowed during the three-hour testing period. The Rome consensus diagnostic threshold of ≥12ppm above baseline was used, with no exclusions or repeat test attempts based on high baseline values. Following these criteria, overall prevalence of SIBO was 17%.

In the third published example, 194 stunted (HAZ<-2) or at-risk-from-stunting (HAZ<-1) children from Dhaka, Bangladesh, underwent glucose BHT before and after a nutritional intervention at around 14 and 18 months of age respectively.(161) The same glucose BHT methods and diagnostic criteria were followed as in the study described above. 15% of participants in the pre-intervention group were described as SIBO-positive.

Building on this precedent, this Chapter describes the methods I followed to develop and appraise a BHT to measure SIBO and for use in the cohort study described in Chapter 5.

Objectives

1. Develop a BHT, suitable for field conditions, to detect SIBO amongst Kenyan infants.
2. Appraise the accuracy, precision and convenience of this BHT as a measure of SIBO in Kenyan infants.

Methods

Method development

Methods for this test were developed following three preparatory activities:

1) A three-day visit to the International Centre for Diarrhoeal Disease Research, Bangladesh Mirpur study site, Dhaka, Bangladesh, in March 2015. There, I observed glucose BHT methods for the ongoing BEED study (Principal Investigator Jeffrey Donowitz, University of Virginia).(238)
2) Literature review (PubMed; search terms "small intestinal bacterial overgrowth" OR "small bowel bacterial overgrowth" OR "glucose hydrogen breath test"): reviewers' recommendations and reported primary research methods for glucose BHT diagnosis of SIBO amongst children in tropical low-income settings (Table 15).
3) A piloting phase at the study site. This was completed in June 2015 at Junju Dispensary in Gongoni village, Kilifi, and involved four participants aged between one and eight months and their caretakers. Each participant underwent one BHT. Oral feedback was given by caretakers during and after each test.

Table 15: Examples of reviewers' recommendations and reported methods for glucose breath hydrogen testing detection of Small Intestinal Bacterial Overgrowth. Blank fields indicate method detail not known or recommendation not given.

Study	Reviewer recommendation or reported methods?	Setting	Age	Fasting before testing	Substrate dose	Water dose	Testing intervals (minutes after baseline)	Testing duration (minutes)	Fasting during testing?	Breath collection method	Breath sensing equipment	Diagnostic criteria
Simren 2006(239)	Reviewer recommendation	N/A	Adult				15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180	180				
dos Reis 2007(158)	Reported methods	Brazil	5-11 years	8 hours	50g	250ml	15, 30, 45, 60, 90, 120, 180	180	Yes	Bag valve	QuinTron micro-analyzer model 12i	Peak minus baseline ≥20ppm
Ledochowski 2008(240)	Reviewer recommendation	N/A	'Paediatric'	14 hours[1]	1g/kg	10ml/kg	15, 30, 45, 60	60	Yes			Peak minus baseline ≥10ppm[2]
Gasbarrini 2009(230)[3]	Reviewer recommendation	N/A	'Paediatric'	Overnight[4]	50g[1]	250ml[1]	15, 30, 45, 60	120	Yes			Peak minus baseline ≥12ppm
Donowitz 2016(160)	Reported methods	Bangladesh	2 years	3 hours	1g/kg	5ml/kg	20, 40, 60, 80, 100, 120, 140, 160, 180	180	Yes	Bag valve	QuinTron BreathTracker SC gas chromatograph	Peak minus baseline ≥12ppm
DeBoer 2018(73)	Reported methods	Tanzania	6-12 months	2 hours	1g/kg	5ml/kg	20, 40, 60, 80, 100, 120	120	Yes	Bag valve	QuinTron BreathTracker SC gas chromatograph	Peak minus baseline ≥12ppm
Gaffar 2019(161)	Reported methods	Bangladesh	12-18 months	3 hours	1g/kg	5ml/kg	20, 40, 60, 80, 100, 120, 140, 160, 180	180	Yes	Bag valve	QuinTron BreathTracker SC gas chromatograph	Peak minus baseline ≥12ppm

1. This is the recommendation for adults. No recommendation for children.
2. Ideally fasting breath hydrogen should not exceed 5-10ppm. If baseline breath hydrogen ≥10ppm, recommend abandon test and re-attempt fasting on another day.
3. The 'Rome Consensus Conference'
4. Also recommended: diet free from non-absorbable carbohydrates the evening before followed by fasting overnight and on morning of BHT

Following the Rome consensus recommendation, I decided on glucose as the substrate due to its specificity to the small intestine.(230) I decided on a glucose solute dose of 1g/kg following the recommendation by Ledochowski et al.(240) I chose a water solvent volume of 2ml/kg as a compromise between risk of under-dosing through participants spitting out part of the solution (with a more concentrated solution, a single 'spit out' during administration would represent a greater proportion of the intended dose) and participant comfort and tolerability (larger, more dilute volumes would take longer to administer, risk vomiting, and could cause participants distress). In addition, very large volumes risk altering normal gastrointestinal physiology including OTT.

Following piloting, I chose a breath testing interval of 20 minutes as a compromise between minimising risk of missing a peak (wider intervals increase this risk) and participant comfort (piloting indicated that shorter intervals were intolerable).

Non-underweight healthy term newborns rarely develop hypoglycaemia during 'demand' breastfeeding which can sometimes involve intervals between feeds of up to four hours or more.(241) This safe interval extends with increasing age amongst well-nourished children. Additionally, the BHT oral glucose load, at 1g/kg, is in fact larger than the recommended dose of oral glucose for management of neonatal hypoglycaemia (200-400mg/kg).(242) Given this, a maximum four-hour 'nil by mouth' fasting duration – with the oral glucose load halfway through equating to a more than double hypoglycaemia correction dose – was presumed safe for all participants who were not severely underweight or severely wasted. A maximum total testing duration of two hours was decided on based on piloting feedback: a longer duration of 20-minute interval sampling was deemed intolerable. So as not to

exceed the maximum total four hours, this left two hours for fasting prior to glucose load. Prior to the start of the four-hour fast, three hours' breastmilk only (or usual milk only, if the participant is not breastfeeding) was decided upon as the maximum feasible following piloting feedback.

For breath collection and measurement, the hand-held battery-powered 'Gastro+™ Gastrolyzer®' hydrogen meter (Bedfont® Scientific Ltd., Maidstone, UK) was decided upon due to its feasibility in the context of absent electricity supply. To date, all published studies amongst children in tropical low-income settings have used various models of the mains-electricity-powered breath hydrogen/methane/carbon dioxide sensor by manufacturer QuinTron (QuinTron Instrument Company, Inc., Milwaukee, USA). The Gastrolyzer® unit has been used in published research studies involving participants not younger than four years(243) but has manufacturer guidance and protocols for all ages, including infants. The QuinTron units require collection of exhaled air using a bag and valve followed by injection of the stored breath sample into the sampler. The Gastrolyzer® allows only for direct measurement of air exhaled directly into the sampler, with no breath storage. It measures hydrogen only, not methane or carbon dioxide.

Oral solution preparation and storage

The oral glucose solution was prepared by pharmacists at KWTRP Research Pharmacy and in line with Good Clinical Laboratory Practice (GCLP). Full methods are described in Appendix 22. An SOP was developed and followed (Appendix 23).

Breath sampling

The first BHT was undertaken on the first Monday or Wednesday following enrolment to the cohort. Subsequent BHTs were scheduled at intervals by calendar month of age until nine months of age: a total of 10 BHTs per participant (0, 1, 2, 3, 4, 5, 6, 7, 8 and 9 months). 1,000 BHTs were therefore scheduled in total. Each month, for each participant, LM test was scheduled to follow BHT by one day (i.e. LM tests took place on Tuesdays and Thursdays).

Glucose administration and breath sampling methods followed a field SOP (Appendix 24). Participants were asked to consume breastmilk only (or their usual milk if not breastfeeding) during the three hours preceding the test. Following this, upon arrival at the Junju dispensary study site, participants underwent a two-hour observed fast (nil by mouth). At the end of the two hours, a baseline breath sample was taken using one of two hand-held hydrogen meters (Gastro+ Gastrolyzer®, Bedfont Scientific Ltd.) connected via a woven mesh infection control filter* to a single-participant-use one-way expiratory valve and personally sized soft plastic facemask. The mask was held firmly but gently against the participant's face to create a seal that persisted during the entire manufacturer-recommended measurement period of 30 seconds. The Gastrolyzer® has a hydrogen detection range of zero to 500ppm, an operating temperature range of 15 to 35^{o}C and an operating humidity range of 10 to 90%. Manufacturer's reported between-sample repeatability is less than 10%. At the beginning of

* This filter was tested by Public Health England's Biosafety Investigation Unit and found to prevent the passage of >99% of aerosolised *Bacillus atrophaeus* spores. Reference: Public Health England report number 14/040 December 2014.

each testing day, participants were assigned to Unit 1 or Unit 2 on a haphazard basis. The same meter (Unit 1 or Unit 2) was then maintained within each test. Breath hydrogen results were displayed and recorded in real time, with no breath sample storage. After this baseline fasting breath hydrogen measurement, participants immediately received a 1g/kg oral glucose dose (2ml/kg of the 500mg/ml glucose solution). Further breath samples were then taken at 20, 40, 60, 80, 100 and 120 minutes following glucose administration. Fasting was maintained throughout. The total fasting duration was therefore four hours (not including the oral glucose dose midway). Results and adherence to protocol were recorded on paper for each BHT (Appendix 25). Following manufacturer's instructions, the infection control filter was replaced on a monthly basis and the two Gastrolyzer® units calibrated monthly in the recommended air-conditioned 18-21^{o}C environment using manufacturer-provided 100ppm hydrogen gas.

Results interpretation

Results were initially interpreted following the Rome categorical criteria.(230) A test where all readings were <12ppm above the baseline value were categorised 'negative'. A test where one or more readings were ≥12ppm above the baseline value were categorised 'positive'. Following the recommendation of Ledochowski et al, superseding these two categories, any test where the baseline value was >5ppm was deemed 'indeterminate'.(240) Examples of curves meeting these three criteria are given in Figure 14.

Figure 14: Example breath hydrogen test readings for a negative, positive and indeterminate result. Negative: green; positive: red; indeterminate: dashed orange. Two 'indeterminate' scenarios are shown: one where subsequent readings are high, one where subsequent readings are low.



Results

Age at first BHT, and missed tests

Age at first BHT ranged between two and 27 days. Three BHTs were missed (by three participants) due to hospitalisation. Three BHTs were missed due to concern over a participant's ability to safely tolerate a four-hour fast without developing hypoglycaemia (the participant was severely underweight). Nine BHTs were missed (by four participants) due to travel away from the study site. 10 participants withdrew early from follow-up, leading to 51 missed BHTs. 934 BHTs were therefore carried out from the planned 1,000.

Adherence to field protocol

For 17 tests, the participant had consumed something other than their usual milk during the three hours preceding testing (Table 16). In all cases this was *uji*. During seven tests, the final reading was omitted in error. No other readings were omitted. Where these omissions were made, for conservative estimation and also reflecting the mode reading, a breath hydrogen concentration of 0ppm was imputed.

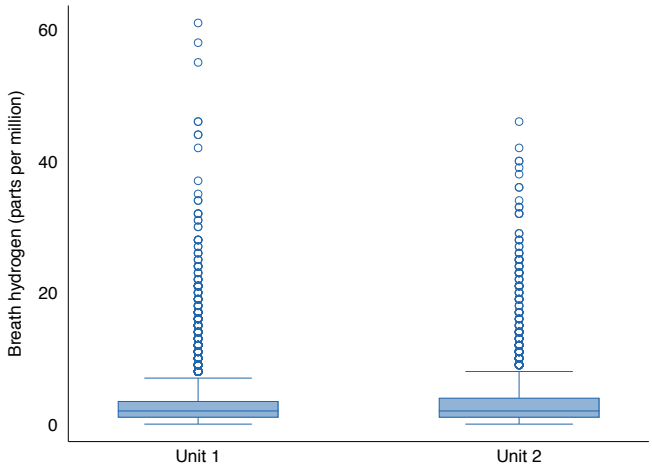
Table 16: Adherence to field protocol for breath hydrogen test.

Field protocol	Number of BHTs that adhered (% of 934)
Usual milk only for three hours preceding test	917 (98)
Nil by mouth for two hours preceding glucose ingestion	934 (100)
Nil by mouth for two hours following glucose ingestion	934 (100)
All breath samples undertaken	927 (99)
All breath samples undertaken within five minutes of target time	839 (90)

Results by hydrogen meter

560 BHTs (3,920 readings) were undertaken using the first unit (Unit 1) and 374 BHTs (2,618 readings) using the second (Unit 2). Fewer tests were carried out using Unit 2 because it was brought into use after the first few months of fieldwork. No difference was observed in readings obtained from either unit (Figure 15).

Figure 15: Box plots demonstrating frequency distribution of breath hydrogen readings obtained by Gastrolyzer® unit used. Wilcoxon rank-sum test of difference between readings by unit: p=0.64. Box midlines denote median, box edges denote 25th and 75th centiles, whiskers denote upper and lower adjacent values, hollow dots denote outlying values.



Baseline breath hydrogen

49 tests (5.3% of 934) showed very high (≥10ppm) baseline (pre glucose ingestion) breath hydrogen. These cases occurred mostly at age under two and six or more months (Figure 16). Very high baseline breath hydrogen was not associated with reported ingestion of foods other than usual milk during the three hours preceding testing (chi squared test p=0.22), nor was it associated with complementary foods having already been introduced in general (chi squared test p=0.20). 106 tests (11.4% of 934) showed moderately high (≥5ppm) baseline breath hydrogen. On a continuous scale, higher baseline breath hydrogen was positively associated with subsequent higher readings (area under the curve; Figure 17).

Figure 16: Baseline breath hydrogen by age.
Red dotted lines indicate suggested thresholds of 5 and 10ppm. This figure excludes one high outlier: age 1.6 months baseline breath hydrogen = 58ppm.

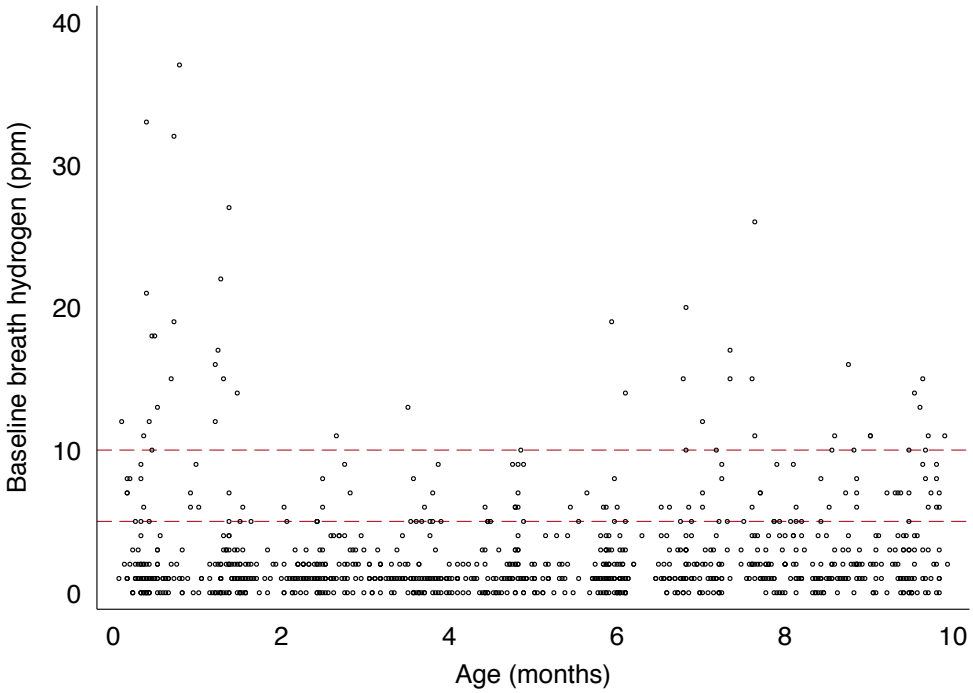
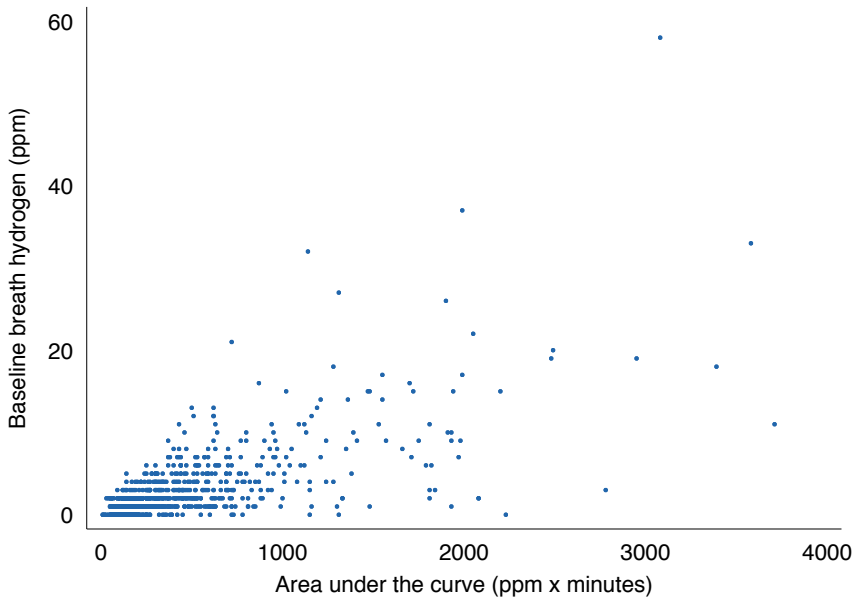


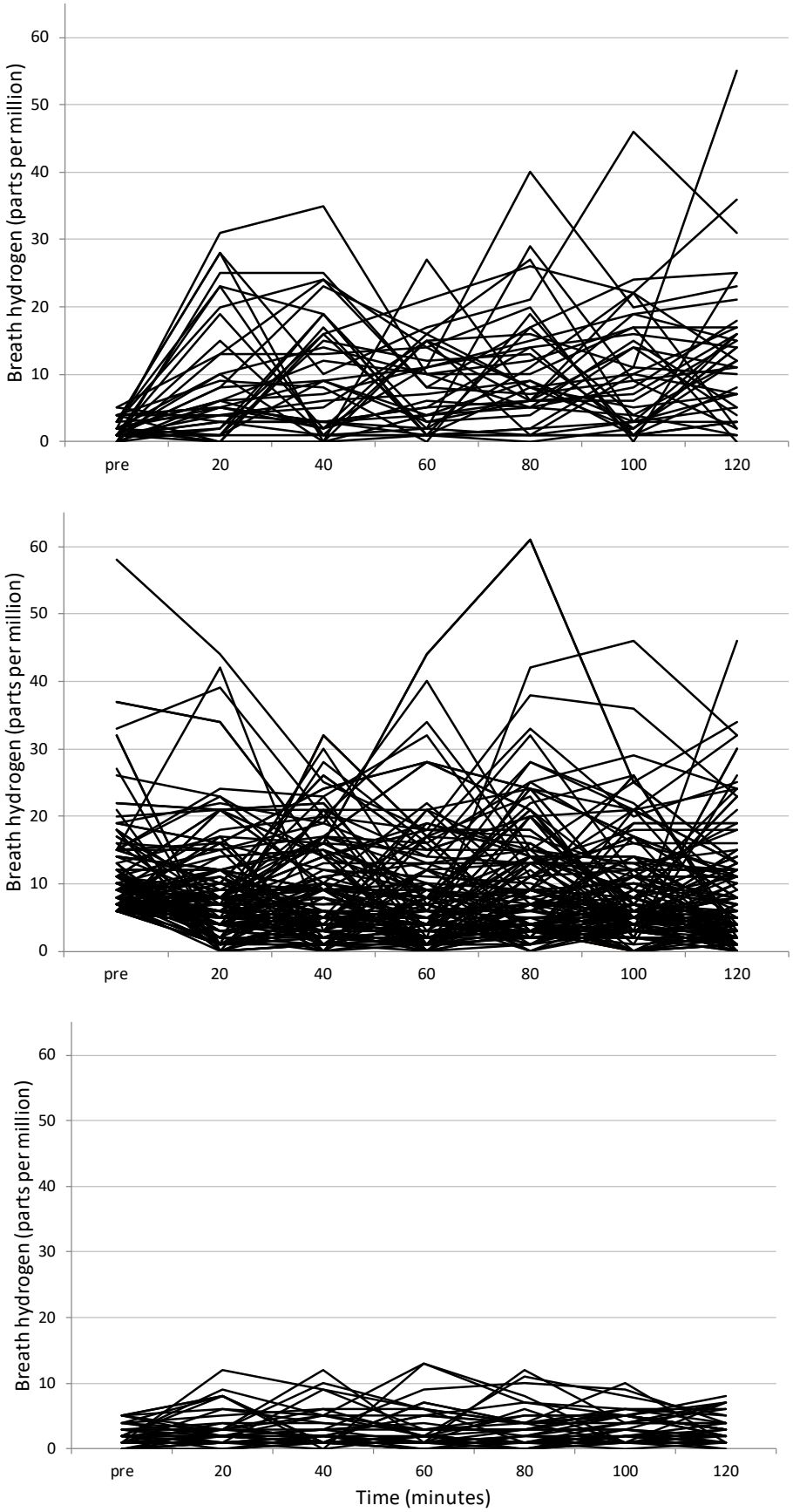
Figure 17: Correlation of baseline breath hydrogen and area under the curve for each test's subsequent 2-hour duration.



Rome criteria results and individual test curves

Following the Rome criteria, 763 tests (82%) were 'negative' (peak <12ppm above baseline and baseline ≤5ppm), 52 (6%) 'positive' (peak ≥12ppm above baseline and baseline ≤5ppm) and 119 (13%) were 'indeterminate' due to high baseline (chosen henceforth as >5ppm). Individual test curves that met these three criteria are shown in Figure 18.

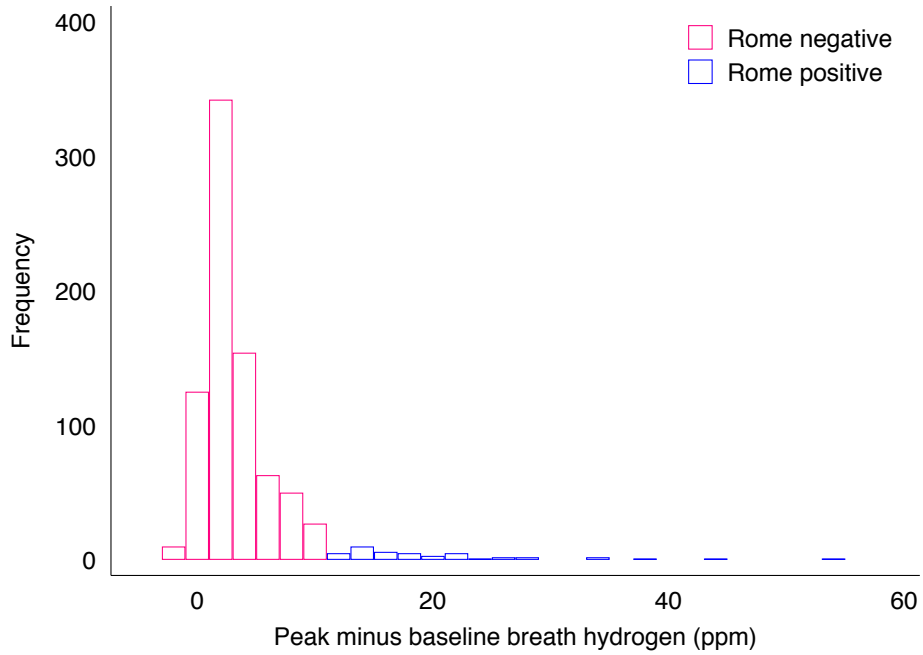
Figure 18: Breath hydrogen test curves for positive, high baseline and negative results.
Positive: top; high baseline: middle; negative: bottom. For negative results, a subset of the 763 curves are shown. All 52 positive and 119 high baseline curves are shown.



Continuous data interpretations

Rome criteria apply a binary categorisation to the value 'peak minus baseline breath hydrogen' amongst those whose baseline breath hydrogen was ≤5ppm. The frequency distribution of this value (again, amongst those with baseline breath hydrogen ≤5ppm) shows that those categorised as 'positive' by Rome criteria were indeed high outliers in this dataset (Figure 19).

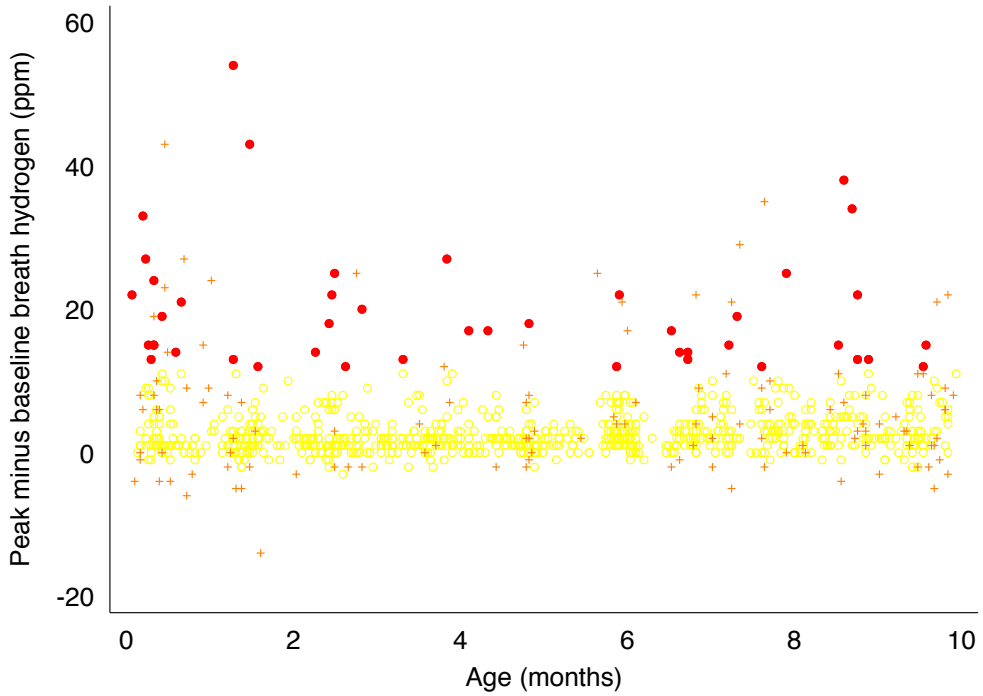
Figure 19: Frequency distribution of difference between peak and baseline breath hydrogen amongst those with a baseline breath hydrogen ≤5ppm.
n=815. Blue bars indicate distribution for those meeting Rome 'negative' criteria (peak minus baseline <12ppm; n=763). Pink bars indicate those meeting 'positive' Rome criteria (peak minus baseline ≥12ppm; n=52).



Plotting this same value (peak minus baseline breath hydrogen) against age, but this time including all results including those for whom baseline breath hydrogen was >5ppm, shows

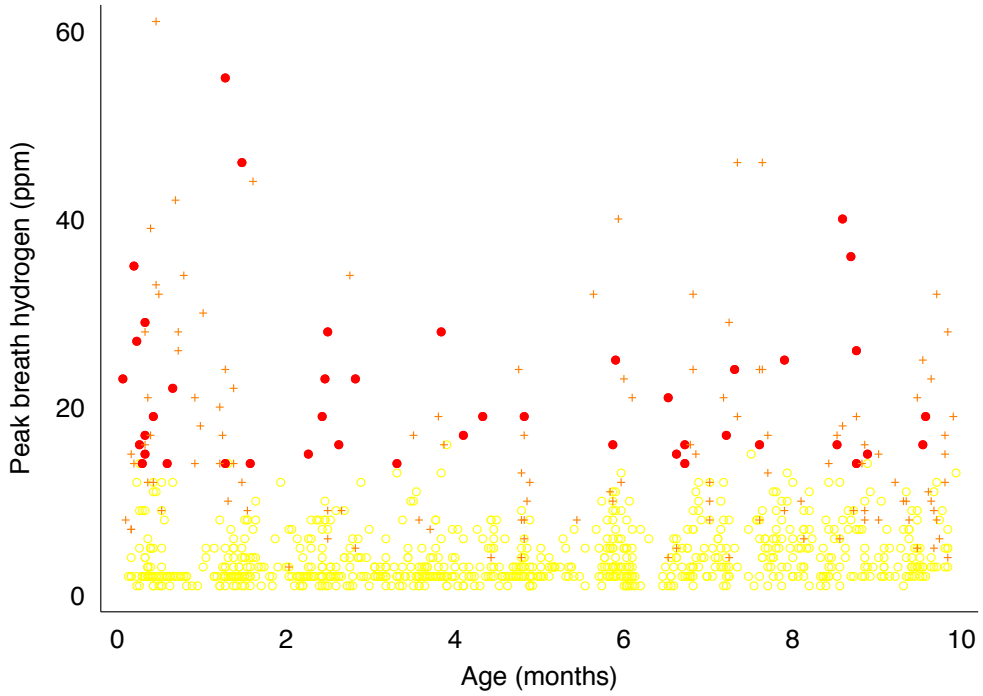
that this value appears to vary widely (although the extent of variation does not appear to vary with age) amongst those meeting 'indeterminate' Rome criteria (Figure 20).

Figure 20: Peak minus baseline breath hydrogen by age.
Yellow circles indicate tests that were categorised as Rome criteria 'negative', orange crosses 'indeterminate', red dots 'positive'.



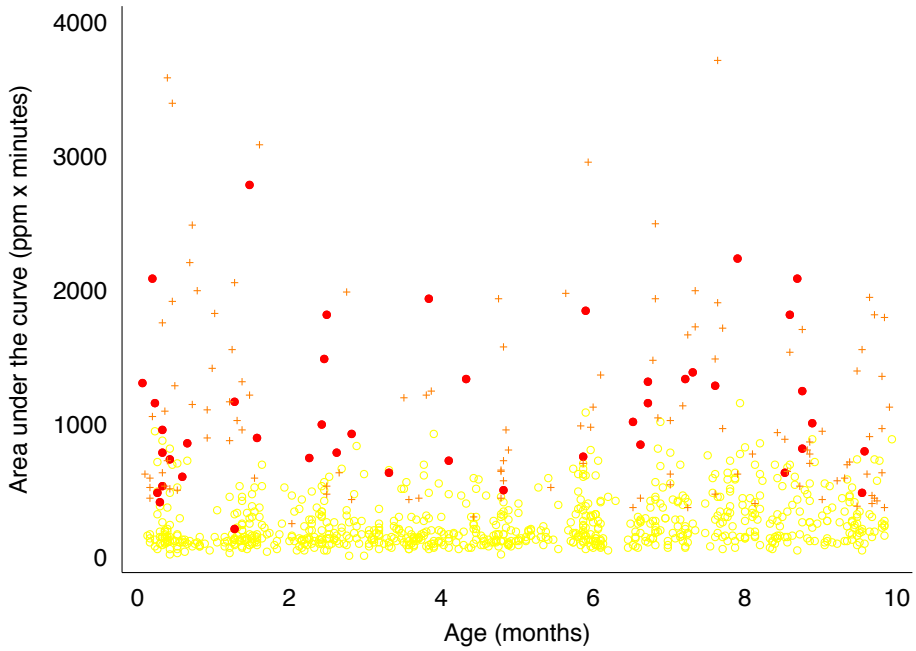
Size of peak alone (amongst breath samples taken after glucose ingestion, i.e. not including the baseline sample) plotted against age shows better correlation with Rome criteria categories (Figure 21).

Figure 21: Peak breath hydrogen by age.
Yellow circles indicate tests that were categorised as Rome criteria 'negative', orange crosses 'indeterminate', red dots 'positive'



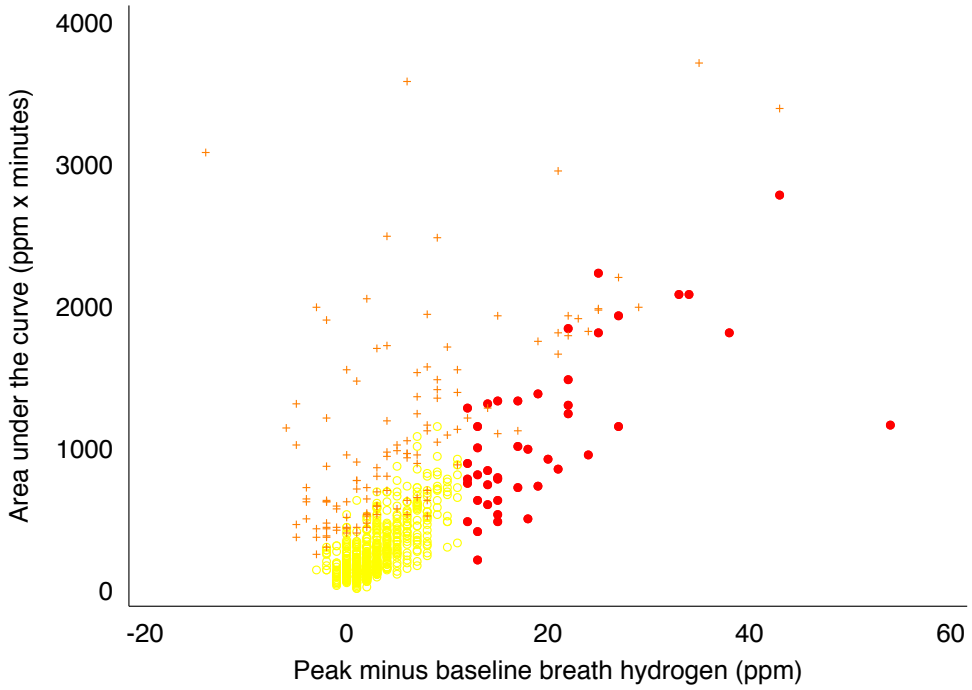
Lastly, area under the curve (AUC) was calculated for each test, using straight lines between data points to create six rhombi (0-20, 20-40, 40-60, 60-80, 80-100, 100-120 minutes) to approximate the AUC for each test.

Figure 22: Breath hydrogen area under the curve by age.
Yellow circles indicate tests that were categorised as Rome criteria 'negative', orange crosses 'indeterminate', red dots 'positive'



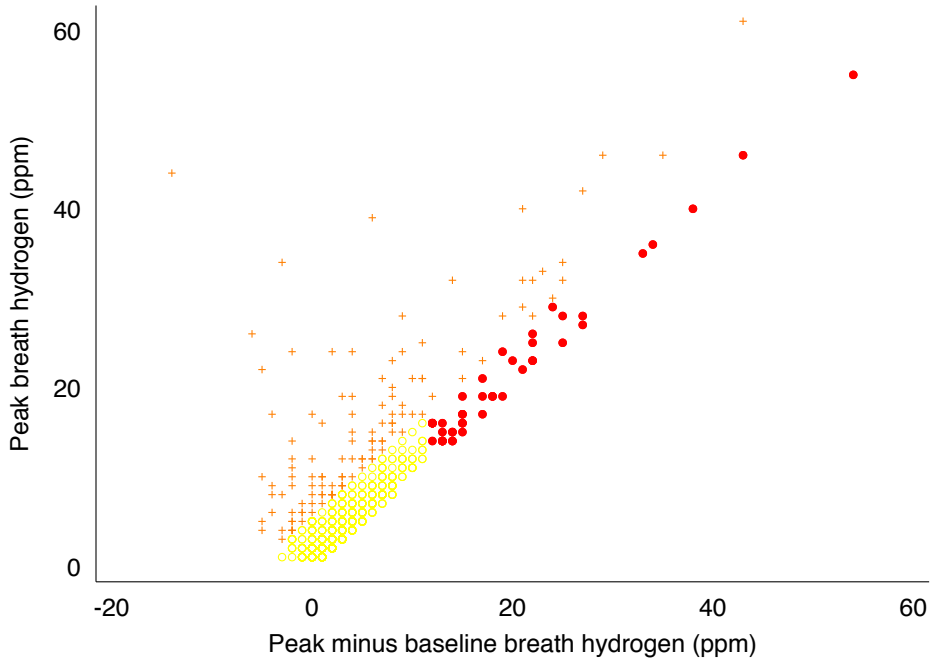
Plotting AUC against 'peak minus baseline breath hydrogen' shows the test curves for which peak minus baseline would give a low or negative result but AUC gives a high result (Figure 23). These test curves, seen in the top left region of Figure 23, all meet Rome 'indeterminate' criteria. Area under the curve and peak minus baseline breath hydrogen are closely correlated.

Figure 23: Area under the curve against peak minus baseline breath hydrogen for all 934 breath hydrogen tests.
Yellow circles indicate tests that were categorised as Rome criteria 'negative', orange crosses 'indeterminate', red dots 'positive'. Pearson's correlation coefficient 0.67.



Plotting peak against peak minus baseline breath hydrogen shows again how both values vary widely amongst 'indeterminate' test curves (Figure 24). Again, correlation between the two values is very high.

Figure 24: Peak against peak minus baseline breath hydrogen for all 934 breath hydrogen tests. Yellow circles indicate tests that were categorised as Rome criteria 'negative', orange crosses 'indeterminate', red dots 'positive'. Pearson's correlation coefficient 0.82.



Peak post-glucose breath hydrogen was chosen as the continuous data interpretation of the breath hydrogen test to be taken forwards into the multivariable regression modelling reported in Chapter 5. The following reasons were behind this decision:

- A continuous measure best preserves power in statistical modelling compared to categorical data such as the Rome criteria.
- Although the Rome categorical criteria derive from peak minus baseline rather than peak by itself, peak by itself has the advantage of being independent of baseline. For

test curves where both baseline and subsequent readings were high, SIBO is a likely diagnosis but peak minus baseline is low. As reported earlier, only 5.3% of tests had very high baseline (≥10ppm).

- There is no loss of data upon log transformation. Log transformation is required for skewed data prior to inclusion in regression models that assume symmetrical distribution of variables. For test curves where peak minus baseline breath hydrogen is less than or equal to zero (172 tests; 18% of 934), attempted log transformation yields a missing data point.
- AUC better distinguishes between sustained high breath hydrogen throughout testing and a single high peak. However, this is in fact a potential weakness in its ability to distinguish SIBO from slow OTT, or a combination of SIBO and slow OTT. As discussed in the introduction to this Chapter, BHT results can be confounded by OTT. High readings will be sustained in prolonged OTT. A single high reading is sufficient to demonstrate increased hydrogen production in the small intestine and to diagnose SIBO. Severity of SIBO is in theory proportional to peak height rather than breadth. AUC is therefore at risk of undue influence by OTT. In cases where SIBO has occurred in whole or part due to slow OTT, this influence is even more likely.

Discussion and conclusions

Glucose BHT in this population of infants in rural Kilifi demonstrates overall a low prevalence of SIBO as defined by the Rome criteria. However, given the nature of the study population and the methods employed, there are several possible causes of false negative results:

- *'Non-hydrogen-producer' status.* Previous studies have suggested that prevalence of this ranges between 2% and 43%.(230) Since glucose is assumed not to pass into the large intestine, it is not possible to identify non hydrogen producers when using glucose as a substrate (i.e. absence of the expected large intestine peak). Within this constraint, measurement of methane alongside hydrogen in response to glucose load could detect cases of SIBO that are missed by hydrogen testing alone due to conversion of hydrogen to methane. Donowitz et al have observed very low prevalence of methane production (Donowitz, personal communication May 2015), suggesting that in their study population (Bangladeshi toddlers), conversion of hydrogen to methane is not occurring at a significant rate. The most populous intestinal bacterial species that converts hydrogen to methane (a methanogen) is *Methanobrevibacter smithii*.(244) Detection of methanogens and their shared genes by next-generation sequencing may be used in screening for non-hydrogen-producers. The gut has its own 'hydrogen economy' where heterogeneity occurs in rates of hydrogen consumption through conversion of hydrogen to methane, acetate or hydrogen sulphide. Severity of SIBO (as defined by the gold standard CFU/ml in small intestinal fluid) may therefore not be predictably associated with breath hydrogen concentration. A case in point is the fact that the differences observed in concentrations of bacteria in the small intestine are on a log-fold scale (10^2 to 10^{11} or higher CFU/ml) whereas differences in observed breath hydrogen concentration are much smaller – ranging from 0 to 60ppm or so. As discussed earlier, sensitivity of BHT compared to intestinal fluid culture ranges from 52 to 62% and specificity 81 to 85%.
- *Sampling of non-alveolar gas.* During normal resting respiration in older children, dead space is normally around 2ml/kg per breath. Assuming a tidal volume of 5ml/kg,

this equates to two fifths of the volume of expired air in each exhalation. In neonates, this figure increases to around half.(230) Only the second half of the volume of each expiration, therefore, is alveolar gas with hydrogen concentration equilibrated to blood. Added to this is the additional dead space present within the face mask and plastic connecting tube. For the equipment used in this study, total dead space was fixed at 73ml (mask 45ml, connector 18ml, infection filter 10ml). The smaller the participant, the more significant this total dead space is in comparison to their tidal volume. These two sources of dead space could have led to false negative results amongst participants of all ages but especially amongst the youngest. Aside from the issue of dead space, participants of all ages were unable to comply with the manufacturer-recommended exhalation technique in which test subjects should inhale maximally, hold their breath for 15 seconds, then exhale slowly and fully into the measurement device. However, it was noted during sampling that hydrogen readings would rise during the infant's first few exhalations then stabilise after around 20 seconds before the measurement period ceased after 30 seconds. Readings would never fall once risen. Measurement of exhaled carbon dioxide could help determine whether sampled air is alveolar or dead space.

- *Hyperventilation*. Crying, a state sometimes associated with hyperventilation, during breath sampling was frequently observed. Hyperventilation *shortly before* breath sampling is known to reduce breath hydrogen through increased opportunity for gas exchange.(230) However, the effect of hyperventilation *during* breath sampling is unknown. Unfortunately, fieldworkers did not record whether a participant was crying during each sample, so it is not possible to comment on the risk of crying leading to false negative results.

- *Inadequate frequency of sampling.* Hydrogen peaks may well have been missed in-between the 20-minute intervals. However, these intervals were taken into consideration when the diagnostic threshold of 12ppm above baseline was decided upon. This study used a slightly longer interval than recommended (15 minutes). This was in response to piloting participant feedback, as more frequent sampling was deemed intolerable.
- *High baseline tests not repeated.* Tests where baseline (fasting pre-glucose) breath hydrogen was >10ppm or even >5ppm could have been abandoned, with a repeat attempt scheduled for the next day. This would have been ideal but was decided against for logistical and long-term participant retention reasons. A proportion of these repeated tests may have gone on to meet categorical criteria for diagnosis of SIBO. As it is, they are categorised as 'indeterminate' and, as already discussed, most demonstrated high subsequent readings likely in keeping with true presence of SIBO.
- *Sensor failure.* This is unlikely to have happened. Both Gastrolyzer® units were used within the manufacturer's recommended temperature and humidity limits and calibrated monthly. There were no problems with any of the calibrations.
- *Glucose under-dosing.* This was avoided through diligent and careful fieldworker glucose administration technique. Accurate concentration of the oral glucose solution is assumed: it was not measured.
- *Slow oro-caecal transit time (OTT).* It is unlikely that the glucose load would have taken more than two hours to reach the caecum in any significant minority of tests – especially amongst infants whose intestinal transit is naturally faster. However, those with slow OTT could have had their SIBO undetected, especially if the overgrowth was confined to the distal small intestine. Testing beyond two hours would capture

these cases, but at the expense of a longer-than-safe fasting period for such young infants and an intolerable experience for participant and caretaker. The recommended testing duration of two hours was met.(230) Indeed, in lactulose breath testing, any peak beyond 60 minutes is considered to originate from the large intestine. Fast OTT, on the other hand, could also increase risk of false negative results if the hydrogen peak occurred and resolved very quickly before the first post-glucose breath sample at 20 minutes.

The list of possible reasons for false positive results is shorter:

- *Incomplete absorption of glucose by small intestine.* Glucose entering the large intestine would be metabolised by the bacteria found there and lead to high breath hydrogen if this occurs within the two-hour testing window. The assumed complete absorption of glucose by the small intestine is a central principle of the glucose breath test and there is no evidence to refute it.
- *Glucose over-dosing.* This may conceivably have arisen through fieldworker error.
- *Unreported non-compliance with fasting.* Given the significant minority of participants with fasting breath hydrogen ≥5ppm, this may have been an issue. However, baseline breath hydrogen was not associated with self-reported non-compliance with the requested minimum three hours of exclusive breastfeeding prior to the start of the two-hour fast (non-compliance itself was rare, too, at 2%). Baseline breath hydrogen was also not associated with the participant having started complementary foods. The two-hour fast was directly observed by fieldworkers, with caretakers not leaving the study facility grounds. Observed compliance was 100%. Alternative explanations for high baseline breath hydrogen are:

- *Hydrogen production by oropharyngeal bacteria.* Antiseptic mouthwash prior to breath testing is recommended by the Rome Consensus.(230) However, use of a mouthwash or infant-appropriate topical mouth preparation was not thought compatible with the public health message priority of exclusive breastfeeding amongst participants aged less than six months.
- *Slow OTT.* Anecdotally, most participants who had started complementary foods would prepare for the BHT by skipping their usual morning meal of *uji* (fermented cereal porridge – a low-absorbable carbohydrate). These participants, whose last complementary food meal would therefore have been the evening before the BHT, would then have consumed nothing but their usual milk for much longer than the requested three hours. However, those with very slow OTT may still have had complementary foods in their stomach or small intestine at the onset of breath testing. The same could also be said for the participant's usual milk. Again anecdotally, participant caretakers, anticipating the four-hour fast ahead, would arrive at the study facility, breastfeed their baby, and then report to the fieldworker to commence the observed two-hour fast. In this way, most participants would start the fast with a full stomach. The same assumption made about glucose exiting the small intestine within the two-hour testing window also applies here, however: any stomach and small intestine contents present at the beginning of the two-hour fast are assumed to have passed beyond the caecum by the time of the baseline breath sample.

Although Rome Consensus criteria suggest low prevalence of SIBO in this study population (6%), continuous measures such as peak breath hydrogen, peak minus baseline and area under the curve do demonstrate that a more sizeable minority of tests are probably in keeping with abnormally high breath hydrogen and a likely diagnosis of SIBO. Indeed, it may be that a lesser degree of SIBO, not necessarily meeting Rome diagnostic criteria, is truly causally associated with EED. Nonetheless, mainly given the relatively large volume of dead space compared to infant tidal volumes and the unknown entity of non-hydrogen producer status, BHT is probably more likely to have underestimated than overestimated prevalence of SIBO in this study. One observation in favour of this study's BHT method being reproducible is the observed stability of SIBO status over time within participants. As described later in Chapter 5, participants appeared to retain their 'positive' or 'high baseline' Rome criteria status in consecutive BHTs over sustained periods of up to four months. This suggests that the BHT method used in this study yielded results that were reproducible over time, even if accurate appraisal of the test's reliability cannot be made.

The BHT method described has proved well tolerated and applicable at scale in a remote setting. However, unanswered questions exist around the significance of false negative results due to non-hydrogen-producer status, non-alveolar breath sampling and variable OTT. Previous studies have not discussed the possible significance of high fasting breath hydrogen. Given the positive association between baseline and subsequent readings, it is possible that indeterminate test results are the result of SIBO. However, residual milk or food in the stomach or small intestine adding to the standardised glucose load cannot be ruled out. Although not a significant problem in this non-inpatient setting, the requirement for a four-hour fast renders this method currently unsuitable for children with SAM and/or acute

severe illness and thus limits the applicability of breath testing amongst these populations in which SIBO may in fact be more common. In the initial inpatient management of complicated SAM, for example, current WHO guidelines recommend a maximum interval between feeds of two hours to reduce the risk of hypoglycaemia.(245)

To my knowledge, at least two ongoing studies are using BHT to measure SIBO amongst children in tropical low-income settings. In the first study, DeBoer et al are using glucose BHT to measure SIBO as a secondary outcome at age six, nine and 12 months amongst a subset of 400 participants of a randomised trial in Tanzania to assess the impact of azithromycin and specific nutritional supplements on subclinical enteropathogen carriage and linear growth.(73) In this study, methane will be measured alongside hydrogen. In the second study, Donowitz et al are administering glucose BHT to Bangladeshi children aged 4-23 months at intervals along with serial measurements of growth, intestinal microbiome, diarrhoea, oral polio vaccine response and intestinal inflammation (Nutrition, Overgrowth and Vaccine Efficacy in Low-income settings; ClinicalTrials.gov NCT02745327).

The BHT has clear logistical advantages over intestinal fluid sampling but its suitability as a reliable measure of SIBO, especially amongst infants, has yet to be confirmed. With increasing accessibility of next-generation sequencing techniques, sampling of small intestinal fluid is subject to renewed interest. In the ongoing BEED study in Dhaka, Bangladesh, a subset of 12-18-month-olds who fail a nutritional intervention against stunting will undergo endoscopy to obtain samples of small intestinal luminal fluid for microbial analysis, as well as BHT.(238) This is an opportunity for direct comparison of breath hydrogen concentrations against the SIBO diagnostic gold standard of intestinal fluid sampling.

It is not yet clear whether elevated breath hydrogen or methane represents small intestinal overgrowth of one or a few dominant bacterial species against a background of healthy overall gut microbiota or, perhaps more likely, a more complex intestinal dysbiosis affecting the whole length of the gut with SIBO as one element leading to disruption of the gut's normal hydrogen pathways. Microbiota analysis of upper and lower intestinal samples may enable better interpretation of the BHT and provide a fuller picture of SIBO amongst children in tropical low-income settings.

Chapter 4: Lactulose mannitol dual sugar absorption test to measure Environmental Enteric Dysfunction amongst infants in Junju, Kilifi

Background

The primary role of the gut is to absorb food whilst minimising passage of non-nutritious foreign substances and pathogens (whole or fragmented). Functional assessment of these two elements yields important information about gut health. Dual sugar absorption testing, of which the lactulose mannitol (LM) test is an example, aims to characterise these two key elements of gut function: absorption and permeability. During a dual sugar absorption test, the subject undergoes a period of fasting before receiving an oral dose of two sugars in water solution: a larger sugar such as lactulose (13 angstrom (Å)) and a smaller sugar such as mannitol (8Å) or rhamnose. Larger sugars such as lactulose have until recently been thought only to be absorbed via abnormal gaps between cells in the intestinal epithelium. Mannitol is an example of a smaller sugar that has until recently been thought to be mainly actively absorbed via transcellular pathways in the small intestinal epithelium in proportion to its absorptive surface area. Sugars, once they have passed through the gut wall, can be measured in the blood or urine. Following oral ingestion, both lactulose and mannitol/rhamnose are excreted intact in urine following minimal metabolism. The proportion of ingested mannitol excreted in the urine is therefore considered positively correlated with intestinal absorptive capacity whilst proportion of ingested lactulose excreted in the urine is considered negatively correlated with intestinal barrier function. Percentage excretion of lactulose and mannitol/rhamnose is calculated by comparing the

mass ingested against the mass present in the bloodstream or excreted in urine over a standardised time window. A dual sugar absorption test therefore aims to measure both gut permeability and gut absorptive capacity. Although these distinctions in sugar uptake are not as fixed as previously thought (see Discussion below), measurement of differential uptake is still one valid model for gut absorptive function and permeability.

The LM ratio is derived by dividing lactulose by mannitol percentage excretion. LM ratio is considered proportional to EED severity, with higher urinary LM ratios (high proportion excreted lactulose, low proportion excreted mannitol) thought to reflect greater abnormalities in one or both functions. An upper range of normal for the LM ratio has historically been quoted as 0.12 based on data from healthy UK children aged 3-15 months. However, the original data for this threshold has not been described.(45) The LM test has been used as a measure of EED in many studies, and a predominant pattern has emerged of higher LM ratios associated with poorer linear growth (see page 11).

Dual sugar absorption testing entails several challenges to accuracy and precision. Test protocols vary in their substrate, oral solution concentrations, fasting regimens, duration and timing of urine collection and method of measurement of substrate in blood or urine. Some protocols give a standard dose of substrate whilst others dose by bodyweight. Some protocols adjust for presence (endogenous or dietary) of substrate in blood or urine before ingestion whilst others do not. Standardised dual sugar preparations are not generally commercially available, and therefore need to be made up by investigators. Bacteria contaminating urine, blood or oral solutions may consume any sugars present. In urinary tract infection, bacteriuria may metabolise sugar in the urine even prior to voiding. Where

sugars are detected in urine rather than blood, inaccuracies can arise from loss of urine volume due to leakage or contamination of urine samples with faeces and, where participants are unable to void on demand, residual bladder volume at the start and end of the timed urine collection period. Residual urine in the bladder at the start of the collection period will not contain any absorbed test substrate and, once mixed with urine produced after substrate ingestion, lead to underestimation of true substrate excretion within the timed period. Residual urine present in the bladder at the end of the collection period will contain absorbed substrate. Failure to collect this residual volume will again lead to underestimation of true substrate excretion. The longer the collection period, the less relatively important these two residual volumes will be in comparison to the total volume of urine obtained. Longer urine collection periods also reduce the risk of test failure due to no post-ingestion urine obtained at all. A shorter urine collection duration, on the other hand, has the advantage of reflecting small intestinal rather than whole gut function. Fasting before and after substrate ingestion is employed in an effort to standardise conditions for substrate absorption in the intestinal lumen. However, lactulose itself can speed up oro-caecal transit which alters opportunity for absorption in an unpredictable way. Lastly, mannitol and lactulose are naturally present in urine in small amounts and, if present, need to be taken into account. Lactulose dietary sources include yoghurt and cereals. Other disaccharides similar to lactulose may be produced during the breakdown of breastmilk or other milks and may interfere with lactulose detection in urine. Mannitol is found in certain food crops but not known to be present in breastmilk.

Sugars have mostly been detected in urine using High Performance Liquid Chromatography (HPLC). Since protocols and platforms for this method vary by laboratory and, given the

variability in urine collection methods described above, comparison of LM test results between studies is difficult. To compare the performance of HPLC against newer mass spectrometry techniques, using a subset of 100 LM test urine samples from the MAL-ED network,(46) Lee et al carried out testing for lactulose and mannitol in three independent laboratories: one using HPLC-PAD (HPLC with Pulsed Amperometric Detection) and two using Liquid Chromatography tandem Mass Spectrometry (LC-MSMS).(78) High agreement was found between urine mannitol concentrations derived from the HPLC-PAD method and those derived from the two LC-MSMS methods, but lower for lactulose concentrations. In comparison to LC-MSMS, the HPLC-PAD platform was found to overestimate where lactulose concentrations were low. Overall, using the institutional LC-MSMS method as the reference, the HPLC-PAD method was found to overestimate LM ratios by 27% and a commercial LC-MSMS method was found to underestimate by 11%.

This Chapter describes method development and quality assessment results for the LM test employed in the cohort described in Chapter 5.

Objectives

1. Develop a dual sugar absorption assay, suitable for field conditions, to measure intestinal permeability and absorptive capacity amongst Kenyan infants
2. Implement this assay to describe the acquisition of EED with age amongst a cohort of Kenyan infants

3. Appraise the performance of this test as a measure of intestinal permeability and absorptive capacity, and as a measure of EED in Kenyan infants

Methods

The LM test was employed as part of a prospective longitudinal birth cohort in Junju, Kilifi, Kenya, during August 2015 to January 2017. Detailed methods for this cohort are described in Chapter 5.

Method development

Methods for this test were developed following four preparatory activities:

1. A two-day visit to the 'WASH Benefits' study site in Kakamega, Western Kenya, in October 2014.(246) There, I observed field urine collection methods.
2. A three-day visit to the International Centre for Diarrhoeal Disease Research, Bangladesh Mirpur study site, Dhaka, Bangladesh, in March 2015. There, I observed field urine collection methods and oral solution preparation for the LM test in the PROVIDE study.(97)
3. Literature review (PubMed; search terms "tropical sprue [MeSH Terms] OR "environmental enteropathy" OR "tropical enteropathy" OR "environmental enteric dysfunction"): reported LM test methods for studies amongst children in tropical low-income published or ongoing before the beginning of my fieldwork in June 2015 (Table 17). Additional studies were identified with reference to a systematic review

of the use of the LM test to evaluate EED amongst under-fives from 2000 to 2010 published by Denno et al in December 2014.(45)

4) A piloting phase was completed in June 2015 at the study site at Junju Dispensary in Gongoni village, Kilifi. This involved five participants aged 1-8 months and their caretakers and helped establish optimum sterile urine collection methods, with trialling of equipment and consumables. To help decide duration of urine collection and fasting, oral feedback was obtained from the five caretakers on the participant experience.

Table 17: Examples of reported methods either published or ongoing on or before June 2015 for urine lactulose mannitol dual sugar absorption testing. Blank fields indicate method detail not known or recommendation not given. Adapted from Denno et al 2014.(45) Blank fields indicate information not reported.

Study	Setting	Age	Fasting before test?	Pre-ingestion urine sample?	Lactulose dose	Mannitol dose	Urine collection duration (hours)	Fasting during urine collection?	Method of lactulose and mannitol detection
Lunn 1991	The Gambia	2-15 months	None	No	400mg/kg	100mg/kg	5	None	
Goto 1999(10)	Guatemala	0-11 months	2 hours	No	400mg/kg	100mg/kg	5	30min NBM then 30min water only	Gas-liquid chromatography
Lunn 1999(26)	The Gambia	0-8 months	No	No	267mg/kg	67mg/kg	5	First 2 hours water only	Enzymatic assay
Thurnham 2000(19)	The Gambia and India	0-11 months	No	No			5	No	
Quadro 2000(247)	Brazil	1-9 years	Overnight	No	5g	1g	5	No	HPLC-PAD
Willumsen 2000(248)	South Africa	0-14 weeks	No	No				No	Enzymatic assay
Zhang 2000(144)	Peru	0-36 months	No	No	400mg/kg	100mg/kg	5	No	Enzymatic assay
Rollins 2000(249)	South Africa	6-60 months	No	No	400mg/kg	100mg/kg	5	No	Enzymatic assay
Rollins 2001(250)	South Africa	1-14 weeks	No	No	400mg/kg	100mg/kg	5	No	Enzymatic assay
Filtaeu 2001(251)	South Africa	0-14 weeks	No	No	400mg/kg	100mg/kg	5	No	Enzymatic assay
Northrop-Clewes 2001(252)	Bangladesh	2-5 years	2 hours	No	4g	1g	5	2 hours	Enzymatic assay
Campbell 2002(11)	The Gambia	2-60 years	No	No	4g	1g	5	No	Enzymatic assay
Goto 2002(27)	Nepal	0-5 years	No	No	400mg/kg	100mg/kg	5	First hour breastfeeding only	Enzymatic assay
Chen 2003(20)	Brazil	2-97 months	No	No	5g	1g	5	No	HPLC-PAD
Campbell 2003(12)	The Gambia / UK	6-36 months	No	No			5	No	Enzymatic assay
Campbell 2003(8)	The Gambia	2-11 months	No	No	400mg/kg	100mg/kg	5	First 2 hours	Enzymatic assay
Rabbani 2004(253)	Bangladesh	5-12 months	3 hours	No	5g	1g	5	No	Enzymatic assay
Campbell 2004(9)	The Gambia	2-15 months	No	No	400mg/kg	100mg/kg	5	First hour	Enzymatic assay
Galpin 2005(169)	Malawi	36-60 months	No	No	5g	1g	3	No	Cation exchange and refractometer

Table 17 continued

Study	Setting	Age	Fasting before test?	Pre-ingestion urine sample?	Lactulose dose	Mannitol dose	Urine collection duration (hours)	Fasting during urine collection?	Method of lactulose and mannitol detection
Lima 2005(254)	Brazil	2-60 months	3 hours	No	400mg/kg	100mg/kg	5	No	HPLC
Darboe 2007(255)	The Gambia	0-12 months	No	No	200mg/kg	50mg/kg	5	No	Enzymatic assay
Williams 2007(256)	The Gambia	4-10 months	No	No	400mg/kg	100mg/kg	5	First 2 hours water only	
Lima 2007(21)	Brazil	6 months to 8 years	3 hours	No	5g	1g	5	No	HPLC-PAD
Vieira 2008(257)	Brazil	2 months to 9 years	Yes; duration not specified	No	400mg/kg	100mg/kg	5	First hour	HPLC-PAD
Trehan 2009(167)	Malawi	3-5 years	Overnight	No	5g	1g	4	No	HPLC-PAD
Goto 2009(28)	Bangladesh	3-15 months	No	No	400mg/kg	100mg/kg	3-6	No	Enzymatic assay
Goto 2009(13)	Bangladesh	3-15 months	1 hour	No	400mg/kg	100mg/kg	3-5	No	Enzymatic assay
Manary 2010(23)	Malawi	3-5 years	Overnight	No	5g	1g	4	No	Enzymatic assay
Lima 2010(22)	Brazil	2 months to 9 years	No	No	400mg/kg	100mg/kg	3-5	No	Enzymatic assay
Weisz 2012(16)	Malawi	2-5 years	Overnight	No	5g	1g	4	4 hours water only	HPLC-PAD
Lee 2014(78)[1]	Peru	3-15 months	2 hours breastfeeding only	No	500mg/kg	100mg/kg	5	30 minutes breastfeeding only	HPLC-PAD and LC-MSMS
Lin 2019(174)[2]	Bangladesh and Kenya	0-23 months	1 hour	Yes	500mg/kg	100mg/kg	5	No	LC-MSMS

1. The 'WASH Benefits' study methods were published after the start of my fieldwork. I learnt of the methods during my visit to their Kenya site in March 2015, when their fieldwork was ongoing.
2. Methods for the multi-site 'Malnutrition and Enteric Diseases' (MAL-ED) study that was ongoing at the time of my fieldwork. This paper reported methods and preliminary results for the Peru site. Methods were harmonised across sites, as later published.

I chose oral lactulose and mannitol doses of 500mg/kg and 100mg/kg in line with the two major ongoing studies at the time of my fieldwork – 'WASH Benefits' and MAL-ED.(78, 174) Since my young study participants could not void their bladders on demand, I chose a five-hour duration of urine collection to minimise the relative importance of residual bladder volume at the start and end of collection. Piloting concluded that any longer duration would be poorly tolerated by participants and caretakers. I chose a one-hour fast beforehand and 'usual milk only' for the five-hour urine collection to be in line with the ongoing 'WASH Benefits' study protocol. A two-hour observed fast would have been impractical to fit in alongside a subsequent five-hour urine collection.

Oral solution preparation, transport and storage

The oral LM solution was prepared by pharmacists at KWTRP Research Pharmacy and in line with Good Clinical Laboratory Practice (GCLP). Full methods are described in Appendix 26. An SOP was developed and followed (Appendix 27).

To compare actual oral solution concentrations against the target 250mg/ml lactulose and 50mg/ml mannitol, a 2ml aliquot was removed from each batch on the day of its preparation, labelled and placed in -20^{o}C storage. Aliquots were then transported frozen on dry ice and subjected to HPLC-PAD detection of lactulose and mannitol at the Royal Cornwall Hospital Laboratories, UK (laboratory methods: Appendix 28), with between two and three repeat measurements undertaken per aliquot and a mean calculated for each.

Urine collection, transport and storage

LM tests were generally carried out by fieldworkers on Tuesdays and Thursdays at the Junju field site. An SOP was followed (Appendix 29). Caretakers were asked to ensure the participant consumed nothing but breastmilk (or, for babies not breastfeeding, their usual milk) for three hours prior to the start of the test. On arrival to the Junju field site, participants were weighed if they had not been weighed the day before. A directly observed one-hour period of fasting would then commence. At the beginning of the hour, a sterile urine collection bag (Hollister U-Bag paediatric 200ml) was applied to each participant's cleaned and dried perineum. Care was taken to ensure good fit to minimise risk of leakage or faecal contamination. If during the hour urine was passed into the bag, the bag was removed and an aliquot of this urine was transferred to a sterile container and labelled with the participant's details and the phrase 'pre-ingestion'. One drop of 2% chlorhexidine (prepared by me in KWTRP Laboratories from 20% Chlorhexidine Digluconate Aqueous Solution (Sigma-Aldrich C9394) and HPLC-grade water (Sigma-Aldrich 7732-18-5)) was added to each aliquot. Aliquots were then placed in 2-8^{o}C storage. Coolbox temperatures were continually monitored and logged at key points. If no urine was passed during the one-hour fast, the test was continued (no additional fasting or waiting for pre-ingestion urine took place). At the end of the one-hour fast, a 2ml/kg dose of oral solution was administered over around two minutes using a syringe. Upon completion of oral LM solution ingestion, the time was noted and a five-hour urine collection started.

During the five-hour urine collection period, participants could freely breastfeed (or, if not breastfed, consume their usual milk) but no other foods or liquids were given. This was

ensured through direct observation, with participants and caretakers remaining within the Junju field site throughout. At any point throughout the one pre-ingestion and five post-ingestion hours, bags observed to contain urine were removed, the urine transferred to a collecting pot, and a fresh bag immediately applied. If a bag became internally contaminated with faeces it was removed and replaced and the contaminated urine discarded. If leakage of urine was noted, the bag was removed, any urine inside the bag collected (provided not contaminated with faeces), and a fresh bag applied. Urine was collected for each participant over the five hours into one large sterile container containing one drop of 2% chlorhexidine. In between bag changes, containers were stored in a coolbox at 2-8^{o}C. If a bag was removed for any reason (urine present, leakage or contamination noted) less than 15 minutes before the end of the five hours, a new bag was not placed and the collection ended early. At five hours the final urine bag was removed, any remaining urine collected, and the test finished.

Fieldworkers recorded the final urine volume to the nearest 2.5ml, as well as the volume or oral solution administered, and any leakages, contamination or protocol deviations in a paper record (Appendix 30). Pre-ingestion and post-ingestion samples were then transported at 2-8^{o}C to KWTRP Clinical Trials Laboratory. There, the pre-ingestion sample was stored in aliquots at -80^{o}C. The post-ingestion container was again assessed for total volume, then stored in aliquots at -80^{o}C.

Detection of lactulose and mannitol in urine

Urine samples were transported to the Royal Cornwall Hospital Laboratories, UK. During transit they were kept frozen using dry ice. On arrival, all samples underwent HPLC-PAD to detect and quantify lactulose and mannitol. This assay was developed and carried out by Dr Simon Fleming. Laboratory methods are detailed in Appendix 28. To assess reproducibility of results, a subset of samples was assayed twice.

Calculation of urine-derived LM ratios

Urine-derived percentage marker (i.e. lactulose or mannitol) excretion was estimated using the following formulae:

$$\%\ \text{marker excretion (unadjusted)} = \frac{\text{excreted marker (mg)}}{\text{ingested marker (mg)}} * 100 = \frac{a * (b / 1000)}{cd} * 100$$

$$\%\ \text{marker excretion (adjusted)} = \frac{\text{excreted marker (mg)}}{\text{ingested marker (mg)}} * 100 = \frac{(a-e) * (b / 1000)}{cd} * 100$$

a = post-ingestion urine marker concentration (mg/L)
b= volume of urine obtained over five-hour collection (ml)
c = volume of oral LM solution ingested (ml)
d = 250 where marker is lactulose, 50 where marker is mannitol
e = pre-ingestion urine marker concentration (mg/L)

Where no data were available for pre-ingestion urine lactulose or mannitol concentrations, values of zero were assumed.

LM ratio was estimated using the following formula:

$$\text{LM ratio} = \frac{\%\ \text{lactulose excretion (adjusted)}}{\%\ \text{mannitol excretion (adjusted)}}$$

Concurrent measurement of plasma- and urine-derived LM ratios

For one LM test per participant, at around six months of age, the usual five-hour urine collection was combined with venesection at 90 minutes after ingestion of oral solution. A 90-minute point was chosen because previous adult studies have shown plasma-derived LM ratios to most closely reflect urine-derived ratios at or after this time, with diminishing returns from waiting beyond 90 minutes.(258) Blood samples were stored at 2-8^{o}C at the study site for up to two hours and then couriered to KWTRP Laboratories also at 2-8 C. Upon arrival at KWTRP Laboratories, plasma was immediately separated. Plasma aliquots were stored at -80^{o}C before transport to Royal Cornwall Hospital Laboratories, remaining frozen during transit in dry ice. There, plasma concentrations of lactulose and mannitol were determined by the same HPLC-PAD method as used for urine and oral solution samples (Appendix 28).

Plasma-derived percentage mannitol and lactulose excretion were then estimated using the following formulae:

$$\text{Lactulose \% excretion} = \frac{tw}{250z} * 100 \qquad \text{Mannitol \% excretion} = \frac{ty}{50z} * 100$$

t = estimated whole body plasma volume (L)
w = plasma lactulose concentration (mg/L)
y = plasma mannitol concentration (mg/L)
z = volume of oral LM solution ingested (ml)

Whole body plasma volume was estimated as (1-haematocrit)*(estimated whole body blood volume). Whole body blood volume was estimated at 80ml/kg. Haematocrit (range 0-1) was measured using the same blood sample, with the exception of two participants for whom full blood count was not possible due to insufficient volume. For these two participants, the cohort median (30%) was imputed.

Results

Quality assessment 1: overall adherence to field protocol

Amongst the 935 LM tests undertaken within age 0-9 months, post-ingestion urine was obtained in the field for 853 (91%) (Table 18). Amongst these 853 post-ingestion urine samples, 14 were found to have extremely low mannitol and lactulose concentrations. For these 14, the post-ingestion urine was assumed to have been obtained during the earliest moments of the five-hour collection (timings of each urine passing event were not recorded),

before the oral LM solution had been absorbed. Results were therefore censored for these tests, leaving 839 tests where an LM ratio could be calculated. Zero-, seven- and eight-month-olds were most likely to be affected by test failure (Table 18).

Table 18: LM test outcome by age.
Figures in brackets are percentage proportions of total by age category.

	Age (integer months)									
	0	1	2	3	4	5	6	7	8	9
LM ratio obtained	82 (78)	90 (95)	94 (95)	89 (96)	88 (92)	73 (90)	92 (93)	74 (84)	79 (86)	78 (90)
LM ratio not obtained	23 (22)	5 (5)	5 (5)	4 (4)	8 (8)	8 (10)	7 (7)	14 (16)	13 (14)	9 (10)
Total	105	95	99	93	96	81	99	88	92	87

Amongst these 839 successful tests (Table 19):

- Two (<1%) were affected by reported ingestion of foods or liquids other than the participant's usual milk during the three or more hours preceding the beginning of the one-hour fast. In both cases, uji (fermented cereal) had been given during these three hours.
- Fasting for one hour before administration of the oral LM solution was directly observed and universally adhered to.
- Collection of urine for the full five hours was universally adhered to.
- Five-hour urine collections were affected by faecal contamination in 203 tests (24%) leading to the discarding of 256 urine collection bags during these tests (range 1-3 bags per affected test).
- Five-hour urine collections were affected by bag leakage in 12 tests (1%), with 13 bags affected in total (range 1-2 affected bags per test).

- Volumes of post-ingestion urine obtained ranged from 0.5 to 132.5ml with a median of 30.0ml.
- A sample of pre-ingestion urine was obtained during the one-hour fast for 683 (80%).

Table 19: LM tests adherence to field protocol.

Field protocol adherence	Denominator		
	All tests within age 0-9 months (n=935)	Tests where post-ingestion urine obtained in field (n=853)	Tests where LM ratio obtained (n=839)
Usual milk only for three hours preceding test	916 (98%)	837 (98%)	837 (100%)
Nil by mouth for one hour preceding oral LM solution ingestion	935 (100%)	853 (100%)	839 (100%)
Pre-ingestion urine sample obtained	742 (79%)	683 (80%)	679 (81%)
Collection of post-ingestion urine for full five hours	935 (100%)	853 (100%)	839 (100%)
Test unaffected by faecal contamination	675 (72%)	646 (76%)	636 (76%)
Test unaffected by urine leakage	919 (98%)	841 (99%)	827 (99%)
Volume of post-ingestion urine obtained (range; median)	0.5-132.5; 30.0	0.5-132.5; 30.0	0.5-132.5; 30.0

Urine volume loss due to contamination or leakage

210 tests (25% of 839) were affected by urine volume loss due to bag leakage or contamination. There was strong statistical evidence for a difference in mean log LM ratios derived in this group compared to log LM ratios derived from the 629 tests unaffected by leakage or contamination (unpaired T test; p=0.02). However, although statistically significant, this difference appears to be very small when plotted graphically (Figure 25).

Tests with a higher number of urine bags lost were indeed more likely to occur amongst older cohort participants (Figure 26). However, comparison of a mixed-effects linear regression model of log LM ratio with age as fixed effect and participant as random effect against the same model also adjusted for urine loss (number of bags) does not demonstrate strong evidence for confounding of the association between age and LM ratio by urine loss (likelihood ratio test p=0.08).

Figure 25: Frequency distribution of LM ratios derived from tests affected and unaffected by urine loss due to leakage or contamination.
Box midlines denote median, box edges denote 25th and 75th centiles, whiskers denote upper and lower adjacent values, hollow dots denote outlying values.

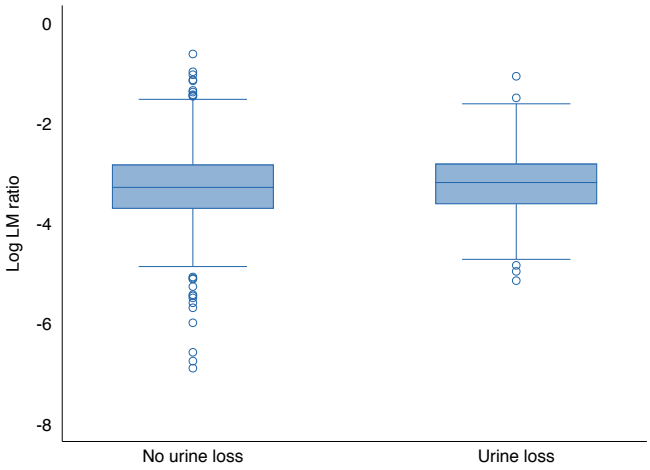
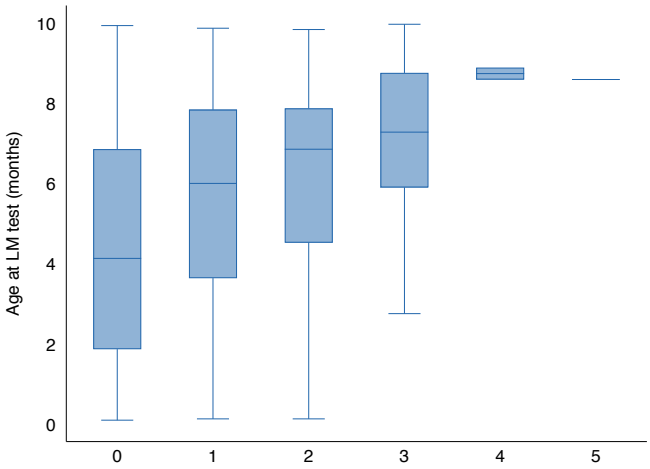


Figure 26: Frequency distribution of age in months stratified by number of urine bags (0 to 5) discarded during LM test due to urine leakage or faecal contamination.
Box midlines denote median, box edges denote 25th and 75th centiles, whiskers denote upper and lower adjacent values, hollow dots denote outlying values.

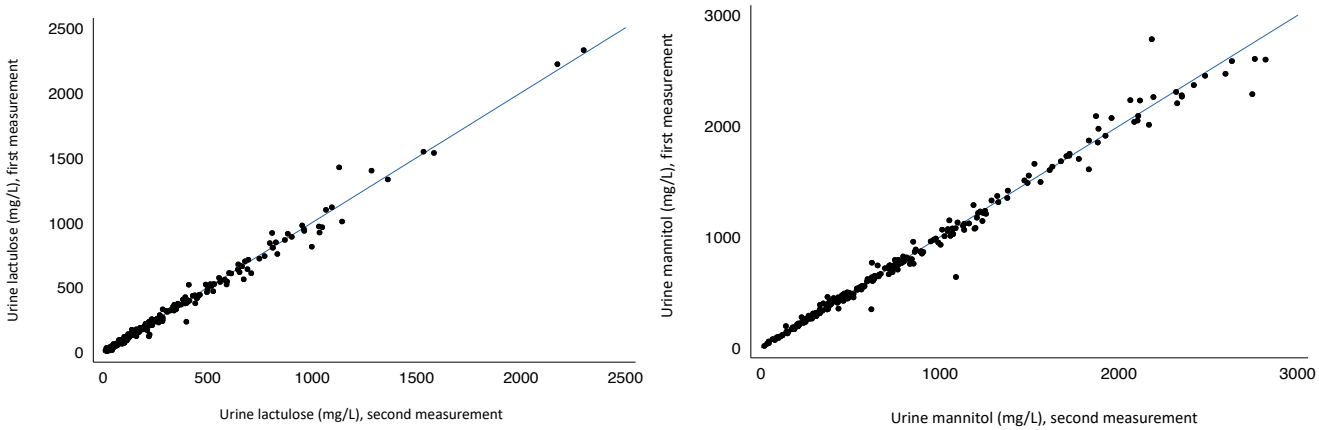


Quality assessment 2: Reproducibility of the lactulose and mannitol assay in urine

At the Royal Cornwall Hospital Laboratories, a random sample of 235 post-ingestion samples underwent mannitol measurement twice, whilst another random sample of 303 post-ingestion samples underwent lactulose measurement twice (232 samples were in both these categories, i.e. underwent repeats of both mannitol and lactulose assays). Across all samples, ranges of observed urine lactulose and mannitol concentrations were 1-2328mg/L and 0-7450mg/L respectively. For mannitol, four samples (2% of 235) had a greater than 25% difference between the first and second mannitol assay results. For lactulose, this figure was 21 (7% of 303). There was good agreement between first and second measurements for those samples that underwent repeat assays (Figure 27). Mean absolute difference between first and second lactulose measurements was 17mg/L (standard deviation 29; range 0-296). Equivalent figures for mannitol were: 38; 68; 0-600. The intra-assay coefficient of variation

ranged between 1.5 and 8.5% depending on lactulose or mannitol concentration. Following this assessment, the first assay result was used wherever lactulose or mannitol had been measured twice, and no further repeat assays were carried out for the remaining 533 samples that had been measured only once.

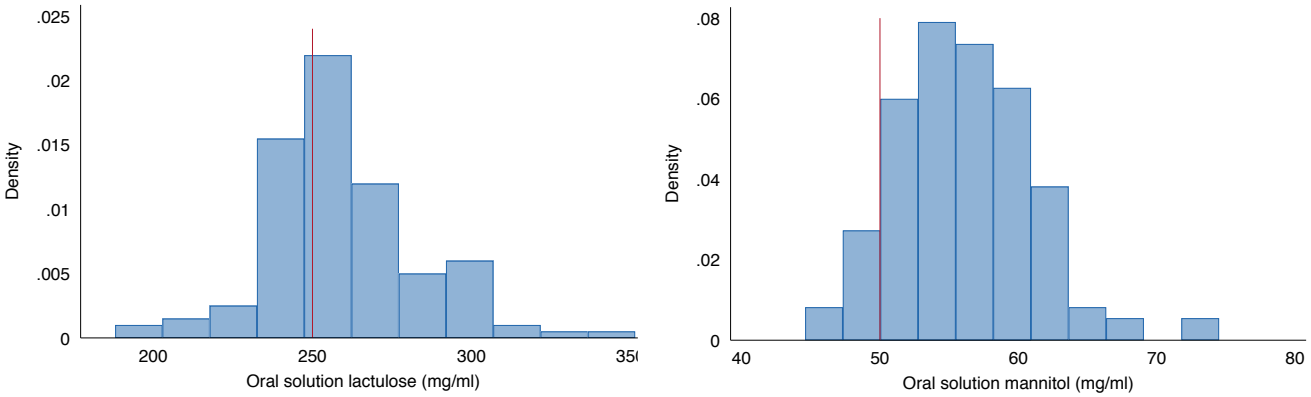
Figure 27: Urine lactulose and mannitol concentrations derived from duplicate measurement of the same samples.
Diagonal lines indicate perfect agreement. Lactulose: n=303; mannitol: n=235. Paired T test comparing log-transformed mannitol concentrations in first and second assays p=0.35; for lactulose p=0.07.



Quality assessment 3: oral lactulose and mannitol solutions

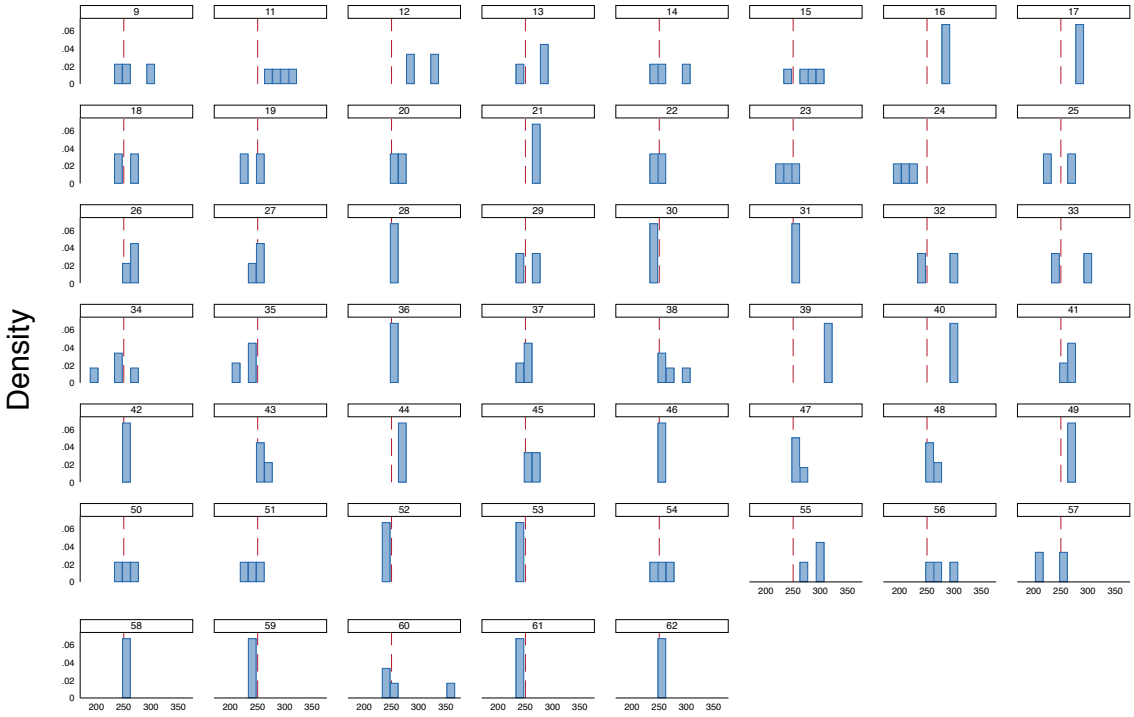
Results were obtained for lactulose and mannitol concentrations in 135 aliquots taken on 135 testing days from 53 batches of oral LM solution. Due to logistical challenges on the relevant testing days, aliquots were not sent from a further nine batches. These nine untested batches occurred at even intervals throughout the study duration. Amongst the 135 aliquots, measured lactulose concentrations ranged 188.0-351.4mg/L and peaked at around the target of 250mg/L (Figure 28). For mannitol, the frequency distribution peak was slightly higher than the target of 50mg/L with a range 44.6-74.5mg/L.

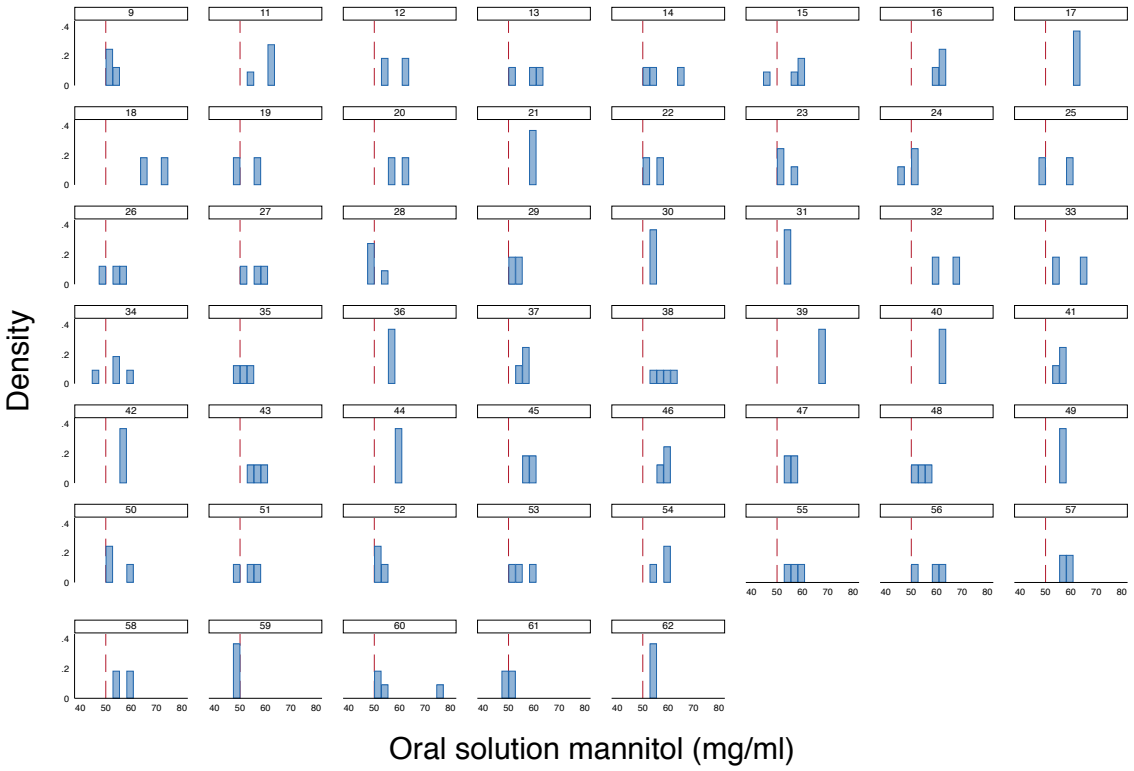
Figure 28: Frequency distribution for lactulose and mannitol concentrations amongst 135 aliquots taken from 53 batches of prepared oral LM solution.
Red lines indicate target concentrations for lactulose (250mg/ml) and mannitol (50mg/ml).



Within each batch, concentrations of both lactulose and mannitol did vary by aliquot (Figure 29).

Figure 29: Frequency distribution histograms for oral solution aliquot lactulose and mannitol concentrations, stratified by batch.
Batch number in clear bar above each histogram. Vertical red dotted lines indicate target concentrations for lactulose (250mg/ml) and mannitol (50mg/ml).





Quality assessment 5: adjustment for pre-ingestion presence of lactulose and mannitol in urine

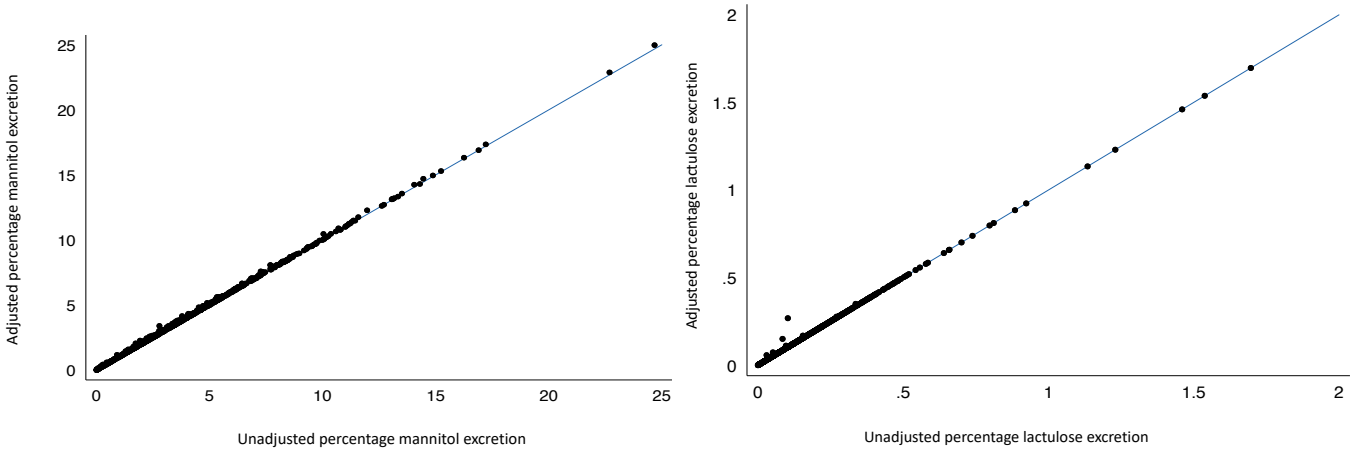
As previously described, for the 839 LM tests where an LM ratio was successfully obtained, pre-ingestion urine was obtained for 679 (81%). Amongst these 679, lactulose was detected in 11 (2%) and mannitol was detected in 632 (93%) (Table 20). Where either sugar was detected in pre-ingestion urine, post-ingestion concentrations were much higher in all cases.

Table 20: Pre-ingestion urine lactulose and mannitol concentrations amongst 679 LM tests where pre-ingestion urine lactulose and mannitol concentrations are known.

Target sugar	n	Pre-ingestion observed urine concentration (mg/L)		
		Number above zero (% proportion)	Interquartile range	Range
Lactulose	679	11 (2)	0 to 0	0 to 138
Mannitol	679	632 (93)	4.8 to 15.4	0 to 94

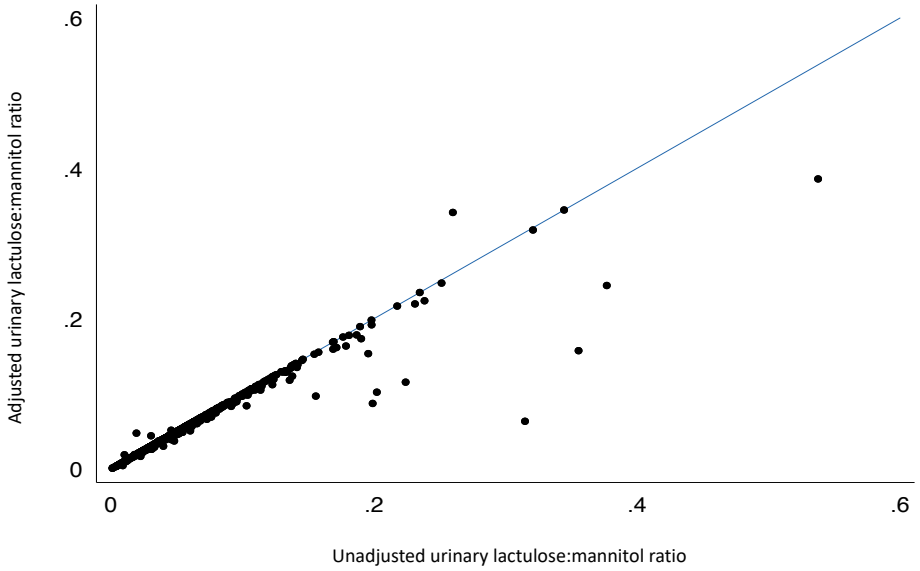
For both lactulose and mannitol, paired T test comparison of log-transformed percentage excretion with and without adjustment for concentration of either sugar in the pre-ingestion urine sample shows strong evidence for difference between adjusted and unadjusted values (lactulose $p=0.03$; lactulose $p<0.0001$). However, scatter plots suggest that these differences, with the exception of two tests for lactulose, are in fact very small (Figure 30). Mean absolute difference between adjusted and unadjusted percentage mannitol excretion was 0.05% (standard deviation 0.07; range 0-0.6).

Figure 30: Scatterplots of percentage lactulose and mannitol excretion adjusted and unadjusted for pre-ingestion urine lactulose and mannitol for the 679 tests where pre-ingestion urine was obtained.
Diagonal lines indicate perfect agreement. Pearson's correlation coefficient for mannitol = 1.00; lactulose = 1.00



LM ratio, calculated using adjusted figures for percentage lactulose and mannitol excretion, ranged 0.001-0.54. For the 679 tests where adjustment was made for pre-ingestion lactulose and mannitol concentrations, paired T test comparison of log transformed LM ratio with and without adjustment for concentration of both sugars in the pre-ingestion urine sample shows very strong evidence for difference between adjusted and unadjusted values ($p<0.0001$). Mean difference between adjusted and unadjusted LM ratios for the 679 tests was 0.002 (standard deviation 0.02; range 0-0.25). Where larger differences were seen, in the majority of cases the adjusted LM ratio was lower than the adjusted LM ratio (Figure 31). Following this, adjusted LM ratio was carried forwards to further analyses for the 679 tests where pre-ingestion urine had been obtained. For the 160 tests where no pre-ingestion urine was obtained, the unadjusted LM ratio was carried forwards.

Figure 31: Scatterplot demonstrating agreement between urine-derived lactulose:mannitol ratios adjusted and unadjusted for pre-ingestion urine concentrations of lactulose and mannitol for the 679 tests where pre-ingestion urine was obtained.
Diagonal line indicates perfect agreement. Pearson's correlation coefficient = 0.95.



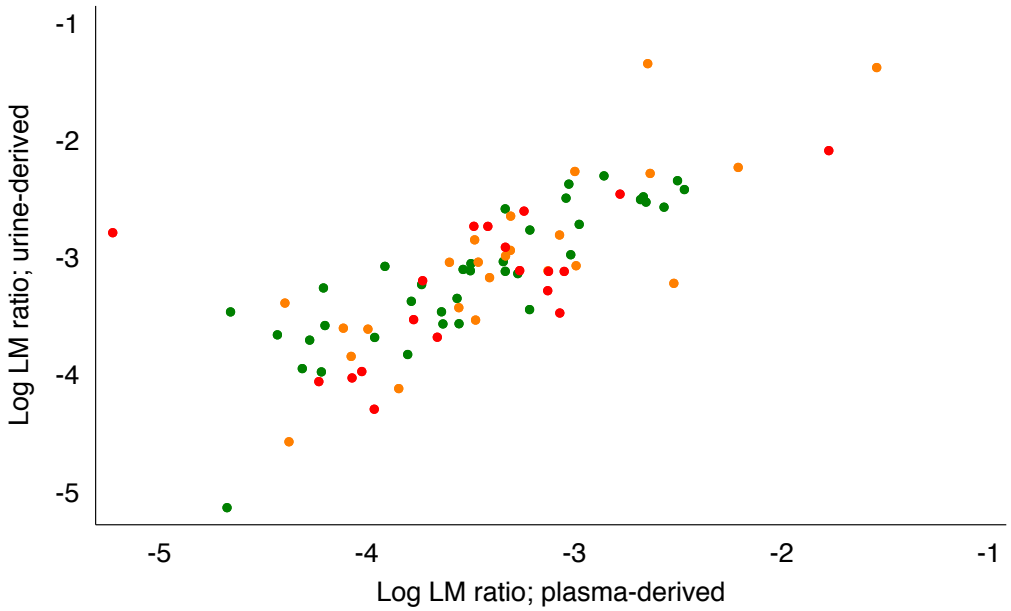
Quality assessment 4: plasma- versus urine-derived LM ratios

Seven participants had already withdrawn from follow-up before the six-month LM test, and one participant did attend for their six-month LM test but failed venesection. Plasma samples were therefore obtained during the six-month LM test for 92 participants. Amongst these, urine derived LM ratios were obtained for 88 during the same LM test (for four participants, although plasma was obtained, post-ingestion urine was not obtained) and pre-ingestion urine obtained for 79 of these 88.

Overall, strong positive correlation was observed between single-time-point plasma- and over-time urine-derived log-transformed LM ratios (Pearson's correlation coefficient 0.75).

For the 84 participants for whom timing of venesection was known, median time between actual and target time for venesection (target was 90 minutes after ingestion of LM oral solution; actual times rounded to the nearest five minutes) was +10, range -10 (10 minutes early) to +90 (90 minutes late). Correlation between plasma- and urine-derived LM ratios is highest where venesection took place 80-95 minutes after ingestion of oral LM solution (Figure 32; Pearson's correlation coefficient 0.85; n=40) or 100-105 minutes after ingestion (coefficient 0.83; n=24) and lower where venesection took place ≥110 minutes after ingestion (coefficient 0.54; n=20).

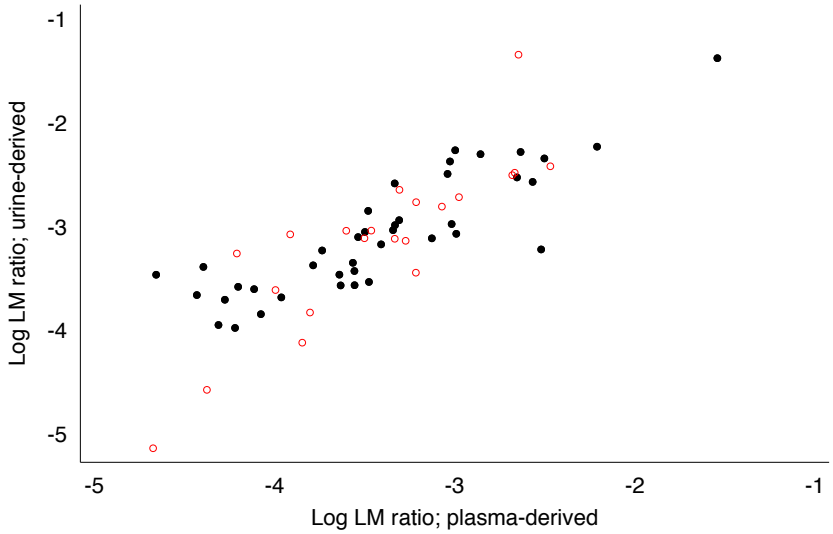
Figure 32: Plasma- and urine-derived log LM ratios where information is available regarding time of venesection.
n=84. Time is rounded to nearest 5-minute interval. Green dots indicate venesection took place between 80 and 95 minutes after ingestion of the oral LM solution. Orange dots indicate venesection took place between 100 and 105 minutes after ingestion. Red dots indicate venesection took place 110 or more minutes after ingestion.



Restricting analysis to the 64 tests where venesection took place 80-105 minutes post ingestion, there is no difference in strength of correlation between urine- and plasma-

derived LM ratios for the 43 tests that were affected by urine volume loss (Figure 33; Pearson's correlation coefficient 0.87) compared to the 21 tests that were affected (coefficient=0.86).

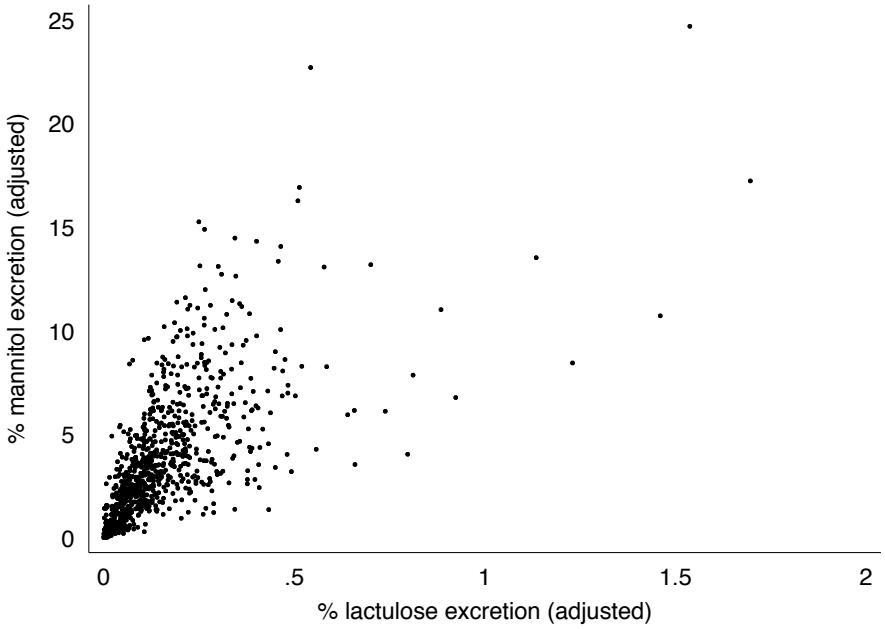
Figure 33: Log-transformed plasma- and urine-derived LM ratios amongst tests where venesection took place between 80 and 105 minutes after oral ingestion of LM solution.
n=64. Black dots indicate where the urine derived LM ratio was not affected by urine volume loss due to faecal contamination or collection bag leakage (n=21). Red circles indicate where the urine derived LM ratio was affected by urine volume loss (n=43).



Correlation of percentage lactulose and mannitol excretion

Lactulose and mannitol percentage excretion were strongly positively correlated (Figure 34).

Figure 34: Scatterplot depicting percentage lactulose and mannitol excretion. Pearson's correlation coefficient 0.66.



Discussion and conclusions

This chapter describes the development of the LM test method assessment of its performance as a measure of intestinal permeability and absorptive capacity amongst the study population.

The LM test failure rate was low, with just 10% of tests failing to yield an eligible post-ingestion urine sample. This is lower than the one example of published LM test failure rate that I have been able to identify, which was 37%.(259) Thus, the possible impact of any age-

related selection bias is small. However, these 10% of tests that failed may have disproportionately represented participants who, had their test succeeded, would have demonstrated higher LM ratios. Test failure was most common amongst the youngest and oldest cohort participants. These same participants demonstrated overall higher LM ratios. Therefore, LM test failure may have led to a minor underestimation in overall prevalence of EED within the cohort. However, balancing this, for the 19% of tests for which no pre-ingestion urine sample was obtained, unadjusted LM ratio may be an underestimate of the true LM ratio had it been adjusted. This may have led to overestimation of summary EED prevalence for the cohort.

25% of tests were affected by urine volume loss. This compares favourably with recent large studies: in the 'WASH Benefits' study 43% of tests amongst three-month-olds and 34% of tests amongst 14-month-olds were affected.(174)

The noted high proportion (93%) of pre-ingestion urine samples containing small amounts of mannitol is in line with other published studies.(260) The presence of mannitol in pre-ingestion urine samples raises the possibility of over-estimation of mannitol excretion for the minority (19%) of LM tests where no pre-ingestion urine was available for adjustment. Amongst the LM tests for which pre-ingestion urine mannitol concentration was known, after adjustment for age, pre-ingestion urine mannitol concentration was higher during the dry season but did not change with exclusive breastfeeding status (Appendix 31). This suggests mannitol is ingested more during the dry season. Other recently published studies have mentioned similar challenges in adjusting for pre-ingestion mannitol and lactulose: the 'WASH Benefits' study obtained pre-ingestion urine during only 377 of 4,101 LM tests.(174)

Some have since proposed replacing mannitol with rhamnose, a monosaccharide that is not present pre dosing.(260)

The oral solution lactulose and mannitol concentrations ranged relatively widely around the targets of 250mg/ml and 50ml/ml respectively. In the case of lactulose, where frequency distribution was centred around the target of 250mg/ml, this is likely to have resulted in reduced accuracy rather than precision. However, in the case of mannitol, since peak frequency distribution was higher than 50mg/ml, the likely result is loss of both accuracy and precision, where oral solutions with higher concentrations of mannitol would have resulted in underestimation of a participant's true percentage mannitol excretion, and thus underestimation of their intestinal absorptive capacity. Loss of accuracy for both sugars may have resulted in type II error (failure to reject a false null hypothesis) in downstream analyses involving percentage lactulose or mannitol excretion or the LM ratio. I could not find any published examples of quality testing of oral LM solution to compare against.

Lee et al recommend LC-MSMS over HPLC-PAD for detection of sugars in urine.(78) However, another group based in Malawi that frequently carries out LM tests has found agreement to be satisfactorily high between methods.(261) I did attempt to work with colleagues at KWTRP Kilifi to develop a protocol for LC-MSMS measurement of lactulose and mannitol in urine. However, challenges with equipment failure and staff turnover were ultimately insurmountable, and costs of sending urine samples for LC-MSMS analysis elsewhere prohibitively high.

Despite the possible sources of bias and inaccuracy inherent to urine sampling techniques, results from the comparison of plasma- and urine-derived LM ratios are reassuring. Not only was correlation between urine- and plasma-derived LM ratios strong overall, but strength of correlation was not affected by post-ingestion urine volume loss. Timed venesection is not subject to the limitations of urine collection. But here urine-derived LM ratios correlate closely. Sampling urine over six hours is onerous for participants, carers and investigators. Timed venesection is quicker but would not have been tolerated on a monthly basis: taking of blood samples is not popular within the local community. It is therefore reassuring that urine-derived LM ratios correlate closely with plasma-derived and that this correlation is not affected by urine volume loss. A 90-minute venesection time is also likely to reflect small intestinal absorption rather than large intestinal. The observed correlation between plasma and urine-derived LM ratios therefore also allays concerns over the longer five-hour urine collection possibly reflecting large intestinal as well as small intestinal function. Recently, investigators in Bangladesh have demonstrated satisfactorily similar LM ratios derived from timed 2-hour compared to 5-hour urine collections from Bangladeshi infants using both HPLC-PAD and LC-MSMS measurement methods.(262) Two-hour urine collections may therefore be a viable way forwards to improve logistical, financial and participant acceptability limitations of the 5-hour test.

One unexpected finding was the close positive correlation between lactulose and mannitol percentage excretion (Pearson's correlation coefficient 0.66). If mannitol excretion was proportional solely to gut absorptive capacity, then it should be inversely proportional to lactulose excretion which is thought to approximate gut leakiness. In EED, poorer absorptive capacity is assumed to occur alongside greater leakiness. In May 2018, investigators from

Malawi published results from modelling of 2,334 LM tests undertaken on 622 Malawian children aged 6-36 months as part of two clinical trials that took place during 2015-2016.(261) A similar positive association between lactulose and mannitol excretion was reported: Pearson's correlation coefficient 0.58. They went on to mathematically model three hypothesised pathways for transfer of lactulose and mannitol across the gut wall: two physiological ('pore' (size 5-10Å) and 'leak' (15-125Å)) and one pathological ('unrestricted' (unknown size, presumed larger)). Due to its larger size of 13Å, lactulose is presumed to only use the leak and unrestricted pathways: i.e. a mixture of physiological and pathological. Smaller 8Å mannitol was hypothesised to pass via all three pathways, and where pathology exists, to pass through the unrestricted pathway in proportion to lactulose. The model predicted that, where pore pathways predominate, mannitol flux is 14 times that of lactulose. Where leak pathways predominate, this ratio falls to 8:1. In pathology, where unrestricted pathways are present, the ratio falls further to 6:1. They also noted inconsistent association between LM ratios and linear growth in published studies worldwide and suggested that percentage lactulose alone be used to measure EED. Despite differences in study participants between the Malawian model and the Kilifi cohort described in Chapter 5 (the Malawian model participants were older at 6-36 months instead of 0-9 months; none of the Malawian participants had acute malnutrition whereas 8% of monthly anthropometry episodes in the Kilifi cohort demonstrated MAM or SAM; none of the Malawian participants had diarrhoea within 48 hours of LM testing whereas this restriction was not placed on the Kilifi cohort), it is possible that these Malawian results are relevant to the Kilifi cohort. Results from sensitivity analyses of multivariable models using lactulose excretion instead of LM ratio to measure EED are presented on pages 250-251.

Mannitol excretion seems to have been used in previous studies as either 1) the denominator in the LM ratio as a normalising factor for lactulose excretion (i.e. as a marker of gut surface area) or 2) to create a composite score of EED – the LM ratio – that simultaneously describes villous blunting and increased permeability. The authors of the Malawian model suggest that normalization of lactulose excretion can instead be done using intestinal length, which can be derived from the child's height using a formula developed and validated from post-mortem data.

In summary, the LM test protocol was well adhered to in this cohort with high acceptability amongst participants and caretakers, no adverse events and a low failure rate. Loss of post-ingestion urine volume may have led to loss of accuracy and/or precision in some tests. However, high agreement between plasma- and urine-derived LM ratios suggests that the net effect of this has been minimal. Residual concerns include the lack of adjustment for pre-ingestion mannitol in 19% of tests, the variability of measured oral solution sugar concentrations and uncertainty in interpretation of percentage mannitol excretion and thus the LM ratio.

Age-adjusted association with linear growth is a desirable attribute for any useful biomarker of EED. Despite their inaccuracies, their expense and their laborious implementation in the field, dual sugar absorption tests remain a useful and relevant measure of the functional impairment seen in EED. However, the LM test measures only one of the five proposed domains of EED (see page 17) and new probes are needed with greater specificity for pathological translocation pathways. Markers addressing other domains are also valuable and consensus has not yet been reached on a single test or group of tests to define EED.

Chapter 5: Acquisition of Environmental Enteric Dysfunction and its putative risk factors amongst infants in Junju, Kilifi: a birth cohort study

Background

The birth cohort study described in this chapter focusses on the epidemiology of stunting, EED and their putative risk factors during the first 10 months of life. During the transition period from exclusive breastfeeding to complementary feeding, which frequently occurs much earlier than the recommended six months of age, infants are at risk of faeco-oral contamination from complementary foods. These may be prepared separately from family foods, stored for longer, and reheated inadequately.(263) As they develop, infants interact more with their environment and bring contaminated objects, soil and faeces to their mouths.(168) It is during these vital early months of maximal growth velocity and immune system development that a convergence of poor nutrition and faeco-oral contamination takes place and EED is seen for the first time.

Review of published data in Chapter 1 and discussion of the limitations of cross-sectional nutritional surveys in Chapter 2 have both outlined the advantages of cohort over cross-sectional study design when investigating aetiology of EED in children. In the MAL-ED cohort study, LM tests were done at three, six, nine and 15 months of age. SIBO was not measured. Stool samples were collected once per month during the first year and quarterly during the

second year.(229) In the WASH Benefits study, LM testing was done at three, 14 and 28 months.(174)

In this cohort, the LM test primarily was used to measure EED and the glucose BHT for SIBO. With the aim of capturing rapidly changing risk factor and outcome statuses during the first ten months of life, shorter intervals between stool sampling (bi-weekly) and LM testing (monthly) were decided upon, with weekly measurement of feeding status and continuous active and passive acute illness and antibiotic case detection.

Hypothesis

The cohort was designed to test the two main hypotheses that 1) enteropathogen exposure causes EED; and 2) EED causes stunting. A hypothesised causal framework was developed including all major relevant factors that were thought necessary to contextualise these two putative associations (Figure 35).[‡] This framework then informed data collection and later statistical interpretation.

At the centre of the framework, intestinal dysbiosis and/or SIBO are hypothesised to cause EED through chronic inflammation: initially in response to pathogens but later possibly self-perpetuating (Figure 35; arrow 1). EED is then hypothesised to cause stunting via loss of

[‡] HIV status of the mother and child has been omitted from this causal framework since HIV infection is rare in the study setting. Examples of other rare stunting risk factors omitted from the framework are congenital cardiac disease, twin/multiple and neurological disability.

surface area for nutrient absorption and/or establishment of a chronic inflammatory state that includes energy diversion and direct growth inhibition via modulation of the IGF-1 pathway (arrow 2). The proposed association between intestinal dysbiosis and/or SIBO may not be entirely mediated via EED (arrow 3).

The framework acknowledges that likely major determinants of stunting during infancy are nutritional status at birth (arrow 4), with prematurity a likely strong risk factor for a lower birth LAZ (arrow 5). Maternal undernutrition is likely a strong risk factor for prematurity, low LAZ/WAZ/WLZ at birth and stunting during later infancy (arrows 6-8). Other genetic factors include sex, chromosomal abnormalities and other inherited conditions, and maternal epigenetics (arrows 6-8). Other proposed proximal risk factors for stunting are diarrhoea and other acute illnesses (arrows 9 and 10). These associations may come about through increased metabolic requirement during acute illness and/or decreased nutrient intake and/or absorption by the infant. Diarrhoea and other acute illnesses may cause EED via a systemic inflammatory response that includes the intestinal epithelium (arrows 11 and 12).

More distally, poor household WASH is proposed to cause contamination of an infant's environment via contaminated water, lack of available soap for hand, body and utensil washing, and unsafe disposal of household faeces (arrow 13). Intestinal dysbiosis is hypothesised to arise from this contamination via chronic high-level enteropathogen ingestion (arrow 14). Introduction of complementary foods may further promote enteropathogen exposure and thus intestinal dysbiosis and/or SIBO if such foods are themselves contaminated (arrow 15). This is more likely to occur if household WASH is poor (effect modification arrow 16).

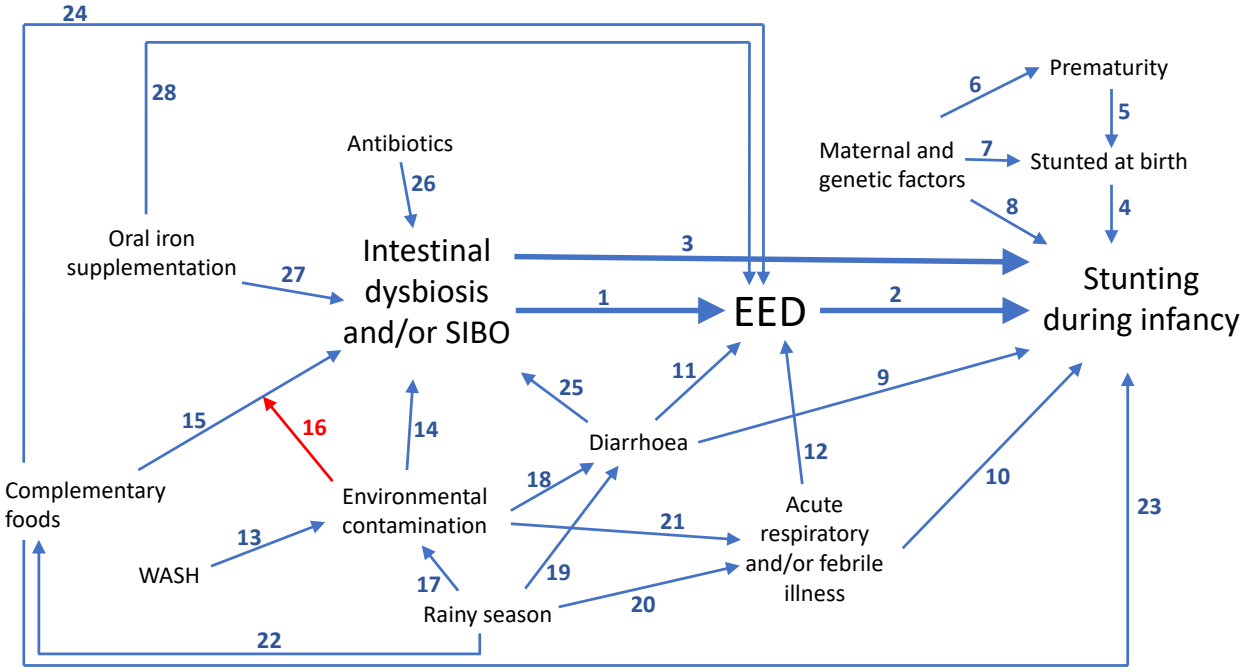
Environmental contamination may either be worse or better during the rainy season (arrow 17). The physical household environment in a traditional rural Junju homestead during rains is less conducive to keeping objects and surfaces clean and, furthest from last harvest, household funds for purchase of soap are lower. On the other hand, for those households without piped water supply, natural water sources are more plentiful and nearby, and may be less contaminated as they have faster flow rates (river or stream) or higher volume (stagnant open water). In support of worse contamination during the rainy season being true, incidence of diarrhoea is greater both locally (anecdotal via Junju dispensary staff) and at the nearby Kilifi County Hospital.(264) Diarrhoea is therefore hypothesised to be promoted not only by environmental contamination (arrow 18) but also rainy season (arrow 19). Transmission of respiratory illness is also hypothesised to increase during the rainy season when people spend more time indoors leading to increased respiratory droplet and faeco-oral indoor spread (arrow 20). This association is hypothesised to be modified by crowding, when the effect of spending more time indoors is magnified (arrow not shown here for simplicity). The outdoor environment during rains is also proposed to be more conducive to respiratory pathogen spread for the same reasons as for diarrhoea since many respiratory pathogens are spread by direct contact (arrows 17 and 21). Lastly, malaria transmission is known to be much higher during the rainy season due to mosquito activity (arrow 20). Season may also affect the type of complementary foods consumed, if household budgets and/or locally available harvested foods vary (arrow 22).

Independent of contamination, type of complementary food may have its own direct association with nutritional status: for example, foods low in energy or micronutrients and/or

high in anti-nutrients such as phytates may lead to stunting (arrow 23). Certain complementary foods may also have either pro- or anti-inflammatory effect on gut epithelium (arrow 24).

Intestinal dysbiosis may be acute or chronic and here refers to an abnormal age-adjusted gut microbiota. Gut microbiota was measured in this study using 16S sequencing but results are not reported in this thesis. An alternative measure of abnormal gut flora, focussing on location of enteropathogens in the small intestine alone, and here measured via hydrogen breath testing, is SIBO. Both states are hypothesised to be promoted by environmental contamination (arrow 14 again) and diarrhoea (arrow 25), with overlap between these two factors. Antibiotics are hypothesised to inhibit intestinal dysbiosis and SIBO (arrow 26). Antibiotics are also hypothesised to inhibit certain acute infectious illnesses (arrow not shown). Oral iron supplementation may promote intestinal dysbiosis via provision of metabolic substrate in the gut lumen (arrow 27), as well as a potential direct inflammatory action of iron on the gut epithelium (arrow 28).

Figure 35: Hypothesised causal framework for cohort study.
Blue arrows indicate hypothesised causation, where a factor induces or potentiates another in the direction of the arrow. One exception to this is for antibiotics, which are hypothesised to inhibit intestinal dysbiosis and SIBO. The red arrow indicates hypothesised effect modification. Larger text size indicates factors of primary focus in this study, and not necessarily proposed relative importance overall.



Aims and objectives

Aim:

- Amongst infants in Junju, describe the distribution of EED and stunting by age. Describe these outcomes' temporal associations with selected preceding risk factors. Infer causality where appropriate.

Objectives:

1. Estimate incidence by age and by season of antibiotic courses, diarrhoea and other acute illnesses.

2. Describe association of undernutrition, SIBO and markers of EED with age and season.
3. Describe association of selected faecal and blood EED biomarkers and SIBO with the primary definition of EED, the LM ratio.
4. Determine factors associated with subsequent linear growth from amongst:
    a. Markers of EED
    b. SIBO
    c. Cessation of exclusive breastfeeding
    d. Diarrhoea and other acute illnesses
    e. Nutritional status at birth
    f. Mother's nutritional status
5. Determine factors associated with subsequent markers of EED from amongst:
    a. SIBO
    b. Cessation of exclusive breastfeeding
    c. Season
    d. Household WASH
    e. Diarrhoea and other acute illnesses
    f. Antibiotics
    g. Iron supplementation

Methods

Overview

The study was a prospective observational cohort. 100 participants were recruited within 14 days of birth and followed until nine months of age. Data collection ceased for each participant between and including the day of turning nine months and the day before turning ten months of age. At enrolment, a questionnaire (Appendix 32) elicited household, mother and baby baseline characteristics. Length, weight and MUAC were measured at enrolment and monthly thereafter. SIBO was measured monthly via BHT. EED was measured monthly via LM test. Participants that completed follow-up would therefore undergo 10 anthropometry, BHT and LM measurements each. Each month, anthropometry, BHT and LM test took place within 1-2 days of each other. At six months of age, plasma lactulose and mannitol were measured via timed venesection during the LM test. Full blood count was also carried out on this six-month blood sample. Participants diagnosed with anaemia (following local guidelines, haemoglobin less than 8g/dL) were given a 14-day course of iron supplementation.

Stool samples were collected approximately bi-weekly. Samples collected 10 or fewer days before a successful LM test underwent ELISA for three biomarkers of EED: alpha-1-antitrypsin, neopterin and myeloperoxidase.

Acute illness events (diarrhoea, breathing difficulty and fever), antibiotic administration, exclusive breastfeeding status and other foods given were captured via weekly home enquiry

(Appendix 33). Attendances for primary and secondary care were captured through passive case detection at Junju dispensary (Appendix 34) and Kilifi County Hospital (Appendix 35).

Participants exited follow-up on the day of their final monthly LM test which took place during their tenth month of life.

Enrolment was preceded by a period of community engagement, and participants and their caretakers were remunerated for their time spent on study activities. Participants benefitted from free transport to local government primary or secondary healthcare facilities when needed. For details of these see Appendix 36.

Sample size

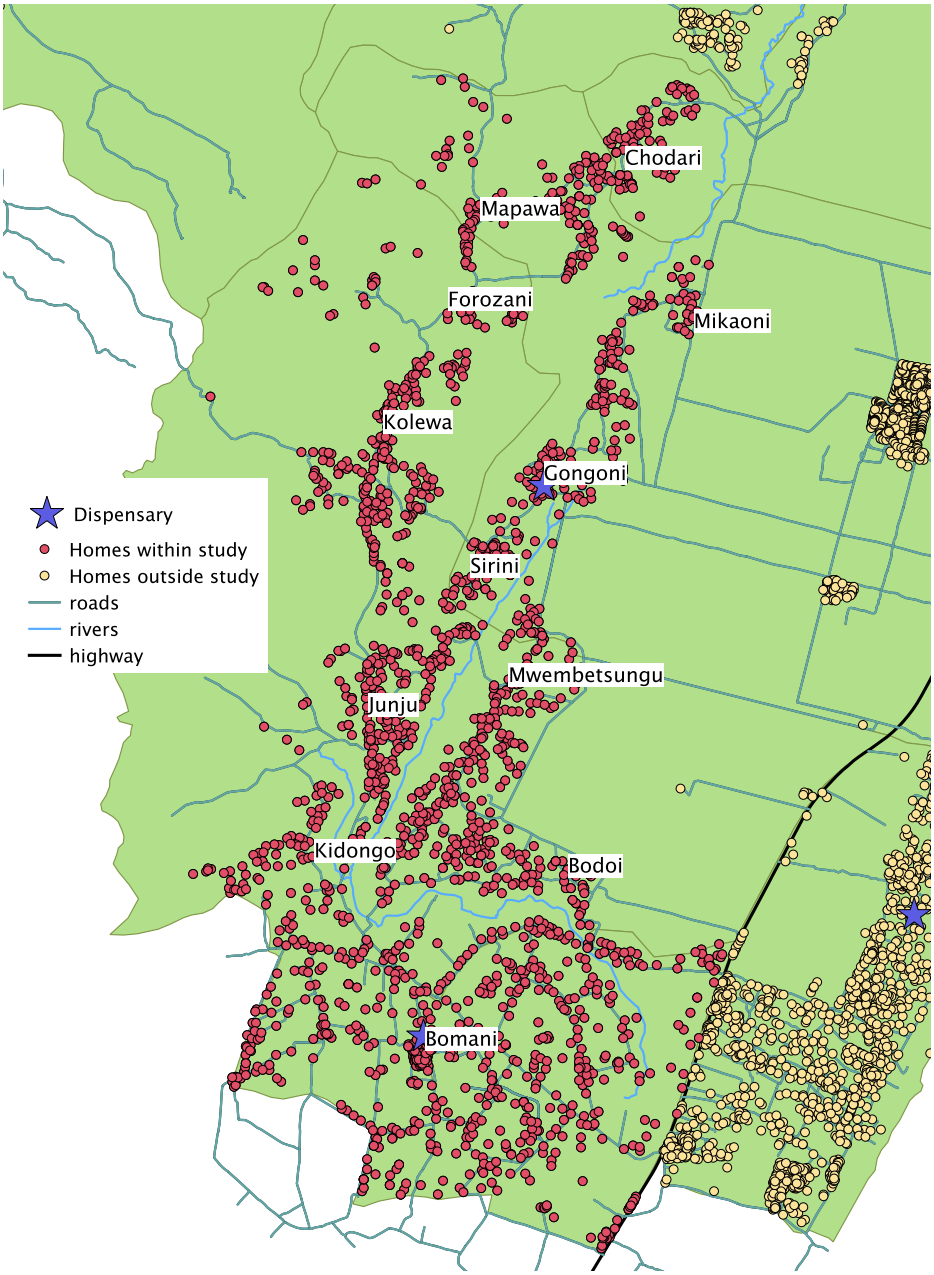
The cohort sample size calculation was based on a statistical significance test comparing a continuous outcome (LM ratio at six months of age) between two groups: exposed and unexposed to SIBO (at least one positive BHT). Previous studies in similar settings have yielded mean infant LM ratios ranging from 0.17 to 0.38 with standard deviations 0.15 to 0.30.(7, 8) The few relevant BHT studies suggested that 10 to 30% of participants may acquire SIBO before six months of age.(158, 237) A sample size of 74 yielded 95% power to detect a two-fold difference in mean LM ratios between SIBO-exposed and SIBO-unexposed groups (alpha 0.05; mean LM ratios 0.2 and 0.4). This number was increased by 15% to allow for possible non-parametric testing(265) (LM ratio distribution is usually right-skewed) and by

15% for possible loss to follow-up. An extra two participants were then added to round up to a total of 100 participants.

Study area

The study area was chosen to represent one contiguous zone surrounding the central facility (Junju dispensary) that could yield 100 participants within a target six-month recruiting period. Assuming 90% of births in the area could be detected and screened, 90% of those screened found to be eligible, and 70% of these would be enrolled to the study, an area generating at least 177 births over six months (354 over a year) would be needed. Ten villages surrounding Junju dispensary in an area measuring 12x26km (Figure 36) were identified from KHDSS records as having yielded 624 births during 2013.

Figure 36: Homesteads within the 10 study area villages.



Inclusion and exclusion criteria

Inclusion criteria were (all required):

- Informed consent;
- Age ≤14 days;

- Birth whilst mother resident within the study area.

Exclusion criteria were (any one):

- Major congenital abnormality;
- Severe life-threatening acute illness evident at screening;
- Weight at screening <1.5kg;
- Imminent relocation away from the study area.

Identification of potential participants

Multiple approaches were used to identify potential participants: at Junju, Bomani and neighbouring Pingilikani dispensaries and Vipingo Sub-County hospital via attendance for antenatal care, delivery, newborn care or BCG immunisation soon after birth; from KHDSS records of current pregnancies; and in the community via formal representatives (KEMRI Community Representatives, Community Health Workers, 'Nyumba ikumi' homestead representatives, members of the Junju and Bomani Dispensary Health Committees) and word-of-mouth.

Enrolment

Following a period of information-giving, which commonly lasted a week or more, fieldworkers obtained written informed consent from those caretakers willing for their child

to participate. Consent forms (Appendix 37) were read through and signed, with a copy given to the caretaker. Consent forms were available in English, Kiswahili and Giryama languages. Translations were carried out by members of the KWTRP Community Liaison Group (CLG), with two members checking each other's work.

Enrolment and subsequent monthly visits for anthropometry, LM test and BHT took place at Junju dispensary, Gongoni village (Figure 36). At enrolment, a questionnaire was administered (Appendix 33). Weight, length and MUAC were measured for the participant following an SOP (Appendix 38) and height and MUAC for the participant's biological mother. A stool sample was taken.

Anthropometry, and screening for acute malnutrition

After enrolment, participants underwent monthly anthropometry following the same SOP (Appendix 38). Weight, length and MUAC were measured. Weight measurements were plotted by the study clinician in real time in participants' government-issue Child Health Record booklets. Caretakers of participants whose weight gain was noted to be faltering (crossing centile lines on the growth chart) were given nutritional advice by the study clinician following local guidelines.

Following national guidelines and local practice, MUAC (amongst those aged six months or more) and clinical examination for signs of kwashiorkor (all ages) were used for real-time screening for SAM for the purposes of participant welfare. Participants meeting real-time

WHO criteria for uncomplicated SAM (amongst over six-month-olds: MUAC less than 11.5cm; amongst all participants: clinical features of kwashiorkor(266, 267)) were referred to their local primary healthcare facility for community-based supplementary therapeutic feeding (those aged six months or more) or breastfeeding or other milk feeding support (those aged less than six months). Also following WHO guidelines(266, 267), participants meeting criteria or concern for complicated SAM (i.e. SAM plus features of severe acute illness), or SAM with inadequate intake (amongst those aged six months or more: potential failure of appetite test; amongst those aged less than six months: failure to adequately breastfeed or other milk feed), were referred to Kilifi County Hospital for further assessment. Research staff other than the study clinician were not involved in SAM screening aside from passing on weight and MUAC measurements.

At the time of fieldwork, there was no local provision (nor national guideline) for treatment of MAM. Participants were therefore not screened in real time for MAM.

Prevalent and incident SAM, MAM, severe and moderate underweight and severe and moderate low LAZ categories were created in Stata using Z-scores (Table 21). These Z-scores were not used for real-time screening.

To assess quality of length measurements, midway through the study, 20 participants were identified to undergo repeat length measurement by all six fieldworkers on the same day. Intra-class correlation coefficient was estimated for these repeat measurements.

Table 21: Z-score definitions for prevalent and incident acute malnutrition, stunting and underweight.

Nutritional status descriptor	Severity	Incident or prevalent	Criteria
Acute malnutrition	Moderate	Prevalent	-3≤WLZ<-2
		Incident	-3≤WLZ<-2 and (previous WLZ<-3 or previous WLZ≥-2 or this is the first measurement at enrolment)
	Severe	Prevalent	WLZ<-3
		Incident	WLZ<-3 and (previous WLZ≥-3 or this is the first measurement at enrolment)
Stunting	Moderate	Prevalent	-3≤LAZ<-2
		Incident	-3≤LAZ<-2 and (previous LAZ<-3 or previous LAZ≥-2 or this is the first measurement at enrolment)
	Severe	Prevalent	LAZ<-3
		Incident	LAZ<-3 and (previous LAZ≥-3 or this is the first measurement at enrolment)
Underweight	Moderate	Prevalent	-3≤WAZ<-2
		Incident	-3≤WAZ<-2 and (previous WAZ<-3 or previous WAZ≥-2 or this is the first measurement at enrolment)
	Severe	Prevalent	WAZ<-3
		Incident	WAZ<-3 and (previous WAZ≥-3 or this is the first measurement at enrolment)

Environmental enteric dysfunction

EED was measured by monthly urine LM test and by ELISA for alpha-1-antitrypsin, myeloperoxidase and neopterin for a selected subset of stool samples.

Participants underwent LM test once per month. Including the enrolment test, for those participants that completed follow-up, a total of 10 tests were carried out per participant with the last test taking place during the tenth month of life. See Chapter 4 for details of LM test piloting, methods and quality assessment.

Stool samples collected 10 or fewer days before a successful LM test were selected for ELISA. Stool samples were thawed in batches for ELISA. Each aliquot was thawed only once, and ELISA for all three biomarkers took place on the same day. ELISA laboratory and statistical interpretation methods were the same as for the cross-sectional survey (see Appendices 12 and 13).

Small intestinal bacterial overgrowth

Participants underwent glucose BHT once per month. Thus, including the enrolment test, for those participants that completed follow-up a total of 10 tests were carried out per participant, with the last test taking place during the tenth month of life. See Chapter 3 for full details of BHT piloting, methods and quality assessment. Results were categorised following the Rome criteria(230). Tests where baseline breath hydrogen exceeded 5ppm were deemed 'indeterminate'. Tests where peak breath hydrogen was ≥12ppm above baseline (where baseline was <5ppm) were deemed 'positive'. Tests where peak breath hydrogen was <12ppm above baseline, and where baseline was <5ppm, were deemed 'negative'. Area under the curve, peak and peak minus baseline values were also calculated for each test.

Diarrhoea surveillance

For active case detection of diarrhoea and other acute illnesses, participants were visited by their designated fieldworker once per week at home. Fieldworkers enquired after episodes of diarrhoea, breathing difficulty or fever since the last home visit (Appendix 33). Duration of reported episodes was recorded, and whether primary or secondary care had been sought. Where diarrhoea was reported, fieldworkers also enquired as to whether blood had been seen in stool. Diarrhoea episodes were also captured via passive case detection on weekdays at the study's designated primary healthcare facility, Junju dispensary, where participants were seen by the study clinician and an acute illness record form filled (Appendix 34). The study clinician also saw all under-fives presenting to Junju dispensary for primary care, regardless of participation in any study.

Diarrhoea was defined as per WHO guidelines as three or more episodes of loose stool (more loose than is usual for the participant) in a 24-hour period(268). During retrospective data analysis, since exact day of onset was not recorded, reported diarrhoea episodes were assumed to have occurred midway between the reporting visit and the previous visit, with start and end dates assigned as symmetrical about that mid-point date, with duration as recorded, in days.

For each diarrhoea episode, attempts were made to obtain a stool sample during the episode. These diarrhoeal samples were aliquoted and stored at -80^{o}C along with non-diarrhoea stool samples, but in addition underwent stool culture at KWTRP Laboratories.

Malaria surveillance

Malaria episodes were identified via active and passive case detection at home and Junju dispensary respectively. Upon weekly home visit, axillary temperature was recorded. If fever was present (at or above 37.5°C), a capillary blood sample was taken for rapid diagnostic test (RDT; CareStart) and a blood film prepared. RDT results were obtained immediately and, if positive, the participant was given a three-day course of artemether-lumefantrine by the fieldworker and the study clinician notified. The blood film was couriered to KWTRP Laboratories and microscopy undertaken on the same day. If the slide was positive and the rapid test negative, the information was relayed to the fieldworker and the participant started on artemether-lumefantrine that same day. If the caretaker reported that the participant had been feverish but temperature upon fieldworker home visit was below 37.5°C, the fieldworker visited the participant at least twice during the next 24 hours, taking an axillary temperature at each visit. If at any point, the temperature was at or above 37.5°C, then blood sampling and treatment took place as above. For passive case detection, any participant brought to Junju dispensary with a history of fever underwent blood sampling for RDT and slide as above.

Other acute illness surveillance

As well as diarrhoea and fever, during weekly home visits fieldworkers enquired specifically about breathing difficulty since last home visit. As for diarrhoea, if breathing difficulty was reported to have been present, its timing was retrospectively assumed to have been

equidistant between that home visit and the previous one. Duration of breathing difficulty was enquired after and start and stop dates presumed to be symmetrical about that mid-point date. Breathing difficulty episodes were also passively captured at Junju dispensary. All other acute illnesses were captured only via passive case detection. Diagnoses were made by the study clinician following WHO Integrated Management of Child Illness syndromic guidelines.(269)

Antibiotics, immunisations and vitamin A supplementation

If antibiotics were prescribed by the study clinician, antibiotic name and course duration were recorded. Fieldworkers also enquired during weekly home visits whether participants had received antibiotics from other providers, and if so, antibiotic name, start date and course duration were recorded.

During weekly home visits, fieldworkers asked whether a participant had received any immunisations or vitamin A supplementation since the last home visit. These responses were later cross-checked against participants' government-issue Child Health Record booklets.

Breastfeeding and complementary foods

During weekly home visits, fieldworkers asked open questions as to which liquids or solids the participant had received since the last home visit. Caretakers were encouraged to list all

items they could recall. Fieldworkers then asked which *one* of these liquids or solids had been the participant's *main* intake during that time.

Statistical analysis

Statistical analysis was carried out using Stata® version 15.1.(219) PCA was used to create summary variables describing participants' households' SEP and WASH statuses. Outcomes of LM ratio and change in LAZ were preserved as continuous: thus, linear regression modelling approaches were used. Highly skewed continuous factors – including LM ratio – were log-transformed prior to inclusion in the models. Where plotting of variables suggested non-linear association, multiple fractional polynomial analysis was carried out to determine whether linear or non-linear curves were the best fit.

To assess generalisability of results to the study population from which participants had been drawn, participant SEP scores were compared against those of the general community from which they were drawn. To do this, eigenvectors were derived via a new PCA of data for the 336 households that participated in the cross-sectional survey, repeated this time using only the household SEP variables that were measured for both cross-sectional survey and cohort participants. A dummy 'first component' was calculated for each cohort participant by summing the product of each variable's binary score and the eigenvector for that variable derived from the cross-sectional survey PCA. Cohort dummy first components were then compared against cross-sectional survey first components.

Seasonality of acute illnesses, antibiotic courses and healthcare seeking was assessed by Poisson regression of longitudinal multiple event (multiple failure) data comparing incidence of each event during rainy versus dry season, adjusted for age. Season was defined via prospective binary recording of rain by week in the local area. Rainy season was deemed November-December 2015, April-July 2016 and November-December 2016 inclusive. Seasonality of factors measured at fixed monthly or bi-weekly intervals (SIBO, EED and nutritional status) was assessed by mixed-effects linear regression with random-effects adjustment for participant and fixed effects of age and season.

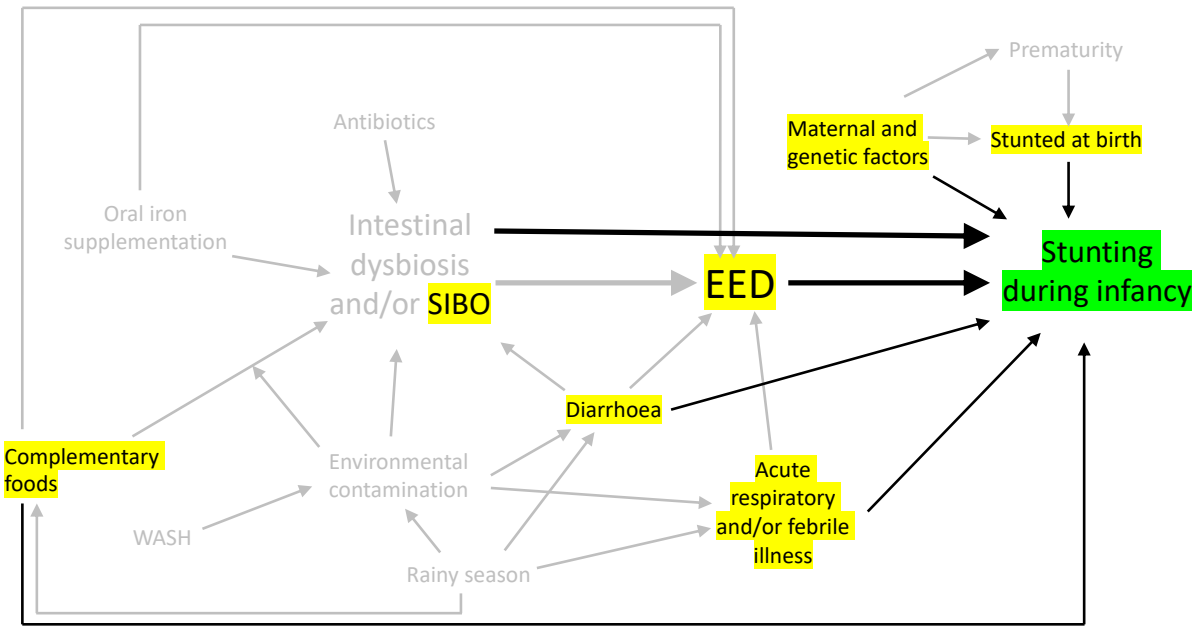
To assess strength of evidence for association between LM ratio and other faecal markers of EED and SIBO, five univariable mixed-effects linear regression models were developed, each with outcome LM ratio and participant as random effect. The fixed effect for each univariable model was in turn faecal alpha-1-antitrypsin, faecal myeloperoxidase, faecal neopterin, peak breath hydrogen and breath hydrogen area under the curve. Age and sex were added to fixed effects in subsequent sensitivity models.

For the final analyses, data were arranged as if for a cross-sectional study where, for analyses with EED as the outcome, each observation was a successful LM test and its preceding and/or contemporaneous events within that participant. For analyses with stunting as the outcome, each observation was a two-month period of linear growth and its preceding and/or contemporaneous events within that participant. Change in LAZ was calculated for each two-month period. In all analyses, participant was included in regression modelling as a random effect to reflect the data structure (multiple events per participant) and adjust for potential clustering of associations that may have occurred as a result of this.

To investigate correlates of linear growth, testing for association between change in LAZ over two months and markers of systemic inflammation (white blood cell count and platelet count), intestinal inflammation (faecal myeloperoxidase and faecal neopterin), intestinal permeability (faecal alpha-1-antitrypsin and urinary lactulose excretion), intestinal absorptive capacity (urinary mannitol excretion, SIBO (peak breath hydrogen and breath hydrogen area under the curve) and anaemia (haemoglobin) was carried out with eight mixed-effects linear regression models. These models aimed to test which of the biomarkers could most closely predict concurrent linear growth. Using a monthly LM test as the starting time point, linear growth was represented by difference between the LAZ one month prior to this time point and LAZ one month after this time point (i.e. change in LAZ over a two-month period symmetrically distributed around the index LM test). Where data were missing for LAZ one month previously (because anthropometry had been missed, or the index LM test was the first at enrolment), this observation was not included in the models. Where data were missing for LAZ one month following the LM test (because anthropometry had been missed, or this was the last LM test before exiting follow-up), this observation was not included in the models. The unit of analysis was therefore a two-month period of person-time. Attempting to adjust for the phenomenon of 'regression towards the mean' and following established practice,(270, 271) LAZ at the beginning of the two-month period (baseline LAZ) was included as a fixed effect in each model. Participant was included as a random effect to acknowledge the clustered nature of the data (multiple observations per participant). Age and sex were added to fixed effects in subsequent sensitivity models.

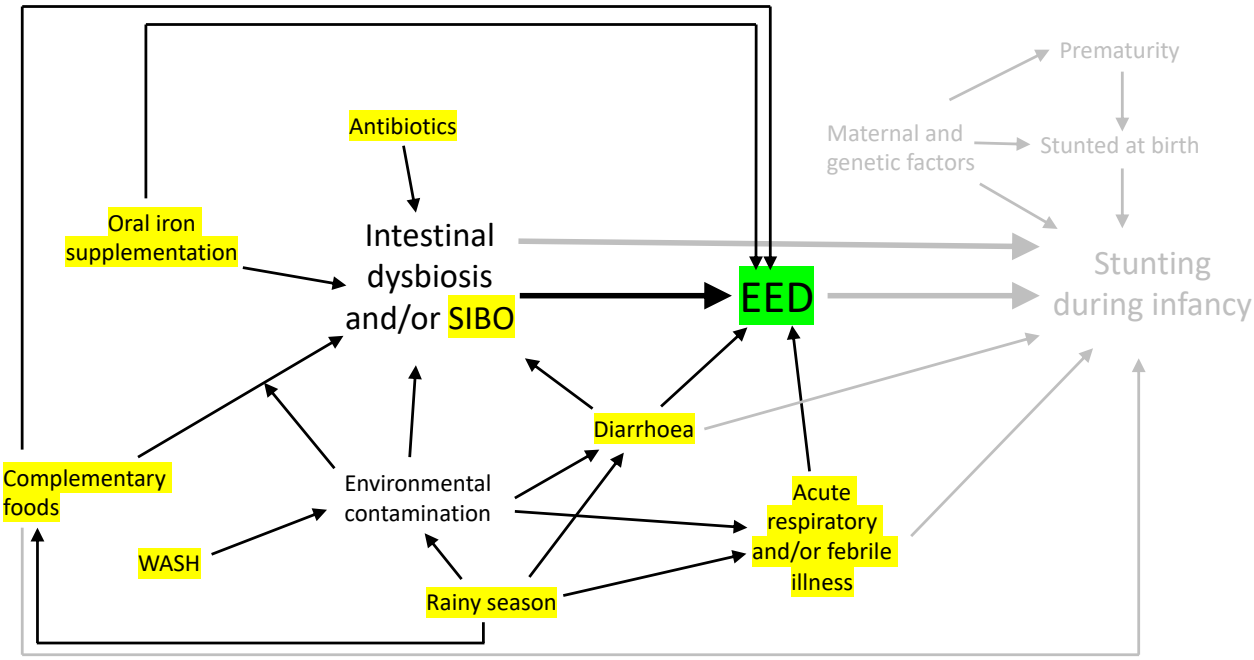
After this, the first multivariable model aimed to assess strength of evidence for association between a wider selection of dependent variables and change in LAZ as per the hypothesised causal framework. Variables were selected based on their direct upstream proximity to stunting in the hypothesised causal framework (Figure 37). As in previous models, each observation in the model related to a two-month period of linear growth symmetrically about a successful LM test, with the outcome difference in LAZ between anthropometry undertaken one month before and one month after this test. Also, as previously, LAZ at the beginning of the two-month period (baseline LAZ) was included to adjust for regression to the mean. Where data was missing for LAZ one month previously or one month following the successful LM test, difference in LAZ could not be calculated thus the observation was not included in the model. Weight at enrolment was used as a proxy for stunting at birth, as length measurement during the first few weeks of life was deemed insufficiently accurate due to safety limitations on full leg extension in neonates (birthweight could not be used as it was missing for 44% of participants). Sex and maternal height were chosen to represent 'maternal and genetic factors'. SIBO was included as a fixed effect and defined by the highest peak reading during the BHT that had taken place within up to three days of the index LM test. Exclusive breastfeeding status was defined by no complementary foods (except prelacteal feeds) reported to be given at all between birth and the date of the home visit immediately following the index LM test. Diarrhoea, breathing difficulty, slide-negative and slide-positive fever were all derived from active and passive case detection methods described earlier and summarised in binary form as having occurred or not occurred during the 30 days preceding the mid-point LM test in each two-month period. Age was included as a fixed effect.

Figure 37: Hypothesised causal framework with factors selected for testing of association with stunting. Green highlighting indicates dependent variable. Yellow highlighting indicates independent variable.



The second multivariable model aimed to assess strength of evidence for association between selected dependent variables and EED as defined by the LM ratio. Working from the hypothesised causal framework, three factors (season, environmental contamination and antibiotics) were included as proxy measures of intestinal dysbiosis despite not being directly upstream of EED in the schematic. Season was defined as previously. Based on an *a priori* assumption, likelihood ratio testing for interaction between season and exclusive breastfeeding status was carried out. Age and sex were included as fixed effects. For diarrhoea, breathing difficulty, fever, antibiotics and iron, where an LM test took place at less than 30 days of age, event occurrence between birth and LM test (rather than during the 30 days preceding LM test) was used. Similar models were developed with the outcomes faecal alpha-1-antitrypsin, myeloperoxidase and neopterin. Here, independent factors were classified with reference to the date of stool sample collection rather than date of LM test.

Figure 38: Hypothesised causal framework with factors selected for testing for association with EED. Green highlighting indicates dependent variable. Yellow highlighting indicates independent variable.

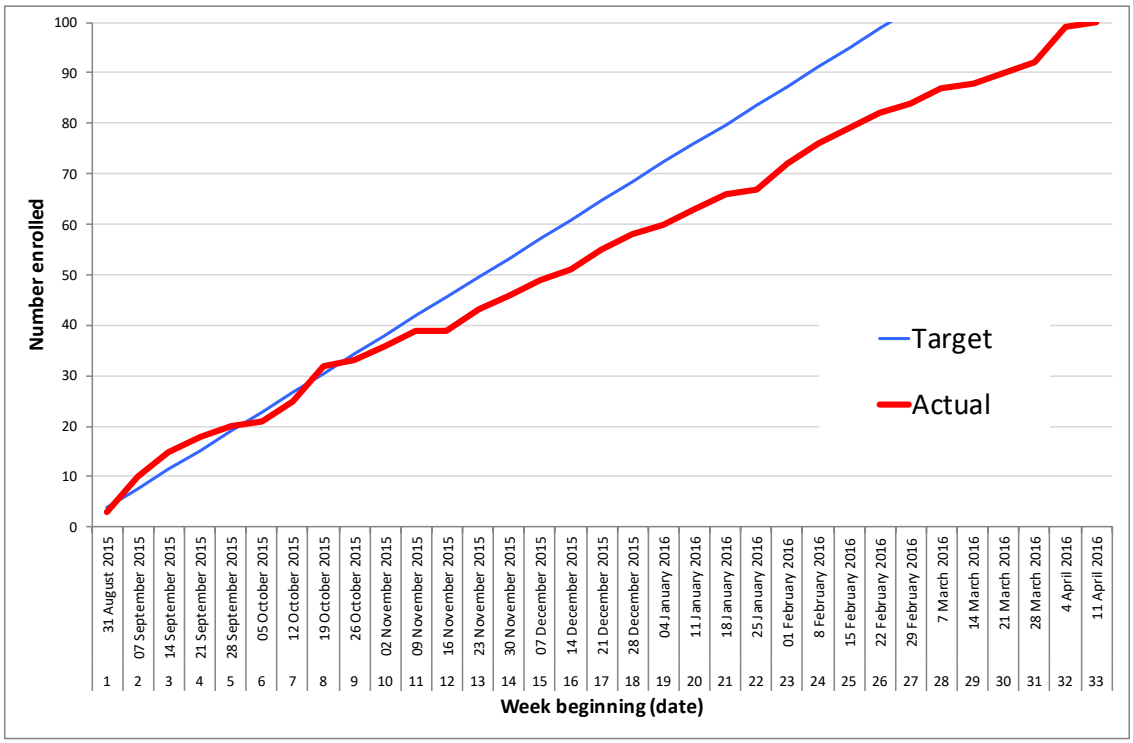


Results

Screening, enrolment and follow-up

Enrolment commenced on 25th August 2015 and lasted 7½ months, with the final participant enrolled on 8th April 2016 (Figure 39). The proportion of eligible neonates recruited to the study was, at 71%, very similar to the predicted proportion of 70%. An additional second programme community barazas was held in November 2015 to boost awareness of the study.

Figure 39: Enrolment rate for the cohort study.

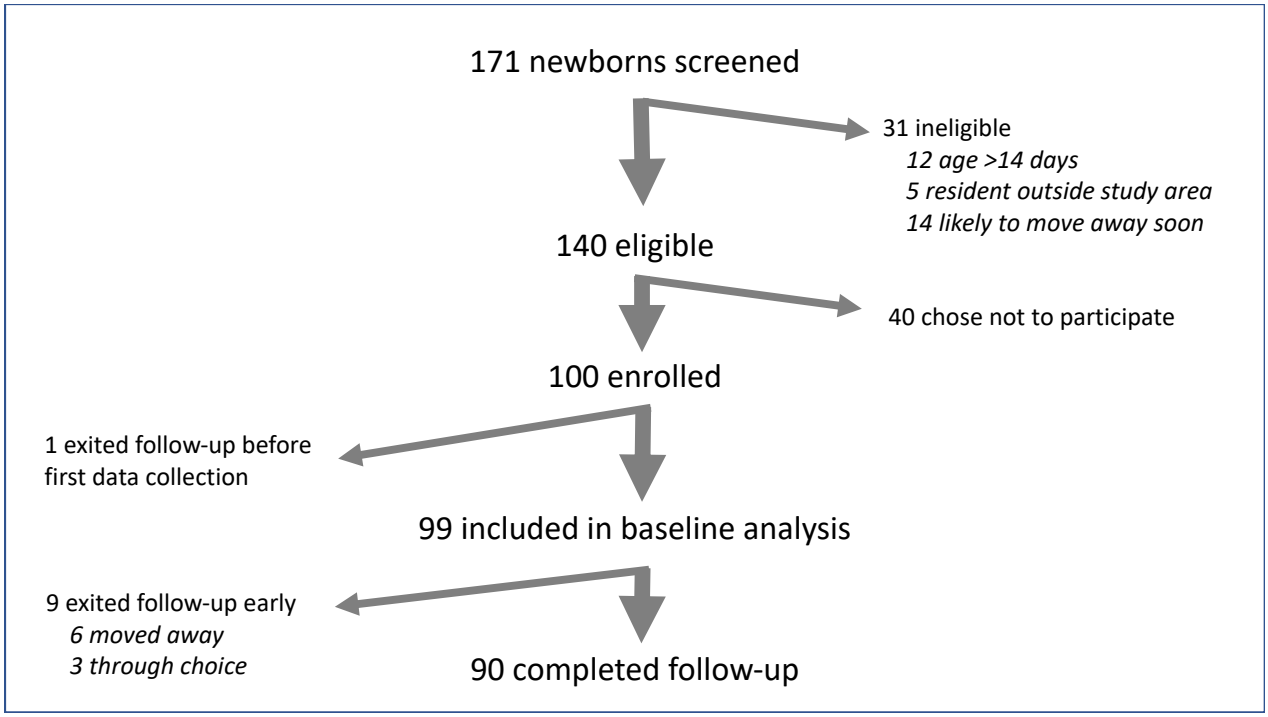


171 newborns were screened (Figure 40). Of these, five were found to be resident outside the geographical boundaries of the study area, 14 were likely to move away from the study area within the intended follow-up time and 12 were aged >14 days by the time of screening. For the remaining 140 newborns, caretakers declined further eligibility screening for 40. Of the 100 newborns undergoing final eligibility screening by the study clinician at Junju dispensary, none weighed <1.5kg and none had severe life-threatening acute illness or major congenital abnormality. All 100 were therefore enrolled.

Participation

Within a week of enrolment one participant exited follow-up due to caretakers' changed preferences. Since no data had been collected beyond the enrolment questionnaire, this participant was not included in any analyses. Three other participants exited follow-up early due to changed preferences and six exited early due to relocation outside the study area. These ten early exits took place at 14, 44, 75, 77, 126, 185, 190, 214, 236 and 245 days of age. For the 90 participants who did not exit early, follow-up was continued until the final LM test and paired BHT, which took place during the 10th month of life. Age at this point ranged 274-306 days. From date of birth to date of exit, for all 100 enrolled participants, total Person Years of Observation (PYO) for the cohort was 75.18, with range per participant 0.12-0.84 years (median 0.79 years).

Figure 40: Screening and enrolment.



3,543 weekly home visits were undertaken. Amongst the 90 participants who did not exit follow-up early, number of visits ranged 31-42 (median 38). Time between consecutive home visits ranged 1-29 days (longer intervals occurred due to hospitalisation or travel away from the study site) with a median of seven days.

Characteristics at enrolment

Baseline sociodemographic and household characteristics, birth and feeding histories are given in Appendix 39. In summary, age at enrolment ranged 0-14 days. 52% were male. Six participants were a twin (i.e. three pairs of twins). Other participants were more distantly related: as first cousins (two pairs) and uncle-nephew (one pair). 4% were thought to be premature; however, none were very (<1.5kg) or extremely (<1kg) low birthweight and only 8% were low (<2.5kg) birthweight. 40% were born at home, none by caesarean section and 3% were exposed to HIV. Only one participant had a primary caretaker who was not their mother. Mothers' ages ranged 16-40 years. All participants were breastfed at least once. 18% received a prelacteal feed, which was defined as any solid or liquid given by mouth before the first breastfeed or other milk feed. These were sugar water (nine participants), sugar salt water (three), water (three) and coconut water (one). 21% of participants had an older sibling (defined here as sharing a mother, not necessarily a father) who had died. The commonest category of monthly household income was US$40-99 equivalent. 58% were from households with unimproved water source, 17% with unimproved sanitation and 75% with no soap.

Participants' household WASH: relative position within cohort study

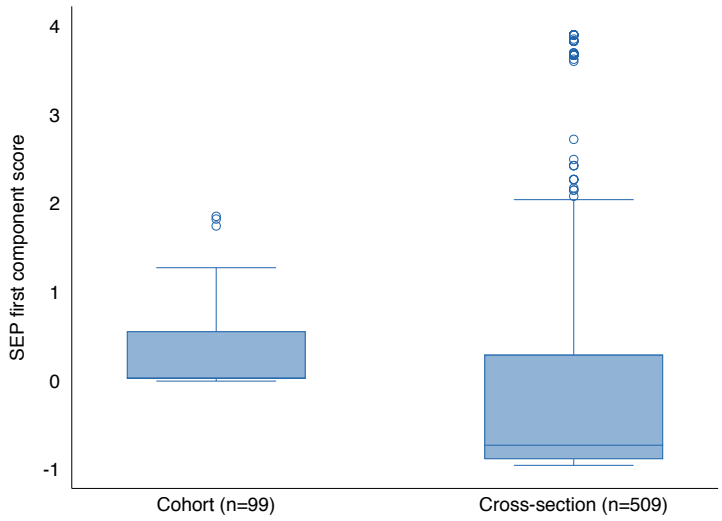
Water source, location, time to fetch water, drinking water treatment, sanitation type, soap availability and presence of animal in the house were selected for PCA to rank cohort participants in order of overall WASH factor variance. All factors were either co-linear or neutral between each other. In the analysis, the first component contributed 24% of the total variability observed. Eigenvectors were all positive except for treatment of drinking water, which is to be expected if drinking water tends to only be treated if caretakers suspect it to be contaminated (Appendix 40). Of note, sanitation type was not inversely correlated with other variables as was seen in the cross-sectional survey. This first component (observed to yield a much higher eigenvalue than the subsequent components – scree plot Appendix 40) was then predicted for each participant.

Cohort participants' household SEP was higher than that of cross-sectional survey participants

Household per capita income, land and livestock ownership and house roof, wall and floor material were selected for PCA to rank cohort participants in order of overall SEP indicator variance. In the analysis, the first component contributed 31% of the total variability observed. Eigenvectors were all positive except for land ownership (Appendix 41). The first component was then predicted for each participant (scree plot: Appendix 41).

Using the same factors and categorisation, PCA-derived first components for SEP for cross-sectional survey participants were generally lower and more widely distributed than those for cohort participants that were manually calculated using the cross-sectional survey PCA-derived eigenvectors (Figure 41; for eigenvectors and an example calculation see Appendix 42).

Figure 41: Comparison of SEP first component summary statistics for cross-sectional survey and cohort participants.
Box plots showing frequency distributions of first components for cross-sectional survey SEP PCA and derived dummy first components for cohort participants using eigenvectors from the cross-sectional survey analysis. Box midlines denote median, box edges denote 25th and 75th centiles, whiskers denote upper and lower adjacent values, hollow dots denote outlying values. Two-sided Z test comparison of means: p=0.0009 (null hypothesis: no difference in means).



Nutritional status: stunting was the commonest form of undernutrition

Of the 990 anthropometry measurement episodes planned for the 99 participants, 937 were undertaken. Forty-five were missed because of early withdrawal from follow-up. Three were

missed due to caretaker choice. One was missed due to temporary travel away from the study area. Four were missed due to hospitalisation. Appraisal of inter-observer variability in length measurement is shown in Appendix 43.

Amongst participants aged six months or more, none met real-time screening criteria for SAM. Amongst participants aged less than six months, one participant was referred to secondary care on two occasions (age three and four months) for severe growth faltering and inadequate breastfeeding. This participant first demonstrated severe growth faltering at two months of age and was referred for further assessment in hospital, where they were deemed unable to breastfeed effectively and were therefore admitted. In hospital, they were treated according to WHO guidelines(266) with first line empirical broad spectrum IV antibiotics, dilute F100 milk, expressed breastmilk via nasogastric tube followed by re-establishment of exclusive breastfeeding and ongoing support. They were discharged after 11 days but readmitted age 3 months again with renewed severe growth faltering plus clinical signs of severe pneumonia. During this second admission they were treated again with the same approach. Under subsequent outpatient follow-up, from age six months onwards, they consumed ready to use therapeutic food (RUTF) at home until eight months of age when they ceased to meet MUAC criteria for SAM.

Incidence of moderate stunting was 0.96 per PYO (Table 22; 95% CI 0.76-0.21). Incidence of severe stunting was 0.40 per PYO (95% CI 0.28-0.57). No participant developed kwashiorkor. The proportion moderately or severely stunted increased particularly from six months of age onwards (Table 23). Amongst the 90 participants who completed follow-up, 30 (33.3%; 95% CI 23.7-44.1) were moderately or severely stunted at exit at nine months of age.

Table 22: Incidence and prevalence of low WLZ, LAZ and WAZ from amongst 937 anthropometry episodes. Length was missing for one episode (data lost). Denominators for acute malnutrition and stunting are therefore 936 and for underweight 937.

Nutritional status descriptor	Severity	Infants experienced ≥1 episode	Incident or prevalent	Total episodes	% proportion of all anthropometry episodes (95% CI)	Incidence rate per PYO (95% CI)
Wasting / wasted	Moderate	32	Prevalent[1]	61	6.5 (5.0-8.3)	-
			Incident[2]	46	-	0.61 (0.46-0.82)
	Severe	12	Prevalent[3]	15	1.6 (0.9-2.6)	-
			Incident[4]	14	-	0.19 (0.11-0.31)
Stunting / stunted	Moderate	47	Prevalent[5]	128	13.7 (11.5-16.0)	-
			Incident[6]	72	-	0.96 (0.76-1.21)
	Severe	18	Prevalent[7]	49	5.2 (3.9-6.9)	-
			Incident[8]	30	-	0.40 (0.28-0.57)
Underweight	Moderate	35	Prevalent[9]	99	10.6 (8.7-12.7)	-
			Incident[10]	49	-	0.65 (0.49-0.86)
	Severe	10	Prevalent[11]	38	4.0 (2.9-5.5)	-
			Incident[12]	16	-	0.210.13-0.35)

1. WLZ<-2 and ≥-3
2. WLZ <-2 and ≥-3, where WLZ was ≥-2 at last measurement (or this is the first measurement at enrolment)
3. WLZ <-3
4. WLZ <-3, where WLZ was ≥-3 at last measurement (or this is the first measurement at enrolment)
5. LAZ<-2 and ≥-3
6. LAZ <-2 and ≥-3, where LAZ was ≥-2 at last measurement (or this is the first measurement at enrolment)
7. LAZ <-3
8. LAZ <-3, where LAZ was ≥-3 at last measurement (or this is the first measurement at enrolment)
9. WAZ<-2 and ≥-3
10. WAZ <-2 and ≥-3, where WAZ was ≥-2 at last measurement (or this is the first measurement at enrolment)
11. WAZ <-3
12. WAZ <-3, where WAZ was ≥-3 at last measurement (or this is the first measurement at enrolment)

Table 23: Proportion moderately and severely stunted, wasted and underweight by age.

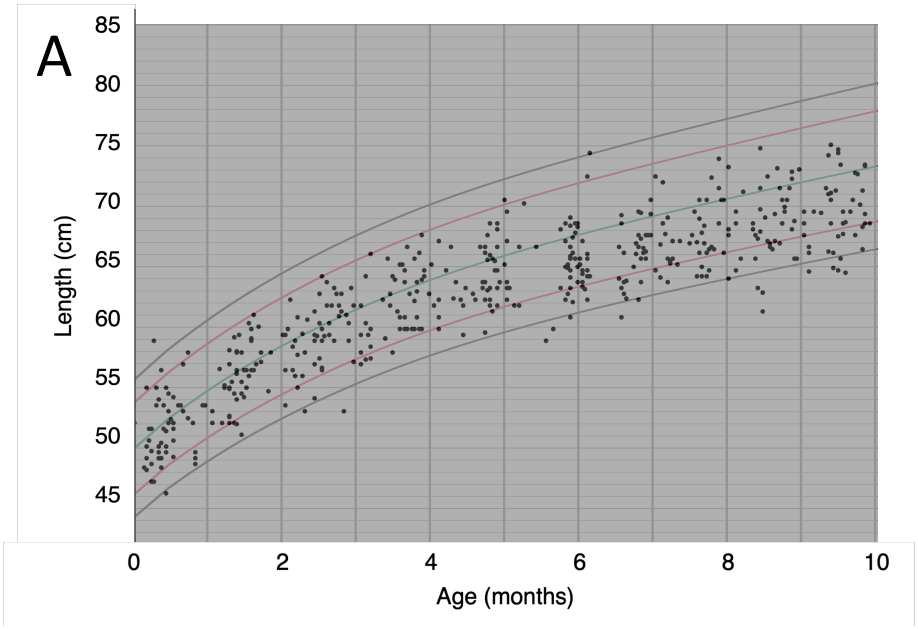
		Moderately or severely wasted (WLZ<-2)		**Moderately or severely stunted (LAZ<-2)**		**Moderately or severely underweight (WAZ<-2)**	
Age (months)	**n**	**% proportion**	**95% CI**	**% proportion**	**95% CI**	**% proportion**	**95% CI**
0-1	201[1]	14.0	9.5-19.6	13.0	8.7-18.5	12.4	8.2-17.8
2-3	197	6.6	3.6-11.0	13.2	8.8-18.8	9.6	5.9-14.7
4-5	180	6.7	3.5-11.4	15.6	10.6-21.7	13.3	8.7-19.2
6-7	182	7.1	3.9-11.9	25.3	13.1-32.2	19.2	13.8-25.7
8-9	177	5.6	2.7-10.1	28.8	22.3-36.1	19.2	13.7-25.8
Total	**937**						

1. Length was missing for one episode in age 0-1 months category (data lost). Denominator for wasting and stunting in this age category is therefore 200 instead of 201.

Nutritional counselling for caretakers was carried out following 113 of 937 anthropometry measurements (12%) due to clinician concern at faltering weight gain as plotted on government child health booklet growth charts. These 113 counselling events were shared amongst 59 of the 99 cohort participants (60%).

Length gain by age in general fell below WHO 2016 growth standards (Figure 42). One participant had consistently and extremely low LAZ (Figures 43; N.B. this participant missed anthropometry at one and two months of age due to caretaker choice). This same participant was treated for SAM as described above. Overall, LAZ declined by a predicted 0.09 Z-scores with each increasing month of age (Figure 43; univariable linear regression of LAZ against age; n=937; coefficient=-0.09; 95% CI -0.12 to -0.06; $p<0.001$). There is weak evidence to suggest that the association between LAZ and age is not linear (likelihood ratio test comparing two univariable linear regression models of LAZ against age, where the first model treats age as continuous and the second categorises age into integer weeks; p=0.04).

Figure 42: Length by age for male and female participants.
Male: A; female: B. Overlaid onto a WHO 2006 Growth Standards growth chart. Green line indicates LAZ=0; red line LAZ=2 or LAZ=-2; black line WAZ=3 or WAZ=-3.



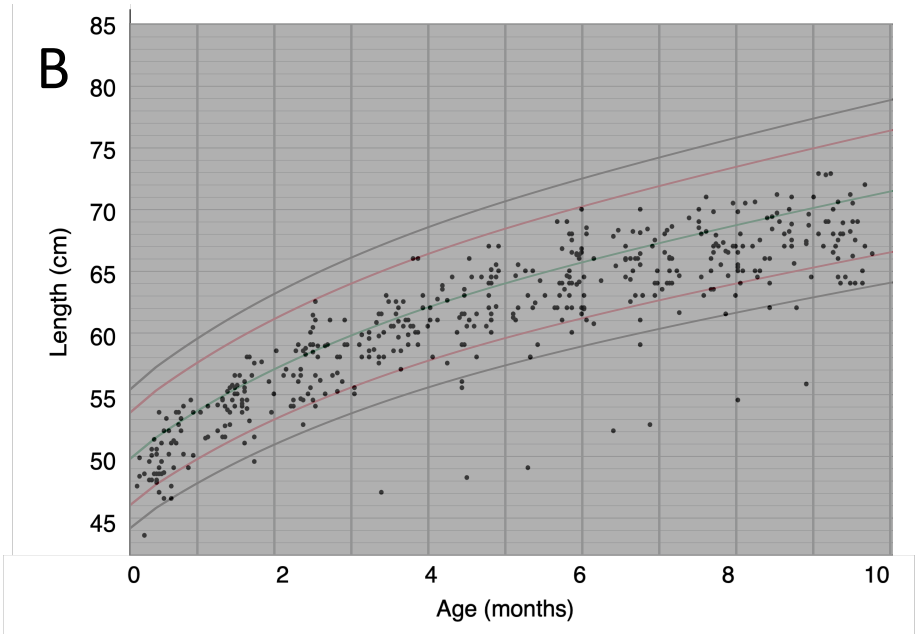


Figure 43: LAZ by age.
Lines connect scores for individual cohort participants.

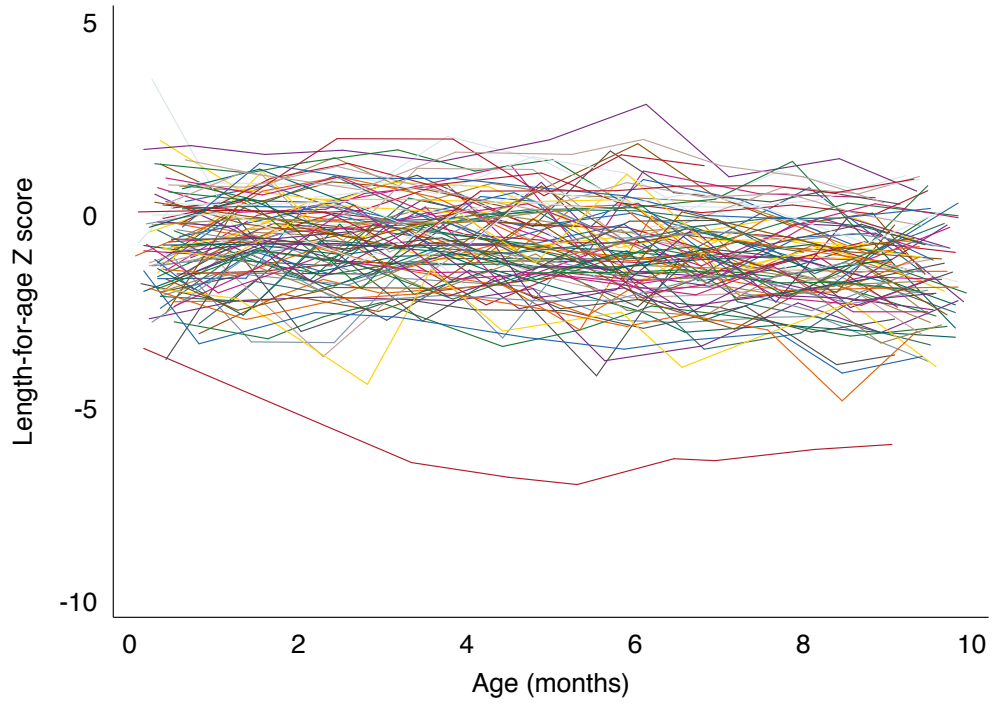
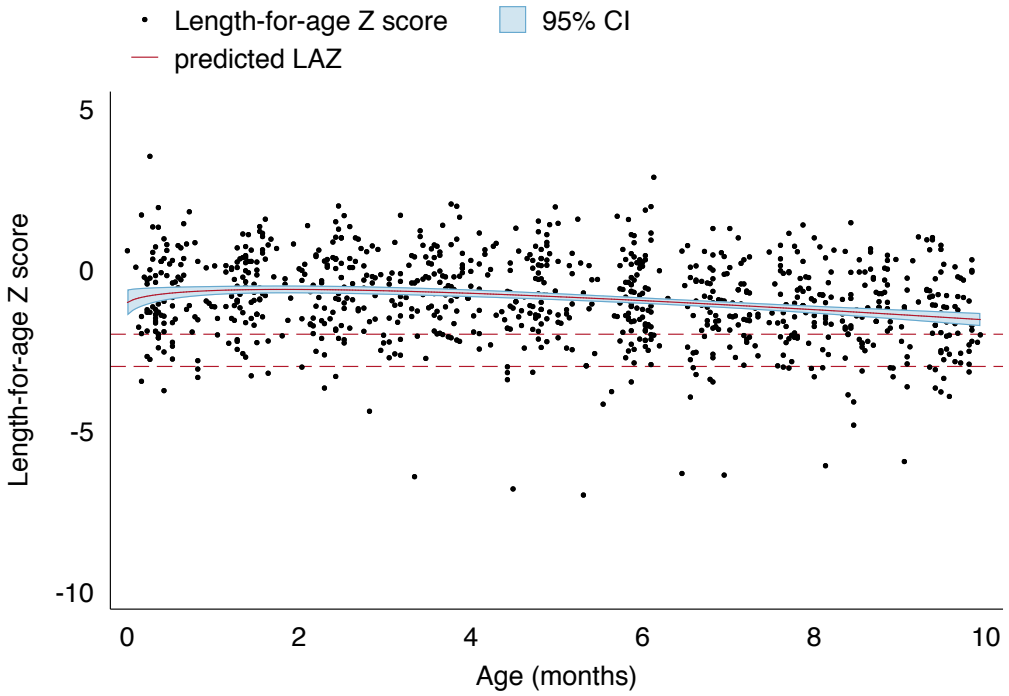
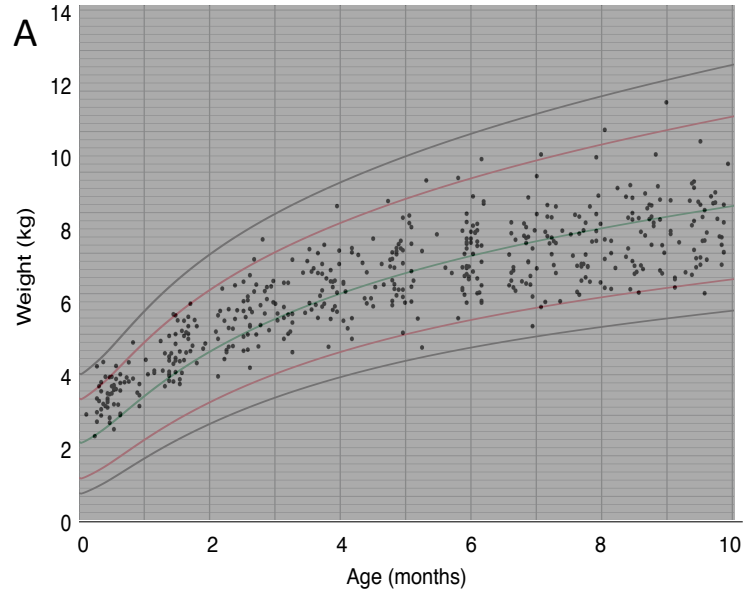


Figure 44: Observed and predicted LAZ by age.
Red dotted lines indicate y axis values at -2 (threshold for moderately stunted) and -3 (threshold for severely stunted).



Weight gain by age fell slightly below WHO 2016 growth standards (Figure 45). WAZ declined by a predicted 0.03 Z-scores with each increasing month of age (Figure 46). There was no evidence to support change in WLZ by age (Figure 47).

Figure 45: Weight by age for male and female participants.
Male: A; female: B. Overlaid onto a WHO 2006 Growth Standards growth chart. Green line indicates WAZ=0; red line WAZ=2 or WAZ=-2; black line WAZ=3 or WAZ=-3.



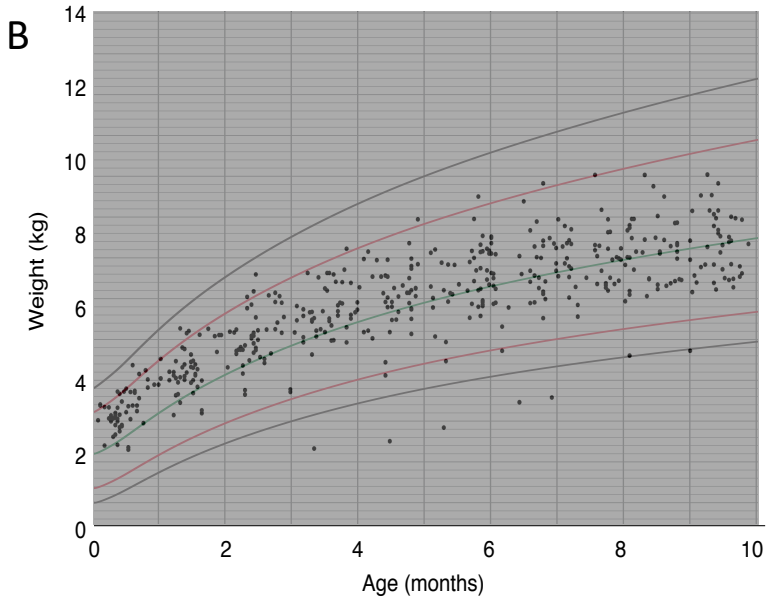


Figure 46: Observed and predicted WAZ by age.
Univariable linear regression of WAZ against age; n=937; coefficient=-0.03; 95% CI -0.06 to -0.01; p=0.01. Red dotted lines indicate y axis values at -2 (threshold for moderately underweight) and -3 (threshold for severely underweight).

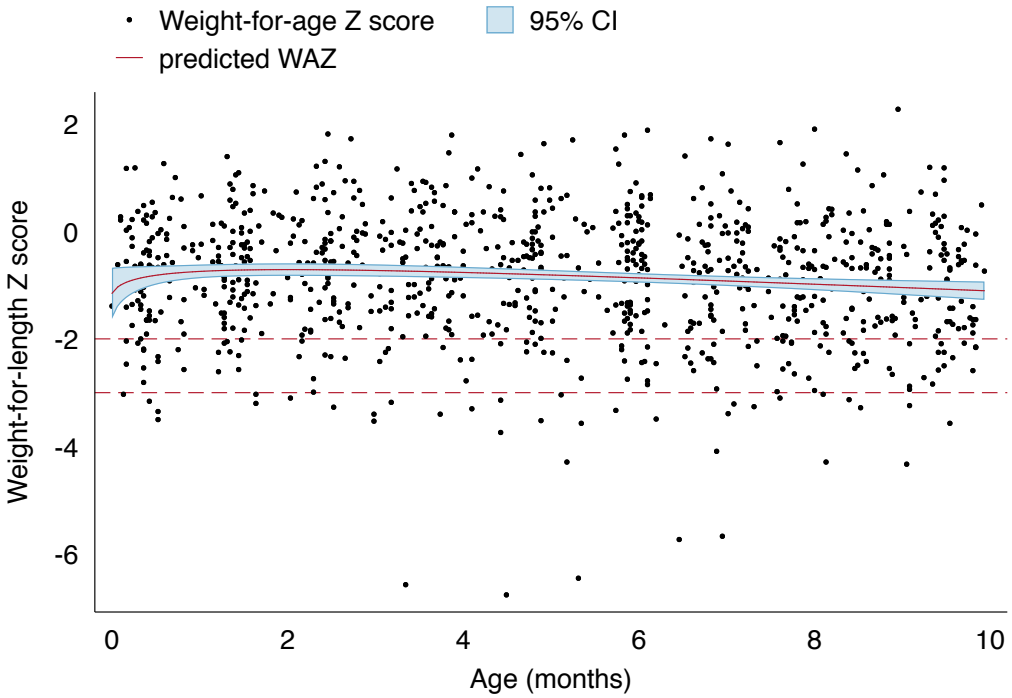
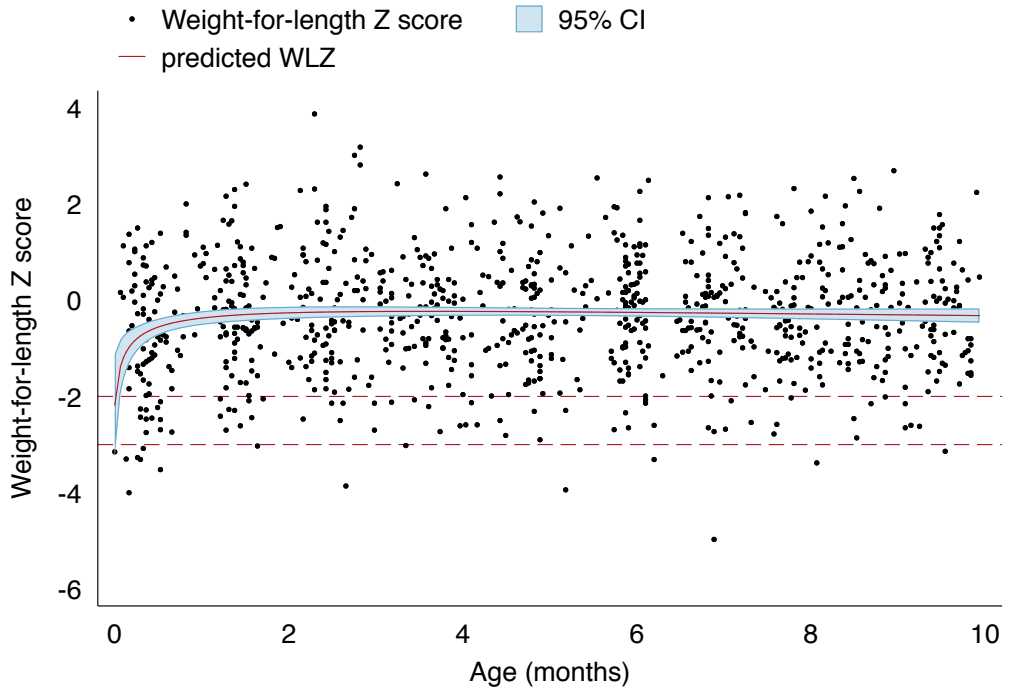


Figure 47: Observed and predicted WLZ by age.
Univariable linear regression of WLZ against age; n=937; coefficient=0.02; 95% CI -0.01 to -0.05; p=0.12. Red dotted lines indicate y axis values at -2 (threshold for moderately wasted) and -3 (threshold for severely wasted).



Diarrhoea was rare

There were 38 episodes of diarrhoea captured amongst 32 participants, resulting in 10 visits to primary care and no hospitalisations. None were bloody (dysentery). Ten were captured by active and passive case detection (attendance to primary care but also recalled during home visit around that time), whilst the remaining 28 were captured by active case detection (home visit) only. Incidence of diarrhoea was 0.51 episodes per PYO (95% CI 0.37-0.69). Longitudinal prevalence of diarrhoea was 0.5% (84 of 27,458 days; 95% CI 0.2 to 0.4%).

Fever was common; malaria was rare

There were 226 episodes of fever captured amongst 87 participants, resulting in 207 visits to primary care and three hospitalisations (occurred amongst three participants; all diagnosed with severe pneumonia). Incidence of all fever was 3.01 episodes per PYO (95% CI 2.64-3.43). Incidence of slide-positive fever was 0.33 per PYO (95% CI 0.22-0.49). Incidence of slide-negative fever was 2.69 per PYO (95% CI 2.34-3.08). Incidence of malaria clinical diagnosis (fever plus positive RDT and/or slide) was 0.61 per PYO (95% CI 0.46-0.82). Further details on fever case detection, RDT and malaria slide microscopy results are in Appendix 44.

Breathing difficulty was very common

There were 367 episodes of breathing difficulty captured amongst 90 participants, resulting in 317 visits to primary care and three hospitalisations amongst three participants (amongst three participants; all diagnosed with severe pneumonia). 317 were captured by active and passive case detection (attendance to primary care but also recalled during home visit around that time), whilst the remaining 50 were captured by active case detection (home visit) only. Incidence of breathing difficulty was 4.88 episodes per PYO (95% CI 4.41-5.41). Attendances to primary care with breathing difficulty resulted in various clinician diagnoses, most commonly upper or lower respiratory tract infection (Appendix 45).

Antibiotic prescribing was very common

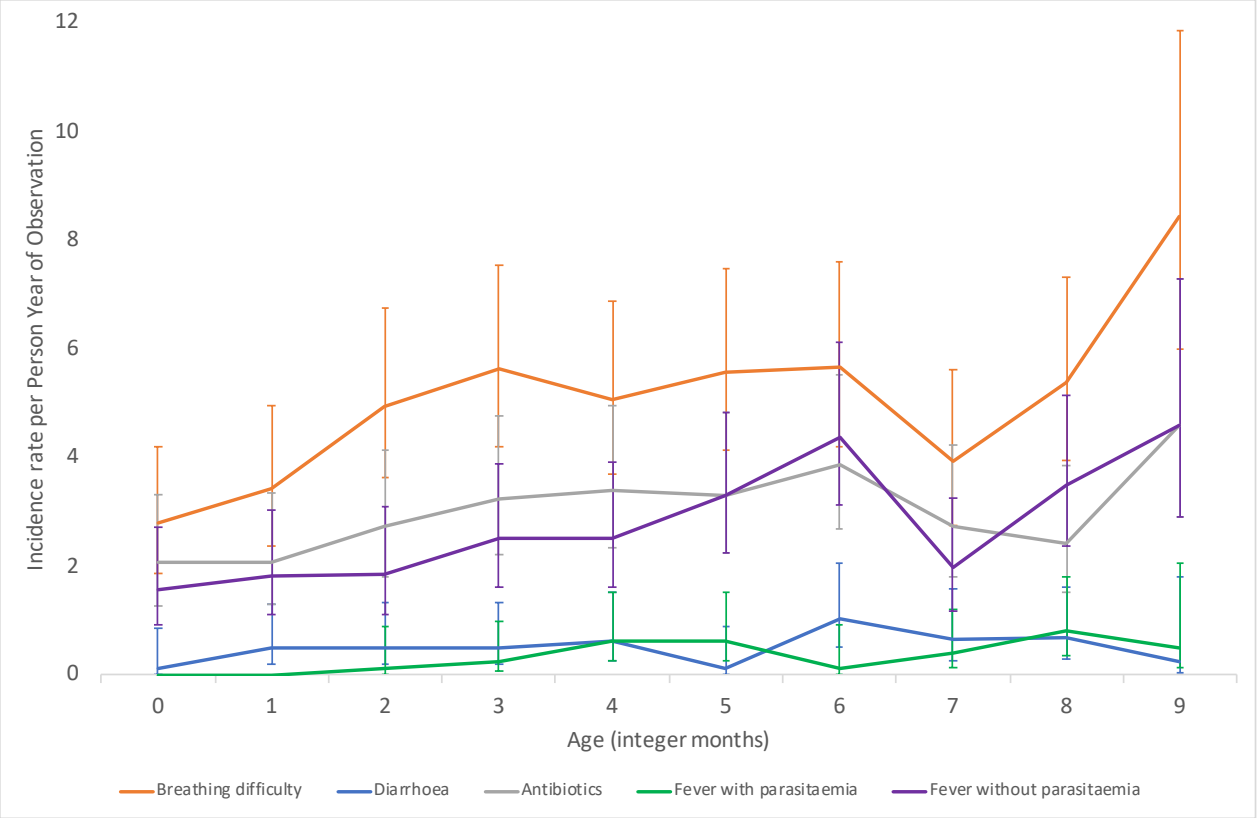
There were 222 antibiotic courses prescribed for 82 participants. Incidence of antibiotic course prescription was 2.95 courses per PYO (95% CI 2.59-3.37). Course duration ranged from 3 to 13 days, with the majority (212; 95%) being five days. The commonest antibiotic prescribed was amoxicillin (Appendix 46). In addition, three participants received daily prophylactic co-trimoxazole throughout follow-up for HIV exposure (Appendix 47). Longitudinal prevalence of short-course antibiotic use (i.e. not including co-trimoxazole prophylaxis) was 4.1% (95% CI 3.9-4.4%; antibiotics taken on 1,130 of 27,458 person days of observation). Longitudinal prevalence of any antibiotic use (short-course and long-term co-trimoxazole prophylaxis for HIV exposure) was 7.1% (95% CI 6.8-7.4%; antibiotics taken on 1,961 of 27,458 person days of observation). These figures do not include daily nevirapine

prophylaxis taken by three HIV-exposed participants until receipt of their negative HIV PCR results at 10, 12 and 12 weeks of age (Appendix 47). No other antivirals or anthelminthics were taken (Kenyan children receive community-wide treatment with mebendazole at one year of age: after participants exited follow-up).

Incidence of antibiotics, non-malarial fever and breathing difficulty increased with age

Incidence rates of breathing difficulty, fever without concurrent slide microscopy-evident parasitaemia and antibiotic prescription appear to rise from zero to six integer months of age before falling briefly until seven to eight months then rising again to nine months (Figure 48). Incidence of diarrhoea and of fever with concurrent parasitaemia are both comparatively low, with an age-related pattern more difficult to discern.

Figure 48: Incidence rates by age for breathing difficulty, diarrhoea, fever and antibiotics.
Rates are unadjusted and estimated by integer age category. Error bars indicate 95% confidence interval. Parasitaemia was diagnosed by slide microscopy.



Seeking primary care was common; referral to secondary care was rare

93 participants sought primary care on one or more occasions during follow-up, totalling 442 episodes and resulting in an incidence of 5.88 episodes per PYO (95% CI 5.36-6.45). 43 further visits were made – either spontaneously or upon clinician invitation – as a follow-up to an initial visit, to check on progress of the same original acute illness. Some primary care visit events resulted in more than one diagnosis. Most frequent diagnoses were upper and lower respiratory tract infection, malaria, fever of unknown focus and skin infection (Appendix 45).

Incidence of referral for inpatient secondary care was very low at 0.07 per PYO (95% CI 0.03-0.16). Five hospital admissions occurred amongst four participants. Indications for hospital admission were: severe pneumonia (n=2), complicated SAM with severe pneumonia (n=1), SAM (n=1) and facial burn (n=1). The two SAM admissions occurred to the same one participant described above.

Adherence to WHO exclusive breastfeeding recommendations was poor

Amongst the 90 participants who did not exit follow-up early, median age at first introduction of complementary foods (excluding prelacteal feeds) was 3.1 months (range 0.7-6.2; interquartile range 2.3-4.3). Amongst all 99 participants, with the exception of 18 participants who received prelacteal feeds before the first breastfeed and one participant who received a solution of sugar and salt in water on one occasion at 25 days of age, all participants consumed nothing but breastmilk during the first 28 days of life. After then, first introduction of complementary foods occurred with no inflection point by age until just beyond six months (Figure 49). Of the 95 participants who completed follow-up to at least six months of age, only three exclusively breastfed continuously without break until six months (3.2%; 95% CI 0.7-9.0%). After first introduction of complementary foods, many participants reverted to receiving nothing but breastmilk for short periods of time although this was rare after six months of age (Figure 50).

Figure 49: Kaplan-Meier survival curve for continuous exclusive breastfeeding status since birth amongst 99 cohort participants.
18 participants received prelacteal feeds. These are nevertheless defined as exclusively breastfeeding for the purposes of this figure. Red dotted line marks six months of age.

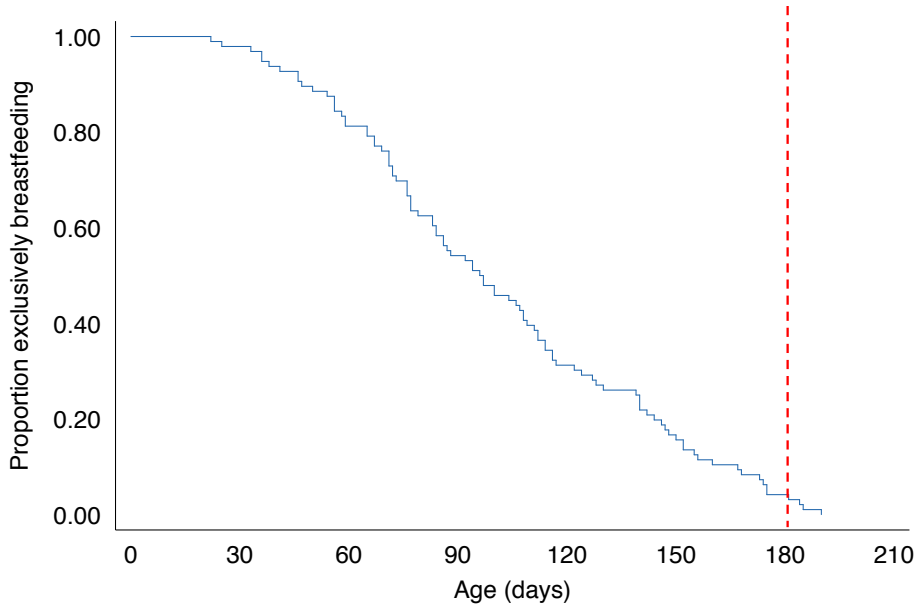
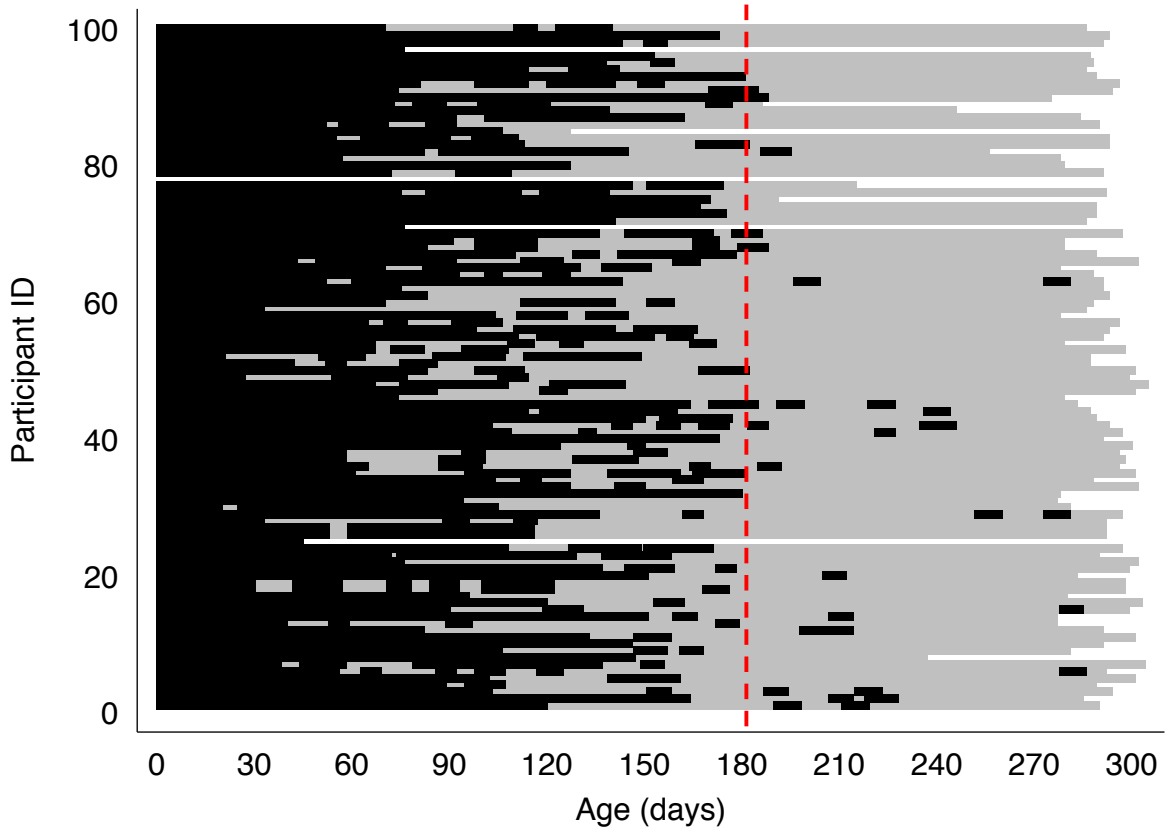
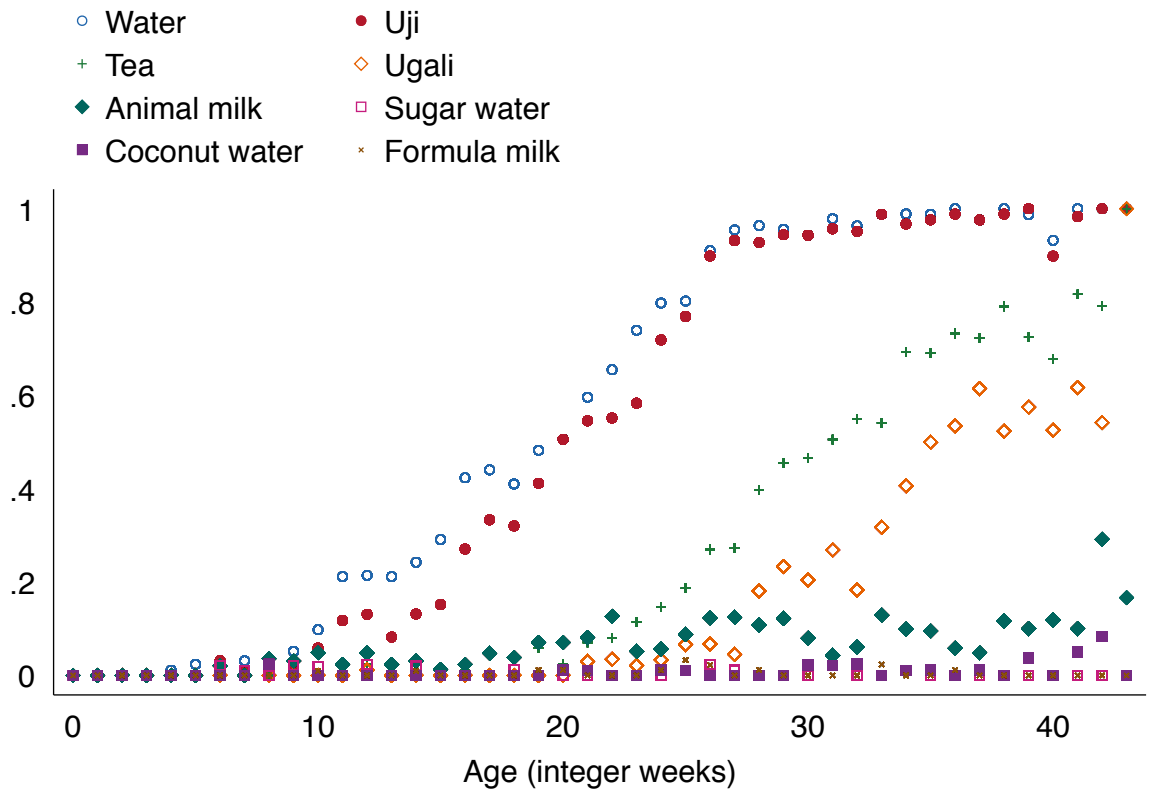


Figure 50: Exclusive breastfeeding status by day of age amongst 99 cohort participants.
Black lines indicate that the participant was reported to have received only breastmilk on that day. Grey lines indicate that complementary foods were given on that day. Total line length differs by participant due to varying duration of follow-up. Vertical red dashed line indicates six months of age.



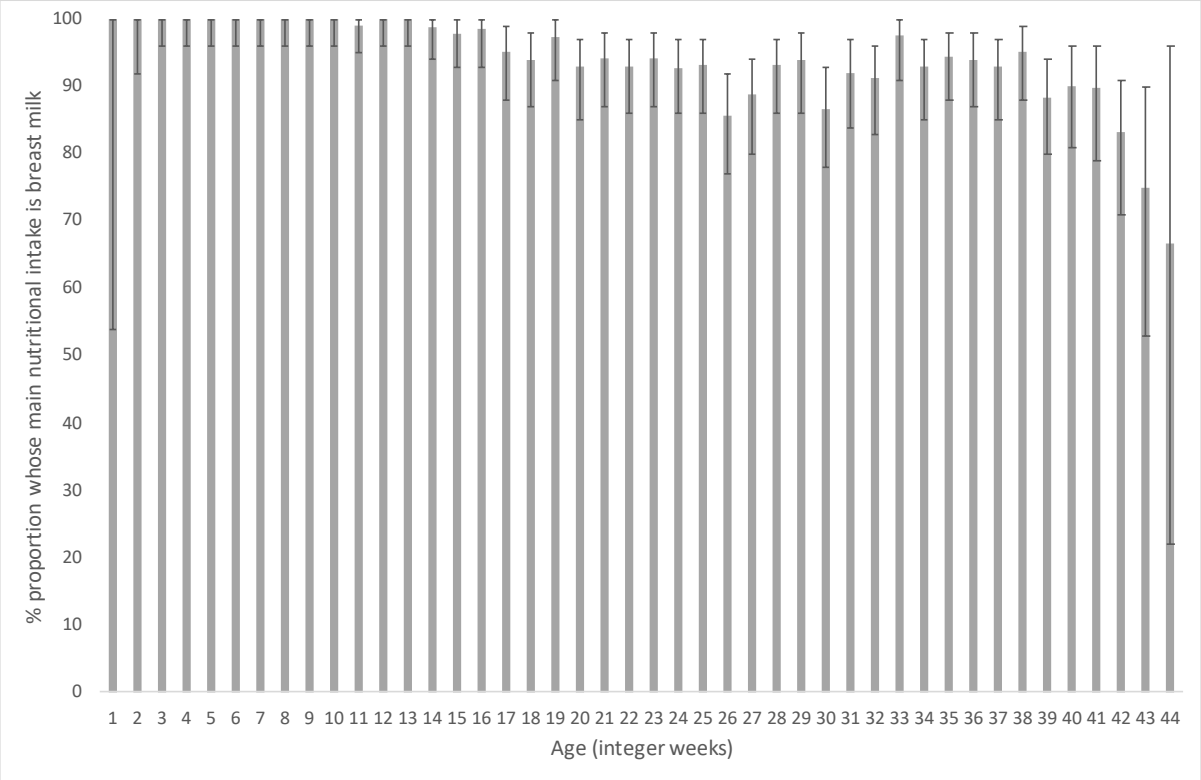
From four weeks of age onwards, the earliest commonly reported solid or liquid other than breastmilk was water, followed closely by 'uji' (a local fermented cereal prepared with warm water) (Figure 51). Then from around 18 weeks of age onwards, tea was introduced, followed from around 20 weeks onwards by 'ugali' (maize porridge prepared with hot water) and, to a lesser extent, animal milk. Other liquids (sugar water, coconut water, formula milk) were occasionally given. A further group of liquids (sugar and salt water) and solids (potato, rice, RUTF, fruit) were reported on fewer than five occasions per item. No other solids or liquids were reported.

Figure 51: Proportion reporting consumption of solids and liquids other than breastmilk by age in integer weeks.
Omitted from figure due to low numbers: sugar and salt water, potato, rice, fruit, RUTF.



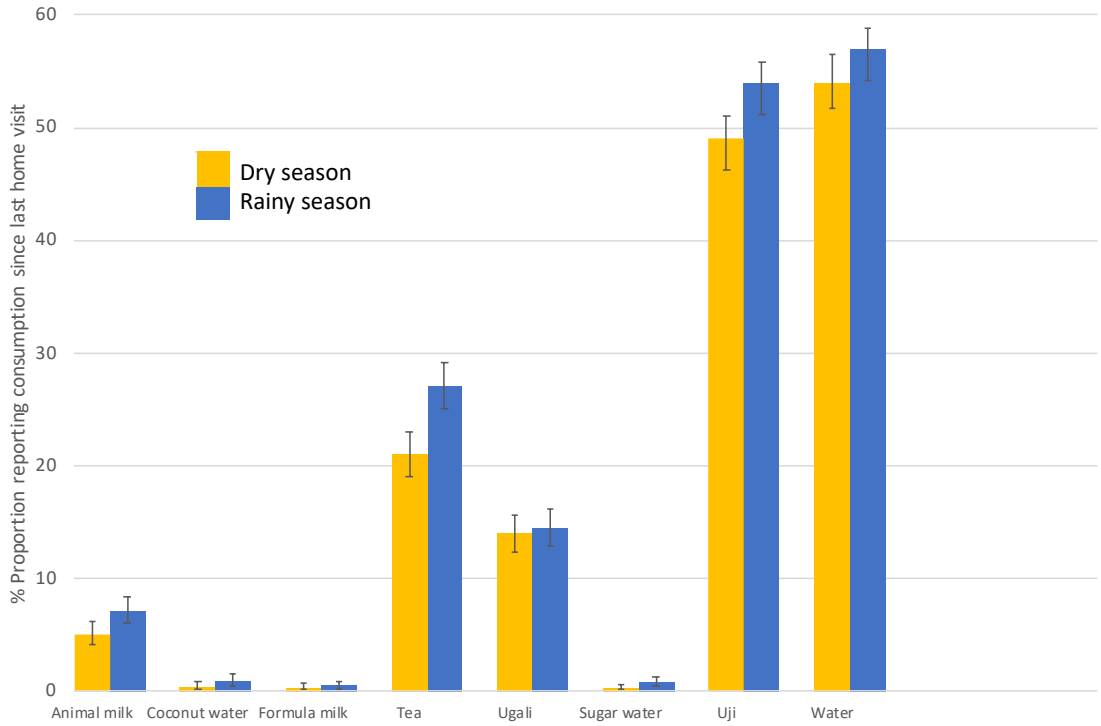
The proportion of participants whose main oral intake was breastmilk remained very high until 12 weeks of age but did not start to drop significantly until 40 weeks - i.e. well beyond six months of age (Figure 52).

Figure 52: Proportion of participants whose main oral intake is breastmilk by age group in integer weeks.



To aid assessment confounding risk in later analyses involving season, a hypothesis that type of food changes with season was tested. Amongst those no longer exclusively breastfeeding, with the exception of a greater proportion of participants consuming tea during the rainy season, unadjusted for age, the type of complementary foods or alternative milks consumed did not change significantly by season (Figure 53).

Figure 53: Proportion reporting consumption of selected complementary foods by season. Peanuts, potato, rice, RUTF and sugar-salt water are omitted due to low numbers. 95% confidence intervals for proportions all overlap except for tea.



Immunisation and vitamin A supplementation

All participants received all primary immunisations as per the Kenya Expanded Programme on Immunisation schedule (Appendix 48).

Kenyan babies should have one vitamin A dose between birth and six months if not breastfed, then another at six months. 61 of 99 participants had no vitamin A dose during follow-up. However, of these, two participants exited follow-up before six months of age and were breastfed so weren't expected to have had a dose during that time. 36 participants had one

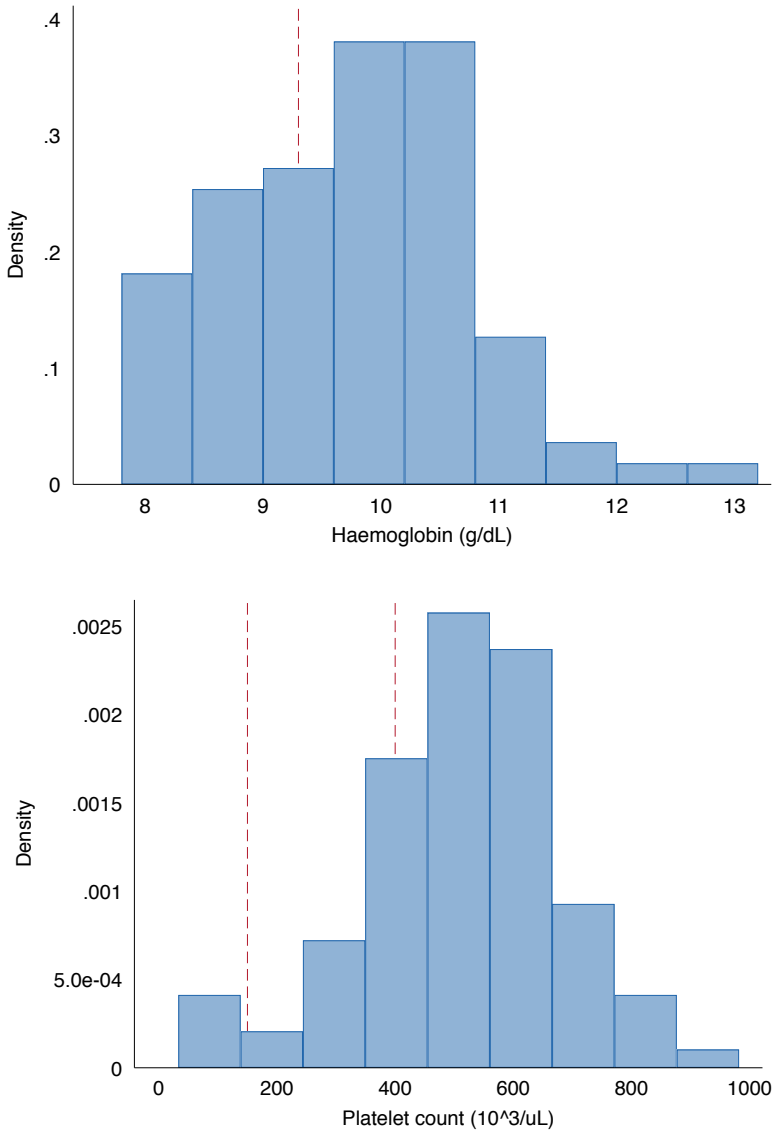
dose during follow-up. Two participants had two doses (both were breastfed so should only have received one).

Anaemia and thrombocytosis were common

By six months of age, 93 participants remained under follow-up. Venous blood samples were obtained from 92 of these (the remaining one participant attended twice but venesection failed). Age at venesection ranged from 5.6 to 6.5 months (median 6.0 months). Of these, 26 (28%; 95% CI 19-39%) met WHO criteria for anaemia(272) with haemoglobin <9.3g/dL (Figure 54). Following local guidelines, those with haemoglobin <8g/dL (31 participants) received 14 days of iron supplementation.

Thrombocytosis (>400,000/μL – a marker of chronic systemic inflammation and also found in iron deficiency anaemia) was found amongst 72 (78%; 95% CI 68-86%) of the 92 participants who underwent venesection (Figure 54).

Figure 54: Frequency distributions of venous haemoglobin and platelet count amongst 92 cohort participants aged 5.6-6.5 months.
Red dotted lines indicate reference normal ranges >9.3g/dL (haemoglobin) and 150-400,000/μL (platelet count) (upper reference range is sometimes 450,000/μL depending on the source). Anaemic participants (Hb<9.3g/dL) were not more likely to have thrombocytosis (>400,000/μL; chi squared test p=0.85).



LM ratio increased with age, largely driven by a decline in mannitol excretion

Of the 990 planned LM tests, 936 were carried out. Two tests were missed (by two participants) due to hospitalisation. One test was missed due to caretaker choice (the participant was undergoing outpatient investigation for severe malnutrition at the time). Five tests were missed (by four participants) due to travel away from the study site. 56 tests were missed due to early withdrawal from follow-up. One test took place after the participant had turned 10 months. 935 tests were therefore subject to further analysis. Of these, results were obtained for 839 (see Chapter 4 for field and laboratory reasons for test failure). Age at first LM test ranged 3-26 days.

Lactulose and mannitol percentage excretion both initially increased until two months of age then decreased until nine months, although mannitol more markedly (Figures 55 and 56). LM ratio increased exponentially with age (Figure 57).

Figure 55: Observed and predicted log-transformed percentage mannitol excretion by age.
Multiple fractional polynomial analysis of log percentage mannitol excretion against age yields a non-linear formula as a superior fit for prediction of log percentage mannitol excretion given age. Where pre-LM urine was obtained, mannitol excretion was adjusted for any presence of mannitol in pre-LM urine.

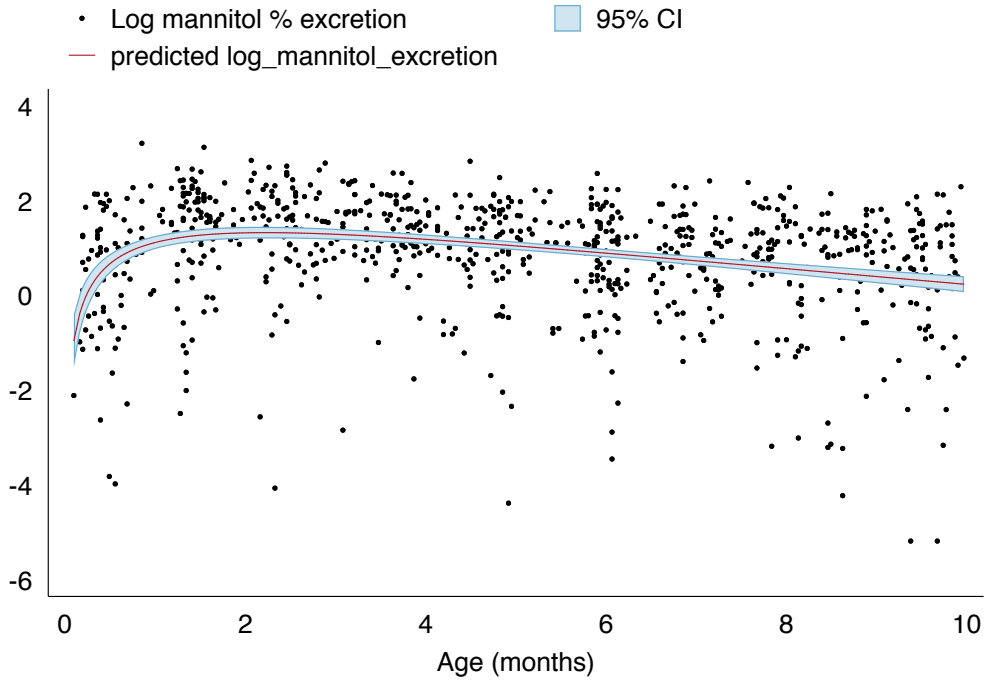


Figure 56: Observed and predicted log-transformed percentage lactulose excretion by age.
Multiple fractional polynomial analysis of log percentage lactulose excretion against age yields a non-linear formula as a superior fit for prediction of log percentage lactulose excretion given age.

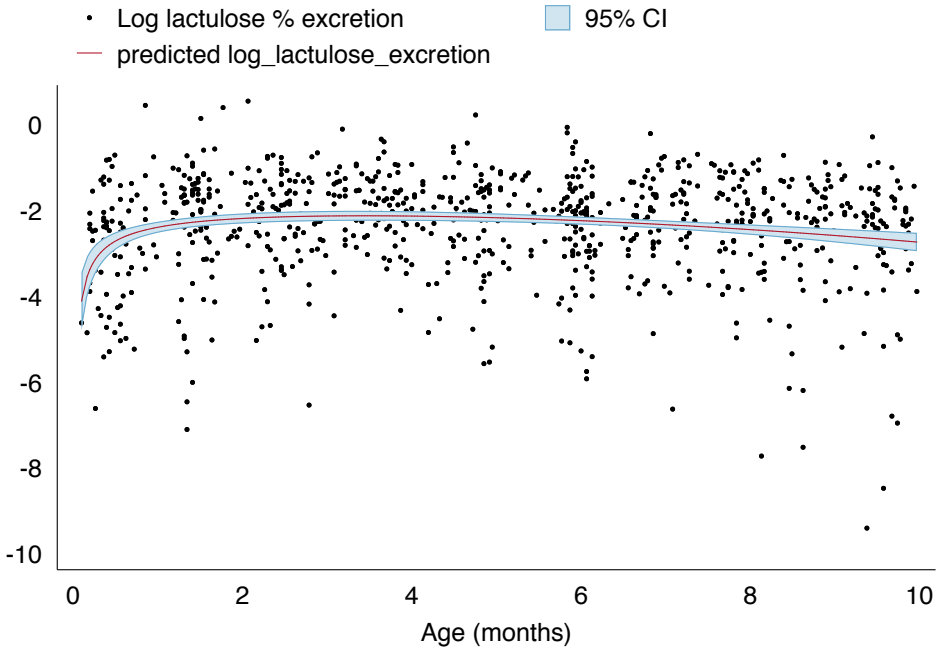
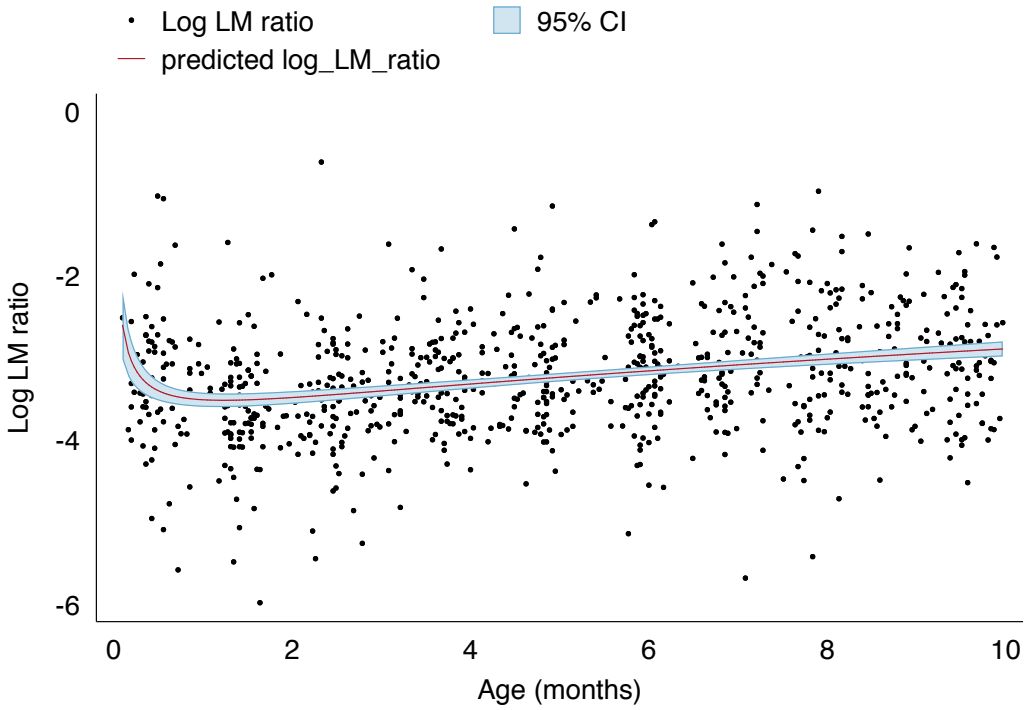


Figure 57: Observed and predicted log transformed LM ratio by age.
Outlying log LM ratio values <-6 are not shown (n=3). Multiple fractional polynomial analysis of log LM ratio against age yields a non-linear formula as a superior fit for prediction of log LM ratio given age.



Univariable linear regression of log transformed LM ratio against age in months with random-effects adjustment for participant shows strong statistical evidence for positive correlation between age and log LM ratio (n=839; groups=99; coefficient=0.0585685; p<0.001). Exponentiation of the coefficient estimated by this model predicts a 6.0% rise in LM ratio with each one-month increase in age (calculation: Appendix 49).

LM ratio trends by individual cohort participant were unstable over time, with outlying high values generally occurring as isolated peaks experienced by many different participants rather than consistently high values amongst a small subset (Figure 58). Trends showed a high degree of temporal concordance within each of the three twin pairs (Figure 59).

Figure 58: Log-transformed LM ratio by age, stratified by cohort participant.

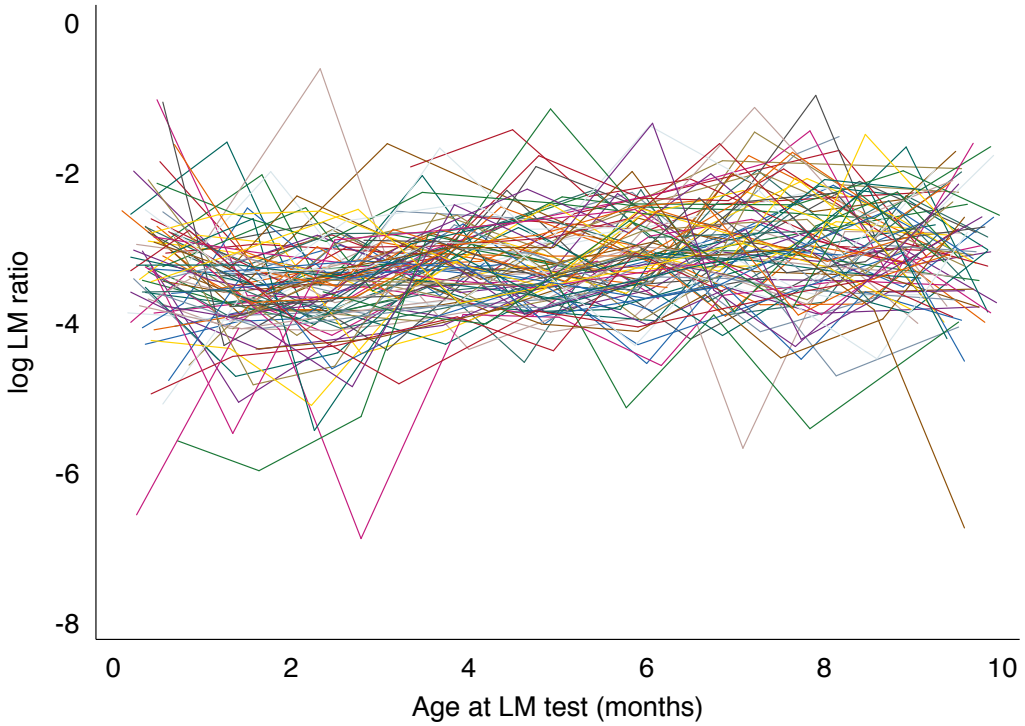
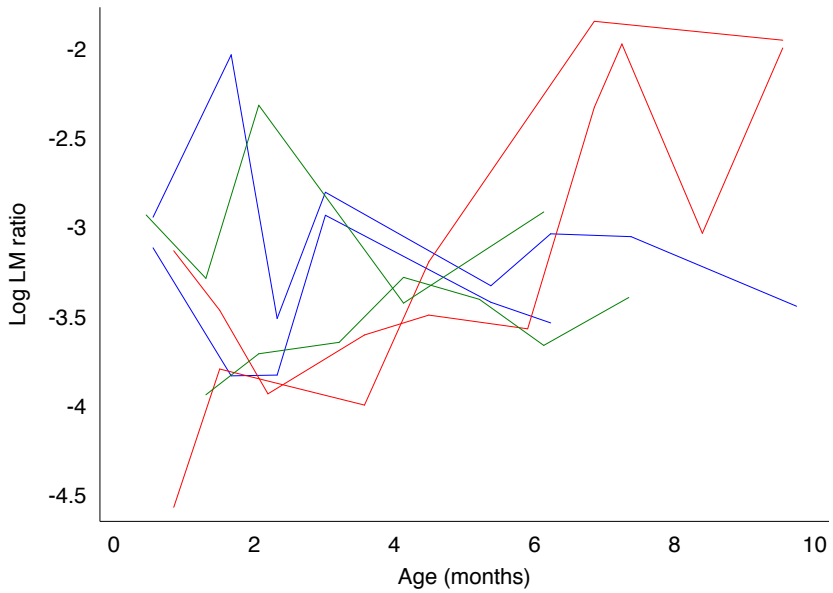


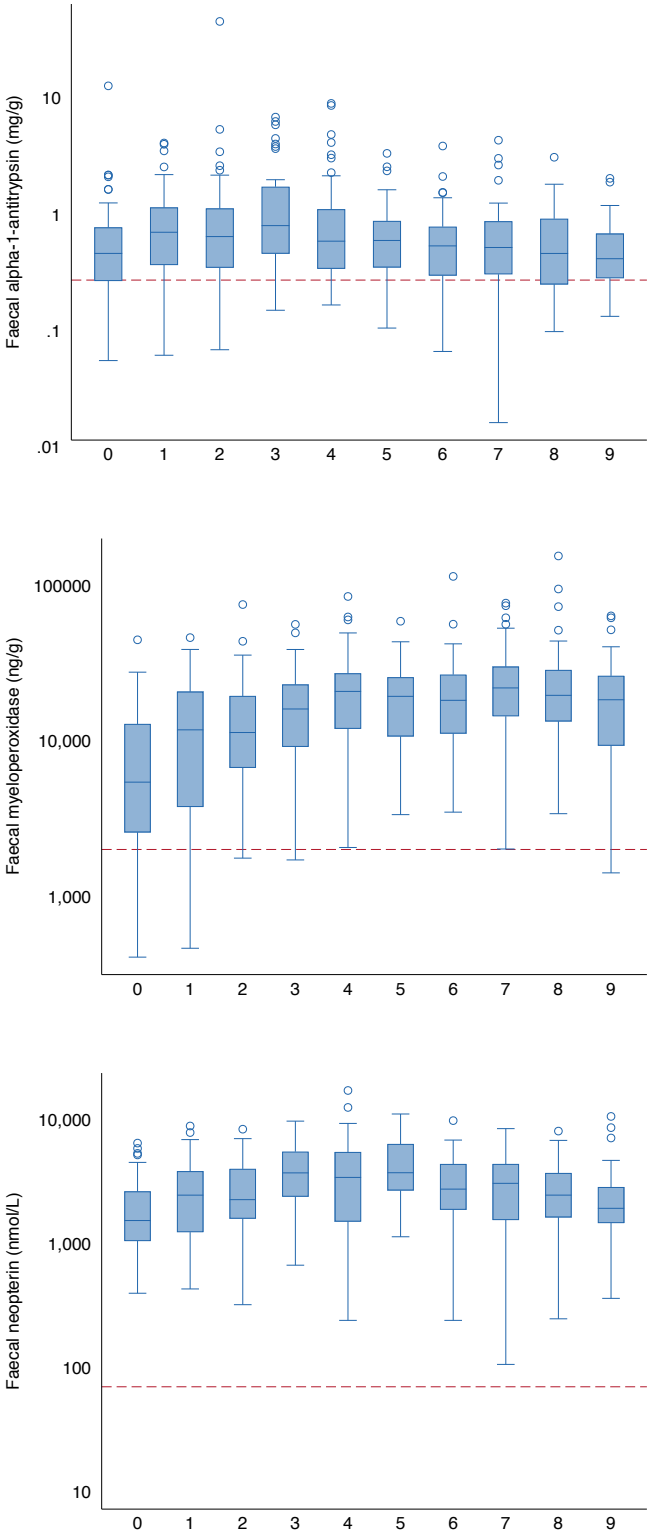
Figure 59: Log LM ratio by age amongst three twin pairs.
Colours indicate twin pairs.



Faecal alpha-1-antitrypsin and neopterin peaked at three to five months whilst myeloperoxidase increased steadily from birth

Of the total 839 successful LM tests, 443 were identified as having been preceded within ≤10 days by one or more stool sample collection dates. These samples were of sufficient volume to obtain results via ELISA for alpha-1-antitrypsin for 432, neopterin for 434 and myeloperoxidase for 434. Faecal alpha-1-antitrypsin peaked at around three to four months of age whilst neopterin peaked at around three to five months and myeloperoxidase showed a slight increase from birth towards a peak at eight months (Figure 60).

Figure 60: Faecal alpha-1-antitrypsin, myeloperoxidase and neopterin by age.
Concentrations of faecal alpha-1-antitrypsin (top; n=432), myeloperoxidase (middle; n=434) neopterin (bottom; n=434) amongst stool samples obtained from 99 cohort participants. Red dotted lines denote threshold normal values (alpha-1-antitrypsin: 0.27mg/g; neopterin: 70nmol/L; myeloperoxidase: 2000ng/g). Note y axis scale is logarithmic. x axes denote age in integer months.

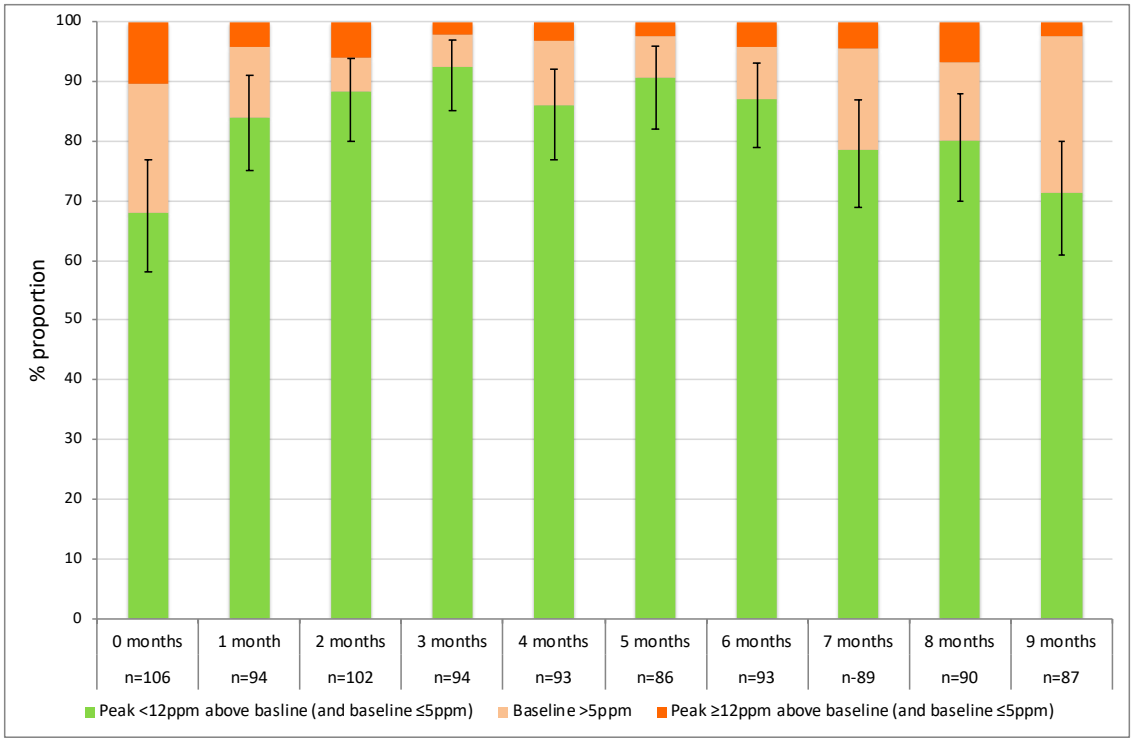


Small intestinal bacterial overgrowth was rare overall, clustered within individuals, and was most common amongst youngest and oldest participants

Of the planned 990, 934 BHTs were undertaken. 45 were missed because of early withdrawal from follow-up. Three were missed due to caretaker choice. One was missed due to temporary travel away from the study area. Five were missed due to hospitalisation. Two were missed due to risk of poor tolerance of the required four-hour fast in a participant recovering from SAM.

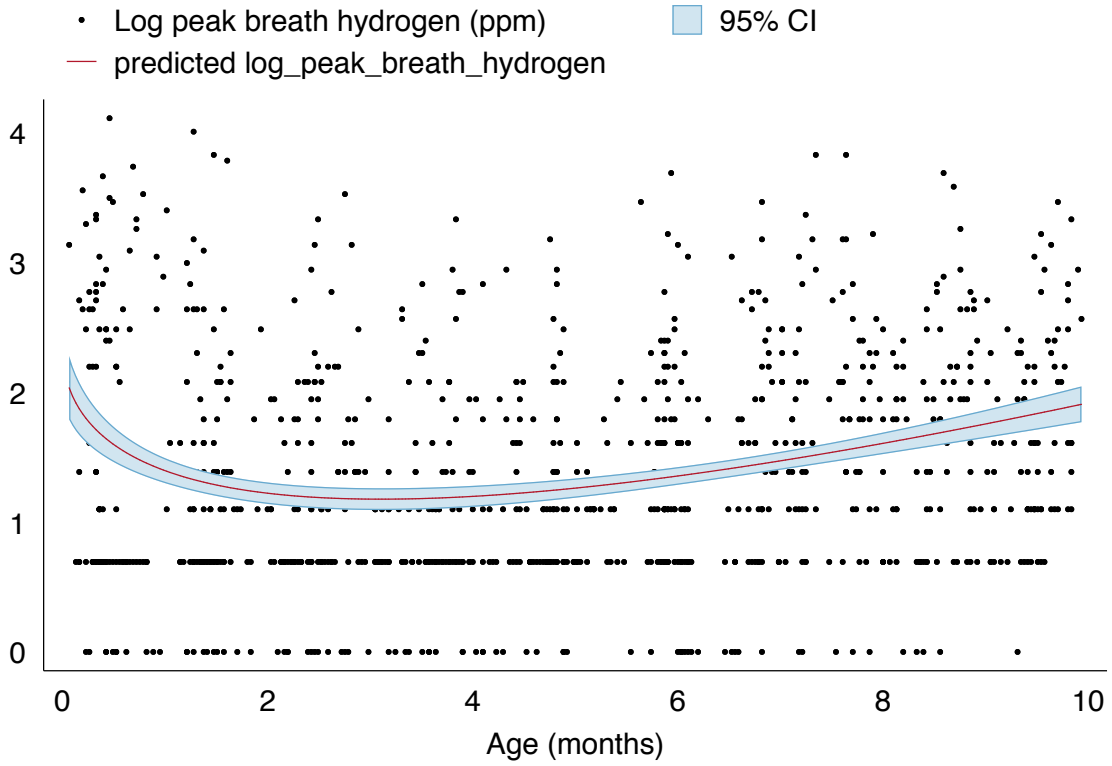
Following the Rome criteria, 763 tests (82%; 95% CI 79-85%) were negative (peak less than 12ppm above baseline), 52 (6%; 4-7) positive (peak 12ppm or more above baseline) and 119 (13%; 11-15) were indeterminate due to high baseline breath hydrogen (>5ppm). The proportion meeting negative Rome criteria appeared to peak at age three to five months (Figure 61). Of the 52 positive tests, 39 were either first test after enrolment or first positive test after a previous negative or indeterminate test. These incident SIBO cases occurred at a rate of 0.52 (95% CI 0.38-0.71) per PYO.

Figure 61: Breath hydrogen test Rome criteria results by age.
Error bars indicate 95% confidence interval for proportion negative.



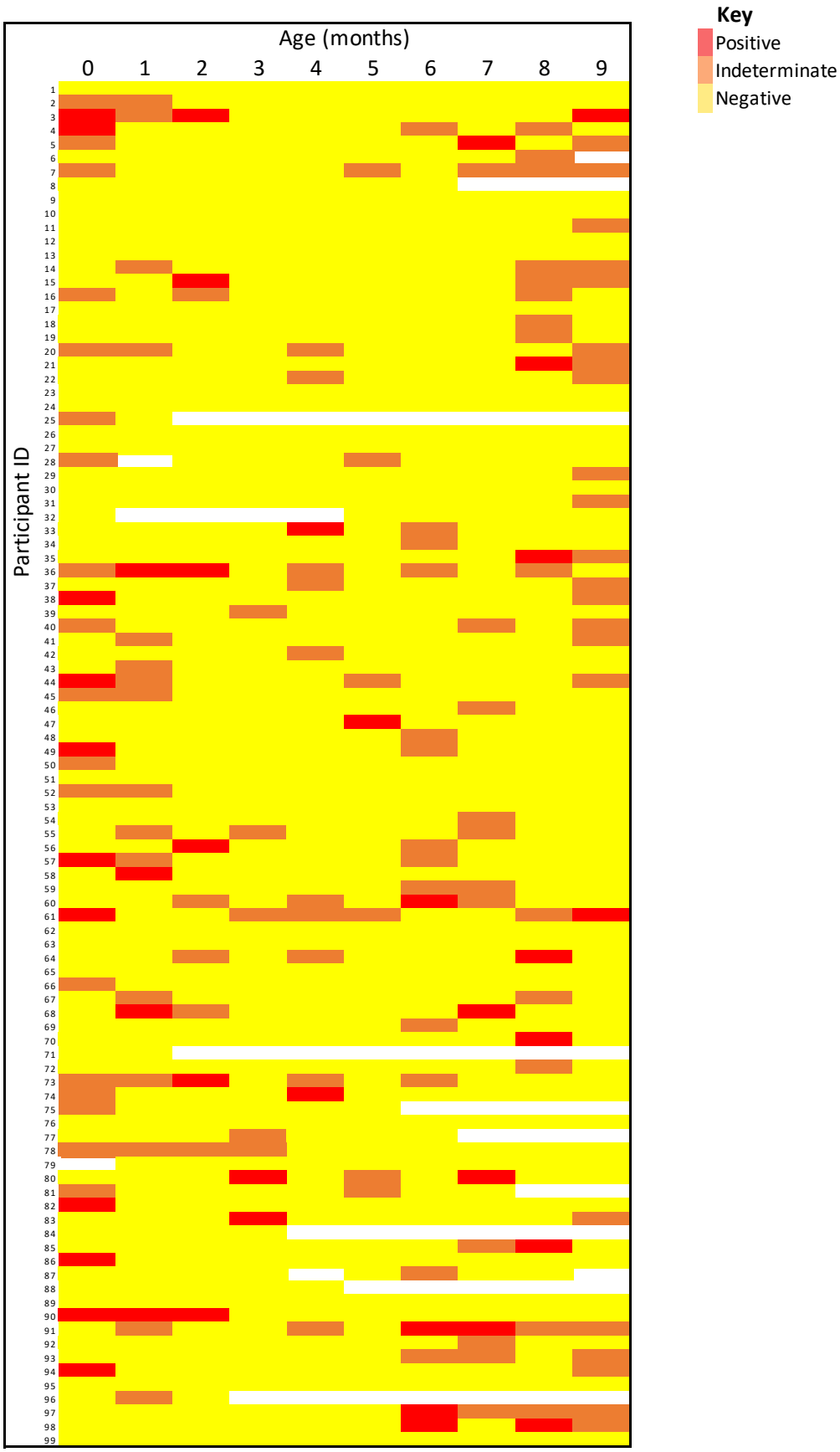
Several alternative continuous-data interpretation methods were compared against Rome criteria categorisation outputs: peak size, peak minus baseline, and AUC. See pages 129-130 for rationale behind the selection of peak size for use in further analyses. Similarly to the Rome criteria results, log peak breath hydrogen appears to be lowest at around two to four months of age, with multiple fractional polynomial analysis confirming a non-linear curve superior fit to linear (Figure 62).

Figure 62: Observed and predicted log-transformed peak breath hydrogen by age.
n=934. Multiple fractional polynomial analysis of log peak breath hydrogen against age yields a non-linear formula as a superior fit for prediction of log peak breath hydrogen given age.



Clustering of positive and indeterminate results was seen within individuals (likelihood ratio test comparing null mixed linear regression models of log peak with and without random-effects adjustment for participant: n=934; $p<0.0001$). Where multiple positive or high baseline BHT results occurred within one individual, those events tended to be consecutive (Figure 63).

Figure 63: Breath hydrogen Rome criteria results by participant and age.
White indicates test not done due to early withdrawal from follow-up, travel away from the study site, participant caretaker's choice or hospitalisation.



The rainy season was characterised by more acute illnesses, healthcare seeking, antibiotics and stunting

All acute illness events, healthcare engagement and antibiotic courses occurred significantly more frequently during the rainy season compared to dry (Table 24). Events with particularly strong evidence for higher incidence during the rainy season were: slide-positive and slide-negative fever, antibiotics and seeking primary care. Amongst nutritional status characteristics, adjusted for age, only stunting shows strong evidence of association with season, with a more negative change in LAZ over time during the rainy season (Table 25). Mean change in LAZ over two months was -0.20 Z-scores (Z test comparison of means 95% CI -0.30 to -0.11) during the rainy season and -0.01 Z-scores (95% CI -0.11 to 0.09) during the dry season: essentially, participants on average experienced a decline in LAZ during the rainy season and no change during the dry season. There was also weak evidence for association of rainy season with higher log LM ratio but not with SIBO or the faecal EED markers.

Table 24: Incidence of acute illness, healthcare use and antibiotic courses by season.
Incidence rate ratios were estimated by Poisson regression with adjustment for age in two-month categories (0-1, 2-3, 4-5, 6-7 and 8-9 months). Avoiding assumption of linearity of association between age and incidence of events, age is included in all models as a categorical variable. Rainy season: November-December 2015, April-July 2016, November-December 2016 inclusive. Dry season PYO = 50.1; rainy season PYO = 25.1.

Event	Number of infants who experienced ≥1 episode	Total episodes	Crude incidence rate (95% CI) per PYO			Incidence rate ratio (95% CI) Rainy versus dry season; adjusted for age	p
			Overall	Dry season	Rainy season		
Diarrhoea[1]	32	38	0.51 (0.37-0.69)	0.35 (0.22-0.57)	0.80 (0.51-1.24)	2.28 (1.20-4.31)	0.01
Breathing difficulty[2]	93	367	4.88 (4.41-5.41)	4.48 (3.88-5.18)	5.36 (4.63-6.19)	1.24 (1.01-1.52)	0.04
Fever, slide negative[3]	84	202	2.69 (2.34-3.08)	2.11 (1.71-2.61)	3.37 (2.81-4.04)	1.75 (1.32-2.32)	<0.001
Fever, slide positive[4]	18	25	0.33 (0.22-0.49)	0.21 (0.12-0.40)	0.61 (0.36-1.03)	2.97 (1.35-6.34)	0.007
Primary care	93	442	5.88 (5.36-6.45)	5.23 (4.58-5.98)	6.67 (5.85-7.60)	1.30 (1.08-1.57)	0.006
Hospital admission	4	5	0.07 (0.03-0.16)	0.02 (0.003-0.13)	0.19 (0.07-0.50)	10.20 (1.14-91.24)	0.04
Antibiotics[5]	82	222	2.95 (2.59-3.37)	2.47 (2.06-2.96)	3.77 (3.11-4.56)	1.53 (1.18-2.00)	0.002

1. ≥3 episodes of loose stool during 24 hours; captured during weekly home visit or at clinic presentation
2. Captured during weekly home visit or at clinic presentation
3. Caregiver recall of fever and/or temperature ≥38.5 on weekly home visit, or clinic visit with fever amongst symptoms, where slide microscopy is negative for *Plasmodium* species
4. Fever as defined above plus positive blood slide microscopy for Plasmodium species; captured during weekly home visit or at clinic presentation
5. IV or oral antibiotics. Antimalarials and antivirals not included. Captured at weekly home visit, clinic and hospital. Excludes three HIV-exposed participants who received long-term prophylactic cotrimoxazole from six weeks of age onwards.

Table 25: Association between season and nutritional status, SIBO and EED.
Five separate mixed-effects linear regression models each examining the association between 1) wasting, 2) stunting, 3) becoming underweight, 4) SIBO or 5) EED with the exposure of interest (season). Each model is also adjusted for age (for SIBO and EED: age on day of breath hydrogen test or LM test; for nutritional outcomes: age at the mid-point of each two-month period; for faecal biomarkers: age at stool sample collection) and sex. Season is defined using the same dates as used for age, where observations with dates failing in the months of April, May, June, July, November and December are classified as rainy season and other observations are classified as dry season. Each model has participant as random effect due to multiple measurements per participant.

Factor	Number of participants	Number of measurement episodes[1]	Median (IQR)	Coefficient Rainy versus dry season	95% confidence interval	p
Wasting[2]	99	732	0.07 (-0.63 to 0.76)	0.10	-0.02 to 0.21	0.10
Stunting[3]	99	732	-0.11 (-0.76 to 0.48)	-0.17	-0.27 to -0.07	0.001
Becoming underweight[4]	99	733	-0.05 (-0.33 to 0.23)	-0.05	-0.10 to 0.01	0.11
SIBO[5]	99	934	1.39 (0.69 to 2.08)	-0.08	-0.18 to 0.03	0.16
Log LM ratio	99	839	-3.26 (-3.69 to -2.82)	0.09	0.00 to 0.18	0.05
Log faecal alpha-1-antitrypsin	97	432	-0.6 (-1.1 to -0.1)	-0.04	-0.13 to 0.22	0.65
Log faecal myeloperoxidase	97	434	9.7 (9.1 to 10.1)	0.08	-0.08 to 0.24	0.31
Log faecal neopterin	97	434	7.9 (7.4 to 8.4)	-0.01	-0.15 to 0.14	0.94

1. For wasting, stunting and becoming underweight, this is the number of two-month measurement pairs. E.g. for a participant who did not skip any measurement episodes and completed follow-up would contribute eight pairs (0-2 months, 1-3, 2-4, 3-5, 4-6, 5-7, 6-8, 7-9). A participant who skipped anthropometry during month 3, for example, would contribute six pairs (0-2, 2-4, 4-6, 5-7, 6-8, 7-9).
2. Change in Weight-for-Length Z (WLZ) score between measurements over the two-month period
3. Change in Length-for-Age Z (LAZ) score between measurements over the two-month period
4. Change in Weight-for-Age Z (WAZ) score between measurements over the two-month period
5. Small Intestinal Bacterial Overgrowth. Log-transformed peak post glucose ingestion breath hydrogen (parts per million)

EED biomarkers and their association with LM ratio: faecal myeloperoxidase most closely predicts LM ratio

In five separate mixed-effects linear regression models, each with random-effects adjustment for clustering by participant and with fixed effect as either log faecal alpha-1-antitrypsin, log faecal myeloperoxidase, log faecal neopterin, log peak breath hydrogen or log breath hydrogen area under the curve – strong evidence for positive association with log LM ratio (derived from LM test undertaken 10 or fewer days after stool sample collection) was found only for myeloperoxidase (Table 26). In a sensitivity model adjusting for age, no associations were found for the five markers (Appendix 50).

Table 26: Association of markers of EED and SIBO with LM ratio.
Five separate mixed linear regression models each with outcome LM ratio and exposure one of: faecal alpha-1-antitrypsin, faecal neopterin faecal myeloperoxidase, peak breath hydrogen, breath hydrogen area under the curve. All factors are log-transformed to achieve normal distribution. To adjust for possible clustering of data and associations arising from multiple observations per participant, participant is included as a random effect in all three models.

Factor	n	Participants	Median (IQR)	Coefficient	95% CI	p
Log faecal alpha-1-antitrypsin (mg/g)	418	97	-0.6 (-1.1 to -0.1)	-0.01	-0.09 to 0.06	0.69
Log faecal myeloperoxidase (ng/g)	420	97	9.1 (9.7 to 10.1)	0.13	0.05 to 0.21	0.001
Log faecal neopterin (nmol/L)	420	97	7.9 (7.4 to 8.4)	0.03	-0.06 to 0.01	0.49
Log peak breath hydrogen (ppm)	837	99	1.4 (0.7 to 2.1)	0.02	-0.04 to 0.07	0.54
Log breath hydrogen area under the curve (ppm minutes)	837	99	5.4 (4.9 to 6.2)	0.03	-0.03 to 0.08	0.32

EED biomarkers and their association with growth: higher mannitol excretion, higher faecal myeloperoxidase and two measures of SIBO are associated with poorer linear growth

In ten separate mixed-effects linear regression models, each with adjustment for clustering by participant and for LAZ at the beginning of each two-month period of observation, there was strong or very strong evidence for inverse associations between percentage mannitol excretion, peak breath hydrogen and breath hydrogen area under the curve and change in LAZ during the two-month period surrounding the measurement (Table 27). There was strong evidence for positive association between faecal myeloperoxidase and change in LAZ. Amongst the 91 participants aged 5.5 to 6.5 months for whom full blood count results were obtained and anthropometry one month before and one month after, there was no evidence for association of venous haemoglobin, total white blood cell count or platelet count with change in LAZ during the two-month period symmetrically surrounding venesection. In sensitivity models adjusting for age and sex, weak evidence remained only for inverse association between percentage mannitol excretion and change in LAZ and no evidence for the remaining factors (Appendix 51).

Table 27: Association of biomarkers of EED with linear growth.
Factors associated with linear growth amongst 92 cohort participants who provided 330 stool samples 10 or fewer days prior to a successful LM test, and where LAZ data exist for the month prior and month after this LM test. Mixed-effects linear regression with inclusion of participant as a random effect. Outcome is difference in LAZ between one month prior to and one month after the stool-linked LM test.

Factor	n	Participants	Median (IQR)	Coefficient	95% CI	p
Log % lactulose excretion	672	97	-2.1 (-2.9 to -1.5)	-0.01	-0.04 to 0.07	0.66
Log % mannitol excretion	672	97	1.2 (-1.5 to 1.7)	0.11	0.06 to 0.17	<0.001
Log faecal alpha-1-antitrypsin (mg/g)	343	93	-0.5 (-1.1 to 0.0)	0.01	-0.10 to 0.11	0.90
Log faecal myeloperoxidase (ng/g)	345	93	9.7 (9.0 to 10.1)	-0.15	-0.26 to -0.03	0.01
Log faecal neopterin (nmol/L)	346	93	7.9 (7.4 to 8.4)	0.02	-0.11 to 0.14	0.78
Haemoglobin (g/dL)	91	91	9.7 (9.0 to 10.5)	-0.03	-0.21 to 0.16	0.78
White blood cells (x1,000 per μL)	91	91	10.3 (8.5 to 12.4)	-0.04	-0.10 to 0.03	0.25
Platelets (x1,000 per μL)	91	91	534 (410-607)	0.00	0.00 to 0.00	0.80
Log peak breath hydrogen (ppm)	731	97	1.1 (0.7 to 2.1)	-0.09	-0.16 to -0.02	0.01
Log breath hydrogen area under the curve (ppm minutes)	731	97	5.3 (4.9 to 6.2)	-0.09	-0.16 to -0.03	0.006

Putative risk factors for stunting: male sex, lower enrolment weight and higher LM ratio are associated with poorer linear growth

In the first multivariable model, after adjustment for clustering by participant, for LAZ at the beginning of each two-month period of observation and for age, there was strong evidence for an inverse association between change in LAZ and 1) male sex and 2) log LM ratio midway through each two-month period (Table 28). There was strong evidence for positive association between enrolment weight and change in LAZ. There was weak evidence for inverse and positive associations between change in LAZ and slide-negative fever and breathing difficulty respectively. Diarrhoea was a rare event, affecting only 5% of the two-month periods. There was no evidence for associations between change in LAZ and SIBO (peak breath hydrogen), exclusive breastfeeding status, diarrhoea or slide-positive fever. Mother's height was found to be strongly co-linear with enrolment weight so dropped from the model.

Table 28: Association of putative exposures with linear growth.
Factors associated with linear growth amongst infants in Junju, Kilifi. Multivariable mixed-effects linear regression with inclusion of participant (groups=97) as a random effect. n=671. Outcome is difference in LAZ between the month prior to and the month after the index (mid-point) LM test. Diarrhoea, fever and breathing difficulty occurred during the 30 days preceding the index LM test.

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Previous (baseline) LAZ			-0.8 (-7.0 to 3.5)	-0.87	-0.94 to -0.80	<0.001
Age (months)			4.9 (0.8-9.0)	-0.09	-0.13 to -0.06	<0.001
Sex		Female	312 (46)	-	-	0.003
		Male	359 (54)	-0.48	-0.16 to -0.80	
Log LM ratio			-3.3 (-6.9 to -0.6)	-0.14	-0.23 to -0.05	0.002
Log peak breath hydrogen during next day's breath hydrogen test (ppm)			1.1 (0.0-4.0)	-0.04	-0.11 to 0.03	0.28
Exclusive breastfeeding		Yes	202 (30)	-	-	0.83
		No	469 (70)	0.02	-0.16 to0.19	
During last 30 days	Diarrhoea	No	638 (95)	-	-	0.18
		Yes	33 (5)	0.18	-0.08 to 0.43	
	Breathing difficulty	No	386 (58)	-	-	0.04
		Yes	285 (42)	0.13	0.01 to 0.25	
	Fever (slide negative)	No	512 (76)	-	-	0.04
		Yes	159 (24)	-0.16	-0.30 to -0.01	
	Fever (slide positive)	No	648 (97)	-	-	0.45
		Yes	23 (3)	-0.12	-0.44 to 0.19	
Weight at enrolment			3.1 (2.1 to 4.2)	1.21	0.83 to 1.58	<0.001

1. For categorical variables: n (% of total). For continuous variables: median (range).

Exponentiation of the coefficient for log LM ratio in this model yields a predicted 12.8% decrease in difference between LAZ over a two-month period with each 1-unit increase in LM ratio after adjustment for other factors in the model (calculation: Appendix 49). Interpreting this figure, after adjustment for other factors in this model, change in LAZ over two months decreases by a predicted 12.8% with each unit increase in LM ratio derived from an LM test midway through the two months.

Lastly, in a linear regression model of difference in LAZ over each two-month period, adjusted only for baseline LAZ and log LM ratio, 14.9% of variability in change in LAZ is explained by

these two independent variables (adjusted R squared = 0.149). This suggests that 14.9% of observed variability in linear growth in the first nine months of life may be accounted for by EED (as measured by LM ratio).

Putative risk factors for EED: exclusive breastfeeding protects against a higher LM ratio but only during the rainy season

The final four multivariable models were developed with reference to the hypothesised causal framework for aetiology of EED, with all factors proximal to EED retained. The outcome of these multivariable mixed-effects linear regression models were LM ratio, faecal alpha-1-antitrypsin, myeloperoxidase and neopterin respectively. Participant was included as a random effect as with previous models. Age was again included in continuous months as a linear factor. Exclusive breastfeeding status was treated as irreversible: excluding prelacteal feeds, once first complementary foods had been introduced a participant was no longer exclusive breastfeeding. Testing an *a priori* assumption that season may modify the association between exclusive breastfeeding status and EED, likelihood ratio test for interaction was carried out comparing models with and without interaction terms between these two independent variables. For the outcome log LM ratio, strong evidence (p=0.01) existed for interaction, so the interaction terms were retained, and coefficients and p-values reported for the effect of exclusive breastfeeding status stratified by season. In this model, adjusted for other factors, there was strong evidence for association between older age and higher LM ratio, and also for association between cessation of exclusive breastfeeding and

higher LM ratio during the rainy season only (Table 29; Figure 64). There was no evidence for interaction for the other three models.

Adjusted for other factors, very strong evidence existed for positive association between complementary feeding and higher log faecal myeloperoxidase and neopterin (Tables 31 and 31) but not for alpha-1-antitrypsin (Table 30).

There was no evidence to support association between SIBO (log peak breath hydrogen) and EED in any of the models.

Given the strong association between exclusive breastfeeding status and age, to assess risk of residual confounding by age, sensitivity models were run where the dependent variable was not linearly associated with age. These were myeloperoxidase and neopterin. Age was categorised as binary (<4 months: associated with lower log myeloperoxidase and neopterin; ≥4 months: higher). For neopterin, weak evidence was seen for association with exclusive breastfeeding status, whilst for myeloperoxidase no appreciable change in model output was seen (Appendix 52). Additionally, model residuals were predicted and plotted against age. For all four outcomes, residuals were not associated with age and appeared to be normally distributed both overall and within age strata (Appendix 53).

In sensitivity models defining exclusive breastfeeding status as a reversible state (a frequent occurrence, as already demonstrated in Figure 50), strong evidence was seen for association between short-term exclusive breastfeeding status and faecal myeloperoxidase but none of the other EED biomarkers (Appendix 54).

Table 29: Association of putative exposures with EED (log LM ratio).
Factors associated with EED (log LM ratio) amongst 99 cohort participants. Multivariable mixed-effects linear regression with inclusion of participant (n=99) as a random effect. n=837. There is strong evidence for modification by season of the effect of breastfeeding on log LM ratio (likelihood ratio test for interaction: p=0.04).

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Age (months)			4.8 (0.1 to 10.0)	0.05	0.02 to 0.07	<0.001
Sex		Male	447 (53)	-	-	0.19
		Female	390 (47)	0.11	-0.05 to 0.27	
Log peak breath hydrogen during next day's breath hydrogen test (ppm minutes)			1.4 (0.0 to 4.1)	-0.01	-0.06 to 0.05	0.85
Dry season	Exclusive breastfeeding		156 (39)	-	-	0.77
	Started complementary foods		245 (61)	0.02	-0.15 to 0.20	
Rainy season	Exclusive breastfeeding		128 (29)	-	-	0.01
	Started complementary foods		308 (71)	0.22	0.05 to 0.40	
WASH principal component analysis first component score			-0.1 (-2.9 to 3.4)	0.02	-0.04 to 0.08	0.60
During 30 days preceding LM test[2]	Antibiotics	No	626 (75)	-	-	0.11
		Yes	211 (25)	-0.10	-0.22 to 0.02	
	Diarrhoea	No	802 (96)	-	-	0.29
		Yes	35 (4)	0.12	-0.10 to 0.35	
	Breathing difficulty	No	505 (60)	-	-	0.51
		Yes	332 (40)	-0.04	-0.15 to 0.07	
	Fever (slide negative)	No	649 (78)	-	-	0.97
		Yes	188 (22)	0.00	-0.12 to 0.13	
	Fever (slide positive)	No	812 (97)	-	-	0.84
		Yes	25 (3)	-0.03	-0.31 to 0.25	
	Iron supplementation	No	792 (95)	-	-	0.63
		Yes	45 (5)	-0.05	-0.25 to 0.15	

1. For categorical variables: n (% of total). For continuous variables: median (range).
2. Where LM test took place at <30 days of age: between birth and LM test

Figure 64: Model-predicted log LM ratio over calendar time stratified by irreversible exclusive breastfeeding status.
Blue circles and red line = exclusive breastfeeding. Green crosses and yellow line = complementary foods introduced. Note higher predicted log LM ratios during the first two rainy seasons but only amongst those no longer exclusively breastfeeding (during the third rainy season all participants had ceased exclusive breastfeeding so no comparison could be made).

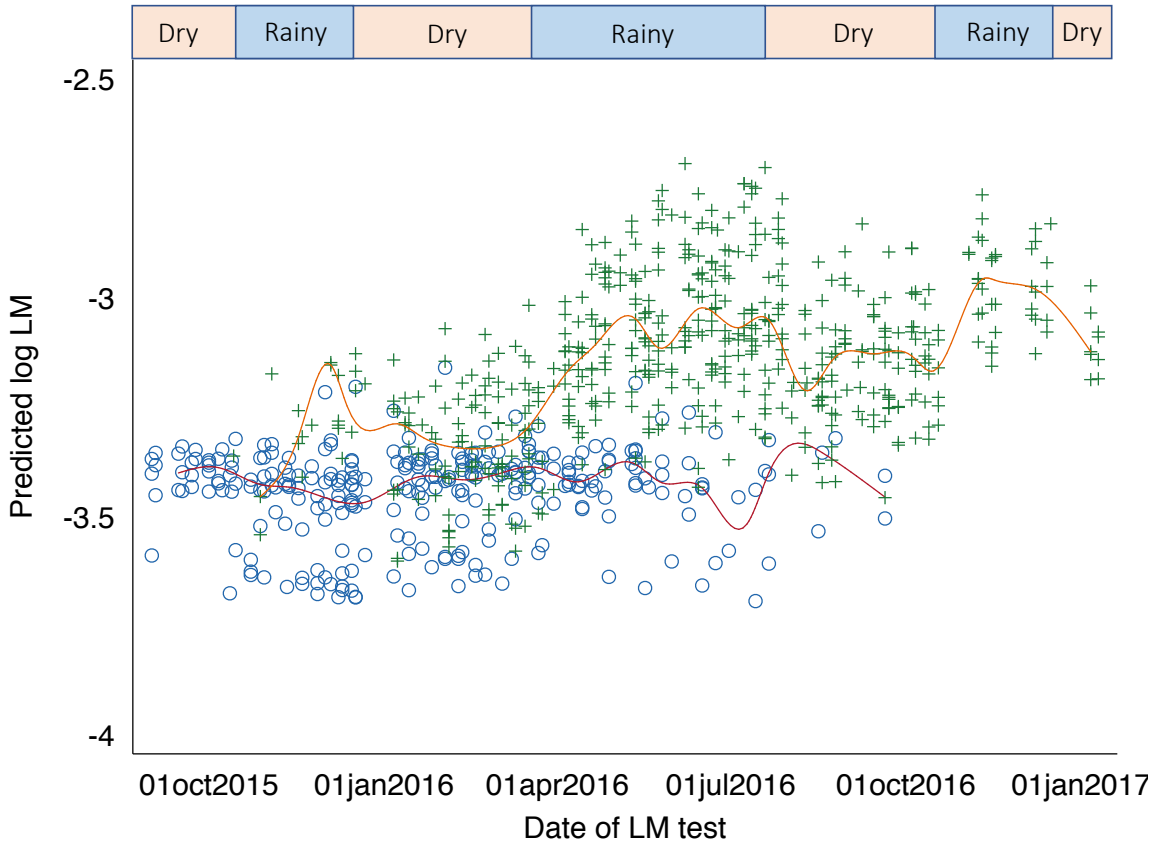


Table 30: Association of putative exposures with EED (log faecal alpha-1-antitrypsin).
Factors associated with EED (log faecal alpha-1-antitrypsin) amongst 97 cohort participants. Multivariable mixed-effects linear regression with inclusion of participant as a random effect. n=431. There is no evidence for modification by season of the effect of exclusive breastfeeding status on log faecal alpha-1-antitrypsin (likelihood ratio test for interaction: p=0.41).

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Age (months)			5.2 (2.5 to 7.5)	-0.05	-0.10 to 0.00	0.04
Sex		Male	235 (55)	-	-	0.77
		Female	196 (45)	-0.03	-0.22 to 0.16	
Log peak breath hydrogen during next day's breath hydrogen test (ppm minutes)			1.4 (0.7 to 2.1)	0.07	-0.03 to 0.17	0.20
Started complementary foods?		No	149 (35)	-	-	0.92
		Yes	282 (65)	0.02	-0.27 to 0.30	
Season		Dry	218 (50)	-	-	0.47
		Rainy	213 (50)	0.06	-0.11 to 0.24	
WASH principal component analysis first component score			-0.1 (-1.1 to 0.9)	-0.06	-0.13 to 0.02	0.12
During 30 days preceding stool sample	Antibiotics	No	320 (75)	-	-	0.76
		Yes	111 (25)	-0.04	-0.27 to 0.20	
	Diarrhoea	No	413 (96)	-	-	0.86
		Yes	18 (4)	0.04	-0.40 to 0.48	
	Breathing difficulty	No	256 (59)	-	-	0.10
		Yes	175 (41)	0.17	-0.03 to 0.38	
	Fever (slide negative)	No	332 (77)	-	-	0.07
		Yes	99 (23)	-0.23	-0.48 to 0.02	
	Fever (slide positive)	No	419 (97)	-	-	0.82
		Yes	12 (3)	-0.07	-0.63 to 0.49	
	Iron supplementation	No	416 (97)	-	-	0.12
		Yes	15 (3)	0.06	-0.41 to 0.53	

1. For categorical variables: n (% of total). For continuous variables: median (range).

Table 31: Association of putative exposures with EED (log faecal myeloperoxidase).
Factors associated with EED (log faecal myeloperoxidase) amongst 97 cohort participants. Multivariable mixed-effects linear regression with inclusion of participant (n=99) as a random effect. n=434. There is no evidence for modification by season of the effect of exclusive breastfeeding status on log faecal myeloperoxidase (likelihood ratio test for interaction: p=0.13).

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Age (months)			5.2 (2.5 to 7.5)	0.04	0.00 to 0.09	0.05
Sex		Male	237 (55)	-	-	0.56
		Female	197 (45)	0.06	-0.14 to 0.27	
Log peak breath hydrogen during next day's breath hydrogen test (ppm minutes)			1.4 (0.7 to 2.1)	0.01	-0.08 to 0.10	0.90
Started complementary foods?		No	150 (35)	-	-	<0.001
		Yes	284 (65)	0.52	0.26 to 0.79	
Season		Dry	219 (50)	-	-	0.33
		Rainy	215 (50)	0.08	-0.08 to 0.23	
WASH principal component analysis first component score			0.1 (-1.1 to 0.9)	0.05	-0.03 to 0.12	0.26
During 30 days preceding stool sample	Antibiotics	No	323 (74)	-	-	0.90
		Yes	111 (26)	0.01	-0.20 to 0.23	
	Diarrhoea	No	415 (96)	-	-	0.78
		Yes	19 (4)	-0.06	-0.45 to 0.33	
	Breathing difficulty	No	258 (60)	-	-	0.33
		Yes	176 (40)	0.09	-0.09 to 0.28	
	Fever (slide negative)	No	335 (71)	-	-	0.36
		Yes	99 (23)	-0.10	-0.33 to 0.12	
	Fever (slide positive)	No	422 (97)	-	-	0.12
		Yes	12 (3)	0.41	-0.10 to 0.92	
	Iron supplementation	No	419 (97)	-	-	0.65
		Yes	15 (3)	0.10	-0.33 to 0.52	

1. For categorical variables: n (% of total). For continuous variables: median (range).

Table 32: Association of putative exposures with EED (log faecal neopterin).
Factors associated with EED (log faecal neopterin) amongst 97 cohort participants. Multivariable mixed-effects linear regression with inclusion of participant as a random effect. n=434. There is no evidence for modification by season of the effect of exclusive breastfeeding status on log faecal neopterin (likelihood ratio test for interaction: p=0.21).

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Age (months)			5.2 (2.6 to 7.5)	-0.03	-0.07 to 0.00	0.07
Sex		Male	237 (55)	-	-	0.22
		Female	197 (45)	0.10	-0.06 to 0.27	
Log peak breath hydrogen during next day's breath hydrogen test (ppm minutes)			1.4 (0.7 to 2.1)	-0.05	-0.13 to 0.03	0.23
Started complementary foods?		No	151 (35)	-	-	0.002
		Yes	283 (65)	0.36	0.13 to 0.59	
Season		Dry	218 (50)	-	-	0.94
		Rainy	216 (50)	-0.01	-0.15 to 0.14	
WASH principal component analysis first component score			0.1 (-1.1 to 0.9)	0.00	-0.06 to 0.07	0.88
During 30 days preceding stool sample	Antibiotics	No	323 (75)	-	-	0.99
		Yes	111 (25)	0.00	-0.19 to 0.19	
	Diarrhoea	No	415 (96)	-	-	0.55
		Yes	19 (4)	0.11	-0.24 to 0.45	
	Breathing difficulty	No	257 (60)	-	-	0.60
		Yes	177 (40)	0.04	-0.12 to 0.21	
	Fever (slide negative)	No	337 (78)	-	-	0.66
		Yes	97 (22)	-0.05	-0.25 to 0.16	
	Fever (slide positive)	No	422 (97)	-	-	0.05
		Yes	12 (3)	0.46	0.00 to 0.91	
	Iron supplementation	No	419 (97)	-	-	0.62
		Yes	15 (3)	0.10	-0.28 to 0.48	

1. For categorical variables: n (% of total). For continuous variables: median (range).

Discussion and conclusions

In this cohort study of 99 infants in rural coastal Kenya, enrolment characteristics confirm the earlier cross-sectional survey results in this same area: this is a very socioeconomically deprived community with many challenging circumstances for child health including poor WASH provision, low dietary diversity and poor food security. Despite this, AM was relatively

rare (8.1% of monthly measurements met AM Z-score criteria), but underweight and stunted statuses were highly prevalent (14.6% and 18.9% respectively). 34% of participants were moderately or severely stunted by exit. This is much higher than the most recent 2014 Kenya DHS proportion amongst age 9-11 months nationally (16.7%) although is likely similar to the equivalent figure for Kilifi County (not calculated by DHS).(210) In the MAL-ED study, prevalence of stunting at birth was 13%, increasing to 41% by 24 months of age (proportion at or near 10 months was not reported).(229) Providing further evidence that stunting is a particular problem for east Africa, the rural Tanzania MAL-ED site demonstrated the highest prevalence of stunting, at 72% by 24 months. Given the known associations of low LAZ, WAZ and WLZ with morbidity and mortality,(1) undernutrition is likely to impact significantly on child health and survival in this coastal Kenyan community.

LAZ declines smoothly from birth to nine months of age with no apparent step-down point. This suggests that whichever risk factors exist for stunting in this age group, and regardless of how long a lag time there may be between their occurrence and resultant stunting, they are occurring (or at least exerting their influence) at a constant rate. One example is cessation of exclusive breastfeeding, which in this cohort occurs between one and six months of age also with no inflection point.

Incidence rates of diarrhoea and slide-positive fever were low (0.51 and 0.33 per PYO respectively; diarrhoea longitudinal prevalence 0.5%). In comparison, using the same definition of diarrhoea as three or more loose stools during 24 hours, incidence amongst infants in a low-income area of urban Bangladesh was much higher at 4.7 per PYO.(273) Adding a minimum two diarrhoea-free days between episodes to the definition of diarrhoea,

the MAL-ED study found an longitudinal prevalence of diarrhoea of 1% amongst under-twos.(274) Using a more restrictive definition, the Global Enteric Multicentre Study found an incidence of moderate-to-severe diarrhoea (three or more loose stools during 24 hours; following a diarrhoea-free period of seven days or more; plus signs of dehydration, hospitalisation or dysentery) amongst infants at its western Kenya site of 0.51 episodes per PYO.(275) Although historically implicated in stunting and malabsorption, diarrhoea is not common enough amongst infants in this community to fully explain EED. For instance, only 32% of participants were affected by diarrhoea on one or more occasion whereas 67% had an LM ratio ≥0.07 on one or more occasion. Also, diarrhoea was associated with neither EED nor stunting. The same observation is true for malaria. This observed lack of association may be a result of limited power but is in line with other recent studies.

Incidence rates of slide-negative fever and breathing difficulty were higher overall (2.69 and 4.88 respectively per PYO) and increased with age. There was weak evidence to suggest slide-negative fever as a risk factor for stunting and breathing difficulty as protective against stunting. Caregiver reported breathing difficulty could be a proxy for increased caregiver attention in general (breathing difficulty was most often diagnosed by the study clinician as a very minor upper respiratory tract infection not requiring antibiotics), thus participants whose caretakers reported breathing difficulty more frequently may have been experiencing a more optimal home environment in general. Acute febrile illness could truly be a risk factor for stunting. However, the weak nature of the statistical evidence for both of these factors suggests that these observed associations are more likely to be the result of measurement error, stochastic variation and/or multiple comparisons. Acute illnesses were not associated with stunting in the MAL-ED study.(89)

Longitudinal prevalence of antibiotics was very high at 7.1%. This figure could be an overestimate of true population prevalence because of the extra contact cohort participants had with healthcare staff and facilities. Antibiotic courses were not associated with EED. This is hopefully a true interpretation, since any potential confounding by health status (participants experiencing greater frequency of acute illness received antibiotics more often) that could have led to type II error was hopefully mitigated by inclusion of acute illnesses in the multivariable model.

Season was extensively influential. All acute illnesses and antibiotics were more common during rainy season. There is also strong evidence for association of rainy season with stunting and weak evidence for association with EED (although not with wasting or becoming underweight and SIBO). Additionally, in the final multivariable model described, season modifies the effect of the only factor with strong evidence for association with EED: introduction of complementary foods. Put differently, there is strong evidence that exclusive breastfeeding is protective against a seasonal change in LM ratio (increase during the rainy season). A true association between rainy season and wasting likely exists but has not been detected in this study due to lack of power: wasting was a rare outcome. Mean change in LAZ was close to zero during the dry season and negative during the rainy season. This means that on average almost all of the linear growth faltering that occurs during the first ten months of life takes place during the rainy season.

There was very poor adherence to WHO exclusive breastfeeding guidelines. Excluding prelacteal feeds, only 3.2% of participants still under follow-up at 6 months exclusively

breastfed from birth to that time. However, the proportion of participants for whom breastmilk was their main nutritional intake remained very high well beyond six months of age. Continuation of breastfeeding alongside complementary foods – even where breastmilk remains the main food – does not therefore appear to protect against EED.

LM ratio increased exponentially with age, increasing by a predicted overall 6.0% every month with no apparent inflection point in rate of increase by age. This is in line with other published findings and suggests that EED risk factors are environmental and intensify with age during infancy. A univariable model of LAZ and LM ratio suggests that 14.9% of the variability observed in linear growth may be accounted for by changes in LM ratio. This is a not insignificant proportion.

Within individuals, LM ratio is unstable over time. This suggests either that the measurement tool (LM test) is inaccurate or, hopefully more likely given the reassuring results of LM test quality assessment (see Chapter 4), gut permeability and absorptive capacity are in rapid flux, likely in response to changing environment.

SIBO is relatively rare overall, and rarest amongst those aged 3-6 months. The few definitively positive cases seen did persist within individuals over time. This clustering by participant and over time suggests that the BHT may have yielded reproducible results both over time and between participants. SIBO (both measured by peak breath hydrogen and area under the curve) is associated in univariable models with poorer linear growth but not with LM ratio or season. However, these associations disappear when adjusted for age. Also, in multivariable models, SIBO is associated with neither stunting nor EED. This suggests that the variability

captured by the hydrogen breath test in this study is perhaps not clinically significant. For full discussion of possible sources of bias and measurement error in the breath hydrogen test see Chapter 3.

Faecal alpha-1-antitrypsin and neopterin peaked at around 3-5 months and did not appear to be associated with concurrent LM ratio or linear growth. Faecal myeloperoxidase peaked at around eight months and demonstrated strong evidence for association with both higher LM ratio and poorer linear growth, although evidence for both these associations weakened after adjustment for age. Poorer linear growth was also very strongly associated with lower mannitol excretion (lower gut absorptive capacity; decreased to weak evidence for association after adjustment for age). No association was seen between linear growth and lactulose excretion (gut permeability), alpha-1-antitrypsin, neopterin, haemoglobin, white blood cell count or platelet count. In conclusion, although it is much more expensive and time consuming, LM ratio – specifically mannitol excretion – appears superior to faecal ELISA in predicting concurrent linear growth in this setting.

In a multivariable hypothesised causal framework model, stunting (change in LAZ over each two-month period) is strongly and positively associated with older age, male sex, lower enrolment weight and EED (higher LM ratio). Of note, strong associations do not exist for diarrhoea (or any acute illness), exclusive breastfeeding status and SIBO. Lack of power may account for some of these observations. Male sex has previously been found to be a risk factor for stunting. The WHO 2016 growth reference standards were developed in optimal conditions and are already sex-specific. Males in this and other studies may therefore have responded more poorly to suboptimal conditions for growth. Many of these results are

reflected in the MAL-ED cohort study: risk factors for stunting at 24 months were lower birth WAZ, shorter maternal height, higher number of enteropathogens in non-diarrhoea stool, lower SEP and lower proportion of energy in food derived from protein.(229) These findings did not vary significantly between the eight sites across South America, sub Saharan Africa and South Asia, including one site in nearby rural Tanzania. Risk factors included in the MAL-ED analysis but not found associated with stunting at 24 months were: caregiver-reported acute illness including diarrhoea; faecal myeloperoxidase, neopterin and alpha-1-antitrypsin; plasma AGP; and LMZ.

In a multivariable hypothesised causal framework model, higher LM ratio is strongly associated with complementary feeding (i.e. continuous exclusive breastfeeding is protective against EED) but only during the rainy season. This finding is repeated when measuring EED using faecal myeloperoxidase and for neopterin also, although without modification by season. Type of complementary food did not change with season so this cannot explain why season modifies the effect of EBF status on EED. However, the quality of water used to prepare complementary foods could explain. These findings are further discussed in Chapter 6. Strong evidence for association of EED with exclusive breastfeeding status persists only for the model defining EED using myeloperoxidase when using a reversible definition for exclusive breastfeeding status. Assuming causality, this suggests that the effect of first initiation of complementary foods on severity of EED does not subside during subsequent brief periods of return to exclusive breastfeeding.

Note that there is no strong evidence for association of SIBO, WASH, acute illnesses or antibiotics with any of the four measures of EED even in the short term (within each participant, exposures and outcomes were no more than two months apart in time).

In the MAL-ED study, during infancy, EED as characterised by faecal alpha-1-antitrypsin, myeloperoxidase and neopterin (LM ratios were not used) was associated with a greater number of pathogens per stool sample (myeloperoxidase), recent consumption of any breastmilk (myeloperoxidase and alpha-1-antitrypsin) and lower SEP (myeloperoxidase and alpha-1-antitrypsin).(89) Recent acute illnesses, including diarrhoea, were not associated with EED and nor were measures of household water or sanitation. Exclusive breastfeeding status was not included in the MAL-ED analysis.

Changes in LM ratio appeared primarily driven by differences in mannitol rather than lactulose excretion, suggesting greater variability in intestinal absorptive capacity rather than permeability. However, as discussed in Chapter 4, concerns have been raised over the interpretability of mannitol excretion. Repeating key models using percentage lactulose excretion instead of LM ratio to represent EED:

- Lactulose excretion was not associated with linear growth in a multivariable model exploring multiple risk factors for stunting (Appendix 55; results for other risk factors were not significantly affected) whereas LM ratio had been (Table 28);
- There was very strong evidence for an increase in lactulose excretion during the rainy season, adjusted for age and sex (coefficient 0.32; 95% CI 0.16-0.48; $p<0.001$) compared to previous weak evidence for increase in LM ratio (Table 25);

- There was weak evidence for positive association between lactulose excretion and faecal neopterin that persisted with adjustment for age and sex (Appendix 56) whereas there had been strong evidence for positive association between LM ratio and faecal myeloperoxidase that did not persist after adjustment for age and sex (Table 26 and Appendix 50);
- There was weak evidence for inverse association between lactulose excretion and SIBO as measured by both peak breath hydrogen and area under the curve, both of which associations became slightly weaker still after adjustment for age and sex (Appendix 54) compared to no association seen with LM ratio (Table 26);
- Exclusive breastfeeding was no longer found to be protective against a rise in EED during the rainy season (Appendix 57; results for other risk factors were not significantly affected; compare to Tables 29-32).

In this cohort, biologically plausible associations consistent with the hypothesis were observed with rainy season, linear growth and exclusive breastfeeding during the rainy season. If mannitol excretion (and therefore, in the Kilifi cohort, since there is minimal variability in lactulose excretion, the LM ratio) is indeed not a helpful measure of EED, an alternative explanation for the observed association of LM ratio with linear growth could be that the LM ratio here may have approximated intestinal length more than pathology. Since pre-ingestion mannitol is known to be higher during the dry season and with complementary feeding in the Kilifi cohort, mannitol excretion (and thus the LM ratio) could also have been influenced by residual confounding by season or breastfeeding status in the 19% of LM tests where adjustment for pre-ingestion mannitol was not done. To avoid this uncertainty in LM ratio interpretation, the authors of the Malawian model suggest as yet unidentified new,

neutral molecules (lactulose and mannitol are charged) for absorption testing that are solely transported via the pathological 'unrestricted' pathway.

A main strength of this study was its thorough capture of acute illnesses, antibiotics and other events via active and passive case detection. It is unlikely that events were missed since the community is geographically remote meaning few alternative opportunities for alternative healthcare or over-the-counter antibiotic purchase. Also, living within the study area, fieldworkers were familiar and accessible to participants' caretakers. The LM test protocol was well adhered to and results likely an accurate reflection of true gut permeability and absorptive capacity (see Chapter 4).

A limitation of this study was sample size and multiple comparisons. Whilst attention was paid for each individual model to minimise risk of sparsity by limiting parameters according to the total number of observations, no adjustment was made for multiple comparisons.

Amongst the 31 participants with venous haemoglobin <8g/dL at six months of age, the 14-day course of iron supplementation they received may have influenced markers of EED. Mice fed iron-deficient diets show lower levels of intestinal inflammation in response to infection.(276) In a randomised controlled trial amongst six-month-old Kenyan infants, iron supplementation was associated with adverse changes in gut microbiome and higher faecal calprotectin.(277) In Bangladeshi children aged 0-23 months, low serum ferritin was associated with higher faecal alpha-1-antitrypsin but not with myeloperoxidase or neopterin.(278) The study authors suggested that increased gut permeability may lead to intestinal ferritin loss. Interplay between intestinal, bloodstream and body iron presence and

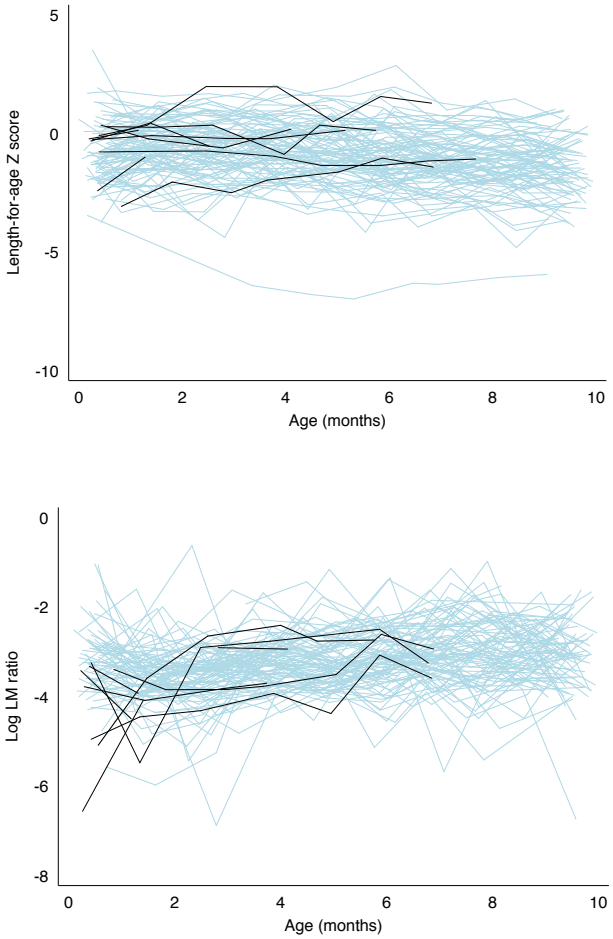
EED is therefore unlikely to be straightforward. The multivariable models exploring factors associated with EED in the cohort study were adjusted for iron supplementation. Iron supplementation was not associated with EED in any model. This observation may have been limited by power: only 5% of LM tests and 3% of stool samples in the multivariable EED models were preceded within 30 days by iron supplementation.

The stunting process is difficult to accurately and precisely measure. Since stunting is a process that occurs over time, it needed to be defined in this study by a difference between two measurements rather than a one-off measurement. Assuming no lag time between cause and effect, a participant who had previously been exposed to conditions that promoted stunting would have a low LAZ. But if their environment changed, they may start to recover and their LAZ increase, although may still be relatively low compared to other participants. If using an outcome of a one-off measurement of LAZ only, lower LAZ could be falsely attributed to those new favourable exposures. A difference in LAZ is therefore the optimum way to describe the stunting process. However, linear growth is slow and length measurement error relatively high in comparison.(271) Between-observer variability in this study was good as measured by the intra-class coefficient but even small length discrepancies are likely to have yielded large differences in LAZ across clinically significant thresholds in this study. Difference in LAZ over a longer time period reduces the relative importance of this measurement error as differences become greater in comparison to the error which remains static. Other studies with greater numbers of participants have used LAZ at exit as the primary measure of stunting. However, summary statistics for exposures and differences in LAZ that occur over a long time (say, 10 months), although minimising measurement error for change in LAZ, risk missing a true association between exposure and

linear growth if that exposure and accompanying growth deficit both occurred over a shorter period within the time frame. A compromise was decided upon with the two-month period of person-time defining observations in models where linear growth was the outcome. One consequence of this choice is that all models using the two-month unit of analysis assume a time lag between exposure and outcome of no longer than two months.

Nine participants exited follow-up early, of whom three were through choice (the other six moved away). For these three, all were due to paternal grandparents' influence, with parents willing to continue but grandparents' wishes followed. Fieldworkers did not enquire about reason for early withdrawal but there had been no specific adverse incidents for these three or indeed any other participant. The main outcomes of interest – LM ratio and LAZ – for the nine early withdrawers were not clearly outlying compared to participants who completed follow-up (Figure 65). This suggests that the likelihood that their early withdrawal led to selection bias is low. Since most of the nine who exited follow-up early withdrew towards the end of the study and not the beginning, resulting in 75.18 person years of follow-up, this represents only a 0.3-10% loss of person time compared to the targeted range of 75.21-83.56 person years of follow-up (based on target exit age range ≥9 to <10 months).

Figure 65: LAZ (top) and log LM ratio (bottom) by age.
Blue lines indicate participants who completed follow-up. Black lines indicate participants who exited follow-up early.



Although efforts were made to identify participants via multiple means so as to avoid selection bias, cohort participants had generally higher SEP compared to the background population. The consent rate was relatively high, and similar to predicted, at 71% of those eligible. Enrolment took slightly longer than predicted (seven instead of six months) because eligible neonates were identified at a slower rate than predicted from the population. This is likely to have occurred because of underestimation of the effect of the local practice of women moving back to their mothers' homesteads to give birth, then returning to their husband's homestead after around a month. These babies missed an opportunity to enrol as they had already exceeded 14 days of age by their return to the study area. However,

anecdotally this practice is equally common across socio-economic and cultural groups within the community so is unlikely to have introduced selection bias.

Nutritional counselling was very common after anthropometry. Infants not participating in this study did not receive this benefit. This could have led to an artificially improved nutritional status of study participants compared to non-participants. However, counselling it is not in excess of national guidelines for growth monitoring. Kenyan national guidelines state that children under the age of one year should have monthly anthropometry with healthcare worker feedback and advice. In any case, studies have shown that counselling alone is rarely effective in improving nutritional status.(279)

Study participants engaged with healthcare staff on a monthly basis. They spent two whole days per month at Junju primary care facility during LM testing and BHT. This, along with free ambulance transfers from home to primary or secondary care for severely unwell participants, meant that some of the usual barriers to healthcare engagement may have been lessened. Incidence of antibiotic prescription may therefore be higher than the background rate, and incidence of non-severe acute illness by passive case detection.

Environmental contamination was not directly measured. This is an important (but assumed) step in the hypothesised pathway between complementary food ingestion and EED. Also, although SIBO was measured, intestinal microbiome was not included in this analysis. Analysis of 16S sequencing data is ongoing and not presented in this thesis.

Of the three HIV-exposed participants, one participant was exclusively breastfed until six months then mixed fed until the end of follow-up. One exclusively breastfed until three months then mixed fed until nine months then stopped breastfeeding altogether at nine months. The last exclusively breastfed until one month, mixed fed until three months then stopped breastfeeding altogether at three months. None of these feeding patterns are exceptional compared to the other 96 participants. However, they did all receive daily cotrimoxazole from age six weeks until the end of follow-up: this is a potentially significant EED risk factor modification. However, cotrimoxazole prophylaxis was included in the definition of recent antibiotics for the EED and stunting models. Sub-group analysis would have been under-powered, as would inclusion of HIV status as a stand-alone putative EED risk factor. It was decided therefore to retain these three HIV-exposed participants in all analyses.

In conclusion, amongst infants in this rural coastal Kenyan community, stunting is common and occurs from around four months of age onwards. EED is a significant health issue in this community: it is highly prevalent and associated with stunting in the short term. The only other factors with strong evidence for association with stunting are male sex and enrolment weight. There is only limited evidence for association of acute illnesses with stunting, and none for their association with EED. SIBO is not an important health problem and is unlikely to play an important role in pathogenesis of EED or stunting. Acute illnesses, antibiotics and stunting are all more common during the rainy season. Finally, adjusted for age and other factors, continuous-since-birth exclusive breastfeeding status (not counting prelacteal feeds) is protective against three out of four selected measures of EED. For LM ratio, this protection only occurs during the rainy season. When measuring exclusive breastfeeding status over the

short term, adjusted for other factors, protection remains only against higher faecal myeloperoxidase and not against other measures of EED.

Chapter 6: Discussion and conclusions

Overview of results

Both the cross-sectional and cohort studies described in this D.Phil. thesis have described a community in which multiple indicators of severe socioeconomic deprivation are present, with poverty, poor WASH, dietary diversity and food security. The prevalence of being stunted (HAZ<-2) was higher than the Kenyan national prevalence, at 35% overall amongst all under-fives compared to 26%.(210) Both studies demonstrated that HAZ declined steadily with age between birth and 24 months of age, followed by stabilisation. Amongst the cohort infants who completed follow-up to nine months of age, 34% were stunted by exit. Acute malnutrition, on the other hand, was rare in both studies.

Amongst under-fives in the cross-sectional study, adherence to WHO exclusive and any breastfeeding duration recommendations was poor. Prevalence of exclusive breastfeeding declined from one to six months with no inflection point. This finding was mirrored in the cohort study: age at cessation of exclusive breastfeeding ranged widely from 0.7 to 6.2 months with participants distributed evenly between these two values. However, although complementary foods were introduced early, the great majority of cohort participants' dietary intake remained breastmilk throughout follow-up until nine months of age. The focus group discussions and the birth cohort study both demonstrated that first complementary foods were *uji* and water, followed by tea, then *ugali*, then animal milk. Type of complementary food did not change with season.

One novel aspect of the cohort study was its measurement of SIBO using the BHT. SIBO as defined by the Rome criteria was diagnosed in only 6% of monthly BHTs, with indeterminate results for a further 13%. The proportion SIBO-positive was greatest at zero and nine months and smallest at 3-5 months. However, high risk of false negative results may have underestimated SIBO prevalence by Rome criteria. Continuous measures of breath hydrogen may give insight into the possible role of lesser degrees of overgrowth that do not meet Rome criteria. However, the fact that in the cohort study peak breath hydrogen was not associated with season, stunting (change in LAZ over two months) or EED (LM ratio, alpha-1-antitrypsin, myeloperoxidase and neopterin) suggests that its observed variability may either be stochastic, or true but clinically insignificant in this population.

Factors independently associated with lower HAZ amongst under-fives in the cross-sectional survey included older age, birthweight <2.5kg, lower SEP, having a deceased sibling, birth in the rainy season and birth interval to younger sibling less than two years. The association between birthweight <2.5kg and lower HAZ was sustained across the first five years of life without any evidence of fade. No association was observed between duration of exclusive or any breastfeeding and HAZ. However, these breastfeeding variables were difficult to assess due to problems with recall bias, missing data and confounding by caretaker perception of general health. Amongst infants in the cohort study, stunting (change in LAZ over two months) was associated with older age, lower enrolment weight, shorter maternal height, male sex and EED (higher LM ratio).

Incidence of diarrhoea amongst infants in the cohort study was low (0.51 per PYO), as was prevalence of helminthiasis on cross-section asymptomatic OCP microscopy at (11%).

Incidence rates of fever and breathing difficulty amongst cohort infants were higher (3.01 and 4.88 per PYO respectively) although severe episodes were rare. Amongst under-fives in the cross-sectional survey, diarrhoea, fever, breathing difficulty and geophagy, plus possibly also intestinal helminthiasis, all occurred most frequently amongst those aged 6-23 months. This age range matches that of the decline in HAZ noted in the cross-sectional survey. In the cohort study, diarrhoea, fever, breathing difficulty, stunting and EED (defined by LM ratio) were all more marked during the rainy season.

Amongst under-fives in the cross-sectional survey, age-related declines in faecal alpha-1-antitrypsin, myeloperoxidase and neopterin reflected the process of stunting. This is especially true for neopterin which additionally demonstrates the more precipitous decline until 24 months of age followed by stabilisation thereafter. Neopterin also demonstrated clustering by village, household and homestead and positive association with HAZ in cross-section. However, both of these observations may have been vulnerable to confounding by age. Amongst infants in the cohort study, EED as defined by the LM ratio worsened with age from four months onwards, with LM ratio increasing by a model-predicted 6.2% every month overall throughout follow-up. Also in the cohort study, faecal alpha-1-antitrypsin, myeloperoxidase and neopterin all increased until around four months of age then stabilised. Amongst infants in the cohort study, of the urine and faecal EED biomarkers assessed, urinary LM ratio (specifically mannitol excretion) and faecal myeloperoxidase are closest associated with concurrent stunting, with LM ratio and faecal myeloperoxidase both inversely associated with change in LAZ over each two-month period. No associations with linear growth were found for faecal alpha-1-antitrypsin in either study.

Factors associated with EED as defined by the three faecal ELISA markers amongst under-fives in the cross-sectional survey included older age (associated with all three markers), unimproved sanitation (neopterin) and breastfeeding (neopterin). As discussed on pages 101-102, a possible explanation for the unexpected positive association of faecal neopterin with breastfeeding is residual confounding by age. When not adjusting for age, sex or breastfeeding status (the latter factor excluded since it is strongly co-linear with age), further emerging factors associated with EED amongst under-fives include geophagy (associated with both alpha-1-antitrypsin and neopterin) and recent acute illness (myeloperoxidase).

Amongst infants in the cohort study, of all potential risk factors assessed, only continuous-since-birth exclusive breastfeeding status is protective against EED. Where EED is defined by LM ratio, this protection only occurs during the rainy season. To my knowledge, only two other cohort studies have measured exclusive breastfeeding alongside EED in a tropical low-income setting. The first study, already described on pages 25-26, involved quantification of 'exclusivity of breastfeeding' in 24 South African infants using deuterium dilution, describing exclusivity on a sliding and reversible scale rather than binary and irreversible.(110) Lower plasma concentrations of the neutrophil-derived protein S100 calcium-binding protein A8 (S100A8, a subunit of calprotectin) and the innate immune pathway mediator interleukin-8 (IL-8) were found amongst those for whom breastmilk formed a greater proportion of nutritional intake. In the second study, 590 Tanzanian children aged six weeks to 12 months participating in a micronutrient intervention trial were recorded as exclusively, predominantly, partially or not breastfeeding at age six weeks and six and 12 months.(111) Not breastfeeding at six weeks compared to exclusive breastfeeding was associated with higher serum anti-flagellin. Results of comparison between exclusive and

predominant/partial breastfeeding were not reported. The WASH Benefits study reported the proportion of participants who exclusively breastfed during the 24 hours preceding each LM test at their Bangladesh site, but did not describe any further analysis including this factor.(174) High rates of exclusive breastfeeding were reported across all arms of the SHINE study(280) but this factor was not assessed in relation to linear growth.(175) Results for SHINE's secondary outcome of EED are awaited. The MAL-ED multi-site study included 'any breastmilk consumption during the past seven days' in its final analyses but not exclusive breastfeeding, either recently or since birth.(89) Suggestions for future research to explore in more detail the role of introduction of complementary foods in development of EED is below.

Notably, in the cohort study no associations were observed between four measures of EED and SIBO, WASH, acute illnesses or antibiotics. For antibiotics, a true effect may have been masked by co-linearity with acute illness (antibiotics were prescribed for illness). Another possible explanation for the lack of association between these factors and EED is insufficient variability within the study population. Regarding acute illnesses, any changes in LM ratio may have been too transient to detect given the chosen measurement method (binary measure: 'did the participant experience an episode of diarrhoea/breathing difficulty/fever during the 30 days preceding the LM test?'). Measurement error may have been an influential factor for SIBO given the limitations of the BHT.

Study strengths and limitations

Main strengths of the two studies described include thorough capturing of events through close interaction with participants (cohort), an accurate and complete sampling frame and high proportion sampled enabling thorough study area characterisation (cross-section), minimal risk of selection bias (cohort) and high participation amongst those approached (both studies – including a low drop-out rate in the cohort study). The LM test success rate compared favourably to previous published studies and urine-derived LM ratios were well supported by plasma-derived ratio quality assessment. Another novel aspect is the combination of contemporaneous cross-sectional and cohort data within the same study population.

Another main novel aspect of the cohort study was its intensive frequency of sampling: monthly LM test and measurement of SIBO, bi-weekly stool sampling. The LM ratio was found to be unstable over time. Assuming this reflects a true trend in intestinal mucosa structure and function rather than measurement stochastic variation, the chosen structuring of data for the main multivariable models exploring risk factors for stunting and EED will have resulted in a focus on short-term associations. Many published cohort studies have used stunting or EED status at exit or at just two to three time points during follow-up and paired these outcomes with risk factors measured either cumulatively throughout follow-up or at around one to three selected time points. This approach is neither inferior nor superior but must be taken into account when interpreting results. Had I followed this latter approach to data analysis, I may have identified different factors associated with stunting and EED.

Limitations to the cross-sectional and cohort studies include limited geographical range with lack of generalisability to study populations that differ significantly to this one: for example, areas with higher HIV prevalence, lower malaria transmission intensity or higher incidence of diarrhoea. The cohort study participants became a more engaged and monitored group than their peers. Efforts were made to mitigate this risk of observer effect by separating clinical care from data collection as far as practically possible. However, there remains a risk that frequency of administration of antibiotics, for example, may have been higher amongst the cohort participants than in the general community. If this were true, the most likely consequence is that the cohort participants were a generally slightly healthier group than their non-participating peers, leading to underestimation of true population prevalence of EED. Associations between outcomes and risk factors are unlikely to have been affected, although statistical power for their detection may have been reduced if outcome prevalence was indeed lower. The opposite appears to be true for LAZ, however: multiple fractional polynomial-predicted LAZ for cohort study participants ranged from -1.38 at just over nine months of age to -1.54 just before 10 months of age. Predicted LAZ for the equivalent age range of cross-sectional survey participants was higher at -0.74 and -0.83 respectively.

An integral limitation of the cohort study is its focus on the two-month period of linear growth symmetrically surrounding each monthly LM test and the presence of risk factors during the month preceding each LM test. This choice targeted an optimum balance between accuracy and precision but nonetheless limits detection of associations to within the two-month time frame and does not allow for shorter or longer time lags between cause and effect.

Lastly, several potentially key risk factors for stunting and EED were not directly measured. These included the intestinal microbiome and microbial contamination of drinking water and complementary foods. Further discussion of their possible role is below.

Challenges encountered

Although field and laboratory work for this study progressed mostly without incident, I encountered some challenges. Firstly, maintaining a high-quality cold chain for samples and minimising time from passage of stool to freezing was difficult. During the cross-sectional and cohort studies, between around one and ten stool samples per day were collected by fieldworkers on motorbikes from homesteads scattered across the 26x13km study area. Many homesteads were isolated and only accessible on foot. Cooperation and patience from participants' caretakers were required. Communication was also a challenge, with most caretakers lacking access to mobile phones. Accessibility was further impeded during the rainy season, with washed-out dirt roads and uncomfortable travelling conditions for study participants and their caretakers. During active raining, the study vehicle was used to collect participants from their homes for monthly LM test and BHT or for clinician assessment in the event of acute illness. This was logistically challenging. After the first six months of fieldwork, time to stool sample freezing was greatly reduced by the installation of a liquid nitrogen dry shipper at Junju dispensary. However, the dry shipper required replenishing with liquid nitrogen on a weekly basis at KWTRP Laboratories, one hour's drive away.

The cohort study required significant investment of time by participants and their caretakers. Risk of participant and fieldworker fatigue was high and mitigated by motivational activities for staff and active engagement and encouragement of participants' caretakers. Care was taken to ensure remuneration and other study 'benefits' adequately compensated this required level of engagement. Careful balance was required so as to minimise risk of inducement: for this, I was supported by the CLG. I learnt to prioritise quality over quantity when collecting data and would have included fewer questions in the cross-sectional survey questionnaire and cohort weekly home visit proforma had I the opportunity to design them again.

Community engagement activities prior to the start of fieldwork alerted me to a lack of local awareness of stunting or moderate malnutrition as a problem for child health. Even more difficult to describe to a lay audience was the concept of EED and its potential implications for the observable outcomes of morbidity and mortality. Following on from this, I occasionally encountered challenges in motivating community members to engage with a study that focussed on a condition that is not yet fully defined, is sub-clinical and has no treatment.

Three sample collection methods were entirely new to the community: stool sampling, the LM test and the BHT. With the exception of one caretaker with mental health difficulties I encountered no acceptability problems with stool sampling. Our long-term relationships with the cohort participants and their caretakers led to gradual understanding and acceptance of the LM test and BHT. Blood sampling has long been unpopular in the community and may

have slowed the recruitment rate to the cohort. At six months, the benefit of checking for anaemia helped with acceptance.

Lastly, regional insecurity three months prior to the start of fieldwork resulted in a one-month delay as I considered relocation. The study area and surrounding coastal Kenya was subject to UK Foreign Office travel advice warnings from then until around a year later, when restrictions were lifted. I was supported through my decision to stay and in optimising the safety of myself and my study team by KEMRI-WTRP and the University of Oxford.

Recommendations for future research

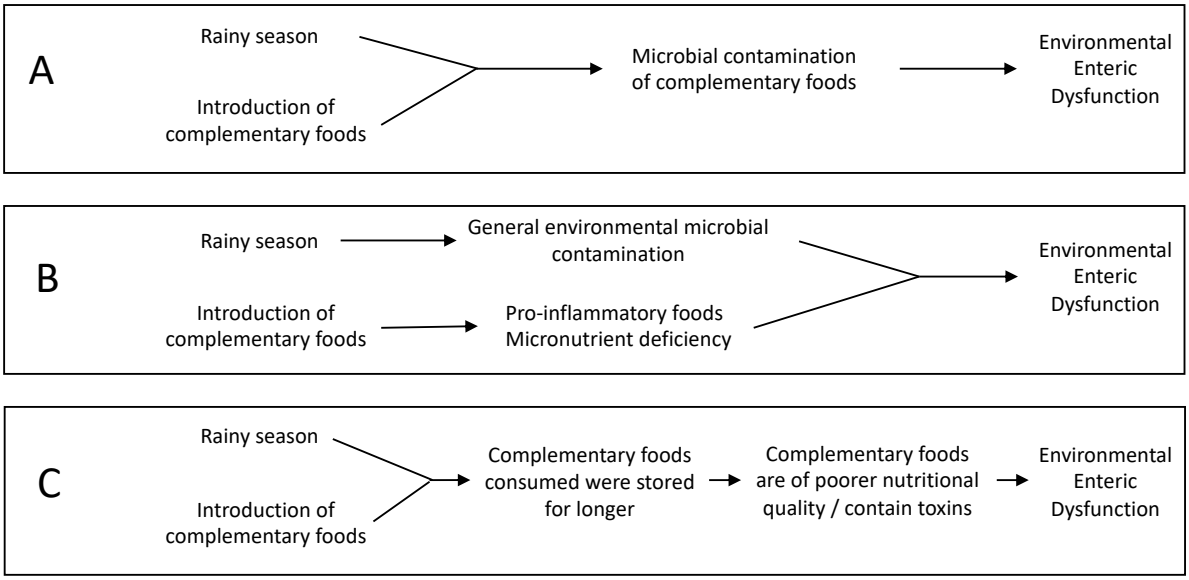
The cohort study has demonstrated that, amongst the assessed putative risk factors for EED in infancy (complementary foods, SIBO, WASH, acute illnesses and antibiotics), aside from older age, it is only complementary food introduction, and then perhaps only during the rainy season, for which strong evidence exists for association with EED. Since type of complementary food does not change with season, it is therefore likely that pathogen contamination of these foods, utensils and caregiver hands is greater during the rainy season and this explains the modification by season of the association between breastfeeding and EED. Furthermore, although incidence of fever and breathing difficulty is higher during the rainy season, overall incidence of diarrhoea (the illness most likely to arise from pathogen contamination of complementary foods and most likely to influence intestinal microbiome) is very low (no difference detected by season but this may have been due to lack of power), and none of these illnesses demonstrated strong evidence for association with EED in the

short term.[§] Therefore, the protective effect of exclusive breastfeeding against EED during the rainy season is more likely mediated via preventing intestinal dysbiosis than via preventing acute symptomatic illness or via the possible poorer nutritional value or pro-inflammatory properties of the complementary food itself (Figure 66; hypothesis A). Another possible explanation for the observed association between complementary foods and EED during the rainy season is that all infants, exclusively breastfed or not, are exposed to an equally higher level of faeco-oral contamination during the rainy season and equally lower during the dry season (Figure 66; hypothesis B). Upon cessation of exclusive breastfeeding, the added insult of the poor nutritional quality of complementary foods is sufficient during the rainy season, with its higher baseline of gut damage due to faeco-oral contamination – to trigger significant worsening of LM ratios. This would fit with a recent mouse model discussed on pages 43-44 that suggested synergy of both is required.(206) A third possible explanation is that, although type of complementary food did not differ between rainy and dry season, duration of food storage may have (Figure 66; hypothesis C). Focus group discussions revealed that local foods consumed during the rainy season had been stored for the longest. Maize is harvested, dried in the sunshine, then stored either in sacks or in an '*uchaga*' – a raised structure made from sticks with a smoke source underneath. Few community residents apply pesticides. Pigeon peas are harvested dry and stored in sealed plastic containers. The two main complementary foods – *uji* and *ugali* – are prepared from dried fermented cereals and maize meal respectively. Both of these are predominantly

[§] N.B. as discussed earlier, in the cross-sectional survey higher faecal myeloperoxidase was associated with diarrhoea, fever or breathing difficulty during the seven days preceding stool sample collection. It may be that EED is worse during and a few days following acute illness but that changes are not sustained beyond this time frame. If this is true, it would be unsurprising that the cohort study final EED multivariable model, which included each acute illness in binary form during the 30 days preceding each LM test, did not detect an association.

grown and stored locally and are vulnerable to loss of nutritional value and contamination with fungal toxins during storage in a humid, hot climate.(281) As discussed on page 30, food mycotoxins have been observed to be associated with markers of EED.

Figure 66: Hypotheses to explain the observed association between EED and introduction of complementary foods during the rainy season.



To further investigate these three hypotheses, we need to quantify microbial contamination of complementary foods in different settings and seasons. This has been done before using classical microbiological field techniques in observational and interventional settings in tropical low-income settings, but not yet in the context of stunting and EED.(282-287) Exclusivity of breastfeeding, even when enquiring on a weekly basis, is vulnerable to recall bias and measurement error. The stable isotope deuterium dilution method objectively quantifies breastmilk intake and also the proportion of energy intake derived from breastmilk. It has been demonstrated as superior to caretaker recall and has no safety or ethical concerns.(288, 289) Future studies of the role of complementary food introduction

in the development of EED would ideally use a technique such as this rather than relying on caretaker recall.

With the exception of myeloperoxidase, the effect of introduction of complementary foods on severity of markers of EED appears to be irreversible. This fits with a hypothesis that changes in gut microbiome and inflammation are irreversible following first exposure to complementary foods and suggests that interventions to support exclusive breastfeeding to prevent EED need to focus on delaying first introduction of complementary foods to the WHO-recommended six months of age. Of note, as discussed on page 212, cohort participants often reverted to brief periods of intake of nothing but breastmilk up to several months after complementary foods had been introduced. These episodes anecdotally occurred as a result of acute illness (when the participant was too unwell to eat other foods), household food insecurity (when scarce food was allocated to non-breastfeeding children) or travel. In studies where exclusive breastfeeding status is allowed to be reversible, therefore, close attention needs to be paid to the risk of confounding by acute health status and household food availability, especially amongst older exclusively breastfed children. Direct comparison cannot be made between (or within) studies with reversible and irreversible definitions for exclusive breastfeeding status.

Lastly, normal values for faecal EED biomarkers such as alpha-1-antitrypsin, myeloperoxidase, neopterin and calprotectin amongst healthy breastfed infants and older children need to be established, as they may be appropriately higher with breastfeeding in certain circumstances. These normal values would need to be developed from multi-site

study of healthy, non-stunted children in optimum care environments but who are genetically similar to those in settings where EED is common.

If further studies confirm that the rainy season is a particularly high-risk time to cease exclusive breastfeeding, interventions against EED could be targeted to this time. Pending or in addition to transformational WASH and major improvements in dietary diversity and food security, such interventions may include specific or enhanced breastfeeding support, food storage improvements, probiotics, antibiotics and nutritional supplements.

In hypotheses A and B in Figure 66 above, higher faeco-oral contamination during the rainy season – either via complementary food ingestion or other routes – is proposed to increase risk of EED not via acute diarrhoea but via alteration of the intestinal microbiome. Diarrhoea was observed to be rare in both the cross-sectional and cohort studies, and not associated with EED. Vital to appraising these hypotheses, therefore, is characterisation of the subclinical intestinal microbiota or microbiome. The possible role of intestinal dysbiosis in the development of EED is outlined on pages 39-41. A selected subset of 401 of the cohort stool samples underwent 16S PCR and subsequent pathogen genome sequencing at KEMRI-WTRP Laboratories.[**] I am currently undertaking analysis of this data along with Dr Edward Parker (London School of Hygiene and Tropical Medicine, UK). See Appendix 58 for methods and results to date. As expected, gut microbiota alpha diversity increased with age. Relative

[**] Acknowledgements: study design was undertaken by myself and Dr Edward Parker (London School of Hygiene and Tropical Medicine, UK) and guided by Professor James Berkley. Stool DNA extraction was undertaken by myself and research assistants Wilson Gumbi and Emily Nyatichi at (KEMRI-WTRP) with guidance from Dr Mami Taniuchi (University of Virginia, USA). 16S PCR, library preparation and sequencing was undertaken by Joyce Ngoi and Zaydah de Laurent at (KEMRI-WTRP). Bioinformatics analysis was undertaken by Dr Parker. Funding was provided by my Wellcome Trust Research Training Fellowship no. 103376/Z/13/Z.

abundance of breastmilk-associated *Bifidobacterium* genus peaked at one month with rarer genera accounting for an increasing proportion from six months onwards. Adjusted for age, there was weak evidence for higher alpha diversity during exclusive breastfeeding. In an unweighted Permutational Multivariate Analysis of Variance (PERMANOVA), a significantly high proportion of variability in beta diversity was attributed to exclusive breastfeeding status amongst those aged 4-6 months. However, relative abundance of specific phyla did not differ with exclusive breastfeeding status (although some changed with season). These interim results overall provide only weak evidence to support a hypothesis that exclusive breastfeeding protects against EED via differences in gut *microbiota*. More comprehensive *microbiome* characterisation using shotgun metagenomic methods may provide further insight.

Another ongoing body of work arising from the cohort study concerns comparison of systemic immune responses in participants affected and unaffected by stunting and EED. The emerging evidence to date for impaired mucosal and systemic immune responses in EED is described on pages 13-16. Stored plasma and peripheral blood mononuclear cells taken from cohort participants at enrolment and six months of age (n=25 and n=92 respectively) will undergo cytokine profiling and flow cytometry to quantify functional general and gut pathogen-specific immune responses by Dr Agnes Gwela at KEMRI-WTRP Laboratories.[††]

[††] Acknowledgements: study design was undertaken by myself and Dr Agnes Gwela (KEMRI-WTRP, Kenya) and guided by Professor James Berkley. Plasma and PBMC preparation was carried out by research assistants Debra Riako and Emily Nyatichi at KEMRI-WTRP Laboratories. Funding was provided by my Wellcome Trust Research Training Fellowship no. 103376/Z/13/Z.

The role of SIBO in the development of EED in this setting is as yet unclear. Use of hand-held breath hydrogen meters for detection of SIBO has the advantage of feasibility in this remote setting but may have led to underestimation of true SIBO prevalence and missing of a possible true association between SIBO and EED in this population. Validation of breath sampling protocols and SIBO diagnostic cut-offs against microbial analysis of directly sampled intestinal fluid should be the next step, and results from the ongoing BEED study discussed on page 137 may provide some insights into this.(238) A challenge will be to obtain paired breath and small intestinal fluid samples in adequate numbers from healthy infants and children in optimal local environments in order to establish accurate breath test reference standards for genetically similar populations. Comparison studies of SIBO prevalence and risk factors between children in these 'optimal' and 'non-optimal' local settings will also be useful.

In this D.Phil. thesis, amongst under-fives in Junju, Kilifi, Kenya, I aimed to describe the epidemiology of undernutrition and EED and their putative risk factors amongst under-fives. Amongst infants, I aimed to describe age-related incidence of EED and stunting and to describe these outcomes' temporal associations with selected preceding and concurrent risk factors. I judge that I have been largely successful in meeting these aims. However, as described above, questions remain, and new hypotheses have been generated. EED is certainly a significant problem for child health in Junju and is amongst multiple health and nutritional outcomes that worsen during the rainy season. A likely major risk factor for EED amongst infants in Junju is a combination of the rainy season and continuous-since-birth exclusive breastfeeding having already stopped. Amongst under-fives, risk factors may additionally include recent acute illnesses and geophagy. In all ages, diarrhoea is rare and

therefore unlikely to play an important role. The effect of SIBO on EED is uncertain. Older age remains a strong risk factor for EED even in multivariable models that aim to include as many age-related factors as possible. This suggests that other unmeasured factors that change with age may be important. Future priorities for EED research should include development of better biomarkers with linkage to histology and normal reference ranges by breastfeeding status and age; international agreement on case and severity definitions; more objective quantification of contributing factors such as environmental microbial and toxin contamination and breastmilk intake; and translational studies focused on interventions against this condition that poses great challenges for child health and survival worldwide.

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Appendix 3: Abbreviations

AGP	α-1-acid Glycoprotein
AM	Acute Malnutrition
AUC	Area Under the Curve
BEED study	Bangladesh Environmental Enteric Dysfunction study
BHT	Breath Hydrogen Test
CFU	Colony-forming Unit
CI	Confidence Interval
CLG	Community Liaison Group
CRP	C-reactive Peptide
DHS	Demographic and Health Survey
DNA	Deoxyribonucleic Acid
EAEC	Enteroaggregative *Escherichia coli*
EDD	Expected Due Date
EED	Environmental Enteric Dysfunction
ELISA	Enzyme-linked Immunosorbent Assay
FANTA	Food and Nutrition Technical Assistance
GCLP	Good Clinical Laboratory Practice
HAZ	Height-for-age Z-score
HIV	Human Immunodeficiency Virus
HPLC	High-performance Liquid Chromatography
HPLC-PAD	High-performance Liquid Chromatography with Pulsed Amperometric Detection
I-FABP	Intestinal Fatty Acid Binding Protein

IgA	Immunoglobulin A
IGF-1	Insulin-like Growth Factor 1
IgG	Immunoglobulin G
IL-1, IL-6, IL-8	Interleukin-1, Interleukin-6, Interleukin-8
K:T ratio	Kynurenine:tryptophan ratio
KHDSS	Kilifi Health and Demographic Surveillance System
KWTRP	Kenya Medical Research Institute Wellcome Trust Research Programme
KWTRP CTL	KWTRP Clinical Trials Laboratory
LAZ	Length-for-age Z-score
LC-MSMS	Liquid Chromatography tandem Mass Spectrometry
LC-PUFA	Omega-3 Long Chain Polyunsaturated Fatty Acid
LM	Lactulose:mannitol
LMZ	Lactulose:mannitol ratio Z-score
LPS	Lipopolysaccharide
MAL-ED study	Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development study
MAM	Moderate Acute Malnutrition
mRNA	Messenger Ribonucleic Acid
MUAC	Mid Upper Arm Circumference
OCP	Ova, Cysts and Parasites
OPV	Oral Polio Vaccine
OTT	Oro-caecal Transit Time
PCA	Principal Components Analysis

PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PMTCT	Prevention of Mother To Child Transmission
ppm	Parts per million
PROVIDE study	Performance of Rotavirus and Oral Polio Vaccines in Developing Countries study
PYO	Person-years of Observation
RDT	Rapid Diagnostic Test
rRNA	Ribosomal Ribonucleic Acid
RUTF	Ready-to-use Therapeutic Food
S100A8	S100 calcium-binding protein A8
SAM	Severe Acute Malnutrition
sCD14	Soluble CD14
SEP	Socio-economic Position
SHINE study	Sanitation Hygiene Infant Nutrition Efficacy study
SIBO	Small Intestinal Bacterial Overgrowth
SOP	Standard Operating Procedure
WASH	Water, Sanitation and Hygiene
WAZ	Weight-for-age Z-score
WHO	World Health Organisation
WHZ	Weight-for-height Z-score

Appendix 4: Article: 'Environmental enteric dysfunction: an overview.'

See next page for the following article:

Crane RJ, Jones KD, Berkley JA. Environmental enteric dysfunction: an overview. *Food and Nutrition Bulletin* 2015; 36(1 Suppl): S76-87.

Environmental enteric dysfunction: An overview

Rosie J. Crane, Kelsey D. J. Jones, and James A. Berkley

Abstract

Background. *Environmental enteric dysfunction (EED) refers to an incompletely defined syndrome of inflammation, reduced absorptive capacity, and reduced barrier function in the small intestine. It is widespread among children and adults in low- and middle-income countries. Understanding of EED and its possible consequences for health is currently limited.*

Objective. *A narrative review of the current understanding of EED: epidemiology, pathogenesis, therapies, and relevance to child health.*

Methods. *Searches for key papers and ongoing trials were conducted using PUBMED 1966–June 2014; ClinicalTrials.gov; the WHO Clinical Trials Registry; the Cochrane Library; hand searches of the references of retrieved literature; discussions with experts; and personal experience from the field.*

Results. *EED is established during infancy and is associated with poor sanitation, certain gut infections, and micronutrient deficiencies. Helicobacter pylori infection, small intestinal bacterial overgrowth (SIBO), abnormal gut microbiota, undernutrition, and toxins may all play a role. EED is usually asymptomatic, but it is important due to its association with stunting. Diagnosis is frequently by the dual sugar absorption test, although other biomarkers are emerging. EED may partly explain the reduced efficacy of oral vaccines in low- and middle-income countries and the increased risk of serious infection seen in children with undernutrition.*

Conclusions. *Despite its potentially significant impacts, it is currently unclear exactly what causes EED and how it can be treated or prevented. Ongoing trials involve nutritional supplements, water and sanitation interventions, and immunomodulators. Further research is needed to better understand this condition, which is of likely crucial importance for child health and development in low- and middle-income settings.*

Key words: Environmental enteric dysfunction, environmental enteropathy, malnutrition, stunting

Introduction

Environmental enteric dysfunction (EED) is a poorly understood condition that may have far-reaching impacts on child growth, health, and development in low- and middle-income country settings. Characterized by small intestinal inflammation and strongly associated with stunting (low height-for-age), it is now the subject of significant research interest as investigators seek to define its causes, pathogenesis, consequences, and possible preventive or curative approaches.

The earliest descriptions of EED, previously known as "tropical enteropathy," date back to the 1960s, when an abnormal microscopic appearance of the small bowel was observed in adults from low- and middle-income countries [1]. It was observed that the villi were blunted and shortened, leading to a decreased surface area for nutrient absorption. Tropical enteropathy was renamed "environmental enteropathy" in the late 2000s in recognition of emerging evidence that the quality of the environment was more important than climate or latitude; EED is not limited to tropical areas, nor does it affect all residents in the tropics. Over the past year, it has been further renamed "environmental enteric dysfunction."

There is no universally accepted case definition or specific diagnostic criteria for EED, and it does not have immediately apparent clinical symptoms. The key

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demonstrable histological features (villous flattening, crypt hyperplasia, and lymphocytic infiltration of the lamina propria [2–5]) are not unique to EED. Tropical sprue (a syndrome of chronic diarrhea, malabsorption, and malnutrition seen in residents of tropical countries) and EED were initially thought to be the same process, with symptomatic tropical sprue the most severe pathology but representing the "tip of the iceberg" of largely unrecognized EED, although this distinction remains unclear [6]. There are also some similarities with other chronic gut inflammatory conditions, such as celiac disease and inflammatory bowel disease (Crohn's disease and ulcerative colitis) [7].

Impact of EED on health and nutrition

Impact on nutrition and development

Childhood EED has been associated with stunting (low height-for-age) for as long as it has been a recognized entity [8–12] although it is not entirely clear which of these two is the primary instigator and which the consequence. A 2008 systematic review of the effectiveness of existing complementary feeding interventions for malnutrition among 6- to 24-month-olds suggested that they had only moderate benefits and that EED may be a key factor limiting effectiveness [13]. Nutritional intervention alone may not be fully addressing the problem of stunting among infants and children in low- and middle-income country settings—with EED possibly explaining this shortfall [14].

There are many ways, other than malabsorption, in which chronic intestinal inflammation could impact growth. One proposed mechanism is appetite suppression, since this—rather than food availability—is often a key factor restricting children's food intake [15]. Proinflammatory cytokines are known to act on appetite centers in the brain [16]. Another proposed mechanism is via negative regulation of systemic inflammatory activation on signaling through the growth hormone–insulin-like growth factor 1 (IGF-1) axis, which has been clearly demonstrated in pediatric Crohn's disease [16, 17]. A similar situation may occur in EED: indeed, higher blood inflammatory markers in Zimbabwean infants were associated with lower levels of IGF-1 and with stunting [18].

Stunting has devastating consequences for child health. Difficult to reverse beyond 2 years of age, it has long-lasting effects on health and development [19, 20]. A meta-analysis in 2007 suggested that each year 200 million children do not reach their developmental potential due to stunting [21]. Certain noncommunicable diseases in adulthood are associated with stunting in childhood, perhaps through epigenetic regulation (which refers to long-term alteration in gene expression) or via chronic inflammation [22].

Impact on immunity

The gut is the site of a highly sophisticated immune surveillance operation, the purpose of which is to detect and destroy potential pathogens and maintain the integrity of host tissues while at the same time avoiding unhelpful immune responses against foods, nonpathogenic microorganisms, and other harmless contents of the gut lumen. The presence of abnormally large numbers of white blood cells in the gut wall of children and adults with EED suggests that this surveillance operation is being stressed. Impaired barrier function (a hallmark of EED) results in luminal contents (including both harmless and potentially pathogenic bacteria) crossing the gut wall itself and activating the immune system. Chronic inflammation is triggered, which may be a direct cause of growth failure (as described above) and may impair antipathogen surveillance capacity. The nature of this chronic inflammation remains poorly understood. This is partly because it is a difficult phenomenon to study, since biopsy samples from children with EED are scarce. However, in recent years there have been a number of fundamental advances in our understanding of mucosal immunity coming from work with mouse models of human disease, and it is possible that approaches utilizing animal model systems will further our understanding of EED and even guide clinical and public health management in the future [23–25]. Lastly, although the exact mechanism is unclear, EED has been implicated in the poor response to oral vaccines (polio and rotavirus) seen in children living in low- and middle-income countries [7, 26–28].

Diagnosis: How do we identify the child with EED?

To confirm the diagnosis of EED beyond doubt requires demonstration of the histological features of EED. This can only be done by endoscopy and small intestinal biopsy. The logistical challenges of this have led investigators to seek simpler biomarkers for EED: less invasive tests that measure proxy biomarkers are used to quantify gut inflammation and reduced barrier function.

The dual sugar absorption tests

To date, the most frequently used tests for EED are the dual sugar absorption tests. The most commonly implemented of these at present is the lactulose:mannitol (L:M) test, although other sugars, such as xylose and rhamnose, can be used. Lactulose is a large sugar that is not normally absorbed by the small intestine. Mannitol is a smaller sugar that is absorbed by the small intestine in proportion to absorptive surface area. In the L:M

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test, after oral ingestion, both lactulose and mannitol are excreted intact in the urine following minimal metabolism. Urinary mannitol therefore gives an index of absorptive capacity, while the presence of lactulose in the urine indicates impaired barrier function. Higher urinary L:M ratios reflect greater abnormalities of one or both functions [8, 29, 30].

The L:M test requires cooperation and understanding from the participant and the carer, which may present a challenge when the test is performed in very young children. Ensuring complete collection of all urine voided over up to 5 hours and avoiding contamination with feces is difficult in infants and requires parental patience. Measurement of lactulose and mannitol in urine can be performed by enzyme-linked immunosorbent assay (ELISA), anion exchange chromatography, or mass spectrometry. All of these methods require centralized laboratory equipment and expertise, and the results are not always comparable between laboratories.

Emerging biomarkers

New biomarkers of EED are being actively investigated. Current markers of gut inflammation are measured in feces: calprotectin [31–34]; myeloperoxidase, neopterin, and α-1-antitrypsin [35]; mRNA [36]; REG1b [37]; lactoferrin [38]. Markers of gut permeability are measured in blood: zonulin; [39] EndoCAb; [11, 12, 18, 39] soluble CD14 [18]. Lastly, a marker of total enterocyte mass is also measured in blood: citrulline [40].

By investigating and testing markers for their hypothesized roles in the establishment and maintenance of EED, our understanding of the processes causing EED will hopefully improve. For example, small intestinal epithelial cells from a child with EED are assumed to differ in the kinds of proteins they produce from those from a child without EED. Certain mRNA transcripts with roles in inflammatory pathways have been shown to be present more often in the stool of Malawian children with abnormal L:M ratios [36].

Most new markers have been evaluated in association with stunting rather than according to histological changes or the L:M test. Future studies may compare new markers against indicators other than anthropometry, including assessing their abilities to closely reflect short-term changes in EED as defined by a gold standard diagnostic test. Such markers would be useful for evaluating the effects of interventions against EED in trials where follow-up is comparatively brief.

Epidemiology: Who has EED? What are its risk factors?

EED was first recognized in adults. A 1971 study of US Peace Corps volunteers is frequently referred to as the first to demonstrate that EED can be acquired and lost according to environment [41]. Participants were assessed during and after placement in India and Pakistan. Gut absorptive function tests and biopsies confirmed abnormalities during residence abroad and recovery within 1 or 2 years after return to the United States. At the time, an environmental cause was suggested. A later study of Zambian adults demonstrated the same histological abnormalities, which varied with season, further implying an environmental cause [4]. In the early 1990s, community-wide studies revealed that EED was a widespread and pervasive problem in infants and children. An abnormal L:M test was almost universally acquired by the end of infancy in The Gambia [12, 42, 43] and was associated with stunting among infants and children in many low- and middle-income country settings, including Zambia, Malawi, Bangladesh, India, Nepal, Brazil, and Guatemala and among Aboriginal communities in Australia [5, 8–12, 30, 31, 40, 42–57]. In 1999, a large study measured intestinal permeability and absorptive capacity among asymptomatic adults in 14 countries across the world [58]. Abnormalities of both intestinal absorption and permeability were, in general, found in tropical rather than temperate countries. However, average intestinal absorptive capacity by country also correlated very closely with national gross domestic product per capita, independently of climate, suggesting that poverty may play a more significant role.

Putting these studies together, a consistent picture has emerged: EED seems highly prevalent in low- and middle-income country settings, is acquired during infancy, and persists into adulthood, but can perhaps be cured or improved with change in environment. It is strongly associated with stunting in infants and children.

Etiology: What causes EED?

Nutritional deficiency

Zinc is known to aid recovery of the intestinal mucosa following diarrhea [48] and is recommended as part of standard therapy for diarrhea by the World Health Organization. Zinc deficiency is associated with abnormal L:M ratio in Malawian children [53]. Vitamin A deficiency is associated with abnormal L:M ratio and stunting in Brazilian children [50]. In The Gambia, the least abnormal L:M ratios are observed during the mango season, when vitamin A intake is highest [49]. However, trials of both vitamin A and zinc have had mixed impacts on growth and/or EED markers among children in low- and middle-income countries [47, 49, 50, 52, 59, 60]. Multiple micronutrient supplementation in Zambian adults has yielded improvements in the histological features of EED [61].

Several other nutritional approaches have been proposed to tackle EED, for example, improving the

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digestibility of food through fermentation, hydrolysis, or enzyme supplementation, or optimizing amino acid profiles to reduce gut inflammation and support repair [62]. Trials of n-3 (omega-3) long-chain polyunsaturated fatty acid (LC-PUFA), a dietary essential fatty acid thought to reduce intestinal inflammation, in The Gambia and of alanyl-glutamine, an essential precursor for rapidly replicating cells such as those lining the small intestine, in Brazil have yielded some positive results (**table 1**) [31, 51]. These interventional studies and others will improve our understanding of the possible nutritional causes of EED.

Microorganisms

Nonspecific feco-oral contamination: Role of WASH

Microorganisms in the gut may be important in the establishment and/or maintenance of EED. Their relative importance may differ according to geographic setting, season, age, and feeding practices. Bangladeshi children from cleaner households were observed to have lower risks of both abnormal L:M ratio and stunting than those from less clean households [11]. In Malawian 3- to 5-year-olds, an abnormal L:M test was associated with lack of latrine access and low household water usage [63]. However, a recent systematic review of 14 trials of water, sanitation, and hygiene (WASH) interventions worldwide on anthropometric outcomes for children aged 0 to 18 years suggested a very modest impact of WASH on stunting (with no effect at all among children aged under 2 years) and no impact on underweight or wasting [65]. Observational study of infants and young children in Zimbabwe suggests that a significant proportion of the burden of feco-oral contamination is likely to come not only from well-characterized sources (food, water, and hands) but also from direct eating of soil and animal feces in the course of play and exploration [66]. Several multisite studies are currently ongoing to investigate the effectiveness of different WASH interventions against EED [67–69].

A recent systematic review concluded that good evidence exists for moderate beneficial effects of antibiotics on growth in prepubertal children in low- and middle-income countries [70]. Several antibiotic studies aimed at particular species of pathogen have included EED in their outcomes (**table 1**).

TABLE 1. Selected published trials conducted among children in low- and middle-income country settings where EED has been explicitly targeted[a]

Author and year	Setting	Age group	Intervention and duration	Results: L:M ratio	Results: anthropometry	Notes
Thurnham et al. 2000 [49]	India; rural	2–15 mo	Vitamin A single dose (inpatients) or for 8 wk (outpatients)	Improvement seen within 30 days	Not reported	
Galpin et al. 2005 [63]	Malawi; rural	3–5 yr	*Lactobacillus* 30 days	No difference	No difference	
Lima 2007 [51]	Brazil; rural	7 mo–7 yr	Alanyl-glutamine 10 days	Improvement	Improved WAZ and WHZ but no difference in HAZ	
Trehan et al. 2009 [64]	Malawi; rural	3–5 yr	Rifamixin 7 days	No difference	Not reported	SIBO itself not measured
Goto 2009 [55]	Bangladesh; urban	3–15 mo	Albendazole 3 monthly and secnidazole monthly for 9 mo	No difference	No difference	Possible reason for failure: high *Giardia* reinfection rate
van der Merwe et al. 2013 [31]	The Gambia; rural	3 mo	n-3 LC-PUFA for 6 mo	No difference	Improved MUAC at 9 and 12 mo	Cognition also examined: no difference
Ryan et al. 2014 [47]	Malawi; rural	1–3 yr	Zinc 14 days	Prevented deterioration	No difference	
			Albendazole single dose	Prevented deterioration	No difference	

EED, environmental enteric dysfunction; HAZ, height-for-age z-score; LC-PUFA, long-chain polyunsaturated fatty acid; MUAC, mid-upper-arm circumference; SIBO, small intestinal bacterial overgrowth; WAZ, weight-for-age z-score; WHZ, weight-for-height z-score; WHO, World Health Organization

a. Searches for key papers and ongoing trials were conducted using PubMed 1966–June 2014, ClinicalTrials.gov, the WHO Clinical Trials Registry, the Cochrane Library, hand searches of the references of retrieved literature, discussions with experts, and personal experience from the field.

During the transition period from exclusive breastfeeding to complementary feeding, which frequently occurs much earlier than the recommended 6 months [71], infants are at risk for feco-oral contamination from complementary (weaning) foods. These may be prepared separately from family foods, stored for longer periods, and reheated inadequately [72]. It is during these vital months of high energy demand and immune system development that the benefits of exclusive breastfeeding are lost and a convergence of poor nutrition and feco-oral contamination takes place. The development of EED is also observed during this time. For example, moving from exclusive breastfeeding to mixed feeding and subsequent cessation of breastfeeding altogether are both seen alongside worsening of the L:M ratio in Nepali infants, and early breastfeeding cessation is associated with worse L:M ratio in Guatemalan infants [9, 45]. Although it is difficult to distinguish from the risk of nutritional deficiency that occurs with early cessation of breastfeeding, a possible cause of these observations may be the poor hygiene often associated with complementary feeding.

Specific pathogens

Linear growth-faltering has been linked to concurrent infection with multiple intestinal parasites (*Cryptosporidium* [73], amoeba [74], roundworm [75], and hookworm [76]). Elevated fecal lactoferrin was found among Ghanaian and Brazilian children with enteroaggregative *Escherichia coli* intestinal infection, and in Brazilian children, linear growth impairment was also found [38, 77]. The abnormal histological findings in EED have been linked to infection with hookworm and the bacterium *Citrobacter rodentium* [4]. The parasite *Giardia duodenalis* has been particularly implicated in abnormal L:M ratios in Nepal [9] and acute growth-faltering in Gambian infants [54]. Acute rotavirus infection has been associated with high L:M ratios in Bangladeshi and Peruvian children [48, 78].

More recent advances in molecular pathogen diagnostics, such as the polymerase chain reaction (PCR), have allowed greater range and sensitivity in detecting pathogens. In urban Bangladeshi infants, associations were found between stunting, diarrhea, and EED (measured by EndoCAb, an antibody formed in the bloodstream in response to movement of gut-resident gram-negative bacteria across a leaky gut wall) [39]. Amoeba, *Cryptosporidium,* and enterotoxigenic *E. coli* were important causes of diarrhea in these infants (associations with EED itself were not examined).

The gut microbiome

An imbalance of gut organisms, rather than the presence or absence of specific pathogens, may be important in EED and nutrition. The gut microbiota differs significantly between African and European infants [79] and between malnourished and well-nourished children [80, 81]. Evidence that the microbiome affects nutritional status comes from an experiment where feces from malnourished children were transplanted into gnotobiotic (germ-free) mice that were fed a typical Malawian diet, resulting in the mice becoming malnourished [82]. There are no published studies to date of the gut microbiome in the specific context of EED.

Helicobacter pylori *and small intestinal bacterial overgrowth*

Helicobacter pylori is a bacterial infection best known as a cause of gastric ulcers. It has also been implicated in allowing the passage of live pathogens beyond the stomach through inhibition of stomach acid secretion [83]. In The Gambia, 15% of 0- to 20-month-olds and 46% of 40- to 60-month-olds had evidence of *H. pylori* infection, with higher prevalence among those acutely malnourished or with chronic diarrhea [84–86]. In urban Peru, a high frequency of *H. pylori* infection was noted among children and was associated with a subsequent higher risk of diarrhea [87].

The small intestine is where the majority of nutrient absorption occurs and where the histological abnormalities of EED are seen. In good health, the small intestine is relatively sterile compared with the large intestine. Small intestinal bacterial overgrowth (SIBO) is a condition that can arise secondary to bowel stasis seen, for example, in muscular disorders [88] The gold standard for the diagnosis of SIBO is sampling and culture of fluid from the small intestinal lumen. In the 1970s and 1980s, small intestinal fluid samples from acutely malnourished Indian adults and Gambian and Nigerian children were found to be heavily contaminated with pathogenic bacteria and yeasts regardless of the presence of diarrhea [89–91]. In The Gambia, SIBO was observed in apparently healthy infants, peaking during the first few months of introduction of complementary foods, as exclusive breastfeeding ceases [92].

Testing the hydrogen content of exhaled air is a reasonable method of detecting the presence of bacteria in the small intestine, since the human body does not produce hydrogen except via metabolically active intestinal bacteria [93]. When measured at intervals following ingestion of a sugar substrate, a large, late peak in the hydrogen content of exhaled air indicates metabolism of the sugar by normally resident bacteria in the large intestine. An earlier, smaller peak indicates abnormal presence of bacteria in the small intestine. This early peak is associated with EED-like small intestinal histologic changes in slum-dwelling Brazilian infants [94], poverty in urban Brazilian children [95], malnutrition and malabsorption in Burmese infants [96], and enteral vaccine failure in Chile [97].

SIBO can be treated successfully with antibiotics [98]. Metronidazole targeted at SIBO (though likely impacting on specific bacterial or parasitic infections, such as *Giardia,* as well) aided recovery from

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malnutrition in Jamaican children [99], but the nonabsorbable oral antibiotic rifamixin, also targeted at SIBO, was found not to improve L:M ratios in Malawian children [64]. Probiotics have also been investigated, although there is very limited evidence for their effectiveness in treating SIBO [88], and they have not been found to improve L:M ratios in Malawian children [63]. The role of SIBO—and its possible promotion via *H. pylori* infection—in the pathogenesis of EED is therefore still unclear.

HIV enteropathy

Enteropathy, with chronic diarrhea and weight loss, is common in severely immune-suppressed people with HIV infection and those who have progressed to AIDS [100]. The microscopic appearance of the gut in HIV infection is similar to that in EED, especially during late-stage illness. The HIV virus itself may cause direct damage to intestinal epithelium, while also permitting injury by other gut pathogens by weakening host defenses against them. This resultant enteropathy may itself drive HIV disease progression.

Which treatments have already been tried?

Table 1 summarizes selected published trials conducted among children in low- and middle-income country settings where EED has been explicitly targeted. The trials described therein have suggested beneficial effects of zinc, vitamin A, n-3 LC-PUFA, and alanyl-glutamine on L:M ratios. Ongoing studies are now building on these results.

How should our present knowledge of EED affect our practice today?

Testing for EED in a therapeutic context, as part of routine management of moderate acute malnutrition (MAM), severe acute malnutrition (SAM), or stunting, is not presently indicated, since its etiology remains unclear and evidence for benefit from targeted treatments is lacking.

The significant impact of poor WASH on the general health of children is already well established, and there is already evidence that WASH interventions can reduce infectious morbidity and mortality in low- and middle-income country settings [101, 102]. A holistic approach to nutritional rehabilitation that includes attention to minimizing feco-oral contamination in the home or hospital environment should therefore already be prioritized. However, our emerging understanding of the possible benefits of these interventions, perhaps mediated via EED, on nutritional and immune status may further enhance the scale and types of WASH approaches undertaken. As new, noninvasive, cheap, and quick biomarkers of EED and evidence for setting-specific etiology and effective treatments emerge, more specific recommendations will follow.

Current research, future priorities

There are several ongoing randomized trials of interventions to prevent or treat EED (**table 2**). Research on EED is a rapidly expanding field. Questions requiring further work include:

- What are the important causes of EED? Is it predominantly due to a specific pathogen, nutritional deficiency, or genetic predisposition?
- How can we diagnose EED using a point-of-use test?
- How prevalent is EED in different settings worldwide?
- What is the impact of EED on child health? How are these effects mediated?
- How can we best prevent EED?
- Once EED is established, is there any role for providing direct (e.g., immunomodulatory) treatment for EED?

Further detailed longitudinal observational studies of EED are needed in varied demographic and geographic settings, focusing on the hypothesized period of establishment of EED (infancy). One ongoing example is the Interactions of Malnutrition & Enteric Infections: Consequences for Child Health and Development (MAL-ED) network: coordinated birth cohorts in Peru, Brazil, Bangladesh, India, Pakistan, Nepal, Tanzania, and South Africa [103]. In addition to repeated measurement of L:M ratios, gut pathogens, and growth, enteral vaccine immunogenicity is being studied within this network.

EED transcends traditional discipline boundaries, so its study requires collaboration among pediatricians, immunologists, gastroenterologists, epidemiologists, water and sanitation experts, nutritionists, and others. The fact that positive findings have been observed in studies investigating a wide range of causes for EED (both nutritional and infective) suggests that EED is likely to be multifactorial in nature and therefore probably not amenable to one single therapy. Understanding this complexity will be crucial to addressing the significant public health issues of childhood malnutrition and susceptibility to infection in low- and middle-income country settings.

Acknowledgments

This paper is an abridged version of a Technical Brief that was originally commissioned and published online via the Community-Based Management of Acute Malnutrition (CMAM) Forum website on 2 September 2014 (available at: http://www.cmamforum.org/briefs).

TABLE 2. Ongoing randomized trials of interventions to prevent or treat EED[a]

Trial name and design	Setting	Age group	Intervention and duration	Outcomes
Mesalazine in Environmental Enteropathy; double-blind, placebo-controlled, randomized trial	Kenya; urban	1–5 yr (SAM and stunting)	Mesalazine 28 days	Safety, growth, EED biomarkers
WASH Benefits Bangladesh; factorial cluster-randomized trial	Bangladesh; urban	Birth–2 yr	Nutrition and WASH interventions	L:M, growth, diarrhea, neurological development
WASH Benefits Kenya; factorial cluster-randomized trial	Kenya; rural	Birth–2 yr	Nutrition and WASH interventions	Above plus fecal neopterin, α-1-antitrypsin, myeloperoxidase
Sanitation, Hygiene, Infant Nutrition Efficacy Project; factorial cluster-randomized trial	Zimbabwe; rural	Birth–18 mo (plus mothers)	Infant nutrition and household WASH interventions	L:M, growth, fecal neopterin, EndoCAb, sCD14, immune activation markers, IGF-1, IGF-1:IGFBP3
Effectiveness of Micronutrient Supplementation and Fish Oil + Micronutrient Supplementation in the Treatment of Environmental Enteropathy; factorial double-blind, placebo-controlled, randomized trial	Malawi; rural	1–3 yr	Multiple micronutrients and highly purified fish oil 6 mo	L:M, growth, fecal human mRNA
n-3 LC-PUFAs for the Healthy Growth and Development of Infants and Young Children in Southwest Ethiopia; factorial double-blind, placebo-controlled, randomized trial	Ethiopia; rural	6–12 mo (plus breastfeeding mothers)	n-3 LC-PUFA 12 mo	Growth, neurological development
Zinc Resistant Starch Project; single-arm, open label	Malawi; rural	3–5 yr	Resistant starch 28 days	L:M, growth, markers of zinc homeostasis
Intervention and Mechanisms of Alanyl-Glutamine for Inflammation, Nutrition and Enteropathy; factorial double-blind, placebo-controlled, randomized trial	Brazil; urban	2 mo–5 yr	Alanyl-glutamine 10 days	L:M, growth, fecal lactoferrin, fecal cytokines, diarrhea, markers of alanyl-glutamine metabolism

EED, environmental enteric dysfunction; LC-PUFA, long-chain polyunsaturated fatty acid; L:M, lactulose:mannitol; SAM, severe acute malnutrition; WASH, water, sanitation, and hygiene

a. Trials were ongoing as of June 2014.

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Appendix 5: Evidence for association of impaired oral vaccine response with EED

The below appraisal is derived from Figure 2 in the review by Church et al 'Exploring the relationship between environmental enteric dysfunction and oral vaccine responses.' Future microbiology. 2018;13:1055-70.

Supporting a hypothesis of a causative association between EED and poor oral vaccine response, poorer seroconversion in response to oral polio vaccine 1 (OPV-1) was associated with a marker of immune tolerance (K:T ratio) in Peru and Tanzania and with a marker of intestinal inflammation (faecal calprotectin) in Bangladesh; poorer clinical efficacy of rotavirus oral vaccine was associated with a marker of intestinal epithelial turnover (faecal Reg-1β - a gene that encodes proteins involved in intestinal repair) in Bangladesh; and lastly poorer immunogenicity of oral cholera vaccine was associated with markers of intestinal inflammation (faecal myeloperoxidase) and translocation (soluble CD14 (sCD14)).

Detracting from this hypothesis, however, higher seroconversion in response to oral rotavirus vaccine was associated with markers of intestinal inflammation (faecal calprotectin and myeloperoxidase) in Nicaragua and intestinal permeability (faecal alpha-1-antitrypsin) in Bangladesh; between oral polio vaccine shedding and markers of intestinal permeability (faecal alpha-1-antitrypsin) and inflammation (faecal calprotectin) in India; between better clinical efficacy of rotavirus oral vaccine and a marker of intestinal inflammation (faecal neopterin) and translocation (sCD14) in Bangladesh; between better OPV-2 and OPV-3 seroconversion and higher intestinal epithelial turnover (faecal Reg-1β) in Bangladesh; and

between better immunogenicity in response to oral cholera vaccine and a marker of intestinal permeability (faecal alpha-1-antitrypsin) in Bangladesh.

Appendix 6: Cross-sectional survey participant information and consent form

See next page for the cross-sectional survey participant information and consent form (English version). Participant caretakers were offered a choice of English, Swahili and Giriama language versions.

KEMRI Wellcome Trust Research Programme: Information Sheet and Consent Form

Junju Child Growth Survey

Prevalence of malnutrition amongst under-fives in Junju, Kilifi: a cross-sectional anthropometry survey

KEMRI-Wellcome Trust Research Programme, Kenya	Rosie Crane (Principal Investigator), Sarah Atkinson, Jay Berkley, Greg Fegan, Evelyn Gitau, Sam Kariuki, Susan Morpeth, Musa Mulongo, Francis Ndungu, Etienne de Villiers, Juliana Wambua.
Royal Cornwall Hospitals NHS Trust, UK	Simon Fleming

Who is carrying out this study?

This study is being carried out by KEMRI. KEMRI is a government organisation that carries out medical research to find better ways of preventing and treating illness in the future for everybody's benefit.

What is this study about?

One problem KEMRI is currently trying to learn more about is malnutrition. In this study, we aim to find out how common malnutrition is in Junju. We are asking a randomly selected group of children in Junju aged under five years to participate in the study. We are also interested in why children become malnourished, and the relationship between malnutrition and the health of the gut (intestines).

We would like children aged under five years in 300 homesteads to participate in this study. The area for this study includes several villages in the Junju area. KEMRI field workers visit all the homes in this area every four months to make a list of people who live in the area. The homesteads invited to participate in this study have been chosen randomly from the list of names in a way that everyone has the same fair chance of participating.

What will it involve for my child(ren) if I agree?

If you allow your child to participate in this research, the following observations will be done on your child(ren) to find out whether they are affected by malnutrition:

- Weight – measured by lying scales (babies) or step-on scales (children).
- Length (for lying babies) or height (for standing children) – measured by tape or height board.
- Mid upper arm circumference (MUAC) – measured by wrapping a small paper tape around your child's upper arm.

These observations will be done once only.

We will also take one stool sample from your child(ren). We will test the stool for microbes and other substances to help us better understand malnutrition in all children. If your child has diarrhoea today, we will wait until a few days after the diarrhoea has stopped before taking the sample. This is because we are interested in the health of children's intestines when they *do not* have diarrhoea. If you are concerned about your child's diarrhoea we recommend that you go to your local dispensary.

Finally, we will ask a short series of questions about your household and all children in the household aged under five years. The measurements, stool sample collection and questionnaire will all together take around 30 minutes.

Are there any risks or disadvantages to my child(ren) in taking part?
Our priority for every participant is their wellbeing. There are no anticipated risks to your child(ren) from taking part.

Are there any advantages for my child(ren) in taking part?
If we find from the measurements today that your child(ren) may have malnutrition, we will inform you and advise your child(ren) attend your local health facility for further assessment. If we find from the stool sample that your child(ren) has a worm infection, we will inform you and advise your child(ren) attend your local health facility for further assessment.

In general, the results of this study will improve understanding of malnutrition and may improve the care of other children and babies with malnutrition in the future.

What happens if I refuse to allow my child(ren) to participate?
All participation in research is voluntary. You are free to decide if you want your child(ren) to take part. If you do agree you can change your mind at any time and withdraw your child(ren) from the research. This will not affect your child(ren)'s healthcare now or in the future.

What happens to the stool sample?

- The sample will be labelled with your child(ren)'s name in the form of a code, to ensure that it can only be linked to your child(ren) by people closely concerned with the research.
- We will test the stool for microbes and other substances to help us better understand malnutrition in all children.
- Most of the research tests that will be done on the sample will be done here in Kilifi. However for some tests that cannot be done in Kenya, part of the sample may be sent to laboratories overseas.
- After the research study has ended, the stool sample will be stored at KEMRI Wellcome Trust laboratories in Kilifi. In the future, new research about child health may be done on the sample. Future research must first be approved by the KEMRI Scientific & Ethics Review Unit to ensure participants' safety and rights are respected.

Who will have access to information about my child(ren) in this research?
All our research records are stored securely in locked cabinets and password protected computers. Only a few people who are closely concerned with the research will be able to access information from participants.

In future, information collected during this study may be used to support new research by other researchers in Kenya and other countries on child health problems. In all cases, we will only share information with other researchers in ways that do not reveal individual participants' identities. For example, we will remove information that could identify people, such as their names and where they live, and replace this information

with number codes. Any future research using information from this study must first be approved by a local or national expert committee to make sure that the interests of participants and their communities are protected.

Who has allowed this research to take place?

A Kenyan committee (the KEMRI Scientific & Ethical Review Unit) has looked carefully at this work and agrees that this study is properly planned, and important and relevant to Kenya. The committee also agrees that the study follows nationally and internationally agreed research guidelines. This includes ensuring that all participants' safety and rights are respected.

What if I have any questions?

You may ask any of our staff questions at any time. You can also contact the research team:
Dr. Rosie Crane (Principal Investigator) or John Muriuki, KEMRI Wellcome Trust Research Programme, P.O. Box 230, Kilifi.
Telephone: 0727416278

If you want to ask someone independent anything about this research please contact:
Community Liaison Manager, KEMRI Wellcome Trust Research Programme, P.O. Box 230, Kilifi.
Telephone: 0723342780 or 0417522063
And
The Secretary – KEMRI Scientific & Ethics Review Unit (SERU), PO Box 54840-00200, Nairobi.
Telephone: 0202722541, 0722205901 or 0733400003

KEMRI-Wellcome Trust Research Programme Consent form for Junju Child Growth Survey

I, [being a parent/guardian of _____
_____
_____
_____
_____(name(s) of child(ren))], have had the research explained to me. I have understood all that has been read/explained and had my questions answered satisfactorily.

Please insert the boxes below where relevant:

- ☐ **I agree to allow my child(ren) to take part in this research**
- ☐ **I agree to samples being stored and used for future research**
- ☐ **I agree to samples being exported**

I understand that I can change my mind at any stage and it will not affect my child in any way.

Parent/guardian's signature: _____ **Date** _____

Parent/guardian's name: _____**Time** _____
(Please print name)

Where parent/guardian cannot read, ensure a witness* observes consent process and signs below:

I attest that the information concerning this research was accurately explained to and apparently understood by the parent/guardian and that informed consent was freely given by the parent/guardian.

Witness' signature: _____ **Date** _____

Witness' name: _____ **Time** _____

**A witness is a person who is independent from the research or a member of staff who was not involved in gaining the consent.*

Thumbprint of the parent/guardian as named above if they cannot write: _____

I have followed the study SOP to obtain consent from the parent/guardian. S/he apparently understood the nature and the purpose of the study and consents to the participation of the child in the study. S/he has been given opportunity to ask questions which have been answered satisfactorily.

Designee/investigator's signature: _____ **Date** _____

Designee/investigator's name : _____**Time** _____
(Please print name)

THE PARENT/GUARDIAN SHOULD NOW BE GIVEN A SIGNED COPY TO KEEP

Appendix 7: Cross-sectional survey questionnaire

See next page for the questionnaire administered to participants, caretakers and their household heads during the cross-sectional survey. N.B. paper copies of questionnaires were not used in the field. Fieldworkers entered responses directly onto an Android tablet using Open Data Kit software.

Junju Child Growth Survey

Household data entry form

	Question	Options / fields	Response *Write code*
1.	Date of interview	DD__ __ /MM __ __ / 2015	
2.	Homestead study ID (fk_hm) *Check DSS print-out*	___ ___ ___	
3.	Household study ID *Please give a unique ID per household within a homestead (01, 02, 03 …)*	___ ___	
4.	GPS coordinates of household	North - ___ ___ . ___ ___ ___ ___ (latitude) East + ___ ___ . ___ ___ ___ ___ (longitude)	
5.	Has informed consent been obtained?	0. No ----------> End 1. Yes ----------> Continue	
6.	What is the sex of the household head?	1. Male 2. Female	
7.	How many people are there in the household?	___ ___ people	
8.	Who is the primary caretaker for the children in this household?	1. Mother 2. Father 3. Grandparent 4. Sibling (aged 15 or younger) 5. Sibling (aged 16 or older) 6. Housemaid 7. Other	
The following questions should be answered by the primary caretaker or mother for this household			
9.	*If the primary caretaker is not the children's mother*: What is the highest education level of this primary caretaker?	1. Didn't go to school 2. Started primary school, but didn't finish 3. Completed primary school 4. Started secondary, but didn't finish 5. Completed secondary 6. Post-secondary	
10.	*If the primary caretaker is not the children's mother*: Does he/she spend most days away from the home for work or other activities?	0. No 1. Yes	

11.	What is the household's **MAIN** source of water for **DRINKING**? *Select one only*	1. Piped to public tap / standpipe 2. Piped to neighbour 3. Piped to compound (outdoors) 4. Piped into dwelling (indoors) 5. Borehole 6. Dug well (covered) 7. Dug well (uncovered) 8. Spring (protected) 9. Spring (unprotected) 10. Rainwater collection 11. Tanker truck 12. Cart with small tank / drum 13. Surface water (river, stream, dam, lake, pond) 14. Bottled water 99. Other (specify)……	
12.	Where is this **MAIN** source of water for **DRINKING** located?	1. Inside the compound (but outside the house) 2. Outside the compound	
13.	How long does it take the water collector to travel to the **MAIN** source of **DRINKING** water?	0. Less than 30 minutes 1. 30-60 minutes 2. More than one hour	
14.	Does the water collector normally have to queue at the **MAIN** source of **DRINKING** water?	0. No 1. Yes	
15.	*If 'Yes' to 14:* For how long does the water collector normally queue?	0. Less than 30 minutes 1. 30-60 minutes 2. More than an hour	
16.	How long in total does it take to go there, fetch water, and come back?	0. Less than 30 minutes 1. 30-60 minutes 2. More than an hour	
17.	How many 20-litre jerricans of water were collected by the whole household yesterday?	___ ___ mitungi (20-litre jerricans)	
18.	Does the household pay for water?	0. No 1. Yes	
19.	*If 'Yes' to 18:* How much per mtungi (20 litre jerrican)?	___ ___ Kenya shillings	
20.	Do you normally treat/boil water before drinking it?	0. No 1. Yes	
21.	*If 'Yes' to 20*: Do you boil the water before drinking it?	0. No 1. Yes	
22.	*If 'Yes' to 20*: Do you add chemicals such as chlorine, aquaguard/waterguard to the water before drinking it?	0. No 1. Yes	
23.	*If 'Yes' to 20*: Do you use a water filter or pass the water through clothes before drinking it?	0. No 1. Yes	
24.	*If 'Yes' to 20*: Do you use traditional herbs or ash to treat water before drinking it?	0. No 1. Yes	

25.	*If 'Yes' to 20*: Do you keep water in the sun to disinfect before drinking it?	0. No 1. Yes	
26.	*If 'Yes' to 20*: Please write any other method that is used to treat water before drinking it?	……	
27.	Where is the drinking water stored?	0. Open container 1. Closed container	
28.	Within this household, what is the **MAIN** toilet facility used by the children's primary caretaker? *Select one only*	1. Pit latrine with uncovered wooden slab 2. Pit latrine with wooden slab covered with soil 3. Pit latrine with concrete slab 4. Ventilated improved pit latrine 5. Flush toilet 6. No facility (bush, open field)	
29.	Is this toilet facility shared with other households? *Skip this question if "No facility"*	0. No 1. Yes	
30.	How are babies' faeces **MAINLY** disposed of?	1. Put in latrine or toilet 2. Throw into nearby bushes 3. Leave in place / scatter within compound 4. Leave for dogs / give to dogs 5. Bury 99. Other (specify) ……	
31.	On which occasions does the children's primary caretaker wash his/her hands? *Select all that apply*	1. After toilet 2. Before handling food or cooking 3. Before eating 4. Before feeding the child (including before breastfeeding) 5. After cleaning child's bottom	
32.	What does the primary caretaker **MAINLY** use to wash his/her hands? *Select one only*	1. Water only 2. Water and soap 3. Water and ash 4. Water and sand	
33.	How many rooms are there in the household for SLEEPING?	___ ___ rooms	
34.	What is the **MAIN FLOOR** material of the rooms in this household? *Seek permission to observe floor* *Select one only*	1. Earth/sand (mchanga) 2. Dung 3. Cement 4. Wood 5. Tiles 6. Carpet 99. Other (specify)……	
35.	What is the **MAIN WALL** material of the rooms in this household? *Observe* *Select one only*	1. Grass/straw/makuti 2. Mud/wood 3. Corrugated iron sheet/ Tin 4. Stone 5. Brick/block 6. Wood planks/shingles 7. No wall	

		99. Other (specify) ……	
36.	What is the **MAIN ROOF** material of the house in this household? *Observe* *Select one only*	1. Grass/Thatch/Makuti 2. Nylon papers/clothes 3. Mud(Manyatta) 4. Corrugated iron (Mabati)/Tins 5. Tiles/Asbesto sheets 6. Concrete 99. Other (specify) ……	
37.	What is the **MAIN** cooking fuel used in this household? *Select one only*	1. Electricity 2. Gas/LPG 3. Paraffin 4. Charcoal 5. Firewood 99. Other (specify) ……	
38.	Does this household own land?	0. No 1. Yes	
39.	*If "Yes" to 38*: How many acres of land does this household own?	___ ___ acres	
40.	How many **cattle** are owned by this household?	___ ___ cattle	
41.	How many **goats and sheep** are owned by this household?	___ ___ goats / sheep	
42.	How many **pigs** are owned by this household?	___ ___ pigs	
43.	How many **donkeys / horses** are owned by this household?	___ ___ donkeys / horses	
44.	How many **chickens and other poultry** are owned by this household?	___ ___ chickens	
45.	How many **rabbits** are owned by this household?	___ ___ rabbits	
46.	Which animals roam (or are kept) **WITHIN the compound**?	1. Cattle 2. Horses/donkeys/mules 3. Goats and sheep 4. Chickens and ducks 5. Pigs 6. Dogs 7. Cats 8. Rabbits	
47.	Which animals may wander **into the HOUSE**?	1. Goats and sheep 2. Chickens and ducks 3. Pigs 4. Dogs 5. Cats 6. Rabbits	

48.	Observe for animal/human faecal material within the compound or immediately surrounding the house, and specify:	1. None / almost none 2. Sparse (kidogo) 3. Very many	

49. Did ANY MEMBER of your household eat any of the following foods yesterday?

1. Cereals and Cereal Products: Maize, rice, pasta, ugali, porridge, bread, biscuits, millet, sorghum, wheat [and any other locally available grains]	0. No 1. Yes	
2. Fish and Sea Foods: fresh or dried fish or shellfish	0. No 1. Yes	
3. Roots and Tubers: Irish potatoes, sweet potatoes, yams, cassava, or foods made from roots or wild roots and tubers	0. No 1. Yes	
4. Vegetables: Sukuma wiki, cabbages, carrots, spinach, and any other locally available vegetables including wild vegetables	0. No 1. Yes	
5. Fruits: Oranges, ripe bananas, mangoes, avocados,	0. No 1. Yes	
6. Meats and Poultry: Camel, beef, lamb, goat, rabbit, wild game, chicken or other birds, liver, kidney, heart or other organ meats or blood-based foods	0. No 1. Yes	
7. Eggs: Chicken, bird eggs	0. No 1. Yes	
8. Pulses / Legumes / Nuts and Seeds: Beans, peas, lentils, nuts, seeds or foods made from these	0. No 1. Yes	
9. Milk and Milk Products: Fresh/fermented milk, cheese, yogurt, or other milk products	0. No 1. Yes	
10. Fats and Oils:Oil, fats, ghee, margarine or butter added to food or used for cooking	0. No 1. Yes	
11. Sugars / Honey and Commercial Juices: Sugar in tea, honey, sweetened soda or sugary foods such as commercial juices, chocolates, sweets or candies	0. No 1. Yes	
12. Miscellaneous: Spices, sweets, unsweetened beverages	0. No 1. Yes	

50.	What is the **main source of the dominant food** item consumed in this household? *Select one only*	1. Own production 2. Purchase 3. Gift from friends/families 4. Food aid 5. Traded/bartered 6. Borrowed 7. Gathered/hunted/wild 99. Other (specify) ……	
51.	During the past 7 days, has ANY member of the household missed a meal due to food shortage?	0. No 1. Yes	

52.	What is the household's MAIN source of income?	1. No income 2. Permanent job 3. Petty trading e.g. small business, sale of firewood 4. Sale of farm/crop products (e.g. maize, beans, potatoes, bananas, coffee, tea etc) 5. Business like retail shop, supermarket/merchadise 6. Brewing alcohol 7. Sale of livestock 8. Sale of livestock products (milk/meat/hides) 9. Fishing 10. Casual labour 11. Remittance 12. Others like sale of personal assets	
53.	What is the household total monthly cash income in Kenya shillings?	1. Less than 1,000 2. 1,000 - 3,999 3. 4,000 - 9,999 4. 10,000 - 19,999 5. 20,000 and above 88. Don't know	
54.	Does this household have bednets?	0. No 1. Yes	
55.	How many children aged under five years are there in the household?	___ ___ under-fives	
56.	Does the mother/caretaker of these children have any children that have died?	0. No 1. Yes	
57.	Do all these children have the same biological mother?	0. No 1. Yes	
If "Yes" to 57: Let the primary caretaker or mother answer all the following questions. If "No" to 57: These questions are specific to each child and they appear in the child questionnaire.			
58.	Is the children's mother alive?	0. No 1. Yes	
59.	*If "Yes" to 58*: Enter current age in YEARS of the mother *If unknown, enter 999*	___ ___ ___ years	
60.	*If "Yes" to 58:* Is the mother usually present in the homestead every day? (Or most days?)	0. No 1. Yes	
61.	*If "Yes" to 58:* Is the mother currently pregnant?	0. No 1. Yes	
62.	*If "Yes" to 58:* Is the mother currently breastfeeding?	0. No 1. Yes	

63.	What is the highest education level of the mother? *If the mother is dead choose the highest education level before she died*	1. Didn't go to school 2. Started primary school, but didn't finish 3. Completed primary school 4. Started secondary, but didn't finish 5. Completed secondary 6. Post-secondary	
64.	What is the marital status of the mother? *If the mother is dead choose the marital status before she died*	1. Single/never married 2. Married and living together 3. Married but not living together 4. Divorced 5. Widowed	
65.	How old IN YEARS was the mother when she delivered her first baby?	___ ___ years	
66.	Do all these children have the same biological father?	0. No 1. Yes	
67.	*If "Yes" to 66:* Is the children's father alive?	0. No 1. Yes	
68.	*If "Yes" to 66:* Does the father have more than one wife?	0. No 1. Yes	

Fieldworker declaration:

I ……[print name] declare that this is true information that I have obtained from the respondent(s).

Signature …… Date ……

Junju Child Growth Survey

Under-fives data entry form

	Question	Options / fields	Response *Write code*
1.	Date of interview	DD___ ___ /MM ___ ___ / 2015	
2.	Homestead study ID (fk_hm) *Check from household questionnaire*	___ ___ ___	
3.	Household study ID *Check from household questionnaire*	___ ___ ___	
4.	Participant study ID *Check DSS print-out*	___ ___ ___ ___	
5.	Has informed consent been obtained from the child's parent or guardian?	0. No ----------> End 1. Yes ----------> Continue	
The following questions should preferably be answered by the child's mother or primary caretaker.			
6.	What is the first NAME of the child (who is under five)?	_ _ _ _ _ _ _ _ _ _	
7.	What is {NAME}'s sex?	1. Male 2. Female	
8.	What is {NAME}'s date of birth? *Enter the date written on the health card. If the health card is not available, enter the recalled date. If the respondent knows the child's age, but not their exact date of birth, work out the year and month of their birth, and enter '15th' for the day.*	DD___ ___ /MM ___ ___ / 20 ___ ___	
If the child belong to a different biological mother, then answer questions 9-18, otherwise skip to 19			
9.	Is {NAME}'s mother alive?	0. No 1. Yes	
10.	*If 'Yes' to 9*: Enter current age in YEARS of {NAME}'s mother *If unknown, enter 999*	___ ___ ___ years	
11.	*If 'Yes' to 9:* Is {NAME}'s mother usually present in the homestead every day? (Or most days?)	0. No 1. Yes	
12.	*If 'Yes' to 9:* Is {NAME}'s mother currently pregnant?	0. No 1. Yes	
13.	*If 'Yes' to 9:* Is {NAME}'s mother currently breastfeeding?	2. No 2. Yes	
14.	What is the highest education level of {NAME}'s mother? *If the mother is dead choose the highest education level before she died*	1. Didn't go to school 2. Started primary school, but didn't finish 3. Completed primary school 4. Started secondary, but didn't finish 5. Completed secondary 6. Post-secondary	
15.	What is the marital status of {NAME}'s mother? *If the mother is dead choose the marital status before she died*	1. Single/never married 2. Married and living together 3. Married but not living together 4. Divorced 5. Widowed	

16.	How old IN YEARS was {NAME}'s mother when she delivered her first baby?	___ ___ years	
17.	Is {NAME}'s father alive?	0. No 1. Yes	
18.	*If 'Yes' to 17:* Does {NAME}'s father have more than one wife?	0. No 1. Yes	
19.	Has {NAME} EVER been breastfed?	0. No 1. Yes	
20.	*If 'Yes' to 19:* Is {NAME} STILL breastfeeding?	0. No 1. Yes	
21.	*If 'No' to 19:* At what age in months did {NAME} STOP breastfeeding?	___ ___ months	
Ask questions 22-29 for babies under 6 months of age. For children aged 6 months or more, skip to question 30			
22.	What is {NAME}'s MAIN food or liquid at the moment? *Select one only*	1. Breast milk 2. Uji / porridge 3. Animal milk (fresh) 4. Infant formula milk (powdered) 5. Other liquid(s) 6. Other solid(s)	
23.	At what age in months did this current MAIN food become {NAME}'s MAIN food?	___ ___ months	
24.	Before this current MAIN food became {NAME}'s main food, did they receive anything else (other than breast milk) as their MAIN intake?	0. No 1. Yes	
25.	*If 'Yes' to 24*: What was {NAME}'s main food BEFORE the current main food was started? *Select one only*	1. Uji / porridge 2. Animal milk (fresh) 3. Infant formula milk (powdered) 4. Other liquid(s) 5. Other solid(s)	
26.	At what age in months did this main food that was given before the current main food become {NAME}'s MAIN food?	___ ___ months	
27.	Other than the current MAIN food, which other foods or liquids does {NAME} take currently? *Select all that apply*	1. Animal milk (fresh) 2. Sugar water 3. Sugar and salt water (home-made) 4. Coconut water 5. Tea / herbal water 6. Plain water 7. Uji / porridge 8. Infant formula milk (powdered) 9. Other liquid(s) 10. Other solid(s)	
28.	*If 'Yes' to 19*: At what age in WEEKS did {NAME} first receive ANY food or liquid other than breast milk? (Even if small amounts)	___ ___ weeks	
29.	*If 'No' to 19:* At what age in WEEKS did {NAME} first receive ANY food or liquid other than their first milk? (Even if small amounts)	___ ___ weeks	

Ask questions 30 and 31 for all children aged 6 months and above			
30.	Yesterday, how many times did you feed {NAME} solid and semi-solid foods?	___ ___ times	
31.	During the past 7 days, have you noticed {NAME} eating soil?	0. No 1. Yes	
Ask the remaining questions for all children			
32.	Has {NAME} EVER received vitamin A dose? *Verify using child health card.*	0. No 1. Yes 88. Card not seen	
33.	*If 'Yes' to 32*: Did {NAME} receive a vitamin A dose within the last SIX months? *Verify using child health card.*	0. No 1. Yes 88. Don't know	
34.	Is {NAME} CURRENTLY taking iron supplements? *Ask to see the medication package to confirm either written name (ferrous sulphate) or tablet appearance.*	0. No 1. Yes 88. Don't know	
35.	Has {NAME} received a deworming treatment within the last six months?	0. No 1. Yes 88. Don't know	
36.	In the past 7 days, has {NAME} had cough or difficult breathing?	0. No 1. Yes	
37.	*If 'Yes' to 36:* Was {NAME} taken to a healthcare facility for this cough?	0. No 1. Yes	
38.	In the past 7 days, has {NAME} had fever?	0. No 1. Yes	
39.	*If 'Yes' to 38:* Was {NAME} taken to a healthcare facility for this fever?	0. No 1. Yes	
40.	In the past 7 days, has {NAME} had diarrhoea? *Diarrhoea defined as passage of 3 or more episodes of loose stool within 24 hours*	0. No 1. Yes	
41.	*If 'Yes' to 40:* Was {NAME} taken to a healthcare facility for this diarrhoea?	0. No 1. Yes	
42.	Is {NAME} currently enrolled in a feeding programme?	0. No 1. Yes	
43.	Did {NAME} sleep under a bednet last night?	0. No 1. Yes	
44.	Does {NAME} have a twin brother or sister?	0. No 1. Yes	
45.	Ask for {NAME}'s health card and enter birth weight. *If not on card, enter 999*	___ . ___ ___ kg	
46.	Was {NAME} born premature?	0. No 1. Yes	
47.	Where was {NAME} born?	0. Home 1. Public health facility 2. Private health facility	
48.	How was {NAME} born?	1. Normal delivery 2. Caesarean	

49.	Does {NAME} have any significant problems with movement, speech or eating, or seems markedly delayed in development of physical skills compared to other children their age?	0. No 1. Yes	
50.	Does {NAME} have a known heart condition?	0. No 1. Yes	
51.	Does {NAME} regularly attend daycare away from home?	0. No 1. Yes	
52.	Where does {NAME} spend most of their time during the day?	1. Indoors at home (e.g. house) 2. Outdoors at home (e.g. garden) 3. Indoors away from home (e.g. school) 4. Outdoors away from home (e.g. shamba)	
53.	Does the child have an older sibling? (alive or dead)	0. No 1. Yes	
54.	*If 'Yes' to 53:* Date of birth of {NAME}'s older sibling (the closest in age to the child, even if now dead)? *Enter the date written on the health card. If the health card is not available, enter the recalled date. If the respondent knows the child's age, but not their exact date of birth, work out the year and month of their birth, and enter '15th' for the day.*	DD___ ___ /MM ___ ___ / 20 ___ ___	
55.	Does {NAME} have a younger sibling?	0. No 1. Yes	
56.	*If 'Yes' to 55:* Date of birth of {NAME}'s younger sibling (the closest in age to the child, even if now dead)? *Use the hint in Q. 54*	DD___ ___ /MM ___ ___ / 20 ___ ___	
57.	Please enter {NAME}'s weight	___ ___ . ___ ___ ___ kg	
58.	Please enter {NAME}'s height or length	___ ___ . ___ cm	
59.	Please enter {NAME}'s MUAC *If **less than 12.5 and over 6 months**, please refer to the local health facility for further assessment*	___ ___ . ___ cm	
60.	Does {NAME} have oedema? *If 'yes', please refer to the local health facility for further assessment*	0. No 1. Yes	
61.	**If {NAME} is under 6 months**, is he/she VISIBLY SEVERELY WASTED? *If 'yes' refer to the local health facility for further assessment*		
62.	Enter the barcode number of {NAME}'s stool	Barcode __ __ __ __ __ __ __ __ __	

Fieldworker declaration:

I …… [print name] declare that this is true information that I have obtained from the respondent(s).

Signature …… Date ……

Appendix 8: Definition of a homestead

A homestead is defined in the KHDSS Manual as *'a cluster of dwellings that are identified by a single descriptive name (often of a person, a homestead head either living or deceased) which are contiguous (not divided by dwellings from other homesteads) but may be separated from each other by up to 30 metres of free land. In most cases a homestead will consist of one or more buildings housing related nuclear families.'*

Appendix 9: Examples of WHO improved and unimproved sanitation facility and drinking water source

Supplementary table 1: Examples of WHO-defined 'improved' and 'unimproved' water and sanitation. Source: Progress on drinking water and sanitation. WHO/UNICEF Joint Monitoring Programme for Water and Sanitation. March 2012.

WASH indicator	Unimproved examples	Improved examples
Water	Unprotected dug well Unprotected spring Surface water (river, dam, lake, pond, stream, canal, irrigation channel) Vendor-provided water (cart with small tank/drum, tanker truck) Bottled water (bottled water is considered improved only when the household use another improved source for cooking and personal hygiene) Tanker truck water	Piped household water connection Public standpipe Borehole Protected dug well Protected spring Rainwater collection
Sanitation	Pit latrines without slabs or platforms or open pit Hanging latrines Bucket latrines Open defecation in fields, forests, bushes, bodies of water or other open spaces Disposal of human faeces with other forms of solid waste Any form of sanitation that is shared between two or more households	Flush toilet (provided flush to an area that hygienically separates waste from human contact e.g. sewerage system or septic tank) Covered pit latrine

Appendix 10: Cross-sectional survey anthropometry SOP

See next page for the cross-sectional survey anthropometry SOP. Illustrations are copied from the WHO 'Job Aid: Weighing and Measuring a Child' available from https://www.who.int/childgrowth/training/jobaid_weighing_measuring.pdf

STANDARD OPERATING PROCEDURE

ANTHROPOMETRY

Study	'Afya tumboni': Epidemiology of Environmental Enteropathy and intestinal microbiota amongst infants In Junju, Kilifi
Author	Rosie Crane
Version	1.0
Date	28/06/2015

Materials required

Step-on scales
Lying scales
Stadiometer (height meter)
Length mat
MUAC tape
Cloth/towel
Cleaning wipe
Pen
Homestead task record

Method: weight

1. *If the child is aged 2 years or older, and can stand,* use the step-on scales. Prepare the scales by removing from their case and placing on level, clean ground. To help level the scales, look at the bubble and try to get it central (this isn't mandatory). Ask the mother to help if the child is scared. Tap the scale. 0.0 should show on the screen. The child should stand in the middle of the scales and keep still. If the weight is 15kg or more, record this weight in the ***Homestead task record*** (it will be to 1 decimal place). If the weight is less than 15kg, do not record but confirm in greater detail by using the lying scales.





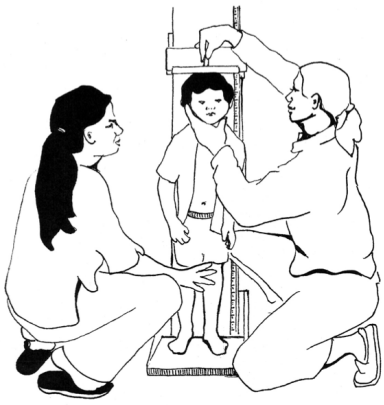
2. *If the child is aged less than 2 years and/or cannot stand and/or they weigh less than 15kg with the standing scales,* use the lying scales. Prepare the scales by removing from their case and placing on level, clean ground. If placing on bare earth, place on top of the case for extra protection from dirt. To help level the scales, look at the bubble and try to get it central (this isn't mandatory). Ask the mother to help if the child is scared. Sit or lie the child on the scale. Older children who weigh less than 15kg may stand on the lying scale, but you must lift them on and off to prevent damage to the scale from children stepping on the edge. Record the weight in the ***Homestead task record*** (it will be to 3 decimal places).



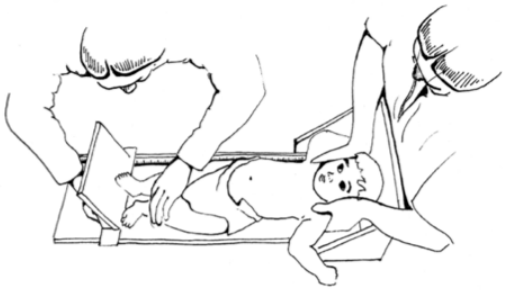
3. *If the child is very scared of both scales,* you can use the Tare method. Weigh the mother on the step-on scales. With the mother still standing on the scales, press the '2 in 1' button. The screen will display 0.0. Pass the child to the mother. Record this weight.

4. Clean both scales – top and underside – using the towel (dry dirt) and wipe (wet dirt, stains). Return to their cases.

Method: height/length



1. *If the child is aged 2 years or older, and can stand,* use the stadiometer. Remove the stadiometer from its case and assemble. Place the stadiometer on level, clean ground. Help the child to stand with feet central, and heels, bottom, shoulders and head against the board. Help the child to hold their head level (not looking down or up). If the child's back is arched, gently push their tummy in so they achieve their full height. Slide the headpiece down level with the top of the child's head. Record the height indicated by the red pointers to the nearest 0.1cm in the ***Homestead task record***.



2. *If the child is aged less than 2 years, and/or cannot stand, and/or is very scared of the stadiometer,* use the length mat. Roll out the mat on a flat, clean surface. Ask the mother to hold the child's head central and level, up against the top of the mat. There should be no space between the child's head and the top of the mat. Position the child's shoulders and bottom centrally on the mat. Using one hand, gently but firmly hold down the child's bottom and knees, so the legs are straight. If the child's back is arched, gently push their tummy in so they achieve their full length. Using your other hand, bring the bottom of the mat up to meet the child's feet. Bend the child's feet to 90 degrees (as if they are standing) and bring the bottom of the mat to the bottom of their feet. Take the measurement here, ensuring the bottom of the mat is level on both sides. (N.B. be careful with the legs of babies aged less than one month – they are delicate and should not be fully straightened). Record the length in the ***Homestead task record*** to the nearest *completed* 0.5cm.



3. Clean the stadiometer and length mat using the towel (dry dirt) and wipe (wet dirt, stains).

Method: MUAC

1. Ask the mother to remove any clothing that may cover the child's left arm. Keep your work at eye level. Sit down when possible. For small children, have them in the mother's lap.

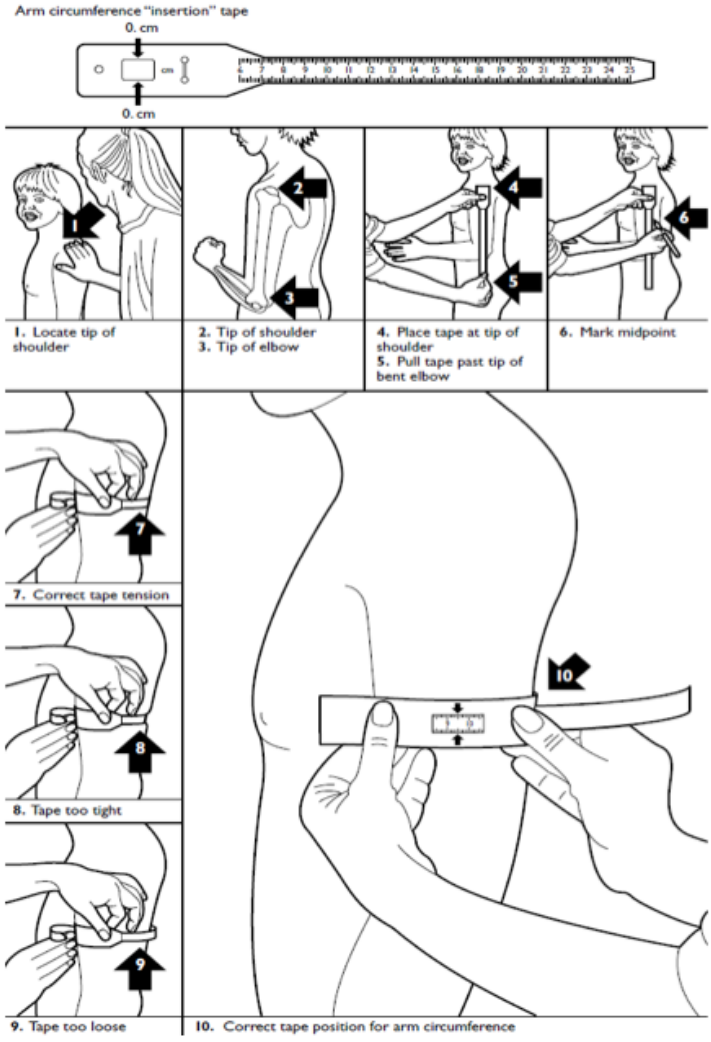
2. Calculate the midpoint of the child's **LEFT** upper arm by first locating the tip of the child's shoulder (Arrows 1 and 2) with your fingertips. Bend the child's elbow to make a right angle (Arrow 3). Place the end of the measuring tape on the tip of the shoulder (Arrow 4) and pull it straight down past the tip of the elbow (Arrow 5). Hold your finger on the location of the length. Bend the tape up in half so that the end of the tape meets the length you measured. Place the tape back on the tip of the shoulder and assistant will be ready with a pen to mark the midpoint on the arm (Arrow 6).

3. Straighten the child's arm and wrap the tape around the arm at midpoint. Make sure the numbers are right side up and the tape is flat around the skin (Arrow 7). Make sure tape passes below window with the zero arrows and is inserted through the small slot to the right of the measuring window.

4. Inspect the tension of the tape on the child's arm. Make sure the tape has the proper tension (Arrow 7) and is not too tight or too loose (Arrows 8-9).

5. When the tape is in the correct position on the arm with the correct tension, read and call out the measurement to the nearest 0.1cm (Arrow 10). Then loosen the tape and record the measurement in the ***Homestead task record***.

6. Children aged 6 months or above with a MUAC of less than 11.5cm should be referred to Pingilikani or Junju dispensaries for further assessment.



Appendix 11: Focus group discussion questions

These questions were written by research assistant John Muriuki.

Theme 1: Food availability

1. What is the food situation in this village at the moment?
2. What are the main food sources in the households?
3. How much does it cost to buy 1kg of the staple from the market?
4. How does this compare to the same month last year?

Theme 2: Food access

1. Explain the main income sources for this community.
2. How do you store food in your households?
3. What do you do when the stored food is finished?

Theme 3: Vulnerability and coping mechanisms during food insecurity

1. What do you understand by the term 'food inadequacy' in your community?
2. Have you faced food inadequacy recently?
3. What do families do when they don't have enough food for three meals a day?
4. In your opinion, which types of households face food shortage in this community? Why?
5. How do you rank household wealth? What makes a household rich, middle or poor?

Theme 4: Water and sanitation

1. What are the main sources of water for drinking in this village?
2. In most cases, is anything done to the water before drinking it? If yes, what is done?
3. How do you store drinking water?
4. On which occasions do most people wash hands?
5. Is there a perception that 'children should eat dirt to get immunity' in your community?
6. What is the proportion of people having toilet facilities in your village?
7. Out of these toilet facilities, how many contain human faeces in a hygienic manner?
8. What are the most common practices of disposing children's faeces?

9. How common is washing of raw foods before eating them?

Theme 5: Perceptions of child health and child care practices

1. What makes a child healthy?
2. What actions do you take to keep the child healthy?
3. What are the main illnesses children experience in this community?
4. Why do they have these illnesses?
5. What are the main ways of caring for the child during each illness?
6. During which seasons are children most vulnerable to sickness in this area?
7. Which children are most vulnerable?
8. How does the community perceive antenatal and postnatal care?
9. Where do most women deliver their children? Why?
10. Do you think the health of the mother before and during pregnancy can affect the growth of a child?
11. What is done by the community to ensure women are healthy before and during pregnancy?
12. What is the minimum interval between births in your village?

Theme 6: Infant and young child feeding practices

1. For how long after birth do mothers breastfeed their children without giving them anything else (even water)?
2. At what age do infants start to receive other solid foods in addition to breastmilk?
3. Which foods/drinks are introduced first to a child?
4. Why are these foods given to children?

Appendix 12: Methods for ELISA detection of alpha-1-antitrypsin, myeloperoxidase and neopterin in stool.

Stool samples were thawed in batches to undergo ELISA for three markers of EED: alpha-1-antitrypsin, myeloperoxidase and neopterin. Each aliquot was thawed only once, and ELISA for all three biomarkers took place on the same day. Commercial kits were used (alpha-1-antitrypsin: ImmunoChrome, GmbH RIC6200; myeloperoxidase: Hycult, Netherlands HK324-02; neopterin: GenWay GWB-286F41). Manufacturers' methods were followed for all kits with no adaptations. Laboratory SOPs were developed for each marker by Dr Agnes Gwela at KWTRP Laboratories (Appendix 13). Sample were assayed in duplicate and percentage discrepancy calculated for each duplicate pair. Where discrepancy exceeded 25%, samples were re-run on subsequent plates. A standard curve was included on every plate. Where optical density was greater than the highest or less than the lowest value on each plate's standard curve, the sample was re-run on a subsequent plate with an appropriately greater or less dilution.

Assay-derived duplicate mean optical densities were converted to biomarker concentrations using four-parameter logistic regression. For this, optical densities for all samples and for the standard curve, blanks and controls (the latter three were supplied within each ELISA kit) were inputted to an online tool found at www.elisaanalysis.com. This tool uses four-parameter logistic regression[‡‡] to fit a non-linear standard curve and EED biomarker concentrations were then inferred from each sample's mean optical density using this curve.

[‡‡] Regression formula and explanation are found in 'The Immunoassay Handbook' 4th Edition by David Wild, published in 2013 by Elsevier, page 327

Appendix 13: Laboratory SOPs for ELISA detection of alpha-1-antitrypsin, myeloperoxidase and neopterin in stool.

See next page for laboratory SOPs for ELISA detection of alpha-1-antitrypsin, myeloperoxidase and neopterin in stool. These were written by Dr Agnes Gwela at KWTRP Laboratories and followed each commercial kit's instructions. No adaptations were made.

SOP FOR THE QUANTITATION OF ALPHA-1-ANTITRYPSIN IN STOOL

Markers: α-1-antitrypsin

Specimens: Stools samples (frozen at -80^{0}C)

Location: Immunology Labs

Persons Responsible: Agnes Gwela, Rosie Crane and Emily Nyatichi

LABORATORY EQUIPMENT AND REAGENTS NEEDED

EQUIPMENT	AVAILABILITY
Fume Hood	Available (chemical Room)
-80^{0}C Freezer	Available
Laboratory Weighing Balance	To be provided
Weighing Boats/ **2ml cap tubes**	Available
ELISA Plate Reader (450nm) Ref λ: 620/690nm	Available
ELISA Plate Washer	Available
Microtitre plate shaker	Available
1.5mL Eppendorf tubes	Available
Vortex Mixer	Available
15mL Falcon Tubes*	Available
Dip sticks*	To be provided by Rosie Crane
ELISA Kits	All Commercial….have been purchased
Refrigerated Centrifuge, 3000xg or 13,000 rpm	Available

ELISA KIT SPECIFICATIONS

KIT	CATALOGUE #	COMPANY
Neopterin ELISA kit	RE59321	IBL International, Hamburg
MPO Human ELISA kit	HK324-02	Hycult, Netherlands
α-1-antitrypsin ELISA kit	RIC6200	ImmunoChrome, GmbH

All ELISA assays will be performed according to the Instructions in each kit.

Kit 1: Alpha-1-antitrypsin ELISA

Components	Descriptions	Amount
MTP	Microtiter plate_coated	96 well
WASHBUF/ Sample Dilution buffer	ELISA wash buffer concentrate 10 fold	100 ml
STD	Standard (1ml) (**0, 3.3, 10, 30, 90 ng/ml**)	5 vials
CTRL	Control 1 & 2 (1ml)	1 vial each
CONJ	Conjugate, peroxidase labelled antibody	15 ml
SUB	TMB substrate (tetramethylbenzidine)	15 ml
STOPP	Stop Solution	7 ml

*****Take ELISA kits out of the fridge and bring all reagents to room temperature before starting assay.**

Reagent Preparations

Prepare buffers on the evening before the assay to save time. During this time, you may also take out the samples to be assayed, record samples numbers without thawing them and keep samples in a designated box.

WASHBUF: Dilute 1:10 in aqua bidest (double distilled H_20). 100 mL WASHBUF + 900 mL ddH_20 This is stable for 14 days at 2-8^{0}C

STANDARDS: Pipette directly into wells

CONTROLS: CTRL1 & CTRL 2 to be used as neat.

90ng/ml	45ng/ml	30ng/ml	10ng/ml	5ng/ml	3.3ng/ml	0ng/ml
STD 1	100ul STD1 + 100ul washbuf	STD 3	STD 4	100ul STD1 + 100ul washbuf	STD 6	BLANK

The kits provides only 5 STDS to be used neat/ as they are. We shall make an extra standard (45ng/ml or 5ng/ml) by diluting the previous high conc. at 1:2 in diluted wash buffer. This should be done immediately before adding standards to designated plate microwells.

STOOL SAMPLES

- **We aim to work on 40 samples per day**
- **Extraction in glass or plastic vials (we shall use 2mL screw cap tubes and dip sticks)**
- **Before picking sample from the stock to weigh, use the dip-stick to mix the sample thoroughly. If possible avoid picking particulates and fibres and pick the 'softer' bits of the stool.**
- **From each stool sample, weigh out 3 portions of (20 mg/ 0.02g each) corresponding to the 3 ELISA tests to be undertaken (1 for Neopterin, 1 for Myeloperoxidase and 1 for a-1-antitrypsin).**
- **Keep the portions on ice at all times.**
- **Be careful to dissolve each portion in the correct extraction buffer corresponding to the specific ELISA kit.**

For Alpha-1-Antitrypsin

Alpha-1-antitrypsin is extracted by the sample dilution buffer (diluted WASHBUF) out of the stool sample.

1. Weigh out 20 mg/ 0.02g of stool and mix with 1ml of wash buffer.
2. Vortex the mixture in a tissue lyser (30 revolutions/s, for 15 sec) until it becomes homogenous.

3. Centrifuge homogenate for 10 min at 10000xg/ 13000 rpm, at 4oC
4. Dilute the supernatant 1:250 with wash buffer in a 'dispenser' 96 well U bottomed plate. 100 µl of the dilution is used in the test per well.

*****For information sake, we shall include an extra 1:100 dilution and a 1:200 dilution in our first run. So we shall have 3 dilutions for a single sample. Which means we shall be able to assay only 38 samples.**

Prepare all sample dilutions in the 'dispenser' plate. Make an excess volume of 220 µl to accommodate any pipetting errors.

- **1:250 (219.12 µl WASHBUF + 0.88 µl stool extract)-recommended kit dilution**
- **1:100 (217.8 µl WASHBUF + 2.2 µl stool extract)**
- **1:200 (218.9 µl WASHBUF + 1.1 µl stool extract)**

You will transfer 100µl per well (in duplicate) from the 'dispenser' plate into the reaction plate according the plate layout.

1. **Washing step:** Take out the plate and wash once with diluted WASHBUF. Remove any residual buffer by tapping the plate on absorbent paper
2. **Sample Incubation:** Pipette 100 µl of STD, CTRL and samples in duplicate into designated wells. Any dilutions of the STD are prepared immediately prior to this step. The reaction starts on pipetting to the antibody pre-coated micro-titre wells therefore pipetting should be as quick as possible. Use a multichannel pipette to ease transfer from 'dispenser' plate into reaction plate.
3. Cover plate and incubate on a shaker for 60 minutes at room temperature.
4. **Washing step:** After 1 hour, discard the contents of the wells and wash 5x with 250 µl of diluted WASHBUF. Remove residual buffer by tapping the plate on absorbent paper after the last wash step.
5. **Incubation Conjugate:** Pipette 100µl of CONJ into each well. Cover plate and incubate on a shaker for 60 minutes at room temperature
6. **Washing step:** After 1 hour, discard the contents of the wells and wash 5x with 250 µl of diluted WASHBUF. Remove residual buffer by tapping the plate on absorbent paper after the last wash step.
7. **Incubation Substrate:** Pipette 100 µl of SUB into each well. Incubate for 10-15min at room temperature in the dark.
8. **Stopping Reaction:** Pipette 50 µl STOPP into each well and mix well.
9. **Reading:** Read absorbance at 450 nm. If the plate reader allows, use a wavelength of 620 nm or 690 nm as reference wavelength.
10. All readings should be done within 5minutes of stopping the reaction. In case the highest standards exceed the range of the reader, the reading should be done at 405 nm against 620 nm/ 690 nm.

Calculation of Analytical Results: Actual concentrations are obtained from the standard curve of OD's.

- The obtained α-1-AT conc. are **multiplied by 12.5**
- Dilution 1: 0.02 g in 1ml corresponds to a factor of 50 (assuming 1g stool = 1ml)
- Dilution 2: **factor 250** (or 100 or 200 if used)
- All conc. obtained from the readings are in ng/ml and since we want to present results as ug/ml, we further divide by 1000
- **Thus Conc. Patient (µg/ml) =[obtained conc. (ng/ml) X 50 X 250]/1000**

Limitations

Stool samples with alpha-1-antitrypsin concentrations above the standard curve should be diluted with WASHBUF and measured again

In cases of strong diarrhea, it is possible that even patients with inflammation in the gut show normal values.

Disposals

The substrate (SUB) must be disposed of as non-halogenated solvent. The stop solution (STOPP) could be neutralized with NaOH and disposed of as a salt solution. The later reaction will generate heat.

Alpha-1-antitrypsin Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1 90ng/ml	STD1 90ng/ml	SPL 1		SPL 9		SPL 17		SPL 25		SPL 33	
B	STD 2 45ng/ml	STD 2 45ng/ml	SPL 2		SPL 10		SPL 18		SPL 26		SPL 34	
C	STD 3 30ng/ml	STD 3 30ng/ml	SPL 3		SPL 11		SPL 19		SPL 27		SPL 35	
D	STD 4 10ng/ml	STD 4 10ng/ml	SPL 4		SPL 12		SPL 20		SPL 28		SPL 36	
E	STD 5 3.3 ng/ml	STD 5 3.3 ng/ml	SPL 5		SPL 13		SPL 21		SPL 29		SPL 37	
F	STD 6 0ng/ml	STD 6 0ng/ml	SPL 6		SPL 14		SPL 22		SPL 30		SPL 38	
G	CTRL 1	CTRL 1	SPL 7		SPL 15		SPL 23		SPL 31		SPL 39	
H	CTRL 2	CTRL 2	SPL 8		SPL 16		SPL 24		SPL 32		SPL40	

SOP FOR THE QUANTITATION OF MYELOPEROXIDASE IN STOOL

Markers: Myeloperoxidase/ MPO

Specimens: Stools samples (frozen at -80^{0}C)

Location: Immunology Labs

Persons Responsible: Agnes Gwela, Rosie Crane and Emily Nyatichi

LABORATORY EQUIPMENT AND REAGENTS NEEDED

EQUIPMENT	AVAILABILITY
Fume Hood	Available (chemical Room)
-80^{0}C Freezer	Available
Laboratory Weighing Balance	To be provided
Weighing Boats/ **2ml cap tubes**	Available
ELISA Plate Reader (450nm) Ref λ: 620/690nm	Available
ELISA Plate Washer	Available
Microtitre plate shaker	Available
1.5mL Eppendorf tubes	Available
Vortex Mixer/ Tissue Lyser	Available
15mL Falcon Tubes*	Available
Dip sticks*	To be provided by Rosie Crane
ELISA Kits	All Commercial….have been purchased
Refrigerated Centrifuge, 3000xg or 13,000 rpm	Available
Double distilled water (ddH_20)	Available

ELISA KIT SPECIFICATIONS

KIT	CATALOGUE #	COMPANY
Neopterin ELISA kit	RE59321	IBL International, Hamburg
MPO Human ELISA kit	HK324-02	Hycult, Netherlands
α-1-antitrypsin ELISA kit	RIC6200	ImmunoChrome, GmbH

All ELISA assays will be performed according to the Instructions in each kit.

Kit 2: Myeloperoxidase (MPO)

Components	Descriptions	Amount
MTP	Microtiter plates pre-coated	96 well X2
Wash buffer 20X/ WB21	ELISA wash buffer concentrate	60 ml
Dilution Buffer A 5X/ DB72	30ml	1 vial
Dilution Buffer B 10X/ DB63	15ml	1 vial

STD	Lyophilized	4 vials
TRACER (biotinylated)	1ml lyophilized	2 vials
Streptavidin Peroxidase 100X/ CON03	250 µL	1 vial
TMB Substrate/ TMB050 or TMB100	22ml	1 vial
STOP Solution/ STOP110	20ml	1 vial

*****Take ELISA kits out of the fridge and bring all reagents to room temperature before starting assay.**

*****This kit contains 2 plates so be careful to prepare only half of the reagents for a single plate**

Reagent Preparations

Prepare buffers on the evening before the assay to save time. During this time, you may also take out the samples to be assayed, record samples numbers without thawing them and keep samples in a designated box.

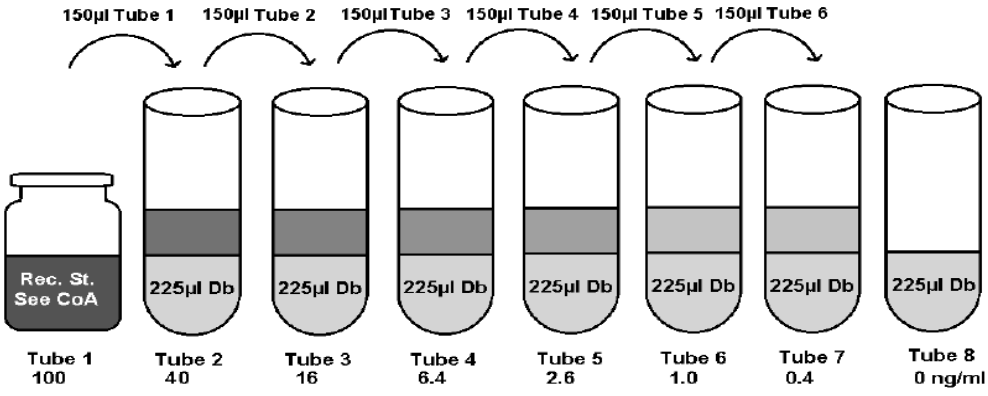
Wash buffer: Dilute 1:20 in double distilled H_20. 30 mL Wash buffer + 570 mL ddH_20.

Dilution Buffer: Mix 15 mL of 5x Dilution buffer A with 22.5 mL of ddH_20 and 7.5 mL of 10X dilution buffer B with 30 mL ddH_20. Combine both solutions equally and mix well. Concentrated dilution buffer may contain crystals. In case these do not dissolve at room temp. within an hour warm the buffer to 37^0C. Do not shake the solution

Standards: Prepare standards just before loading the test plate. They must be used within an hour of preparation.

First reconstitute supplied standard (54.1ng) in 541µL of dilution buffer to obtain a conc. of 100ng/ml. This is **STD1** and is diluted to obtain the other standards (range 100-0.4ng/ml). Pipette 225 µl of dilution buffer into 7 polypropylene tubes. Add 150µl of STD1 into the 1st tube and the serially transfer 150µl into each subsequent tubes except the blank.

100ng/ml	40ng/ml	16ng/ml	6.4ng/ml	2.6ng/ml	1.0ng/ml	0.4ng/ml	0ng/ml
STD 1 (neat)	**150ul STD1 + 225ul dilution buffer**	**150ul STD2 + 225ul dilution buffer**	**150ul STD3 + 225ul dilution buffer**	**150ul STD4 + + 225ul dilution buffer**	**150ul STD5 + 225ul dilution buffer**	**150ul STD6 + + 225ul dilution buffer**	**Blank 225ul dilution buffer**



Tracer:

Reconstitute with 1mL of ddH_20. Dilute further by adding 11 mL of dilution buffer in a 15mL falcon tube.

Streptavidin Peroxidase Solution: Dilute 1:100. 250µL of 100X stock is supplied for 2 plates. For 1 plate, prepare 125µL of peroxidase + 12.375 mL of Dilution buffer.

STOOL SAMPLES

- **We aim to work on 40 samples per day**
- **Extraction in glass or plastic vials (we shall use 2mL screw cap tubes and dip sticks)**
- **Before picking sample from the stock to weigh, use the dip-stick to mix the sample thoroughly. If possible avoid picking particulates and fibres and pick the 'softer' bits of the stool.**
- **From each stool sample, weigh out 3 portions of (20 mg/ 0.02g each) corresponding to the 3 ELISA tests to be undertaken (1 for Neopterin, 1 for Myeloperoxidase and 1 for a-1-antitrypsin).**
- **Keep the portions on ice at all times.**
- **Be careful to dissolve each portion in the correct extraction buffer corresponding to the specific ELISA kit.**

<u>For Myeloperoxidase</u>

MPO is extracted with 1X wash buffer out of the stool sample.

1. Weigh out 20 mg/ 0.02g of stool and mix with 1ml of wash buffer. (1:50 dilution)
2. Vortex the mixture in a tissue lyser (30 revolutions/s, for 60 sec) until it becomes homogenous.
3. Centrifuge homogenate for 10 min at 10000xg/ 13000 rpm, at 4^0C
4. Dilute the supernatant 1:10 with wash buffer in a 'dispenser' 96 well U bottomed plate. 100 µl of the dilution is used in the test per well.
   This gives a total dilution of 1:500

*****For information sake, we shall include an extra 1:100 dilution and a 1:200 dilution in our first run. So we shall have 3 dilutions for a single sample. Which means we shall be able to assay only 38 samples.**

Prepare all sample dilutions in the 'dispenser' plate. Make an excess volume of 220 µl to accommodate any pipetting errors.

- **1:10 (198 µl WASHBUF + 22 µl stool extract)-recommended kit dilution**
- **1:20 (209 µl WASHBUF + 11 µl stool extract)**
- **1:100 (217.8 µl WASHBUF + 2.2 µl stool extract)**

You will transfer 100µl per well (in duplicate) from the 'dispenser' plate into the reaction plate according the plate layout.

Take out the plate and let it warm to room temperature.

1. **Sample Incubation:** Pipette **100 µl of Standards and samples** in duplicate into designated wells. Any dilutions of the Standards are prepared immediately prior to this step. The reaction starts on pipetting to the antibody pre-coated micro-titre wells therefore pipetting should be as quick as possible. Use a multichannel pipette to ease transfer from 'dispenser' plate into reaction plate.
2. Cover plate and incubate on a shaker for 60 minutes at room temperature.
3. **Washing step:** After 1 hour, discard the contents of the wells and wash 5x with 250 µl of diluted wash buffer. Remove residual buffer by tapping the plate on absorbent paper after the last wash step.
4. Add of **100 µl of diluted tracer** to each well. Cover plate and incubate for 1 hour at room temperature.
5. **Washing step:** Discard the contents of the wells and wash 5x with 250 µl of diluted wash buffer
6. **Incubation Conjugate:** Pipette **100µl of diluted streptavidin peroxidase** into each well. Cover plate and incubate on a shaker for 60 minutes at room temperature
7. **Washing step:** After 1 hour, discard the contents of the wells and wash 5x with 250 µl of diluted Wash buffer. Remove residual buffer by tapping the plate on absorbent paper after the last wash step.
8. **Incubation Substrate:** Pipette **100 µl of TMB substrate** into each well. Incubate for 30min at room temperature in the dark.
9. **Stopping Reaction:** Pipette 100 µl Stop solution into each well and mix well.
10. **Reading:** Read absorbance at 450 nm.

Calculation of Analytical Results: Actual concentrations are obtained from the standard curve of OD's.

- The obtained MPO conc. are **multiplied by 0.5**
- Dilution 1: 0.02 g in 1ml corresponds to a factor of 50 (assuming 1g stool = 1ml)
- Dilution 2: **factor 10** (or 20 or 100 if used)
- All conc. obtained from the readings are in ng/ml and since we want to present results as ug/ml, we further divide by 1000
- **Thus Conc. Patient (µg/ml) =[obtained conc. (ng/ml) X 50 X 10]/1000**

SOP FOR THE QUANTITATION OF NEOPTERIN IN STOOL

Markers: Neopterin (NEO)

Specimens: Stools samples (frozen at -80^{0}C)

Location: Immunology Labs

Persons Responsible: Agnes Gwela, Rosie Crane and Emily Nyatichi

LABORATORY EQUIPMENT AND REAGENTS NEEDED

EQUIPMENT	AVAILABILITY
Fume Hood	Available (chemical Room)
-80^{0}C Freezer	Available
Laboratory Weighing Balance	Available
Weighing Boats/ **2ml screw cap tubes**	Available
ELISA Plate Reader (450nm) Ref λ: 620/690nm	Available
ELISA Plate Washer	Available
Microtitre plate shaker	Available
2Ml screw cap tubes	Available
Vortex Mixer or tissue lyser	Available
15Ml Falcon Tubes*	Available
Dip sticks*	Available
ELISA Kits	All Commercial….have been purchased
Refrigerated Centrifuge, 3000xg or 13,000 rpm	Available
Double distilled water (ddH_20)	Available
Glassware for buffer preparation	Available
Micropipettes. Volume 10, 100, 200, 1000	Available

ELISA KIT SPECIFICATIONS

KIT	CATALOGUE #	COMPANY
Neopterin ELISA kit	RE59321	IBL International, Hamburg
MPO Human ELISA kit	HK324-02	Hycult, Netherlands
α-1-antitrypsin ELISA kit	RIC6200	ImmunoChrome, GmbH

All ELISA assays will be performed according to the Instructions in each kit.

Kit 3: Neopterin (NEO)

Components	Descriptions	Amount
MTP	Microtiter plates pre-coated	96 well X1
WASHBUF CONC **(20X)**	ELISA wash buffer concentrate	50 ml
ASSAYBUF	Ready to use	21mL
CAL A-F/ Standards	**0, 1.35, 4.0, 12.0, 37.0, 111** nmol/L	6 X 1.5 mL
CONTROL 1	**1.5mL ready to use**	1 vial
CONTROL 2	**1.5mL ready to use**	1 vial
ANTISERUM	**Neopterin antiserum**	8mL
ENZCONJ	Ready to use (protect from light)	13mL
TMB SUBS	TMB Substrate Solution	19mL
TMB STOP	TMB Stop Solution (1M H_2SO_4)	19mL
FOIL	Adhesive foil	1

*****Take ELISA kits out of the fridge and bring all reagents to room temperature before starting assay.**

*****Lights in the lab should be turned off for the Neopterin Sample Prep and ELISA steps as this reaction is light sensitive**.

Reagent Preparations

Prepare buffers on the evening before the assay to save time and keep in the cold room. During this time, you may also take out the samples to be assayed, record samples numbers without thawing them and keep samples in a designated box in the -80^{0}C freezer.

0.9% Sodium Chloride (NaCl): Prepare by dissolving 9g of Sodium Chloride in ddH_20. First dissolve solute in 900mL of ddH_20, pour solution into 1L measuring cylinder, then top up to 1L with ddH_20.

Wash buffer: Dilute 1:20 in double distilled H_20. 50 mL conc. wash buffer + 950 mL ddH_20.

Dilution Buffer: There is an Assay Buffer provided but samples will be diluted in 0.9% NaCl as in the Wash Benefits Study.

Standards: Ready to use.

Controls: Ready to Use

STOOL SAMPLES

- **We aim to work on 40 samples per day**
- **Extraction in glass or plastic vials (we shall use 2mL screw cap tubes and dip sticks)**

- **Before picking sample from the stock to weigh, use the dip-stick to mix the sample thoroughly. If possible avoid picking particulates and fibres and pick the 'softer' bits of the stool.**
- **Place the tube labelled NEO for the first sample on the balance and tare so that the balance reads 0.000. Using a sterile wooden applicator stick, measure between 20mg/ 0.02g of stool into the tube. (Allow an error range of 0.002g) so that the maximum amount to be weighed is 0.022g). Place the stool in the screw cap tube and keep on ice. Place the original stool aliquot sample back on ice. Record the actual weight of stool transferred to the tube.**
- **From each stool sample, weigh out 3 portions of (20 mg/ 0.02g each) corresponding to the 3 ELISA tests to be undertaken (1 for Neopterin, 1 for Myeloperoxidase and 1 for a-1-antitrypsin).**
- **Keep the portions on ice at all times.**
- **Afterwards, be careful to dissolve each portion in the correct extraction buffer corresponding to the specific ELISA kit.**

For Neopterin

NEO is extracted with 0.9% NaCl out of the stool sample.

1. Weigh out 20 mg/ 0.02g of stool and mix with 1ml of 0.9% NaCl. (1:50 dilution)
2. Vortex the mixture in a tissue lyser (30 revolutions/s, for 60 sec) until it becomes homogenous.
3. Centrifuge homogenate for 10 min at 10000xg/ 13000 rpm, at 4^{o}C
4. Dilute the supernatant 1:20 with 0.9% NaCl. in a 'dispenser' 96 well U bottomed plate. 20 µl of the dilution is used in the test per well.
   This brings the total dilution to 1:1000

Prepare all sample dilutions in the 'dispenser' plate. Make an excess volume of 220 µl to accommodate any pipetting errors.

- **1:20 dilution (57 µl 0.9% NaCl + 3µl stool extract)**
- **1:20 dilution (57 µl Assay buffer + 3µl stool extract) Total dilution= 1:500**
- **1:40 dilution (58.5 µl 0.9% NaCl + 1.5µl stool extract) Total dilution= 1:2000**

You will transfer 20µl per well (in duplicate) from the 'dispenser' plate into the reaction plate according the plate layout.

1. Take out the plate and let it warm to room temperature.
2. Pipette **20 µl of standard, controls and samples** in duplicate into respective wells of the microtiter plate. Use a multichannel pipette to ease transfer of samples from 'dispenser' plate into reaction plate as quickly as possible
3. Pipette **100 µl of Enzyme Conjugate** into each well.
4. Pipette **50 µl of Neopterin Anti-serum** into each well.
5. Cover plate with black adhesive foil and incubate on a shaker (500rpm) for **90 minutes** at room temperature in the dark.

6. Remove adhesive foil. Discard incubation solution. Wash plate 4 X 250µl diluted wash buffer. Remove residual buffer by tapping the plate on absorbent paper after the last wash step.
7. For adding of Substrate and Stop solution, use a multi-channel micropipettor. Pipetting should be carried out in the same time intervals for Substrate and Stop solution. Use positive displacement and avoid formation of air bubbles. If any air bubbles appear, poke them with a pipette tip being careful not to cross-contaminate.
8. Pipette **150µl** of **TMB Substrate Solution** into each well.
9. Incubate **10 minutes** at room temperature (18-25^{0}C)
   (this incubation time may be variable, so keep watching for color change and use your intuition; BUT once a stop time is determined, this should be consistent for all the subsequent plates)
10. Stop the substrate reaction by adding **150 µl** of **TMB Stop Solution** into each well. Briefly mix contents by gently shaking the plate.
11. Measure optical density with a photometer at 450 nm. (Reference-wavelength: 600-650 nm) within 5-10 minutes.

Calculation of Analytical Results: Actual concentrations are obtained from the standard curve of OD's.

- The obtained NEOPTERIN conc. are **multiplied by 10**
- Dilution 1: 0.02 g in 1ml corresponds to a factor of 50 (assuming 1g stool = 1ml)
- Dilution 2: **factor 20** (or 40 if used)
- All conc. obtained from the readings are in nmol/L
- **Thus Conc. Patient (**nmol/L**) =[obtained conc. (**nmol/L**) [X 50 X 20]**

Limitations

Stool samples with alpha-1-antitrypsin concentrations above the standard curve should be diluted with WASHBUF and measured again

In cases of strong diarrhea, it is possible that even patients with inflammation in the gut show normal values.

Disposals

The substrate (SUB) must be disposed of as non-halogenated solvent. The stop solution (STOPP) could be neutralized with NaOH and disposed of as a salt solution. The later reaction will generate heat.

Appendix 14: PCA of measures of household SEP in the cross-sectional survey

Focus group discussion participants agreed that 'rich' householders are those that own vehicles, land and cattle, have permanent jobs, permanent houses, higher education levels, electricity and tap water. They described 'middle' householders as those with permanent jobs, who own small businesses or can access casual labour, own land, a motorbike, a TV, goats and chickens, have enough food, have completed secondary education, and have semi-permanent houses, electricity and tap water. 'Poor' householders were thought to have mud walls and thatch roofs that are often in disrepair, to be casual labourers with primary education only, to own chickens but no other livestock, to have infrequent meals, to not own land, obtain food from shops instead of their own farm, make and sell brooms from coconut leaves, not practice family planning and suffer ill health. 'Very poor' householders were thought to have no income and rely heavily on borrowed food or food aid, drink alcohol in excess, to sometimes be disabled, have no education and poorly maintained houses.

Arising from these discussions, six factors thought to discriminate between socio-economic groups in the local area were selected: house building materials (floor, wall and roof), household land and livestock ownership and household per capita income. Frequency distribution of these characteristics is summarised in Supplementary table 2. In a PCA of these six variables, the first component contributed 37% of the total variability observed. This first component (observed to yield a much higher eigenvalue than the subsequent components – see scree plot, Supplementary figure 1) was then predicted for each household and divided into quartiles. Noting that eigenvectors were positive for all factors

but nevertheless making the assumption that in the majority of cases all factors were indeed associated with higher true SEP, these quartiles were labelled 'poorest' (lowest quartile of first component), 'poorer', 'richer' and 'richest' (highest quartile of first component) households.

Supplementary table 2: Frequency distribution of wealth indicators used in principal components analysis for socio-economic position.
Factors are given by household (n=336). No data are missing.

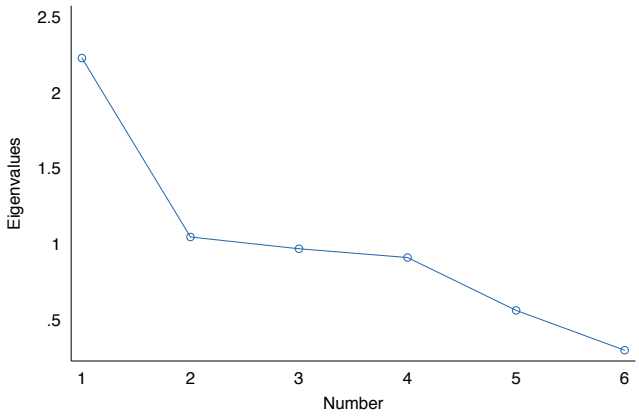
Factor	Category	Distribution[1]
Floor material	Earth	294 (88)
	Cement/carpet	42 (12)
Roof material	Thatch	241 (72)
	Corrugated iron or tiles	95 (28)
Wall material	Mud/wood/corrugated iron	280 (83)
	Stone/brick	56 (17)
Land ownership, acres		2 (0.25-31)
Household per capita monthly income (Kenya shillings)		1500 (181-10000)
Livestock ownership score[2]		0.10 (0-49.5)

1. *For categorical variables: n (% of total). For continuous variables: median (range).*
2. *1 point per cow or equivalent (1 cow is considered, based on local market sale value, equivalent to 2 pigs, 20 goats or 60 chickens)*

Supplementary table 3: Eigenvectors derived from a principal components analysis of 336 households that participated in the cross-sectional survey.

Factor	Cross-sectional survey eigenvector for first principal component
Household per capita income (Kenya shillings) (≥2,000 versus <2,000)	0.1087
Household owns land	-0.0077
Household owns livestock	0.0306
Roof material (corrugated iron versus *makuti* (grass thatch))	0.5307
Wall material (stone/brick versus *makuti*/mud/wood)	0.6082
Floor material (cement versus earth)	0.5793

Supplementary figure 1: Scree plot of eigenvalues derived from Principal Component Analysis of household socio-economic position indicators.



Appendix 15: PCA of measures of household water provision in the cross-sectional study

Following focus group discussions, 10 factors pertaining to water, sanitation and hygiene were considered for PCA to rank households in order of overall WASH standard. Collinearity was present in the expected direction between all variables with the notable exception of sanitation type and presence of domestic animals in the house. Factors pertaining only to water were therefore chosen for PCA. In the PCA, the first component contributed 27% of the total variability observed (eigenvectors and scree plot: Supplementary table 5 and Supplementary figure 2). This first component was then predicted for each household and the predicted first components divided into quartiles and, making the same assumptions as for SEP, labelled 'lowest', 'lower', 'higher' and 'highest' water quality and availability.

Supplementary table 4: Frequency distribution of water indicators amongst 336 households in Junju, Kilifi. No data are missing.

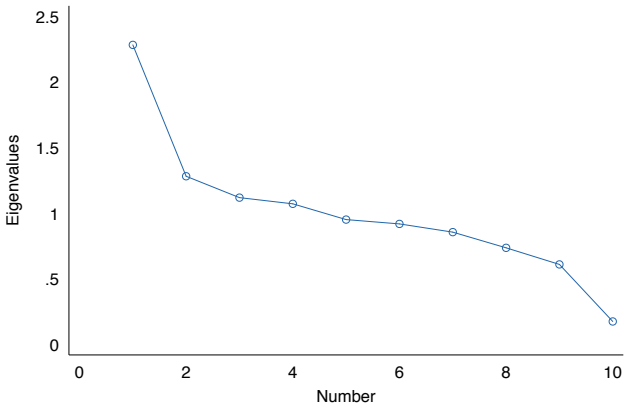
Factor	Category	Distribution (n(%))	Included in PCA?
Water source	WHO unimproved	153 (46)	Yes
	WHO improved	183 (54)	
Time to fetch water and return (minutes)	≥30	79 (24)	Yes
	<30	257 (76)	
Pay for water?	Yes	164 (49)	Yes
	No	172 (51)	
Water treated[1]	No	302 (90)	Yes
	Yes	34 (10)	
Drinking water storage	Open container	23 (7)	Yes
	Closed container	313 (93)	
Water use per person per day (litres)	<15	234 (70)	Yes
	≥15	102 (30)	

1. Water treatment method: 25 chlorination, 19 boiling, 5 filtration.

Supplementary table 5: Eigenvectors derived from a principal components analysis of 336 households that participated in the cross-sectional survey.

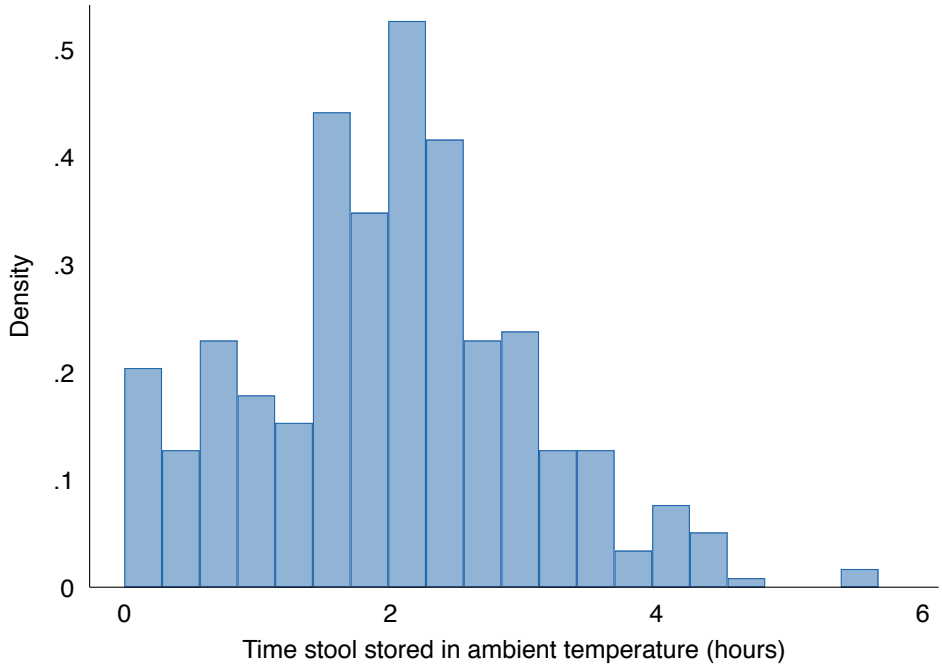
Factor	Cross-sectional survey eigenvector for first principal component
Household water source (improved versus unimproved)	0.6851
Water storage (closed versus open container)	0.1762
Time to fetch water and return (minutes) (<30 versus ≥30)	0.6556
Household drinking water treated?	-0.0114
Volume of water used per person per day (≥15 litres versus <15 litres)	0.2641

Supplementary figure 2: Scree plot of eigenvalues derived from Principal Component Analysis of household water indicators.

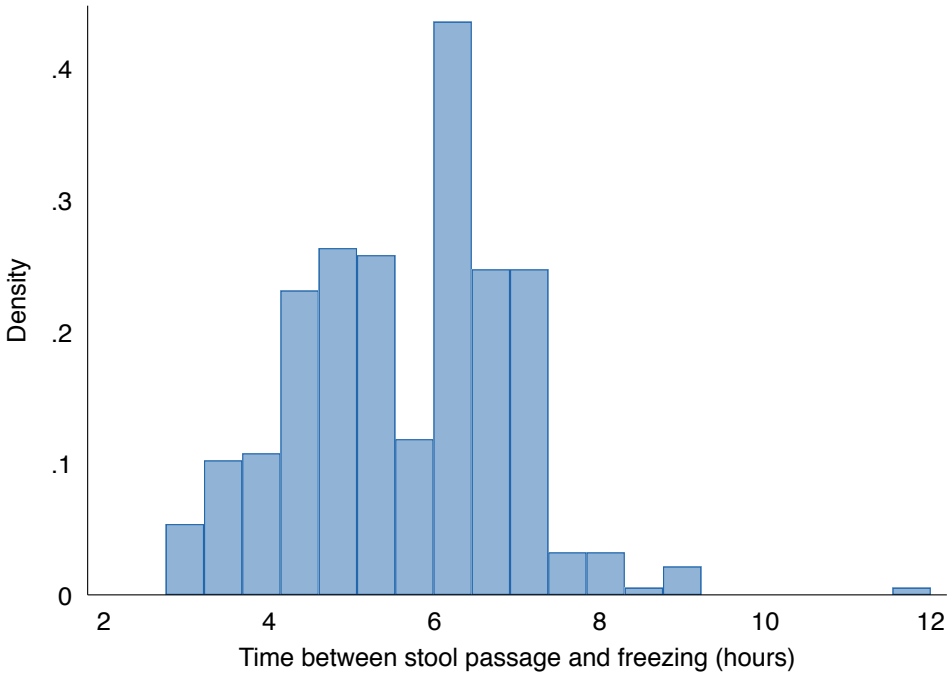


Appendix 16: Cross-sectional survey stool sampling cold chain: time in ambient temperature and time between passage and freezing

Supplementary figure 3: Frequency distribution of time from stool passage to collection by fieldworker.



Supplementary figure 4: Frequency distribution of time between stool passage and freezing.



Appendix 17: Methods for selection of variables for modelling of factors associated with HAZ and EED in the cross-sectional survey.

From a list of 46 potential exposure variables, 20 exposures were shortlisted for inclusion as fixed effects in multivariable models exploring risk factors for low HAZ and EED. Variables were selected following the decisions below.

Prematurity was discarded due to perceived poor quality. Fieldworkers felt that caretakers' recall was poor, due dates were unknown, and community perception of what constitutes a premature birth was widely variable.

Presence of domestic animals inside the house was discarded due to perceived poor quality. Fieldworkers found it difficult to confirm responses with direct observation.

Availability of soap within the household was discarded due to perceived instability over time. Fieldworkers noticed through personal and anecdotal experience that availability of soap is transient depending on local availability and household budget, and that all households in the study area typically would experience shortage on occasion.

Recalled duration of exclusive breastfeeding and any breastfeeding were dropped due to perceived poor quality. These variables were deemed too vulnerable to recall bias. Further discussion of this decision is found in the Discussion section below.

The following factors were discarded due to missing data: mother's age at the time of the index child's birth or at the time she had her first baby (11% and 9% missing respectively). Missingness did not occur at random in both these cases: missingness correlated with place of delivery since birthweight was generally not measured if a baby was born at home. Mother's age was generally not known in cases where the mother had died or travelled away.

Household dominant food source was intended for descriptive purposes only and not intended for the model.

Selected factor pairs were checked for collinearity. Selections were based on *a priori* suspicion of possible correlation. The following factors were discarded due to strong collinearity with another factor: sex of household head (co-linear with father alive), firstborn (co-linear with birth interval to older sibling).

The following binary factors were discarded due to highly skewed distribution (less than 5% in one category): mother's marital status, ever breastfed, mother alive, child enrolled on a feeding programme, neurological condition, chronic neurological and cardiac problems, birth route, birthplace, father alive.

Household size, number of under-fives in household and crowding (number of household residents per sleeping room) were found to be highly co-linear. Households almost always had just one sleeping room which varied greatly in size. In addition, in this rural area, anecdotal observation revealed that much use was made of spacious outdoor areas during waking hours in all participating households. Number of residents per sleeping room was

therefore not deemed to be a good measure of crowding. Also, number of residents per sleeping room was not thought to be a relevant health determinant in this rural study population. The crowding variable was therefore dropped. To approximate the care or attention a participant may have received during their life, household size and number of under-fives were dropped in favour of mother-specific birth interval spacing.

From the remaining 23 variables, cough, fever and diarrhoea over the past seven days were amalgamated to one factor: any of the three acute illnesses during the past seven days. Due to concern over confounding by healthcare access barriers, primary care attendance for these illnesses was dropped.

Appendix 18: Factors associated with HAZ in the cross-sectional survey: sensitivity model excluding SEP and deceased sibling

Supplementary table 6: Factors associated with HAZ amongst 508 children aged 0-59 months in Junju, Kilifi.
A mixed-effects linear regression model. Analysis is adjusted for clustering by homestead (229 groups).

Factor	Category	Distribution[1]	Coefficient	95% CI	p
Age (months)		30 (16 to 45)	-0.01	-0.02 to 0.00	0.24
Sex	Male	284 (56)	-	-	0.66
	Female	224 (44)	-0.05	-0.25 to 0.16	
Birth weight (kg)	≥2.5kg	177 (35)	-	-	0.0001
	<2.5kg	15 (3)	-1.36	-1.98 to -0.73	
	Unknown	316 (62)	-0.12	-0.36 to 0.11	
Birth interval to younger sibling	≥24 months or no younger sibling	50 (10)	-	-	0.03
	<24 months	257(51)	-0.03	-0.39 to 0.32	
	Cannot evaluate (age <24 months)	201 (40)	0.51	0.11 to 0.91	
Birth interval to older sibling	≥24 months or firstborn	425 (84)	-	-	0.74
	<24 months	83 (16)	0.05	-0.23 to 0.33	
Season of birth	Dry	295 (58)	-	-	0.08
	Rainy	213 (42)	-0.19	-0.40 to 0.02	
Slept under a bednet last night	Yes	388 (76)	-	-	0.03
	No	120 (24)	-0.31	-0.58 to -0.03	
Mother's education	Completed primary or higher	120 (24)	-	-	0.34
	None, or some primary	389 (76)	-0.13	-0.40 to 0.13	
Primary caretaker	Mother	470 (93)	-	-	0.83
	Not mother	38 (7)	0.03	-0.38 to 0.48	
Household water position PCA[2] first component score		0.1 (-0.6 to 0.9)	0.00	-0.12 to 0.08	0.68
Household sanitation type	WHO improved	83 (16)	-	-	0.77
	WHO unimproved	425 (84)	-0.05	-0.37 to 0.27	
Household dietary diversity	High	262 (52)	-	-	0.38
	Low or medium	246 (48)	-0.10	-0.33 to 0.13	
Household food security	No missed meal during past 7 days	440 (87)	-	-	0.83
	Missed meal during past 7 days	68 (13)	-0.04	-0.37 to 0.29	

1. For categorical variables: n (% of total). For continuous variables: median (interquartile range). 2. Principal Component Analysis

Appendix 19: Association between HAZ and faecal EED biomarkers in the cross-sectional survey: sensitivity model excluding age and sex

Supplementary table 7: Biomarkers associated with HAZ amongst 418 under-fives. Three separate mixed linear regression models.

Factor	n	Median (interquartile range)	Coefficient	95% CI	p
Log faecal alpa-1-antitrypsin (mg/g)	418	-1.1 (-2.0 to -0.4)	0.02	-0.07 to 0.11	0.73
Log faecal myeloperoxidase (ng/g)	418	8.8 (7.8 to 9.9)	0.06	-0.02 to 0.14	0.14
Log faecal neopterin (nmol/L)	418	6.1 (5.1 to 7.0)	0.15	0.07 to 0.23	<0.001

Appendix 20: Factors associated with markers of EED in the cross-sectional survey: sensitivity model excluding age, sex and current breastfeeding status

Supplementary table 8: Factors associated with log faecal alpha-1-antitrypsin, myeloperoxidase and neopterin amongst 419 children aged 0-59 months in Junju, Kilifi. Three separate mixed-effects linear regression models, one for each faecal EED biomarker outcome. Analysis is adjusted for clustering via inclusion of homestead as a random effect (205 groups).

Factor	Category	Distribution[1]	Log faecal alpha-1-antitrypsin (mg/g)			Log faecal myeloperoxidase (ng/g)			Log faecal neopterin (nmol/L)		
			Coefficient	95% CI	p	Coefficient	95% CI	p	Coefficient	95% CI	p
Fever, cough or diarrhoea during past seven days	No	204 (49)	-	-	0.07	-	-	0.04	-	-	0.58
	Yes	215 (51)	0.24	-0.01 to 0.49		0.32	0.02 to 0.61		0.08	-0.21 to 0.37	
Geophagy noted during past seven days	No	350 (84)	-	-	0.02	-	-	0.51	-	-	<0.001
	Yes	69 (16)	0.42	0.08 to 0.76		0.13	-0.26 to 0.53		0.78	0.39 to 1.17	
Faecal ova, cysts and parasites	Negative	375 (90)	-	-	0.10	-	-	0.53	-	-	0.74
	Positive	44 (11)	0.35	-0.07 to 0.76		0.16	-0.32 to 0.64		0.08	-0.39 to 0.55	
Water PCA[2] first component score		0.1 (-1.4 to 1.4)	0.10	0.00 to 0.21	0.06	0.00	-0.12 to 0.13	0.94	0.16	0.03 to 0.29	0.01
Household sanitation WHO type	Improved	77 (18)	-	-	0.42	-	-	0.10	-	-	0.06
	Unimproved	342 (82)	-0.14	-0.47 to 0.19		-0.32	-0.71 to 0.07		0.38	-0.01 to 0.78	
Household dietary diversity	High	220 (53)	-	-	0.12	-	-	0.73	-	-	0.88
	Low / medium	199 (47)	-0.21	-0.47 to 0.05		0.05	-0.25 to 0.36		-0.02	-0.34 to 0.29	
Household food security: missed meal during past seven days	No	363 (87)	-	-	0.66	-	-	0.14	-	-	0.71
	Yes	56 (13)	0.08	-0.29 to 0.46		0.33	-0.11 to 0.76		0.08	-0.35 to 0.52	

1. For categorical variables: n (% of total). For continuous variables: median (interquartile range).
2. PCA: Principal Component Analysis

Appendix 21: Combinations of household water and sanitation characteristics in the cross-sectional survey

Supplementary table 9: Number of households with WHO improved and unimproved water and sanitation characteristics. Orange shaded cells indicate unimproved water and sanitation; green cells indicate improved water and sanitation; yellow cells indicate discordance between water and sanitation categories (improved water and unimproved sanitation or unimproved water and improved sanitation).

		Sanitation facility					
		Bush (no facility)	Trench	Unimproved (uncovered) latrine	Ventilated Improved Pit latrine	Flush toilet to river	Flush toilet to sewer
Drinking water source	Surface (river, dam)	5	7	84	4	0	20
	Rainwater collection	0	0	0	0	1	0
	Dug well (uncovered)	2	12	87	0	2	18
	Dug well (covered)	2	6	23	0	0	0
	Borehole	0	0	0	0	2	0
	Piped to public tap	1	23	72	0	0	13
	Piped to neighbour	3	15	69	0	4	10
	Piped to compound	3	3	5	3	2	4
	Piped to inside house	0	0	0	0	1	0

Appendix 22: Methods for preparation of oral glucose solution at KWTRP Research Pharmacy

Preparation was undertaken in weekly batches. Glucose powder (D+ Glucose; QuinTron) was added to sterile water ('Water for injection'; local clinical supply) to make a solution of 500mg/ml glucose. The prepared solution was stored at 2-8^{o}C in a labelled sterile Duran bottle in the KWTRP Research Pharmacy refrigerator until the morning of BHT, when 15ml aliquots were poured into sterile screw cap containers and each container labelled with details of the solution plus batch number. Containers were then transferred immediately into a coolbox with ice packs and a thermometer probe with continual external display. On each BHT testing morning, the courier (either myself or a senior fieldworker) would record the internal coolbox temperature at the start and end of transfer, and visually monitor temperatures during transfer. If temperature approached or exceeded 8^{o}C during transit, fresh ice packs were added. Upon arrival at the testing site, temperature was again continually monitored, and recorded by fieldworkers at the moment the first container was removed for administration to the first participant. Solutions were visually inspected before administration to participants to ensure no frozen elements or visible contamination, and manually shaken prior to opening.

Sterility was promoted during preparation through use of gloves and lab coat, spraying of work surfaces with 70% ethanol, non-touching of key parts, and use of sterile measurement aids such as needles, syringes and weighing boats. The KWTRP Research Pharmacy fridge was subject to GCLP standard temperature monitoring 24 hours a day maintaining temperatures between 2 and 8^{o}C. Reagents were securely stored in 24-hour air conditioning at approximately 18-21^{o}C.

Appendix 23: Glucose oral solution preparation SOP for use in pharmacy

See next page for SOP for preparation of oral glucose solution for the BHT, for use in KWTRP Research Pharmacy.

STANDARD OPERATING PROCEDURE

PREPARATION OF ORAL GLUCOSE SOLUTION FOR BREATH HYDROGEN TEST

Study scientific title	Epidemiology of Environmental Enteropathy and intestinal microbiota amongst infants in Junju, Kilifi
Lay title	Afya tumboni
SERU protocol number	2983
Authors	Rosie Crane & Jimmy Shangala
Reviewer	Johnstone Thitiri
Approver	Rosie Crane
Version	1.2
Date	24/09/2015

Materials

Water for injection, in ampoule/bottle
Glucose powder
Gloves, non-sterile
Lab coat
Needles, sterile G19 x2
Sterile spatula
Weighing scale
70% ethanol in spray bottle
Paper tissue
Duran bottle, 500ml autoclaved sterile with screw lid
Syringes, sterile 10ml or 20ml or 50ml x2
Falcon tubes 15ml or 50ml / 30ml sterile universal containers
Marker pen
Glucose solution quality assessment / quality control log (Appendix)

Method

1. Wear a lab coat and gloves.

2. Prepare your work surface and weighing scales by spraying with 70% ethanol. Ensure a clean workspace. Spray gloves with 70% ethanol at intervals throughout.

3. Remove the screw cap from a 500ml sterile autoclaved Duran bottle and place it face up on the work surface – do not touch the inside of the cap.

4. Place the opened bottle (without cap) on scales and tare.

5. Open the glucose powder bottle and place the screw cap face up – do not touch the inside of the cap. Using a sterile spatula, weigh the desired amount of glucose powder directly into the Duran bottle. Do not touch the inside of the glucose powder bottle. If glucose powder exceeds the desired weight, do not return to the bottle but withdraw the desired amount from the Duran bottle and discard. Return the screw cap to the glucose powder bottle.

6. Using a sterile G19 needle and sterile 10/20/50ml syringe, withdraw the desired volume of water for injection from the ampoule/bottle and add to the Duran bottle. Return the screw cap to the Duran bottle.

7. Mix manually by gentle shaking for 2 minutes.

8. Using a fresh sterile G19 needle and a fresh 10/20/50ml sterile syringe, transfer desired aliquots of the glucose/water solution into sterile 15ml or 50ml Falcon tubes.

9. Label each Falcon tube with today's date, your initials, the aliquot volume and 'glucose 500mg/ml' or 'glucose 50%'

10. Store at 2-8^{o}C. Prepared glucose solution has a shelf life of 3 months.

11. Complete the ***Glucose solution quality assessment / quality control log***.

To make a 500mg per ml (1g per 2ml; 500g per litre) solution:

Batch volume	Water for injection	Glucose powder
100 ml	100 ml	50 g
200 ml	200 ml	100 g
300 ml	300 ml	150 g
400 ml	400 ml	200 g
500 ml	500 ml	250 g

Quality control

Remove aliquots on the first and last day of use for each batch. Use a sterile G19 needle and sterile syringe to remove a 1.8ml aliquot from one of the Falcon tubes (that tube can still be used for testing). Transfer this aliquot to a 1.8ml tube and label with sample type, batch number and today's date. Transfer to Clinical Trials Laboratory for storage at -20 or -80^{o}C.

Appendix

See overleaf for ***Glucose solution quality assessment / quality control log***.

Appendix 24: BHT SOP for use in the field

See next page for SOP for breath sampling in the BHT, for use at the field study site. Images are copied from the Gastrolyzer product manual.

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STANDARD OPERATING PROCEDURE

BREATH HYDROGEN TEST

Study scientific title	Epidemiology of Environmental Enteropathy and intestinal microbiota amongst infants in Junju, Kilifi
Lay title	Afya tumboni
SERU protocol number	2983
Author	Rosie Crane
Reviewer	Johnstone Thitiri
Approver	Rosie Crane
Version	1.2
Date	24/09/2015

Materials required (per test)

BHT record sheet (Appendix 1)
Cover sheet (Appendix 2)
Task tracker (Appendix 3)
Oral solutions transport log (Appendix 4)
Pen
70% ethanol spray
Tissues
Demonstration ('demo') doll
Demo 5ml syringe
Demo face mask
Demo connecting tube
Demo D piece
Glucose solution 500mg/ml*
Sterile new 5ml syringe
Face mask (for participant)
Connecting tube (for participant)
D piece**
Gastrolyzer***
Spare batteries for Gastrolyzer (3x AA)

** Glucose solution will have been prepared in KWTRP Clinical Trials Pharmacy (see SOP **Preparation of oral glucose solution for breath hydrogen test**) and come in sterile labeled 15-30ml containers; in a cool box with ice packs and thermometer; transported under cold chain that morning from KWTRP Clinical Trials Pharmacy fridge. Cold chain and custody will be recorded in an **Oral solutions transport log** (Appendix 4). Containers will be labeled with solution name, batch number and date, and pharmacist initials.*
*** D piece should be changed at least once per month. The Principal Investigator will ensure this is done by keeping a **Breath hydrogen meter D piece change log** (Appendix 5).*
**** The Gastrolyzer should be calibrated at least once per month. The Principal Investigator will ensure this is done by keeping a **Breath hydrogen meter calibration log** (Appendix 5) and will follow manufacturer's instructions (Appendix 6).*

Method

1. Complete the ***BHT record sheet*** as you go along. Check the participant's initials and study number against their ***Cover sheet***. Record today's attendance in the participant's ***Task tracker***.

2. Confirm verbally with the caretaker that they are still happy to proceed. If they need reminding, demonstrate the procedure using the doll and the demo syringe, demo face mask, demo connecting tube and demo D piece.

3. The participant should have undergone exclusive breastfeeding only for the past 3 hours. Check that this is the case. If this is not the case, continue anyway but note on the ***BHT record sheet***.

4. Explain to the caretaker that for the next 2 hours, ideally the participant should not be fed ('nil by mouth'). Note the time of the start of fasting. Allow feeding only if the participant becomes very distressed. Ask the caretaker to stay close by during the fast and provide regular reminders and encouragement for them not to feed the participant. If the participant is fed, continue anyway but document in the ***BHT record sheet***.

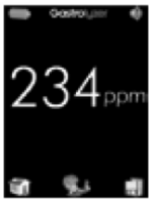
5. During the 2-hour fast:
    - Weigh the participant (see SOP ***Anthropometry***).
    - Calculate the amount of glucose solution you will need to give to the participant by multiplying their weight today (in kg) by 2 then rounding up or down to the nearest 0.1ml. For example, a 4.532kg participant requires 4.532 x 2 = 9.064ml rounded to 9.1ml.
    - Locate the participant's specific stored mask and connecting tube. Clean the mask using 70% ethanol and tissue. Clean the connecting tube also, with the exception of the paper valve.

6. 5 minutes before the end of the 2-hour fast, switch on the Gastrolyzer using the round black switch at the top. Don't connect the D piece yet. Wait for the home screen to show:



7. Connect the participant's mask and connecting tube to the D piece (don't connect to the Gastrolyzer yet). Get into a comfortable position in front of the participant.
    - *Position for sleeping/small participants: in caretaker's arms, facing sideways. Caretaker holds participant's body. Caretaker/assistant holds participant's head. Tester holds mask and Gastrolyzer.*
    - *Position for larger participants: on caretaker's lap, facing forwards. Caretaker holds participant's head. Caretaker/assistant holds participant's arms. Tester holds mask and Gastrolyzer.*

8. Press the blue mask picture on the Gastrolyzer screen. A screen like this should appear (and read less than 5ppm when sampling room air – if the reading is higher than this contact the Principal Investigator before proceeding):



9. Gently but firmly apply the mask over the participant's nose and mouth. Ensure a good airtight seal by observing the inside of the mask for condensation and checking the sides of the mask for gaps. Allow the participant to breathe into the mask until the sand-timer animation appears on the bottom of the screen. This shouldn't take more than 30 seconds. Provided the caretaker is comfortable, continue applying the mask even if the participant cries. Reassure the caretaker that the participant is able to breathe freely via the Gastrolyzer exhaust port (see manufacturer's instructions for location) and connecting tube pressure release valve. As soon as the sand-timer appears, remove the mask from the participant's face and remove the D piece, connecting tube and mask from the Gastrolyzer. Wait for the reading to finalise (once the sand timer icon disappears) and record it.

10. Record the current, minimum and maximum temperature of the coolbox containing the glucose solution.

11. Wash your hands. Withdraw the required volume of glucose solution from the container using a sterile 5ml syringe. Administer the whole volume slowly to the participant's mouth, ensuring no spillage.

Record the time when the participant has completed the dose. Aim to have completed the dose within 10 minutes of the baseline breath sample that you recorded in step 9.

12. Fill the ***BHT record sheet*** with target times for the remaining breath measurements: starting 20 minutes after the time of glucose dose completion, every 20 minutes thereafter, until and including 2 hours since glucose dose.

13. Take readings at these times. Record actual time of readings you take to the nearest 5 minutes. Aim to take each reading within 5 minutes of its target time. If readings are more than 5 minutes before or after the target time, do not adjust subsequent target times. Keep to the original target times. Throughout, encourage the caretaker to continue fasting. Caretakers and participants are free to walk around between measurements. Between readings, return the Gastrolyzer to its protective case. If the Gastrolyzer is not needed for 10 minutes or more, switch it off to conserve battery life. Do not handle the Gastrolyzer with dirty or wet hands. Consult the Principal Investigator in the event of any Gastrolyzer error messages or malfunctions.

14. After the final reading, the test is complete and the fasting period is over. Inform the caretaker that the fasting period has been completed. Ensure the ***BHT record sheet*** is filled and place in the 'Completed forms' box.

Appendix

See overleaf for:

1. BHT record sheet
2. Cover sheet
3. Task tracker
4. Oral solutions transport log
5. Breath hydrogen meter D piece change log & Breath hydrogen meter calibration log
6. Gastrolyzer Operating Manual

Appendix 25: BHT record of results and adherence to protocol for use in the field

See next page for the data entry form used during the BHT at the field study site to record results and adherence to the field protocol.

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BHT record sheet

Participant initials: ____ ____ ____ Participant study ID number: ____ ____ ____

Date of test: Day ____ ____ / Month ____ ____ / 201____

At least 3 hours' <u>exclusive breastfeeding</u> before start of fast? Yes [ ]
No [ ] → ___ hours ___ ___ mins

Time of <u>start of 2-hour fast</u>: ____ ____ : ____ ____

Weight ____ ____ . ____ ____ kg → glucose solution dose = weight x 2 = ____ ____ . ____ ml

= ____ x 5ml syringes plus ___ . ___ ml in the last syringe

Glucose solution batch number ____ ____

Gastrolyzer machine number used: 1 [ ] 2 [ ]

Reading	**Target** time	**Actual** time	**ppm**
Baseline	*= 2 hours **after time of start of fast*** ____ ____ : ____ ____	____ ____ : ____ ____	____ ____
*Give glucose solution immediately **after** baseline reading. Time of **completion** of glucose dose:* ____ ____ : ____ ____			
20 minutes	*= 20 minutes **after glucose solution*** ____ ____ : ____ ____	____ ____ : ____ ____	____ ____
40 minutes	*= 40 minutes **after glucose solution*** ____ ____ : ____ ____	____ ____ : ____ ____	____ ____
1 hour	*= 1 hour **after glucose solution*** ____ ____ : ____ ____	____ ____ : ____ ____	____ ____
1 hour 20 minutes	*= 1 hour 20 minutes **after glucose solution*** ____ ____ : ____ ____	____ ____ : ____ ____	____ ____
1 hour 40 minutes	*= 1 hour 40 minutes **after glucose solution*** ____ ____ : ____ ____	____ ____ : ____ ____	____ ____
2 hours	*= 2 hours **after glucose solution*** ____ ____ : ____ ____	____ ____ : ____ ____	____ ____

Fasting for the full 4 hours? Yes [ ]
No – less than 4 hours [ ] → _____ hours _____ _____ mins

Comments *If fast was broken, indicate times baby was fed*

Fieldworker initials ____ ____ ____

Data entry clerk initials ____ ____ ____

Appendix 26: Methods for preparation of oral LM solution at KWTRP Research Pharmacy.

Preparation was undertaken in weekly batches. The oral solution was prepared by pharmacists at KEMRI-WTRP Research Pharmacy. Mannitol powder (D-mannitol; ≥98%; Sigma M4125) and lactulose liquid (Lactulose; 3.3g/5ml; Qualitest) were added to sterile water ('Water for injection'; local clinical supply) to make a solution of 50 mg/ml mannitol and 250mg/ml lactulose. The prepared solution was stored in a labelled sterile Duran bottle until the morning of LM testing, when 15ml aliquots were poured into sterile screw cap specimen containers (one for each participant, plus two spare) and each container labelled with details of the solution plus batch number. Specimen containers were then transferred promptly into a coolbox with ice packs and a thermometer probe with continuous external display. On each LM testing morning, the courier (either myself or a senior fieldworker) would record the internal coolbox temperature at the start and end of transfer, and visually monitor temperatures during transfer. If temperature approached or exceeded 8^{o}C during transit, fresh ice packs were added. Upon arrival at the testing site, temperature was again continually monitored, and recorded by fieldworkers as the first container was removed for administration to the first participant. Solutions were visually inspected before administration to participants to ensure no frozen elements or gross contamination, and manually shaken prior to opening. Sterility was promoted during preparation through use of gloves and lab coat, spraying of work surfaces with 70% ethanol, non-touching of key parts, and use of sterile measurement aids such as needles, syringes and weighing boats. The KWTRP Research Pharmacy fridge was subject to GCLP standard temperature monitoring 24 hours a day. Reagents were stored under 24-hour air conditioning at approximately 18-21^{o}C.

Appendix 27: Lactulose mannitol oral solution preparation SOP for use in pharmacy

See next page for SOP for preparation of oral lactulose mannitol solution for the LM test, for use in KWTRP Research Pharmacy.

STANDARD OPERATING PROCEDURE

PREPARATION OF ORAL LACTULOSE & MANNITOL SOLUTION FOR L:M TEST

Study scientific title	Epidemiology of Environmental Enteropathy and intestinal microbiota amongst infants in Junju, Kilifi
Lay title	Afya tumboni
SERU protocol number	2983
Author	Rosie Crane & Jimmy Shangala
Reviewer	Johnstone Thitiri
Approver	Rosie Crane
Version	1.2
Date	24/09/2015

Materials

Water for injection, in ampoule/bottle
Mannitol powder
Lactulose liquid 3.3g/5ml
Gloves, non-sterile
Lab coat
Needles, sterile G19 x3
Sterile pastette
Scalpel blade
Weighing scale
Weigh boat
70% ethanol in spray bottle
Paper tissue
Duran bottle, 500ml autoclaved with cap
Syringes, sterile 10ml or 20ml x3
Falcon tubes 15ml & 30ml / 30ml sterile universal container
LM solution quality assessment / quality control log (Appendix)

Method

1. Wear a lab coat and gloves.

2. Prepare your work surface and weighing scales by spraying with 70% ethanol. Ensure a clean workspace. Spray gloves with 70% ethanol at intervals throughout.

3. Remove the screw cap from a 500ml sterile autoclaved Duran bottle and place it face up on the work surface – do not touch the inside of the cap.

4. Spray a weigh boat with 70% ethanol, wipe with tissue and allow to completely dry. Using a new sterile scalpel blade, cut the pastette into a 'spatula' shape. Open the mannitol powder bottle and place the screw cap face up – do not touch the inside of the cap. Using the cut pastette, weigh the desired amount of mannitol powder into the weigh boat. Do not touch the inside of the mannitol powder bottle. Once weighing is complete, immediately return the screw cap to the mannitol powder bottle. If you remove too much mannitol powder from the bottle, do not return it to the bottle – discard it.

5. Using a fresh sterile G19 needle and a fresh sterile 10/20ml syringe, withdraw the desired volume of water for injection from the ampoule/bottle and add to the Duran bottle. Return the screw cap to the Duran bottle.

6. Mix manually by gentle shaking for 1 minute.

7. Open the lactulose liquid bottle and place the screw cap face up – do not touch the inside of the cap. Using a fresh sterile G19 needle and fresh sterile 10/20ml syringe, withdraw the desired volume of lactulose liquid from the lactulose bottle and transfer to the Duran bottle. Ensure any part of the syringe that touches the inside of the lactulose bottle has remained sterile. If you remove too much lactulose from the bottle, do not return it – discard it.

8. Mix manually by gentle shaking for 1 minute.

9. Pour desired aliquots of the L:M solution from the Duran bottle into sterile 15ml or 50ml Falcon tubes.

10. Label each Falcon tube with today's date, batch number and 'lactulose 250mg/ml, mannitol 50mg/ml' or 'L:M solution'.

11. Store at 2-8^{o}C. Prepared L:M solution has a shelf life of 3 months.

12. Complete the ***L:M solution quality assessment / quality control log***.

To make a 250mg/ml lactulose, 50mg/ml mannitol solution:

Batch volume	Water for injection	Lactulose liquid 3.3g/5ml	Mannitol powder
100 ml	62.1 ml	37.9 ml	5 g
200 ml	124.2 ml	75.8 ml	10 g
300 ml	186.4 ml	113.6 ml	15 g
400 ml	248.5 ml	151.5 ml	20 g
500 ml	310.5 ml	189.5 ml	25 g

Quality control

Remove aliquots on the first and last day of use for each batch. Use a sterile G19 needle and sterile syringe to remove a 1.8ml aliquot from one of the Falcon tubes. Transfer this aliquot to a 1.8ml tube and label with sample type, batch number and today's date. Transfer to Clinical Trials Laboratory for storage at -20 or -80^{o}C.

Appendix

See overleaf for ***L:M solution quality assessment / quality control log***.

Appendix 28: Laboratory methods for the detection of lactulose and mannitol in urine and in the oral solution

The below text was written by Dr Simon Fleming, Royal Cornwall Hospital Laboratories.

"The sugar probes, mannitol and lactulose used in the intestinal permeability procedure which were collected in timed urine and a select number of serum samples, were analysed by high pressure anion exchange chromatography with pulsed amperometric detection based on the methods as described by Fleming et.al.[§§] Urine and serum aliquots were stored frozen at -80C before analysis.

Urine was diluted 1:20 with deionised water and internal standard (melibiose 100mg/l) was added. The mixture was then desalted with mixed ion exchange resins (Amberlite 120H and IRA 400 Cl)and then centrifuged at 3500g. The supernatant (30ul) was injected onto the HPLC system ,and sugars separated on a CarboPac PA1 (Dionex Thermo-Fisher) 250mm x 4.6mm anion exchange column, using an isocratic eluent of 120mM NaOH with 0.5mM zinc acetate.

For serum samples 200ul of serum was added to 200ul internal standard (melibiose 50mg/L) and to this 200ul of ice-cold 5-sulfosalicylic acid (35g/L) was added to precipitate the serum proteins. After standing on ice for 20min samples were centrifuged at 3,500g for 5 minutes each supernatant was desalted with the mixed ion-exchange resin and re-centrifuged. Thirty microliters of supernatant were injected onto the HPLC system and eluted under the same conditions as the urine samples.

[§§] Fleming SC, Duncan A, Russell RI, Laker MF. Measurement of sugar probes in serum: an alternative to urine measurement in intestinal permeability testing. Clinical chemistry. 1996;42(3):445-8.
Fleming SC, Kapembwa MS, Laker MF, Levin GE, Griffin GE. Rapid and simultaneous determination of lactulose and mannitol in urine, by HPLC with pulsed amperometric detection, for use in studies of intestinal permeability. Clinical chemistry. 1990;36(5):797-9.
Kynaston JA, Fleming SC, Laker MF, Pearson AD. Simultaneous quantification of mannitol, 3-O-methyl glucose, and lactulose in urine by HPLC with pulsed electrochemical detection, for use in studies of intestinal permeability. Clinical chemistry. 1993;39(3):453-6.

Analytes were detected using pulsed amperometric detection. Peak heights were measured, and quantitation was by internal standardisation against known prepared standards.

Analytical validity of the method has been described previously. The assay is linear up to 125mg/L mannitol and 40mg/L lactulose in pre-diluted samples. Analytical recovery ranges between 89% and 105% for all analytes. Overall coefficients of variation range between 1.5% and 8.5% at various sugar concentrations."

Appendix 29: LM test SOP for use in the field

See next page for the SOP used at the field study site for the LM test.

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Afya tumboni

STANDARD OPERATING PROCEDURE

L:M TEST URINE COLLECTION

Study scientific title	Epidemiology of Environmental Enteropathy and intestinal microbiota amongst infants in Junju, Kilifi
Lay title	Afya tumboni
SERU protocol number	2983
Author	Rosie Crane
Reviewer	Johnstone Thitiri
Approver	Rosie Crane
Version	1.2
Date	24/09/2015

Materials required (per test)

L:M test record sheet (Appendix 1)
Cover sheet (Appendix 2)
Task tracker (Appendix 3)
Oral solutions transport log (Appendix 4)
Urine sample request form (Appendix 5)
Sample transport log (Appendix 6)
Breath and urine tests appointments diary
Pen
Demo doll (male or female)
70% ethanol spray
Gloves, non-sterile
Paper tissue
Sterile water
Cotton wool balls
Urine bag x6
Chlorhexidine solution 2% (stored at 2-8^{o}C)
Sterile pastette x10
Sterile tube 5ml
Falcon tube 50ml x2
L:M solution*
Sterile 5ml syringe
Ziploc bag

** L:M solution will have been prepared in KWTRP Clinical Trials Pharmacy (see SOP **Preparation of oral lactulose & mannitol solution for L:M test**) and come in sterile labeled 15-30ml containers; in a coolbox with ice packs and thermometer; transported under cold chain that morning from KWTRP Clinical Trials Pharmacy fridge. Cold chain and custody will be recorded in an **Oral solutions transport log** (Appendix 3). Containers will be labeled with solution name, batch number and date, and pharmacist initials.*

Method

1. Complete the ***LM test record sheet*** as you go along. Check the participant's initials and study number against their ***Cover sheet***. Record today's attendance in the participant's ***Task tracker***.

2. Confirm verbally with the caretaker that they are still happy to proceed. If they need reminding, demonstrate the procedure using the demo doll (choose the same sex doll as the participant).

3. The participant should have undergone exclusive breastfeeding only for the past 3 hours. Check that this is the case. If this is not the case, continue anyway but note on the ***L:M test record sheet***.

4. Look up the LM solution dose in the ***Breath and urine tests appointments diary*** – it will have been calculated during yesterday's breath hydrogen test. If the dose is not available, weigh the participant (see SOP ***Anthropometry***) and calculate the dose at 2ml per kg bodyweight (see SOP ***Breath hydrogen test***).

5. Explain to the caretaker that for the next 1 hour, ideally the participant should not be fed ('nil by mouth'). Note the time of the start of fasting. Allow feeding only if the participant becomes very distressed. Ask the caretaker to stay close by during the fast and provide regular reminders and encouragement for them not to feed the participant. If the participant is fed, continue with testing but document in the ***L:M test record sheet***.

6. During the 1-hour fast, collect a pre-L:M 'spot' urine sample:
   - Clean work surfaces using 70% ethanol and paper tissue. Wear gloves (powder-free when applying urine bags). Change gloves and wash hands regularly.
   - Undress the participant and clean the genital and perineal area using sterile water and cotton wool. Allow the area to dry. Apply a urine bag over the participant's urethral opening (for girls – over the labia majora; for boys – over the penis), ensuring a thorough seal and that the participant's anus remains uncovered, outside the urine bag.
   - Keep checking the urine bag <u>at least</u> every 15 minutes.
     - *If faeces are seen, even if there is no urine:* remove the bag, clean the participant, and place a fresh bag. Discard urine from bags that have been contaminated on the inside by faeces.
     - *If only urine is seen:* remove the bag and, using a sterile pastette, transfer 5ml of the urine from the bag to a 5ml sterile tube. Add one drop of 2% chlorhexidine solution. Label the 5ml tube with the participant's study number and initials, today's date and 'pre-LM'. Discard the bag and excess urine. Store the 5ml tube at 2-8^{o}C. If the sample is the first that day to go into the coolbox, press 'reset' on the thermometer. Do not store oral solutions in the same coolbox as urine or any other biological samples. Use two separate coolboxes if necessary.
     - *If nothing is seen:* <u>check the seal is still good</u> and look again later.
   - Continue to wait until the participant has completed the one-hour fast. Only one 'spot' urine sample needs to be collected during this one hour, so once you have collected one pre-LM urine sample, you don't need to collect another.
   - If you don't manage to collect a urine sample during the 1-hour fast, don't worry, just progress to the next step (and keep the urine bag in place).

7. At the end of the 1-hour fast, give the L:M solution:
   - Record the current, minimum and maximum temperature of the coolbox containing the L:M solution.
   - Wash your hands.
   - Withdraw the required volume of L:M solution using a sterile 5ml syringe.
   - Administer the whole volume slowly to the participant's mouth, ensuring no spillage.
   - Record the time when the participant has completed the dose.

8. If a urine bag is not already in place from before, apply one immediately after completing the L:M solution dose. Collect <u>all urine</u> passed over the next 5 hours:
   - Transfer <u>all urine</u> collected during these 5 hours to one 50ml Falcon tube labeled with the participant's study number, your initials, today's date and 'post-LM'.

- Add one drop of 2% chlorhexidine to the Falcon tube along with the first urine sample (only add chlorhexidine with this first sample – not others).
- Keep the Falcon tube stored at 2-8^{o}C between additions.
- If the participant produces more than 50ml urine during the 5 hours, use 2 Falcon tubes (don't forget to add a drop of 2% chlorhexidine to the first sample in the second Falcon tube).
- Document all episodes of urine leakage and faecal contamination in the ***L:M test record*** sheet.
- Allow free exclusive breastfeeding during these 5 hours, but no other foods or liquids. Document any feeding during this time in the ***L:M test record*** sheet.
- If a new urine bag is needed less than 15 minutes from the end of the 5-hour collection, end the collection early and do not place a new bag.

9. At the end of the 5 hours, remove the bag from the participant and clean if necessary. Inform the caretaker that the test is now complete. Transfer any remaining urine in the last bag to the 50ml Falcon tube.

10. Record the total volume of the 5-hour urine collection – to the nearest marker line on the Falcon tube (N.B. these lines are every 2.5ml. Do not try to estimate volumes between the lines. Recorded volumes should be 5, 7.5, 10, 12.5, 15, 17.5ml etc.. If the volume is below the 5ml line, estimate to the nearest 1ml).

11. Store the 50ml (post-LM) and 5ml (pre-LM) tubes from the same participant inside one Ziploc bag inside the coolbox, along with a filled ***Urine sample request form***.

12. Ensure the ***LM test record sheet*** is complete.

13. Return the 2% chlorhexidine to the fridge.

14. Keep custody of the coolbox containing urine samples until formal handover to the courier at the end of the testing period. If you notice the temperature start to approach 8.0^{o}C, rearrange ice packs and samples to ensure better insulation against heat. On handover to courier, complete the ***Sample transport log*** then reset the thermometer.

Appendix

See overleaf for:

1. L:M test record sheet
2. Cover sheet
3. Task tracker
4. Oral solutions transport log
5. Urine sample request form
6. Sample transport log

Appendix 30: LM test field record

See next page for the data entry form used during the LM test at the field study site to record results and adherence to the field protocol.

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L:M test record sheet

Participant initials: ____ ____ ____ Participant study ID number: ____ ____ ____

Date of test: Day ____ ____ / Month ____ ____ / 201____

At least 3 hours' exclusive breastfeeding before start of fast? Yes [ ]
No [ ] → ___ hours ___ ___ mins

Time of start of 1-hour fast: ____ ____ : ____ ____

LM solution dose (see diary – use yesterday's weight x2): ___ ___ . ___ ml

LM solution batch number ____ ____

Pre-LM spot urine sample obtained during 1-hour fasting period? Yes [ ]
No [ ]

Fasting for 1 hour before LM solution given? Yes [ ]
No – less than 1 hour [ ] → _____ hours _____ _____ mins

Time L:M solution given (start of 5-hour urine collection): ____ ____ : ____ ____

Post-LM 0-5 hours urine sample obtained? Yes [ ] → total volume ____ ____ . ____ ml
No [ ]

Were any urine samples discarded due to faecal contamination? Yes [ ] → ____ samples
No [ ]

Were any urine samples missed due to leakage? Yes [ ] → ____ samples
No [ ]

Urine collected for the full 5 hours? Yes [ ]
No – less than 5 hours [ ] → _____ hours _____ _____ mins

Exclusive breastfeeding for all of this time? Yes [ ]
No [ ] → _____ hours _____ _____ mins

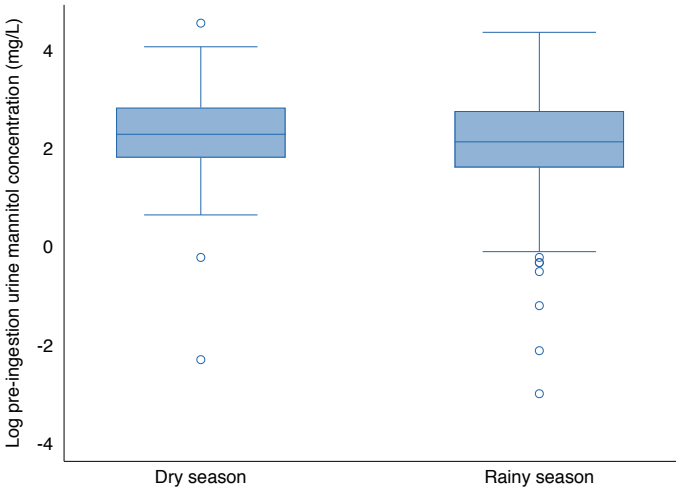
Comments

Fieldworker initials ____ ____ ____

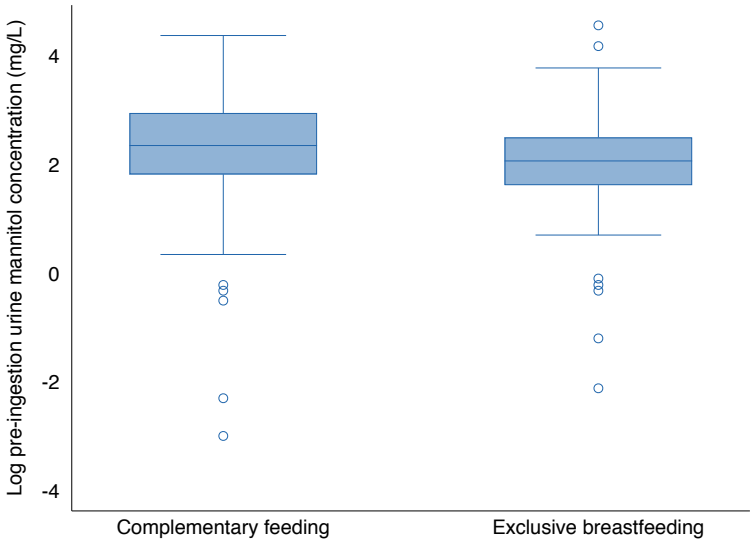
Data entry clerk initials ____ ____ ____

Appendix 31: Pre-ingestion urine mannitol by season and by exclusive breastfeeding status

Supplementary figure 5: Box plots depicting frequency distribution of mannitol concentration in 624 urine samples obtained during the one hour prior to oral LM solution ingestion as part of the LM test during the rainy and dry seasons. Adjusted for age, pre-ingestion mannitol concentrations were higher during the dry season (linear regression of log mannitol concentration with age in months and season as fixed effects: coefficient for rainy season versus dry = -0.19; 95% CI -0.31 to -0.06; p=0.03). Box midlines denote median, box edges denote 25th and 75th centiles, whiskers denote upper and lower adjacent values, hollow dots denote outlying values.



Supplementary figure 6: Box plots depicting frequency distribution of mannitol concentration in 624 urine samples obtained during the one hour prior to oral LM solution ingestion as part of the LM test amongst exclusively breastfed and complementary feeding cohort participants. Adjusted for age, there was no evidence to suggest difference in pre-ingestion mannitol concentration by exclusive breastfeeding status (linear regression of log mannitol concentration with age in months and exclusive breastfeeding status as fixed effects: coefficient for exclusive breastfeeding versus complementary feeding = -0.13; 95% CI -0.34 to 0.07; p=0.22). Box midlines denote median, box edges denote 25th and 75th centiles, whiskers denote upper and lower adjacent values, hollow dots denote outlying values.



Appendix 32: Cohort enrolment questionnaire

See next page for the questionnaire administered to new participants at enrolment to the cohort study.

Afya tumboni

Cohort enrolment questionnaire

Section 1: To be filled by CLINICIAN

Eligibility - final check

	Yes	No
Is the baby born to a mother who is a resident of the study area?*	**[ ]**	[ ]
Is the baby aged 14 days or younger?	**[ ]**	[ ]
Within the next 9 months, is the baby unlikely to relocate to outside the study area?	**[ ]**	[ ]
Is the baby's weight at screening 1.5kg or more? *Screening weight* = ____ . ____ ____ *kg*	**[ ]**	[ ]
Is the baby free from any major congenital abnormality?	**[ ]**	[ ]
Is the baby free from any severe life-threatening acute illness?	**[ ]**	[ ]
Has informed consent been given?	**[ ]**	[ ]

** If the mother is no longer present, but the baby is being cared for in a homestead within the study area, respond 'Yes'*

If **all** of the above responses are 'Yes', the baby is eligible for enrolment.
If **any** of the responses above are 'No', do not enroll.

Mother

01. *How many live births has the baby's mother had before this baby?* [Do not include stillbirths or miscarriages]

_____ _____ births

02. *Has this baby's mother had any children or babies that have died?* [Do not include stillbirths or miscarriages]

Yes [ ] → *How many?* ___ ___ deaths
No [ ]

Pregnancy and delivery

03. *How many antenatal care appointments did the mother attend for this baby?* [Check card. Tick one only]

None [ ]
1-4 [ ]
5 or more [ ]
Don't know [ ]

04. *Was the mother tested for HIV during this baby's pregnancy?* [Check card. Tick one only]

Yes, positive [ ]
Yes, negative [ ]
Not tested during pregnancy [ ] → Recommend mother seek testing now
Don't know [ ]

05. *What was the mother's expected date of delivery?* [Check card]

Day ___ ___ *or* day unknown [ ]
Month ___ ___ *or* month unknown [ ]

06. *Where was the baby delivered?* [Tick one only]

Home [ ]
Private facility [ ] Specify _____
Public facility [ ] Specify _____

07. *Were there any complications during labour and/or delivery?* [Tick all that apply]

Bleeding before delivery [ ]
Mother high blood pressure [ ]
Fever before, during or shortly after delivery [ ]
Prolonged/obstructed labour [ ]
None of the above [ ]

08. *What kind of delivery was it?* [Tick one only]

Normal [ ]
Caesarean [ ]

09. *Did the baby cry immediately after birth?* [Tick one only]

Yes [ ]
No [ ]
Don't know [ ]

10. *What did the baby weigh at birth?* [Refer to card. Do not record unless documented on card]

Weight recorded on card [ ] →____ . ____ ____ kg
Weight not recorded on card [ ]

Baby

11. *Has the baby had any of the following problems since birth?* [Tick all that apply]

Breathing difficulty [ ]
Diarrhoea* [ ] →How many days of diarrhoea? ___ ___ days
Fever [ ]
None of the above [ ] →Go to 13
Don't know [ ] →Go to 13

* = Three or more unusually loose or liquid stools in one day

12. *How was this/these problem(s) dealt with?* [Tick all that apply]

Home-based care / none [ ]
Dispensary visit [ ] →Name of dispensary _____
→Name(s) of any antibiotic(s) given _____
Hospital outpatients [ ] →Name of hospital _____
→Name(s) of any antibiotic(s) given _____
Hospital admission [ ] →Name of hospital _____
→Name(s) of any antibiotic(s) given _____

13. *Is the baby currently taking any long-term medications?* [Tick one only. Check treatment card if available]

Yes [ ] Names _____
No [ ]
Don't know [ ]

Clinician initials ____ ____ ____

Section 2: To be filled by FIELDWORKER AT ENROLMENT

Household

14. Details of all household members, including the participant. [Enter the head of the household first]

First name	Age (years)	Male or female?	Relationship to head of household*
		M [ ] F [ ]	01
		M [ ] F [ ]	
		M [ ] F [ ]	
		M [ ] F [ ]	
		M [ ] F [ ]	
		M [ ] F [ ]	
		M [ ] F [ ]	
		M [ ] F [ ]	
		M [ ] F [ ]	
		M [ ] F [ ]	
		M [ ] F [ ]	
		M [ ] F [ ]	
		M [ ] F [ ]	
		M [ ] F [ ]	
		M [ ] F [ ]	

*Code for relationship to head of household:

01 Head	06 Parent	11 Niece / Nephew
02 Wife / Husband	07 Parent-In-Law	12 Other relative
03 Son / Daughter	08 Brother / Sister	13 Adopted / Foster / Stepchild
04 Son-In-Law / Daughter-In-Law	09 Brother-In-Law / Sister-In-Law	14 Not related
05 Grandchild	10 Uncle / Aunt	98 Don't know

Main caretaker

15. *Who is the main caretaker of the baby?* [Tick one only]

Biological mother	[ ]
Biological father	[ ]
Step-mother	[ ]
Step-father	[ ]
Grandmother	[ ]
Grandfather	[ ]
Aunt/uncle	[ ]
Older sibling – aged less than 16 years	[ ]
Older sibling – aged 16 years or more	[ ]
Friend	[ ]
Other	[ ] Specify _____

16. *What is/was this main caretaker's highest level of education?* [Tick one only]

None	[ ]
Some primary	[ ]
Completed primary	[ ]
Some secondary	[ ]
Completed secondary	[ ]
Some higher	[ ]
Completed higher	[ ]

17. *What is the main occupation of this main caretaker?* [What they spend most of their time doing. Tick one only]

No occupation / domestic tasks within this homestead	[ ]
Farming	[ ]
Fishing	[ ]
Petty trade (e.g. market stall)	[ ]
Employed (professional e.g. teacher)	[ ]
Employed (casual e.g. labourer)	[ ]
In full-time education	[ ]

Household

18. *What is the main source of income for the household?* [Tick one only]

No income	[ ]
Permanent job	[ ]
Petty trading e.g. sale of firewood	[ ]
Sale of own crop/livestock products	[ ]
Business	[ ]
Brewing alcohol	[ ]
Fishing	[ ]
Casual labour	[ ]
Remittance	[ ]
Other	[ ] Specify _____

19. *How many rooms in the house are used for sleeping?*

____ ____ rooms

20. *What is the household's main source of lighting?* [Tick one only]

Electricity (mains or battery)	[ ]
Solar lamp	[ ]
Tin / lantern / pressure lamp	[ ]
Fire / candles	[ ]
Other	[ ] Specify _____

21. *What is the household's main cooking fuel?* [Tick one only]

Electricity (mains or battery)	[ ]
Gas / LPG	[ ]
Paraffin	[ ]
Charcoal	[ ]
Wood	[ ]
Other	[ ] Specify _____

22. *Does the household have:* [Tick all that apply]

Electricity	[ ]
Mobile phone	[ ]
Motorbike	[ ]
Radio	[ ]
Television	[ ]
Bicycle	[ ]
Animal-drawn cart	[ ]
Vehicle/car	[ ]
None of the above	[ ]

23. *What is the main source of drinking water for the baby's main caretaker?* [Tick one only]

Piped to public tap / standpipe	[ ]
Piped to neighbour	[ ]
Piped to compound (outdoors)	[ ]
Piped into dwelling (indoors)	[ ]
Borehole	[ ]
Dug well (covered)	[ ]
Dug well (uncovered)	[ ]
Spring (protected)	[ ]
Spring (unprotected)	[ ]
Rainwater collection	[ ]
Tanker truck	[ ]
Cart with small tank / drum	[ ]
Surface water (river, stream, dam, pond)	[ ]
Bottled water	[ ]
Other	[ ] Specify _____

24. *Where is that water source located?* [Tick one only]

Inside the household/homestead compound	[ ]
Outside the household/homestead compound	[ ]

25. *How long does it take to go to that source, get water, and come back?* [Tick one only]

Less than 30 minutes	[ ]
More than 30 minutes	[ ]
Don't know	[ ]

26. *Does the household do anything to the water to make it safer to drink?* [Tick one only]

Yes	[ ]
No	[ ] → Go to 28
Don't know	[ ] → Go to 28

27. *What does the household usually do to make water safer to drink?* [Tick all that apply]

Boil	[ ]
Add bleach / chlorine	[ ]
Strain through a cloth	[ ]
Use water filter	[ ]
Solar disinfection	[ ]
Traditional herbs / ash	[ ]
Other	[ ] Specify _____

28. *What does the baby's main caretaker usually use to wash their hands?* [Tick one only]

Water only	[ ]
Water and soap	[ ]
Water and ash/mud/sand	[ ]

29. Does any member of this household own agricultural land? [Tick one only]

Yes	[ ] → How many acres? ___ ___ acres
No	[ ]

30. *Does any member of this household own the following livestock?* [Indicate numbers – enter 00 if none]

Cattle	___ ___
Horse / donkey	___ ___
Goats / sheep	___ ___
Pigs	___ ___
Chickens / ducks	___ ___
Rabbits	___ ___

31. *Within the homestead, where are animals free to roam?* [Tick all that apply]

	Not present	Nearby compound	Within compound	Within house
Cattle	[ ]	[ ]	[ ]	
Horses / donkeys	[ ]	[ ]	[ ]	
Goats/ sheep	[ ]	[ ]	[ ]	[ ]
Pigs	[ ]	[ ]	[ ]	[ ]
Chickens / ducks	[ ]	[ ]	[ ]	[ ]
Rabbits	[ ]	[ ]	[ ]	[ ]
Dogs	[ ]	[ ]	[ ]	[ ]
Cats	[ ]	[ ]	[ ]	[ ]

32. *What is the household total monthly cash income? (in Kenya shillings)* [Tick one only]

<1,000	[ ]
1,000 - 3,999	[ ]
4,000 - 9,999	[ ]
10,000 - 19,999	[ ]
20,000 +	[ ]
Don't know	[ ]

Mother

33. *How old is the baby's mother?* [Refer to mother's ID card if available. If age unknown, enter estimate]

____ ____ years

34. *Just before this baby was born, how old was the youngest <u>live</u> child of this baby's mother?*

____ ____ years ____ ____ months *or* No other live children [ ]

Baby

35. *Who looks after the baby during most of the daytime?* [Tick one only]

Main caretaker	[ ]
Someone else:	
<u>Adult</u> (aged 16 years or older)	[ ]
<u>Younger</u> (aged less than 16 years)	[ ]

36. *Where does the baby spend <u>most</u> of the daytime?* [Tick one only]

Within main caretaker's homestead:
- Indoors [ ]
- Outdoors [ ]

Outside of main caretaker's homestead:
- Outdoors (e.g. shamba / fields) [ ]
- Indoors (e.g. nursery / other house) [ ]

37. *Did the baby sleep under a mosquito net <u>last night</u>?* [Tick one only]

Yes [ ]
No [ ]
Don't know [ ]

38. *Has the baby ever been breastfed?* [Tick one only]

Yes [ ]
No [ ] →Go to 40
Don't know [ ] →Go to 40

39. *How old was the baby when they first breastfed?* [Tick one only]

Under one hour [ ]
1-24 hours [ ]
More than 24 hours [ ]
Don't know [ ]

40. *Did the baby receive anything by mouth before the very first milk feed?* [Tick one only]

Yes [ ]
No [ ] →Go to 42
Don't know [ ] →Go to 42

41. *What did the baby receive before the very first milk feed?* [Tick all that apply]

Medicine (Western) [ ]
Medicine (traditional/local) [ ]
Sugar water [ ]
Sugar and salt water (home-made) [ ]
Coconut water [ ]
Tea / herbal water [ ]
Plain water [ ]
Other [ ] Specify _____

42. *What is the baby's <u>main</u> food or liquid at the moment?* [Tick one only]

Breast milk [ ]
Uji / porridge [ ]
Animal milk (fresh) [ ]
Infant formula milk (powdered) [ ]
Other [ ] Specify _____

43. *Since the very first milk feed, has the baby also received any of the following?* [Tick all that apply]

Breast milk	[ ]
Animal milk (fresh)	[ ]
Sugar water	[ ]
Sugar and salt water (home-made)	[ ]
Coconut water	[ ]
Tea / herbal water	[ ]
Plain water	[ ]
Uji / porridge	[ ]
Infant formula milk (powdered)	[ ]
Other	[ ] Specify _____

Anthropometry

For MOTHER (if present):

44. Mid upper arm circumference: ____ ____ . ____ cm

45. Height ____ ____ ____ . ____ cm

For BABY:

46. Length ____ ____ . ____ cm

47. Mid upper arm circumference ____ ____ . ____ cm

48. Head circumference ____ ____ . ____ cm

Enrolment fieldworker initials ____ ____ ____

Section 3: To be filled by FIELDWORKER AT HOUSEHOLD

50. Homestead GPS coordinates: [Decimal degrees] South ____ ____ . ____ ____ ____ ____ ____
East ____ ____ . ____ ____ ____ ____ ____

51. *What is the main toilet facility used by the baby's main caretaker?* [Observe the facility. Tick one only]

Pit latrine with uncovered wooden slab	[ ]
Pit latrine with wooden slab covered with soil	[ ]
Pit latrine with concrete slab	[ ]
Ventilated improved pit latrine	[ ]
Flush toilet	[ ]
No facility (bush, open field)	[ ]

52. *What is the main floor material of the homestead buildings that are used for everyday living?* [Observe the floor. Tick one only]

Earth/sand (mchanga)	[ ]
Dung	[ ]
Cement	[ ]
Wood	[ ]
Tiles	[ ]
Carpet	[ ]
Other	[ ] Specify _____

53. *What is the main wall material of the homestead buildings that are used for everyday living?* [Observe the wall. Tick one only]

Grass / straw / makuti	[ ]
Mud / wood	[ ]
Corrugated iron / tin (mabati)	[ ]
Stone	[ ]
Brick / block	[ ]
Wood planks / shingles	[ ]
Other	[ ] Specify _____

54. *What is the main roof material of the homestead buildings that are used for everyday living?* [Observe the roof. Tick one only]

Grass / straw / makuti	[ ]
Mud (manyatta)	[ ]
Corrugated iron / tin (mabati)	[ ]
Tiles / asbestos sheets	[ ]
Concrete	[ ]
Other	[ ] Specify _____

Household fieldworker initials ____ ____ ____

Enrolment fieldworker observations (if any):

Initials ___ ___ ___ Day ___ ___ Month ___ ___ Year 201 ___

Household visiting fieldworker observations (if any):

Initials ___ ___ ___ Day ___ ___ Month ___ ___ Year 201 ___

Clinician observations (if any):

Initials ___ ___ ___ Day ___ ___ Month ___ ___ Year 201 ___

Data entry clerk observations (if any):

Initials ___ ___ ___ Day ___ ___ Month ___ ___ Year 201 ___

Principal Investigator observations (if any):

Initials ___ ___ ___ Day ___ ___ Month ___ ___ Year 201 ___

Appendix 33: Cohort weekly home visit questionnaire

See next page for the data entry form used by fieldworkers during weekly home visits to cohort study participants.

Weekly home visit data entry form

Afya tumboni

01. Date of visit: DD ___ ___ / MM ___ ___ / 201 ___

02. Outcome of visit:
Seen [ ]
Not seen [ ] specify reason (e.g. funeral, travelled) _____

03. Currently, what is the baby's main food or liquid? [Tick one only.]

Breast milk	[ ]
Uji / porridge	[ ]
Animal milk (fresh)	[ ]
Infant formula milk (powdered)	[ ]
Other liquid(s)	[ ] Specify _____
Solid food(s)	[ ] Specify _____

04. Since the last home visit, has the baby also received any of the following? [Tick all that apply. *In case of the first home visit, ask for anything received SINCE ENROLMENT.]

Breast milk	[ ]
Animal milk (fresh)	[ ]
Sugar water	[ ]
Sugar and salt water (home-made)	[ ]
Coconut water	[ ]
Tea / herbal water	[ ]
Plain water	[ ]
Uji / porridge	[ ]
Infant formula milk (powdered)	[ ]
Other liquid(s)	[ ] Specify _____
Solid food(s)	[ ] Specify _____

05. *If the baby is aged 6 months or more:* Since the last home visit, has the baby received a vitamin A dose? [Confirm using the child health card]

Yes	[ ] → [ ] Day ___ ___ Month ___ ___ / 201___
No	[ ]
Baby is not yet 6 months old	[ ]

06. Since the last home visit,* has the baby received any vaccinations? [If yes, check card. *In case of the first home visit, ask for vaccinations given SINCE BIRTH.]

No [ ]
Yes [ ] →

BCG	[ ] Day ___ ___ Month ___ ___ / 201___
OPV (polio oral)	[ ] Day ___ ___ Month ___ ___ / 201___
IPV (polio injection)	[ ] Day ___ ___ Month ___ ___ / 201___
Pentavalent	[ ] Day ___ ___ Month ___ ___ / 201___
Pneumococcal	[ ] Day ___ ___ Month ___ ___ / 201___
Rotavirus	[ ] Day ___ ___ Month ___ ___ / 201___
Measles	[ ] Day ___ ___ Month ___ ___ / 201___

07. Since the last home visit,* has the baby had diarrhoea** or bloody stool? [*In case of the first home visit, ask about SINCE ENROLMENT. **Three or more unusually loose or liquid stools in one day.]

Yes – diarrhoea	[ ] →How many days of diarrhoea? ___ ___ days
Yes – bloody stool	[ ] →How many days of bloody stool? ___ ___ days
No	[ ] →Go to 09
Don't know	[ ] →Go to 09

08. How was the diarrhoea/bloody stool dealt with? [Tick all that apply]

Home-based care / none	[ ]
Dispensary visit	[ ] →Name of dispensary _____
Hospital outpatients	[ ] →Name of hospital _____
Hospital admission	[ ] →Name of hospital _____

09. Since last home visit,* has the baby had breathing difficulty (with or without cough)? [Tick one only. *In case of the first home visit, ask about SINCE ENROLMENT.]

Yes	[ ]
No	[ ] →Go to 11
Don't know	[ ] →Go to 11

10. How was the breathing difficulty dealt with? [Tick all that apply]

Home-based care / none	[ ]
Dispensary visit	[ ] →Name of dispensary _____
Hospital outpatients	[ ] →Name of hospital _____
Hospital admission	[ ] →Name of hospital _____

11. Does caretaker say the baby has a 'hot body'? Yes [ ] No [ ]

12. Temperature of baby: ___ ___ . ___ ^{o}C

13. Has the baby had a rapid malaria test done already this week? Yes [ ] No [ ]

14. Was a rapid test and film taken now? Yes [ ] No [ ] ➔ Go to 18

15. Rapid test result now:

P. falciparum and *P.* others	[ ]
P. others only	[ ]
Negative	[ ]
Indeterminate	[ ]

16. Rapid test kit used:

CareStart	[ ]
OptiMAL	[ ]

17. Was the baby treated with Co-artem? Yes [ ] No [ ]

18. Visit conclusion

Baby to be seen next week	[ ]
Return after 6-12 hours	[ ]
Refer to dispensary	[ ]
Refer to Kilifi County Hospital	[ ]
Other (record in notes)	[ ]

Fieldworker initials ____ ____ ____

Data entry clerk initials ____ ____ ____

Appendix 34: Cohort acute illness form for use at Junju primary health care facility

See next page for the data entry form used by the study clinician at Junju primary healthcare facility for episodes of acute illness amongst cohort study participants.

Afya tumboni

Acute illness clinical data entry form (dispensary)

To be completed by clinician seeing the participant at the dispensary

01. *Today's date:* DD ___ ___ / MM ___ ___ / 201 ___

02. *Is this a new problem, or a follow-up?* New problem [ ]
Follow-up [ ]

02. *What are the baby's problems?* [Tick all that apply]

Breathing difficulty and/or cough	[ ] → *How many days?* ___ ___ days
Diarrhoea*	[ ] → *How many days?* ___ ___ days
Vomiting	[ ]
Blood in stool	[ ]
Worms in stool	[ ]
Fever	[ ] → *How many days?* ___ ___ days
Seizure	[ ]
Poor feeding	[ ]
Other	[ ] → Specify _____

* = Three or more unusually loose or liquid stools in one day

03. *Is the baby currently taking any long-term medications?* [Check treatment card if available]

Yes [ ] specify name(s) _____
No [ ]
Don't know [ ]

04. Have any tests been done? [Either at this dispensary, or elsewhere within the current illness. Tick all that apply]

Rapid Malaria Test [ ] result: Negative [ ] Positive [ ]
Malaria blood slide [ ] result: Negative [ ] Positive [ ]
Other test [ ] specify _____ result: _____
No tests done [ ]

05. Was a stool sample collected? [Remember to collect a stool sample for ALL cases of diarrhoea and/or bloody stool amongst study participants.]

Yes [ ]
No [ ]

06. What is your working diagnosis? [Tick all that apply]

UPPER respiratory tract infection (e.g. cold / sore throat / ear infection / tonsillitis)	[ ]
LOWER respiratory tract infection (e.g. pneumonia / TB / bronchiolitis)	[ ]
Gastroenteritis (including diarrhoea and/or vomiting and/or blood in stool)	[ ]
Ascariasis / intestinal worms	[ ]
Malaria	[ ]
Neurological infection (e.g. meningitis / encephalitis)	[ ]
General febrile illness, unknown focus	[ ]
Malnutrition	[ ]
Micronutrient deficiency (e.g. iron deficiency anaemia, rickets)	[ ]
Other (specify) _____	[ ]
Don't know	[ ]
No diagnosis – well baby	[ ]

07. Have you prescribed any of the following treatments? [Tick all that apply]

Anti-malarial	[ ]	specify name(s) _____
Antibiotic (oral, not topical)	[ ]	specify name(s) _____ days ___ ___
Anti-worming	[ ]	specify name(s) _____
Iron supplement	[ ]	
None of the above	[ ]	

08. What is the outcome of your assessment?

Sent home, no follow-up	[ ]	
Sent home, return for follow-up	[ ]	date requested to return: Day ___ ___ Month ___ ___
Referred to Kilifi County Hospital	[ ]	
Referred to other facility	[ ]	specify facility _____
Other outcome	[ ]	specify outcome _____

Clinician initials: ___ ___ ___

Fieldworker observations (if any):

Signature _____ initials ___ ___ Day ___ ___ Month ___ ___ Year 201 ___

Supervisor (senior fieldworker) observations (if any – including, if sample taken, results of stool sample OCP and culture, whether these were fed back to participant's caretaker and clinician, and any action taken):

Signature _____ initials ___ ___ Day ___ ___ Month ___ ___ Year 201 ___

Clinician observations (if any):

Signature _____ initials ___ ___ Day ___ ___ Month ___ ___ Year 201 ___

Principal Investigator observations (if any):

Signature _____ initials ___ ___ Day ___ ___ Month ___ ___ Year 201 ___

Appendix 35: Cohort acute illness form for use at Kilifi County Hospital

See next page for the data entry form used by the study clinician or Principal Investigator (myself) at Kilifi County Hospital for episodes of acute illness amongst cohort study participants.

Afya tumboni

Acute illness clinical data entry form (Kilifi County Hospital)

To be completed by Principal Investigator using KCH electronic and paper admission records

1. Date of admission: DD ___ ___ / MM ___ ___ / 201 ___

2. PID: ___ ___ ___ ___ ___ ___

3. Serial: ___ ___ ___ ___ ___ ___

4. Anthropometry on admission:

   Weight: ___ ___ . ___ ___ kg

   Length: ___ ___ . ___ cm

   MUAC: ___ ___ . ___ cm

   Head circumference: ___ ___ . ___ cm

5. Breastfeeding status <u>immediately prior</u> to acute illness:

Exclusively breastfed	[ ]
Predominantly breastfed	[ ]
Partially breastfed	[ ]
Not breastfeeding	[ ]

6. *What were the baby's problems on admission?* [Tick all that apply]

Breathing difficulty and/or cough	[ ] ➔ *How many days?* ___ ___ days
Diarrhoea*	[ ] ➔ *How many days?* ___ ___ days
Vomiting	[ ]
Blood in stool	[ ]
Worms in stool	[ ]
Fever	[ ] ➔ *How many days?* ___ ___ days
Seizure	[ ]
Weight loss / poor weight gain	[ ]
Other	[ ] Specify _____
None of the above	[ ]

* = Three or more unusually loose or liquid stools in one day

7. *On admission, was the baby taking any <u>long-term</u> medications?*

Yes	[ ] specify name(s) _____
No	[ ]
Don't know	[ ]

8. *Which of the following tests were done during the KCH admission?* [Tick all that apply]

Malaria blood slide	[ ] ➔	result: negative [ ] positive [ ]
HIV antibody test	[ ] ➔	result: negative [ ] positive [ ]
Chest X-ray	[ ]	
Blood culture	[ ]	
Stool culture	[ ] ➔	□□□□□□□ _____
Stool virus ELISA	[ ] ➔	□□□□□□□ _____
Urine dipstick	[ ] ➔	□□□□□□□ _____
Urine microscopy	[ ] ➔	result: WBCs ____ ____ ____ per mm^3
Urine culture	[ ]	
Lumbar puncture	[ ] ➔	result: WBCs ____ ____ ____ per mm^3 RBCs ____ ____ ____ per mm^3 Glucose ____ . ____ mmol/L Protein ____ ____ . ____ mg/dL
Other test(s)	[ ]	specify _____ result: _____ specify _____ result: _____ specify _____ result: _____ specify _____ result: _____

9. *If a blood culture was done, what was the result?* [Tick one only]

No growth	[ ]	
Growth – likely contaminant	[ ] ➔	□□□□□□□ _____
Growth – likely clinically significant	[ ] ➔	□□□□□□□ _____
No result	[ ]	
Test not done	[ ]	

10. *If a urine culture was done, what was the result?* [Tick one only]

No growth	[ ]	
Growth – likely contaminant	[ ] ➔	□□□□□□□ _____
Growth – likely clinically significant	[ ] ➔	□□□□□□□ _____
No result	[ ]	
Test not done	[ ]	

11. *If CSF culture was done, what was the result?* [Tick one only]

No growth	[ ]	
Growth – likely contaminant	[ ] ➔	□□□□□□□ _____
Growth – likely clinically significant	[ ] ➔	□□□□□□□ _____
No result	[ ]	
Test not done	[ ]	

12. *Were additional stool samples collected for research purposes?* [Collect an additional research sample for ALL cases of diarrhoea and bloody stool. If a participant has no diarrhoea or bloody stool but is due their weekly asymptomatic stool collection, take the weekly sample(s) throughout admission]

Yes – asymptomatic	[ ] ➔	□date collected MM ___ ___ / DD ___ ___ / 201 ___
Yes – diarrhoea/bloody	[ ] ➔	□date collected MM ___ ___ / DD ___ ___ / 201 ___
No	[ ]	

13. *What was the diagnosis made by the admitting clinician?* [Tick all that apply]

UPPER respiratory tract infection (e.g. cold / sore throat / ear infection / tonsillitis)	[ ]
LOWER respiratory tract infection (e.g. pneumonia / TB / bronchiolitis)	[ ]
Gastroenteritis (including diarrhoea and/or vomiting and/or blood in stool)	[ ]
Ascariasis / intestinal worms	[ ]
Malaria	[ ]
Neurological infection (e.g. meningitis / encephalitis)	[ ]
General febrile illness, unknown focus	[ ]
Malnutrition	[ ]
Micronutrient deficiency (e.g. iron deficiency anaemia)	[ ]
Other (specify) _____	[ ]
Don't know	[ ]
No diagnosis – well baby	[ ]

14. *What was the length of stay?*

___ ___ days

15. *What treatment was given?*

Antibiotic 1	[ ] ➔ specify _____	duration ___ ___	days
Antibiotic 2	[ ] ➔ specify _____	duration ___ ___	days
Antibiotic 3	[ ] ➔ specify _____	duration ___ ___	days
Antibiotic 4	[ ] ➔ specify _____	duration ___ ___	days
Antibiotic 5	[ ] ➔ specify _____	duration ___ ___	days
Other drugs	[ ] ➔ specify _____		
Surgery	[ ] ➔ specify _____		

16. *What was the diagnosis made by the discharging clinician?* [Tick all that apply]

UPPER respiratory tract infection (e.g. cold / sore throat / ear infection / tonsillitis)	[ ]
LOWER respiratory tract infection (e.g. pneumonia / TB / bronchiolitis)	[ ]
Gastroenteritis (including diarrhoea and/or vomiting and/or blood in stool)	[ ]
Ascariasis / intestinal worms	[ ]
Malaria	[ ]
Neurological infection (e.g. meningitis / encephalitis)	[ ]
General febrile illness, unknown focus	[ ]
Malnutrition	[ ]
Micronutrient deficiency (e.g. iron deficiency anaemia)	[ ]
Other (specify) _____	[ ]
Don't know	[ ]
No diagnosis – well baby	[ ]

17. *According to review of the notes, which diagnoses are likely to apply?* [Tick all that apply]

UPPER respiratory tract infection (e.g. cold / sore throat / ear infection / tonsillitis)	[ ]
LOWER respiratory tract infection (e.g. pneumonia / TB / bronchiolitis)	[ ]
Gastroenteritis (including diarrhoea and/or vomiting and/or blood in stool)	[ ]
Ascariasis / intestinal worms	[ ]
Malaria	[ ]
Neurological infection (e.g. meningitis / encephalitis)	[ ]
General febrile illness, unknown focus	[ ]
Malnutrition	[ ]
Micronutrient deficiency (e.g. iron deficiency anaemia)	[ ]
Other (specify) _____	[ ]
Don't know	[ ]
No diagnosis – well baby	[ ]

18. *What was the outcome of the admission?*

Discharged alive	[ ]
Referred to other facility	[ ] ➔ specify _____
Died	[ ]
Still KCH inpatient at end of study	[ ]
Unknown	[ ]

19. Comments: _____
_____
_____
_____
_____
_____
_____

Principal Investigator: Initials ___ ___ DD ___ ___ / MM ___ ___ / 201 ___

Data entry clerk: Initials ___ ___ DD ___ ___ / MM ___ ___ / 201 ___

Appendix 36: Cohort community engagement and participant remuneration and benefits

Initial ideas for the study, its potential acceptability and strategies for community engagement were explored with KWTRP CLG through establishment of a dedicated Community Advice for Studies group consisting of CLG members and project managers with experience of the Junju area. Following this, presentations were made to Kilifi Sub-County Health Management Team, Junju and Bomani Dispensary Health Committees, to KEMRI Community Representatives and finally to the community as a whole through a series of 'baraza' meetings held in six of the 10 villages and hosted by the area sub-Chief and village elders. Finally, during a piloting phase in the month before enrolment began, BHT and LM test administration methods were developed with the assistance of 11 local volunteer mothers and their babies. These mothers gave feedback on acceptability and participant experiences of these two procedures that were new to the community. The six fieldworkers who between them carried out all study procedures and home visits were all long-term residents of the study villages. Caretakers of participants were reimbursed for time spent on study activities on LM and BHT testing days. Reimbursement was at the rate of local unskilled labour and in line with guidelines from the KWTRP CLG. Local public transport fares – for scheduled or unscheduled trips from home to Junju dispensary – were reimbursed. Free ambulance transfer from home Junju dispensary and on to Kilifi County Hospital was arranged for severely acutely unwell participants. Transfers were arranged by fieldworkers, who lived within the same communities as participants. Costs arising from admission to secondary care at Kilifi County Hospital and Vipingo Sub-County Hospital were paid by the study.

Appendix 37: Cohort participant information and consent form

See next page for the cohort study participant information and consent form (English version). Participant caretakers were offered a choice of English, Swahili and Giriama language versions.

KEMRI Wellcome Trust Research Programme: Information Sheet and Consent Form

Junju Baby Growth Study

Epidemiology of Environmental Enteropathy and intestinal microbiota amongst infants in Junju, Kilifi.

KEMRI-Wellcome Trust Research Programme, Kenya	Rosie Crane (Principal Investigator), Sarah Atkinson, Jay Berkley, Greg Fegan, Evelyn Gitau, Sam Kariuki, Susan Morpeth, Musa Mulongo, Francis Ndungu, Etienne de Villiers, Juliana Wambua.
Royal Cornwall Hospitals NHS Trust, UK	Simon Fleming

Who is carrying out this study?

This study is being carried out by KEMRI. KEMRI is a government organisation that carries out medical research to find better ways of preventing and treating illnesses in the future for everybody's benefit.

What is this study about?

One problem KEMRI are currently trying to learn more about is child growth. In this study, we aim to find out how different kinds of infections can affect the early development of a baby's stomach and intestines, and how this can in turn affect how babies grow during the first year of life.

We are asking the parents or guardians of every newborn baby in the Junju area for their permission for their baby to participate in the study. For each baby, the study will last from enrolment until they reach nine months of age. We would like 100 babies to participate.

What will it involve for my baby if I agree?

If you allow your baby to participate in this research, the following observations will be done on your baby:

- On the day of enrolment: in a quiet location, a fieldworker will ask you some questions about your baby, your household, and yourself. Your baby will have their length, weight and upper arm and head circumference measured. The fieldworker will also obtain a stool sample from your baby. If she is present, the baby's mother's height and upper arm circumference will be measured.
- Once per week: a fieldworker will visit your home to ask a few questions about your baby's general health and feeding. During some of these home visits, this fieldworker will also collect a sample of your baby's stool.
- Twice per month: you will be asked to bring your baby to Junju dispensary in the morning for two tests: a urine test and a breath test.
    - For the urine test (once per month), we will give your baby a very small amount of sugar water to drink. We will then collect the urine that your baby produces for the next five hours by taping a small plastic bag to your baby's nappy area. We will ask you not to feed your baby for one hour before the start of the test. You and your baby will be free to return home in the afternoon.
    - For the breath test (once per month), we will give your baby a very small amount of sugar water to drink, and then ask you to hold your baby whilst we place a soft plastic mask over their mouth and nose to measure the air that they breathe out. We will hold the mask gently to your baby's mouth and nose for a few seconds, once every 20 minutes, for two hours. We will ask you not to feed your baby for two hours before the start of the test and

during the two hours of breath sampling: four hours in total. This is not harmful for your baby. You and your baby will then be free to return home in the afternoon.

- On two occasions during the study: we will take a small sample of blood from your baby's arm. Each time, the volume will be less than 5ml (one teaspoon). The first test will be done at Junju dispensary at enrolment. The second test will be done when your baby reaches six months of age.
- If your baby develops diarrhoea (three or more loose stools in one day): we will ask you to bring your baby to Junju dispensary so that an extra stool sample can be collected. If you cannot attend the dispensary, a fieldworker can visit your home to collect the stool sample. However, we will still recommend visiting the dispensary so your baby can be seen and treated by a nurse or clinical officer.
- If your baby becomes unwell with fever or any other illness:
    - Inside normal working hours, we recommend that you attend Junju dispensary where your baby will be seen by a nurse or clinical officer.
    - Outside of normal working hours, we recommend that you contact your local fieldworker, who will measure your baby's temperature. If your baby's temperature is high, the fieldworker will test for malaria and provide treatment if the test is positive. The fieldworker will also talk to the study clinical officer and arrange for referral to Kilifi County Hospital if needed.

Are there any risks or disadvantages to my baby in taking part?

- Our priority for every participant is their wellbeing.
- For the urine collection test, there is a chance that the sugar solution may cause your baby's next stool to be slightly looser than usual. This will not harm your baby, and will get better by itself within a day.
- For the blood tests, the baby will experience some discomfort. There is also a small risk of bruising. This is not harmful and will heal by itself. There is a very small chance of an infection at the needleprick site. However, this is extremely rare. We will use a clean technique to minimise this chance.

Are there any advantages for my baby in taking part?

By having a weekly visit by the KEMRI fieldworker your baby will have a regular check to see if they are unwell. If they have malaria they will receive immediate treatment and if they have other illnesses they will be referred to the local dispensary to be seen by a nurse or clinical officer. In order to make sure that children with malaria or other illnesses have good access to care on days when the fieldworker is not visiting, KEMRI have placed extra nursing and clinical officer staff at the local dispensary. This benefits people in this community whether they are in the study or not in the study.

On the days once per month that you attend Junju dispensary with your baby for the urine and breath tests, lunch will be provided and your travel expenses (any necessary transport costs for you and your baby to travel from home to Junju dispensary) will be reimbursed according to local rates. Since the monthly dispensary visit is long, and therefore prevents you from working that day, we will also offer an amount of 350 Kenya shillings at each monthly scheduled dispensary visit.

The results of this study will improve understanding of poor growth and may improve the care of other children and babies with growth problems in the future.

What happens if I refuse to allow my baby to participate?

All participation in research is voluntary. You are free to decide if you want your baby to take part. If you do agree you can change your mind at any time and withdraw your baby from the research. This will not affect your baby's healthcare now or in the future.

What happens to the samples?

- Individual names are removed from all samples and replaced by codes, to ensure that samples can only be linked to the participants by people closely concerned with the research.
- Most of the research tests that will be done on the sample will be done here in Kilifi. However for some tests that cannot be done in Kenya, part of the samples will be sent to laboratories overseas. For example, the urine samples will be sent to the UK for specialist testing.
- After the research, a small portion of the blood and stool samples will be stored at KEMRI Wellcome Trust laboratories in Kilifi. In the future, new research about child health may be done on these samples. Future research must first be approved by the KEMRI Scientific & Ethics Review Unit to ensure participants' safety and rights are respected.

Who will have access to information about my baby in this research?

All our research records are stored securely in locked cabinets and password protected computers. Only a few people within KEMRI who are closely concerned with the research will be able to access information that identifies individual participants by name.

In future, information collected during this study may be used to support new research by other researchers in Kenya and other countries on child health problems. In all cases, we will only share information with other researchers in ways that do not reveal individual participants' identities. For example, we will remove information that could identify people, such as their names and where they live, and replace this information with number codes. Any future research using information from this study must first be approved by a local or national expert committee to make sure that the interests of participants and their communities are protected.

Who has allowed this research to take place?

A Kenyan committee (the KEMRI Scientific & Ethical Review Unit) has looked carefully at this work and agrees that this study is properly planned, and important and relevant to Kenya. The committee also agrees that the study follows nationally and internationally agreed research guidelines. This includes ensuring that all participants' safety and rights are respected.

What if I have any questions?

You may ask any of our staff questions at any time. You can also contact the research team:
Dr. Rosie Crane (Principal Investigator), KEMRI Wellcome Trust Research Programme, P.O. Box 230, Kilifi.
Telephone: 0727416278

If you want to ask someone independent anything about this research please contact:
Community Liaison Manager, KEMRI Wellcome Trust Research Programme, P.O. Box 230, Kilifi.
Telephone: 0723342780 and 0417522063
And
The Secretary - KEMRI/Scientific & Ethics Review Unit, P.O. Box 54840-00200, Nairobi,
Telephone: 0202722541, 0722205901 or 0733400003

KEMRI-Wellcome Trust Research Programme Consent form for Junju Baby Growth Study

I, [being a parent/guardian of _____ (name of child)], have had the research explained to me. I have understood all that has been read/explained and had my questions answered satisfactorily.

Please tick the boxes below where relevant:

☐ **I agree to allow my child to take part in this research**

☐ **I agree to samples being stored and used for future research**

☐ **I agree to samples being exported**

☐ **I agree to take part in this research (Only the mother can tick this box. Leave blank if the mother is not present.)**

I understand that I can change my mind at any stage and it will not affect my child in any way.

Parent/guardian's signature: _____ **Date** _____

Parent/guardian's name: _____**Time** _____
(Please print name)

Where parent/guardian cannot read, ensure a witness* observes consent process and signs below:

I attest that the information concerning this research was accurately explained to and apparently understood by the parent/guardian and that informed consent was freely given by the parent/guardian.

Witness' signature: _____ **Date** _____

Witness' name: _____ **Time** _____

**A witness is a person who is independent from the research or a member of staff who was not involved in gaining the consent.*

Thumbprint of the parent/guardian as named above if they cannot write: _____

I have followed the study SOP to obtain consent from the [parent/guardian]. S/he apparently understood the nature and the purpose of the study and consents to the participation [of the child] in the study. S/he has been given opportunity to ask questions which have been answered satisfactorily.

Designee/investigator's signature: _____ **Date** _____

Designee/investigator's name: _____**Time** _____
(Please print name)

THE PARENT/GUARDIAN SHOULD NOW BE GIVEN A SIGNED COPY TO KEEP

Appendix 38: Cohort anthropometry SOP

See next page for the SOP used by fieldworkers for measuring height and weight amongst cohort participants. Illustrations are copied from the WHO 'Job Aid: Weighing and Measuring a Child' available from https://www.who.int/childgrowth/training/jobaid_weighing_measuring.pdf

KEMRI | Wellcome Trust

Afya tumboni

STANDARD OPERATING PROCEDURE

ANTHROPOMETRY

Study scientific title	Epidemiology of Environmental Enteropathy and intestinal microbiota amongst infants in Junju, Kilifi
Lay title	Afya tumboni
SERU protocol number	2983
Author	Rosie Crane
Reviewer	Johnstone Thitiri
Approver	Rosie Crane
Version	1.2
Date	24/09/2015

Materials required

Lying scales (Seca 336)
Step-on scales (Seca 876)
Stadiometer (Seca 213)
Length board
MUAC tape
Head circumference tape
Cleaning wipe
Paper tissue
Monthly anthropometry form (Appendix 1) or ***Cohort enrolment questionnaire***
Weight tracker (Appendix 2)
Pen

Weight (for participants)

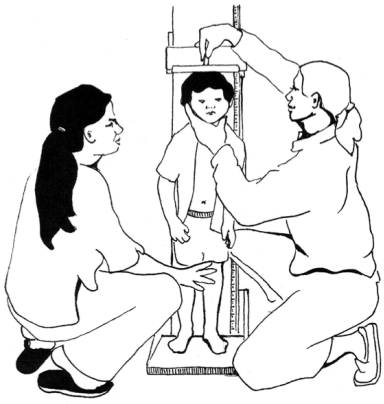
1. Prepare the scales by removing from their case and placing on a level, clean surface. To help level the scales, look at the bubble and try to get it central by twisting the scale's legs up or down. Wipe the scales clean and place a tissue. Ensure the room is draft-free by closing windows and doors. Ask the caretaker to undress the baby completely. Press 'Start' on the scale. Wait for 0.000 to appear on the screen. Ask the caretaker to sit or lie the baby on the scale. Wait until the display settles. Record the weight (it will be to 2 decimal places since the last decimal place in the kg display is always zero). Ask the caretaker to pick up the baby. Remove and discard the tissue. Wipe the scales clean again.

2. *If an older, mobile baby is very scared of the lying scales and will not stay on them,* you can use the Tare method. Weigh the caretaker on the step-on scales. With the caretaker still standing on the scales, press the '2 in 1' button. The screen will display 0.0. Pass the baby to the caretaker. Record this weight.



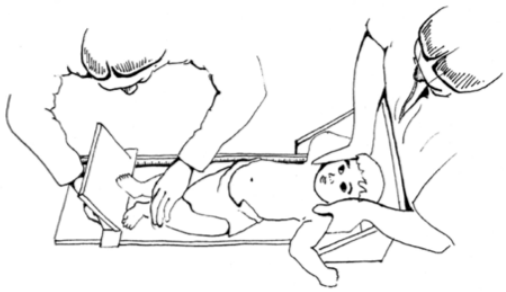
Height (for participants' mothers)

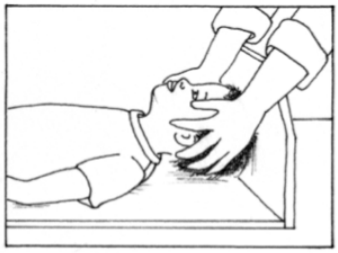
Use the stadiometer. Remove the stadiometer from its case and assemble. Ensure it is clean. Place the stadiometer on level, clean ground. Help the mother to stand with her feet central, and heels, bottom, shoulders and head against the board. Help her to hold her head level (not looking down or up). If you notice her back is arched, ask her to draw in her tummy. Slide the headpiece down level with the top of her head. Record the height indicated by the red pointers to the nearest 0.1cm.



Length (for participants)

Use the length board. Clean the board and place a tissue. Ask the caretaker to hold the baby's head central and level, up against the top of the board. There should be no space between the baby's head and the top of the board. Position the baby's shoulders and bottom centrally on the board. Using one hand, gently but firmly hold down the baby's bottom and knees, so the legs are straight. Small babies' legs may not fully straighten – do not press down too hard. Using your other hand, bring the bottom of the board up to meet the baby's feet. Bend the baby's feet to 90 degrees (as if they are standing) and bring the bottom of the board to the bottom of their feet. In small babies you may need to stroke the underside of their foot to encourage it to bend upwards. Take the measurement here, ensuring the bottom of the board is level on both sides. Record the length to the nearest 0.1cm. Clean the board when finished.





Head circumference (for participants)

Assemble the head circumference tape as shown:



Ask the caretaker to sit the baby up and support its head upright. Place the tape just above the baby's eyebrows at the front, and at the back, over the occiput (the widest part of the back of the baby's head). This should make the largest horizontal circle possible around the baby's head. Take care not to scratch the baby's skin with the edge of the tape. Make sure the tape isn't too tight or too lose. Record the measurement to the nearest 0.1cm.



MUAC (for both participants and their mothers)

1. Remove any clothing that may cover the baby/mother's left arm. Keep your work at eye level. Sit down when possible. Have babies in the caretaker's lap.

2. Use the MUAC tape for babies. Use the head circumference tape for mothers since the MUAC tape is too small.

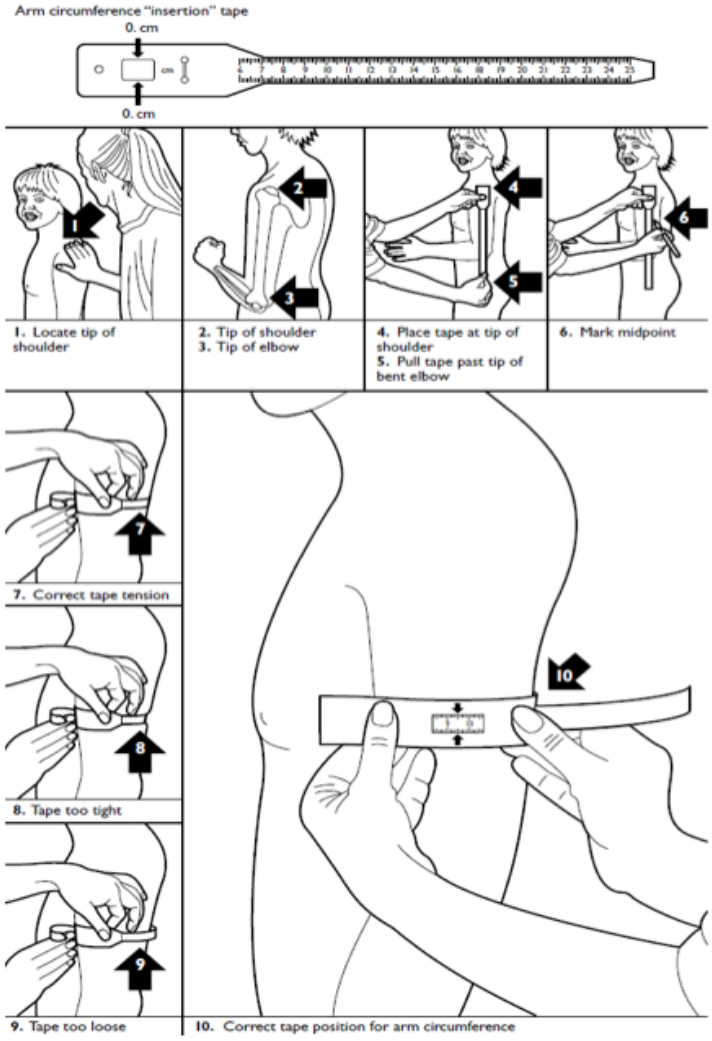
3. Calculate the midpoint of the baby/mother's **LEFT** upper arm by first locating the tip of the baby/mother's shoulder (Arrows 1 and 2) with your fingertips. Gently bend the baby/mother's elbow to make a right angle (Arrow 3). Place the end of the measuring tape on the tip of the shoulder (Arrow 4) and pull it straight down past the tip of the elbow (Arrow 5). Hold your finger on the location of the length. Bend the tape up in half so that the end of the tape meets the length you measured. Place the tape back on the tip of the shoulder and assistant will be ready with a pen to mark the midpoint on the arm (Arrow 6).

4. Straighten the baby/mother's arm and wrap the tape around the arm at midpoint. Make sure the numbers are right side up and the tape is flat around the skin (Arrow 7). Make sure the tape passes below the window with the zero arrows and is inserted through the small slot to the right of the measuring window.

5. Inspect the tension of the tape on the baby/mother's arm. Make sure the tape has the proper tension (Arrow 7) and is not too tight or too loose (Arrows 8-9).

6. When the tape is in the correct position on the arm with the correct tension, read and call out the measurement to the nearest 0.1cm (Arrow 10). Then loosen the tape and record the measurement.

7. Babies aged 6 months or above with a MUAC of less than 12.5cm and mothers with a MUAC of less than 23.0cm should be referred to the study clinician for further assessment.



Documentation and review

- Document measurements in the ***Cohort enrolment questionnaire*** for new participants and in the ***Monthly anthropometry form*** for existing participants.
- Document participants' weights also in their ***Weight tracker***. Hand this to the study clinician for review.

Appendix

See overleaf for:

1. Monthly anthropometry form
2. Weight tracker

Appendix 39: Cohort participant characteristics at enrolment

Supplementary table 10: Frequency distribution of characteristics of 99 cohort participants at enrolment.

Factor	Category	Distribution[1]
Age at enrolment (days)		8 (5-11)
Sex	Male	51 (52)
	Female	48 (48)
Number of antenatal visits	None	4 (4)
	1-4	72 (73)
	≥5	23 (23)
Mother's HIV status during pregnancy	Negative	96 (97)
	Positive	3 (3)
Complications during labour or delivery	None	97 (98)
	Fever	1 (1)
	Excessive bleeding	1 (1)
Estimated gestation at birth (weeks)	<37	4 (4)
	≥37	80 (81)
Season of birth	Dry	75 (76)
	Rainy	25 (25)
Place of birth	Home	40 (40)
	Public facility	51 (52)
	Private facility	8 (8)
Birth route	Vaginal	99 (100)
	Caesarean	0 (0)
Cried immediately at birth	No	5 (5)
	Yes	94 (95)
	Unknown	15 (15)
Birthweight	Normal (≥2500g)	47 (48)
	Low (1500-2499g)	8 (8)
	Very low (1000-1499g)	0 (0)
	Extremely low (<1000g)	0 (0)
	Missing	44 (44)
Twin	Not twin	93 (94)
	Twin	6 (6)
Received prelacteal feed[2]	No	81 (82)
	Yes	18 (18)
Age at first breastfeed (days)	0	42 (42)
	1	51 (52)
	≥2	6 (6)
Birth order		3 (2-6)
Birth interval to older sibling (months)	<24	12 (12)
	≥24	67 (68)
	Firstborn	20 (20)
Participant slept under bednet last night?	No	16 (16)
	Yes	83 (84)

1. For categorical variables: n (% of total). For continuous variables: median (interquartile range).
2. Type of prelacteal feed given: 9 sugar water, 3 sugar salt water, 3 water, 1 coconut water

Supplementary table 10 continued

Factor	Category	Distribution[1]
Primary caretaker	Mother	98 (99)
	Other relative aged ≥16 years	0 (0)
	Other relative aged <16 years	1 (1)
Occupation of primary caretaker	None	31 (31)
	Farming	49 (50)
	Fishing	5 (5)
	Petty trade (e.g. market stall)	8 (8)
	Employed, casual (e.g. labourer)	4 (4)
	Employed, professional (e.g. teacher)	2 (2)
Education of primary caretaker	None	22 (22)
	Some primary	53 (54)
	Completed primary	16 (16)
	Some secondary	4 (4)
	Completed secondary	4 (4)
Mother alive	No	0 (0)
	Yes	99 (100)
Mother's age (years)		25 (21-31)
Mother's height (cm)		154.4 (151.2-158.6)
Mother's mid upper arm circumference (cm)	<23	13 (13)
	23-29.9	83 (84)
	≥30	3 (3)
Ever breastfed	No	0 (0)
	Yes	99 (100)
Current main food	Breastmilk	99 (100)
Older sibling has died	No	78 (79)
	Yes	21 (21)
Number of under-fives in household		2 (1-2)
Number of household residents per sleeping room		2.0 (3.0-4.0)
Household per capita monthly income (Kenya shillings (approximate US dollar equivalent)[3]	<2,000 ($20)	75 (76)
	≥2,000 (≥$20)	24 (24)
Household land ownership (acres)	None	7 (7)
	1-4	85 (86)
	≥5	7 (7)
Household livestock ownership[4]	No livestock	17 (17)
	Equivalent of <1 cow	69 (70)
	Equivalent of ≥1 cow	13 (13)
Building roof material	Makuti (grass thatch)	72 (73)
	Corrugated iron	27 (27)
Building wall material	Makuti / wood / mud	88 (89)
	Stone / brick	11 (11)

1. *For categorical variables: n (% of total). For continuous variables: median (interquartile range).*
3. *Household income unknown for 13 participants. Mode of 4,000-9,999 Kenya shillings was imputed.*
4. *1 point per cow or equivalent (1 cow was considered, based on local market sale value, equivalent to 2 pigs, 20 goats or 60 chickens)*

Supplementary table 10 continued

Factor	Category	Distribution[1]
Building floor material	Earth	93 (94)
	Cement	6 (6)
Household water source[5]	Unimproved	57 (58)
	Improved	42 (42)
Household water location[5]	Outside house/compound	93 (94)
	Inside house/compound	6 (6)
Time to fetch water and return (minutes)	<30	79 (80)
	≥30	20 (20)
Household drinking water treated	No	85 (86)
	Yes	14 (14)
Household sanitation type[5]	No facility (bush)	16 (16)
	Uncovered pit latrine	1 (1)
	Covered pit latrine	81 (82)
	Ventilated improved pit latrine	2 (2)
Household soap availability[5]	No	74 (75)
	Yes	25 (25)
Animal exposure inside house	No	75 (76)
	Yes	24 (24)

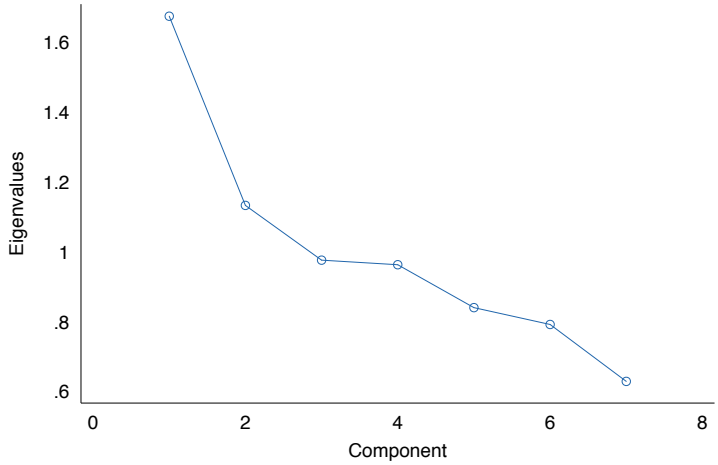
1. *For categorical variables: n (% of total). For continuous variables: median (interquartile range).*
5. *These factors were confirmed by fieldworker's visual inspection of the participant's homestead*

Appendix 40: PCA of household WASH characteristics for the cohort study: eigenvectors and scree plot

Supplementary table 11: Eigenvectors generated by principal components analysis of selected water, sanitation and hygiene exposures amongst 99 participants.

Factor	Eigenvector
Improved household water source (vs. unimproved)	0.52
Household water located inside house/compound (vs. inside)	0.46
Time to fetch water and return <30minutes (vs. ≥30)	0.46
Household drinking water treated (vs. untreated)	-0.04
Household sanitation provided by latrine (covered or uncovered) (vs. no facility)	0.24
Primary caretaker uses soap to wash hands (vs. does not use soap)	0.27
Animals not present inside house (vs. present)	0.42

Supplementary figure 7: Scree plot of eigenvalues derived from Principal Component Analysis of household water, sanitation and hygiene indicators for cohort participants.

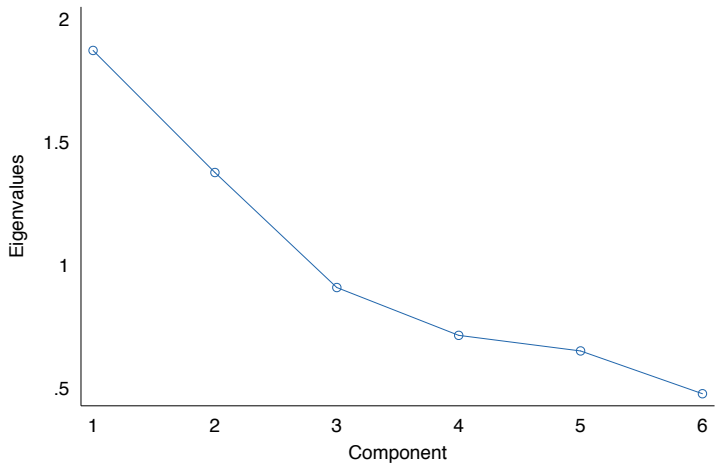


Appendix 41: PCA of household SEP characteristics for the cohort study: eigenvectors and scree plot

Supplementary table 12: Eigenvectors generated by principal components analysis of selected socio-economic position exposures amongst 99 participants.

Factor	Eigenvector
Household per capita income ≥2,000 Kenya shillings (vs. <2,000)	0.11
Household land ownership (vs. no land owned)	-0.01
Household livestock ownership (vs. no livestock owned)	0.03
Roof material corrugated iron (vs. makuti)	0.53
Wall material stone/brick (vs. makuti/mud/wood)	0.61
Floor material cement (vs. earth)	0.58

Supplementary figure 8: Scree plot of eigenvalues derived from Principal Component Analysis of household socio-economic position indicators for cohort participants.



Appendix 42: Example manual calculation of dummy first principal component for household SEP for cohort participants using eigenvectors generated by the equivalent cross-sectional survey PCA

From Supplementary table 13: Eigenvectors derived from a principal components analysis of 336 households that participated in the cross-sectional survey.

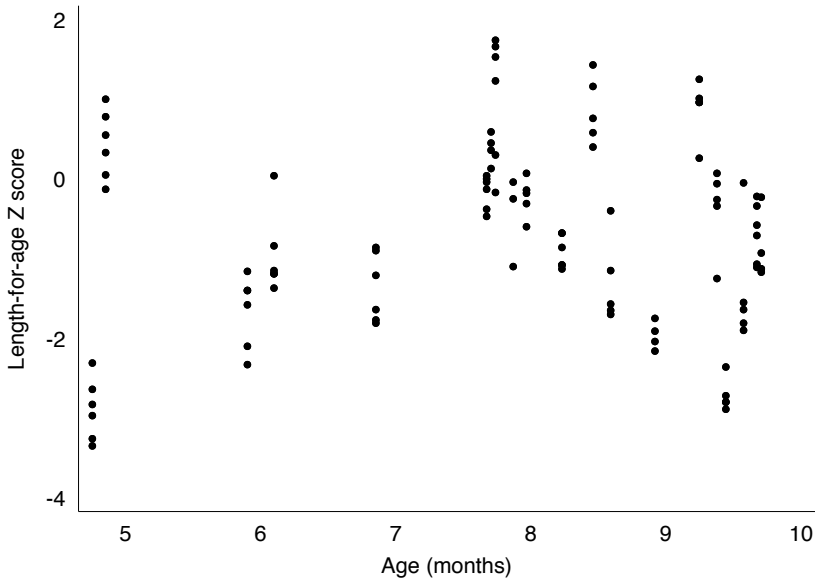
Factor	Category	Binary score	Cross-sectional survey eigenvector for first principal component
Household per capita income (Kenya shillings)	<2,000	0	0.1087
	≥2,000	1	
Household land ownership	No	0	-0.0077
	Yes	1	
Household livestock ownership	No	0	0.0306
	Yes	1	
Roof material	*Makuti* (grass thatch)	0	0.5307
	Corrugated iron	1	
Wall material	*Makuti*/mud/wood	0	0.6082
	Stone/brick	1	
Floor material	Earth	0	0.5793
	Cement	1	

Referring to the eigenvectors generated by PCA of household SEP indicators for the cross-sectional survey in Supplementary table 1 (reproduced above), the dummy principal component is the sum of the product of the binary score and the eigenvector for each factor. In the example of a cohort participant whose household has a per capita income less than 2,000 Kenya shillings, does not own land but does own livestock, whose house has *makuti* roof, stone walls and cement floor: PC1 = (0x0.1087) + (0x-0.0077) + (1x0.0306) + (0x-0.0326) + (0x0.5307) + (1x0.6082) + (1x0.5793) = 1.2181.

Appendix 43: Appraisal of inter-observer variability in measurement of length amongst cohort participants

20 cohort participants were identified for repeat length measurement on the same day by all six fieldworkers. Results for one fieldworker were excluded from intra-class coefficient analysis since that fieldworker only measured nine participants. Another fieldworker measured 17 participants, another measured 19 and the remaining three fieldworkers measured all 20 participants. A mixed-effects intra-class coefficient model was therefore built with five observers (fieldworkers), 17 targets (participants), fieldworker as fixed effect and participant as random effect. Individual intra-class coefficient was high at 0.92 (95% CI 0.85-0.97). However, despite this good statistical agreement, derived LAZs ranged approximately one Z-score within individual participants (Supplementary figure 9). The extent of inter-observer variability did not appear to vary with age (Supplementary figure 9).

Supplementary figure 9: Derived LAZ for 20 participants that underwent repeat measurement on the same day by different fieldworkers. Each participant is represented by a vertical cluster of data points at the same age, with one data point for each fieldworker measurement.



Appendix 44: Characteristics of fever episodes amongst cohort participants

207 were captured by active and passive case detection (attendance to primary care but also recalled during home visit around that time), whilst the remaining 64 were captured by active case detection (home visit) only. Of the passive case detection episodes, 44 were by caretaker recall (of which 18 were confirmed by fieldworker as ≥37.5°C on the day of home visit) and the remaining 20 involved a participant with temperature ≥37.5°C despite no caretaker perception of current fever.

46 of the febrile episodes observed during the cohort were accompanied amongst 23 participants by positive malaria RDT, and 25 of these 46 also with positive slide microscopy for either *Plasmodium falciparum* or *P. malariae* (Supplementary table 14). No slide was positive for *P. ovale*. There was no occurrence of a positive slide with negative RDT.

Supplementary table 14: Characteristics of the 226 observed fever episodes.

Malaria rapid diagnostic test result	Malaria slide result	Case detection type	n
Negative	Negative	Passive	171
		Active	9
Positive	Negative	Passive	14
		Active	0
	Result not obtained	Passive	7
		Active	0
	Plasmodium falciparum	Passive	21
		Active	3
	Plasmodium malariae	Passive	1
		Active	0
Total			**226**

Appendix 45: Acute illness and injury clinician diagnoses in primary care for the cohort study participants

Supplementary table 15: Passive case detection of acute illness via attendance for primary care; clinician diagnoses.

Clinician diagnosis	n
Upper respiratory tract infection	198
Lower respiratory tract infection	114
Malaria	41
Fever, unknown focus	39
Skin infection	38
Gastroenteritis	20
Conjunctivitis	14
Allergic reaction	6
Oral candidiasis	6
Neonatal sepsis	6
Severe acute malnutrition	4
Colic	3
Neonatal jaundice	2
Intestinal helminthiasis	2
Urinary tract infection	1
Trauma	1
Umbilical cord infection	1
Chickenpox	1
Burn	1

Appendix 46: Antibiotics prescribed to cohort study participants

Supplementary table 16: Antibiotic courses prescribed for 99 cohort participants from age zero to 10 months; excluding long-term co-trimoxazole prophylaxis for three HIV-exposed participants.

Antibiotic(s) name(s)	Route	Number of courses prescribed
Amoxicillin	Oral	110
Co-trimoxazole	Oral	54
Cloxacillin	Oral	26
Erythromycin	Oral	20
Co-amoxiclav	Oral	5
Ampicillin & gentamicin	Intravenous	4
Metronidazole	Oral	1
Ceftriaxone	Intravenous	1
Ceftriaxone & azithromycin	Intravenous & oral	1
TOTAL		222

Appendix 47: HIV-exposed cohort participants

Three cohort participants were HIV-exposed (mother HIV infected). These participants and their mothers were managed by the Junju Dispensary nurse according to national Prevention of Mother-To-Child Transmission (PMTCT) guidelines and 'Option B+' from the 2013 WHO PMTCT guidelines.[***] Participants' mothers had received three-drug antiretroviral therapy during pregnancy and would continue to do so for the study duration and beyond. For participants, prophylactic cotrimoxazole was started at around six weeks of age (six weeks, five weeks six days, and six weeks respectively) and continued throughout follow-up (national guidelines are to continue until two weeks after cessation of breastfeeding). Nevirapine was started at birth for all three participants and blood taken for HIV PCR at six weeks of age. PCR results were negative for all three participants. Nevirapine was stopped upon receipt of negative PCR result. This occurred at 10, 12 and 12 weeks of age respectively.

[***] Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection. World Health Organisation. 2013.

Appendix 48: Immunisation schedule followed by cohort participants

All 99 cohort participants received Bacillus Calmette-Guerin (BCG) at birth; OPV at six, 10 and 14 weeks; pentavalent diphtheria / pertussis / tetanus / Hepatitis B / *Haemophilus influenzae* type B at six, 10 and 14 weeks; pneumococcal vaccine at six, 10 and 14 weeks; rotavirus vaccine at six and 10 weeks and measles vaccine at nine months.

Appendix 49: Exponentiation of coefficients derived from regression modelling of log LM ratio and difference in LAZ over time and risk factors in the cohort study

Univariable regression of log LM ratio and age

% increase in LM ratio with each 1-month increase in age = (e^coefficient – 1) * 100

= (e^0.0585685 – 1) * 100

= 6.0%

Multivariable regression of difference in LAZ over a two-month period and multiple putative stunting risk factors

% change in difference in LAZ with each 1-unit increase in LM ratio, adjusted for other factors

= (e^coefficient – 1) * 100

= (e^-0.1374785 – 1) * 100

= -12.8%

Appendix 50: Association of markers of EED and SIBO with LM ratio in the cohort study: sensitivity model adjusting for age

Supplementary table 17: Association of markers of EED and SIBO with LM ratio.
Five separate mixed linear regression models each with outcome log LM ratio and exposure one of: faecal alpha-1-antitrypsin, faecal neopterin faecal myeloperoxidase, peak breath hydrogen, breath hydrogen area under the curve. All factors are log-transformed to achieve normal distribution. To adjust for possible clustering of data and associations arising from multiple observations per participant, participant is included as a random effect in all three models. Each model is adjusted for age in continuous months.

Factor	n	Participants	Median (IQR)	Coefficient	95% CI	p
Log faecal alpha-1-antitrypsin (mg/g)	418	97	-0.6 (-1.1 to -0.1)	0.02	-0.05 to 0.09	0.64
Log faecal myeloperoxidase (ng/g)	420	97	9.1 (9.7 to 10.1)	0.05	-0.02 to 0.13	0.17
Log faecal neopterin (nmol/L)	420	97	7.9 (7.4 to 8.4)	0.03	-0.06 to 0.11	0.55
Log peak breath hydrogen (ppm)	837	99	1.4 (0.7 to 2.1)	-0.01	-0.07 to 0.03	0.62
Log breath hydrogen area under the curve (ppm minutes)	837	99	5.4 (4.9 to 6.2)	-0.01	-0.07 to 0.04	0.65

Appendix 51: Association of biomarkers of EED with linear growth in the cohort study: sensitivity model adjusting for age and sex

Supplementary table 18: Association of biomarkers of EED with linear growth.
Factors associated with linear growth amongst 92 cohort participants who provided 330 stool samples 10 or fewer days prior to a successful LM test, and where LAZ data exist for the month prior and month after this LM test. Mixed-effects linear regression with inclusion of participant as a random effect and age and sex as fixed effects. Outcome is difference in LAZ between one month prior to and one month after the stool-linked LM test. Sensitivity models were not run for white blood cell count, haemoglobin and platelet count because there was no significant variation in age for these exposures (range 5.6 to 6.5 months).

Factor	n	Participants	Median (IQR)	Coefficient	95% CI	p
Log % lactulose excretion	672	97	-2.1 (-2.9 to -1.5)	0.00	-0.06 to 0.05	0.87
Log % mannitol excretion	672	97	1.2 (-1.5 to 1.7)	0.05	0.00 to 0.11	0.07
Log faecal alpha-1-antitrypsin (mg/g)	343	93	-0.5 (-1.1 to 0.0)	-0.02	-0.12 to 0.08	0.66
Log faecal myeloperoxidase (ng/g)	345	93	9.7 (9.0 to 10.1)	-0.08	-0.20 to 0.03	0.16
Log faecal neopterin (nmol/L)	346	93	7.9 (7.4 to 8.4)	0.02	-0.09 to 0.15	0.69
Log peak breath hydrogen (ppm)	731	97	1.1 (0.7 to 2.1)	-0.04	-0.10 to 0.03	0.26
Log breath hydrogen area under the curve (ppm minutes)	731	97	5.3 (4.9 to 6.2)	-0.03	-0.09 to 0.03	0.34

Appendix 52: Association of putative exposures with EED in the cohort study: sensitivity models with binary age classification

Supplementary table 19: Association of putative exposures with EED (log faecal myeloperoxidase).
Factors associated with EED (log faecal myeloperoxidase) amongst 97 cohort participants. Multivariable mixed-effects linear regression with inclusion of participant (n=99) as a random effect. n=434. There is no evidence for modification by season of the effect of exclusive breastfeeding status on log faecal myeloperoxidase (likelihood ratio test for interaction: p=0.12).

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Age (months)		<4	165 (38)	-	-	0.15
		≥4	269 (62)	0.18	-0.07 to 0.43	
Sex		Male	237 (55)	-	-	0.55
		Female	197 (45)	0.06	-0.14 to 0.27	
Log peak breath hydrogen during next day's breath hydrogen test (ppm minutes)			1.4 (0.7 to 2.1)	0.02	-0.08 to 0.11	0.73
Started complementary foods?		No	150 (35)	-	-	<0.001
		Yes	284 (65)	0.57	0.31 to 0.84	
Season		Dry	219 (50)	-	-	0.25
		Rainy	215 (50)	0.09	-0.06 to 0.25	
WASH principal component analysis first component score			0.1 (-1.1 to 0.9)	0.05	-0.03 to 0.13	0.23
During 30 days preceding stool sample	Antibiotics	No	323 (74)	-	-	0.93
		Yes	111 (26)	0.01	-0.20 to 0.22	
	Diarrhoea	No	415 (96)	-	-	0.87
		Yes	19 (4)	-0.03	-0.42 to 0.36	
	Breathing difficulty	No	258 (60)	-	-	0.29
		Yes	176 (40)	0.10	-0.09 to 0.29	
	Fever (slide negative)	No	335 (71)	-	-	0.37
		Yes	99 (23)	-0.10	-0.33 to 0.12	
	Fever (slide positive)	No	422 (97)	-	-	0.11
		Yes	12 (3)	0.42	-0.09 to 0.93	
	Iron supplementation	No	419 (97)	-	-	0.73
		Yes	15 (3)	0.08	-0.35 to 0.50	

1. For categorical variables: n (% of total). For continuous variables: median (range).

Supplementary table 20: Association of putative exposures with EED (log faecal neopterin).
Factors associated with EED (log faecal neopterin) amongst 97 cohort participants. Multivariable mixed-effects linear regression with inclusion of participant as a random effect. n=434. There is no evidence for modification by season of the effect of exclusive breastfeeding status on log faecal neopterin (likelihood ratio test for interaction: p=0.21).

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Age (months)		<4	165 (38)	-	-	0.72
		≥4	269 (62)	-0.04	-0.26 to 0.18	
Sex		Male	237 (55)	-	-	0.23
		Female	197 (45)	0.10	-0.06 to 0.26	
Log peak breath hydrogen during next day's breath hydrogen test (ppm minutes)			1.4 (0.7 to 2.1)	-0.06	-0.15 to 0.02	0.12
Started complementary foods?		No	151 (35)	-	-	0.06
		Yes	283 (65)	0.23	-0.01 to 0.47	
Season		Dry	218 (50)	-	-	0.80
		Rainy	216 (50)	-0.02	-0.16 to 0.12	
WASH principal component analysis first component score			0.1 (-1.1 to 0.9)	0.00	-0.06 to 0.06	0.93
During 30 days preceding stool sample	Antibiotics	No	323 (75)	-	-	0.97
		Yes	111 (25)	0.00	-0.19 to 0.19	
	Diarrhoea	No	415 (96)	-	-	0.64
		Yes	19 (4)	0.08	-0.26 to 0.43	
	Breathing difficulty	No	257 (60)	-	-	0.69
		Yes	177 (40)	0.03	-0.13 to 0.20	
	Fever (slide negative)	No	337 (78)	-	-	0.59
		Yes	97 (22)	-0.06	-0.26 to 0.15	
	Fever (slide positive)	No	422 (97)	-	-	0.06
		Yes	12 (3)	0.44	-0.01 to 0.90	
	Iron supplementation	No	419 (97)	-	-	0.60
		Yes	15 (3)	0.10	-0.06 to 0.06	

1. For categorical variables: n (% of total). For continuous variables: median (range).

Appendix 53: Association of putative exposures with EED in the cohort study: model residuals and their association with age

Supplementary figure 10: Kernel density plots of residuals predicted from models described in Tables 28-31.

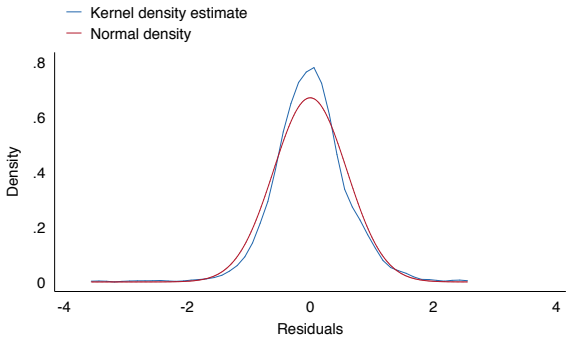


Table 28 (outcome: log LM ratio)

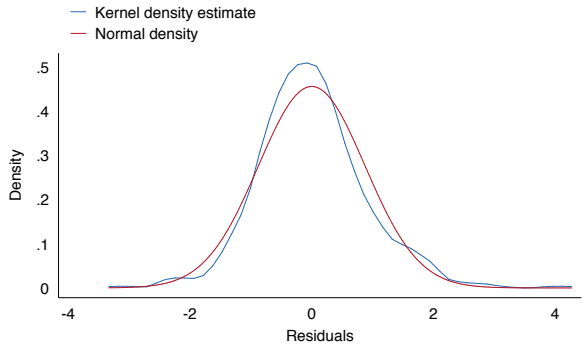


Table 29 (outcome: log faecal alpha-1-antitrypsin)

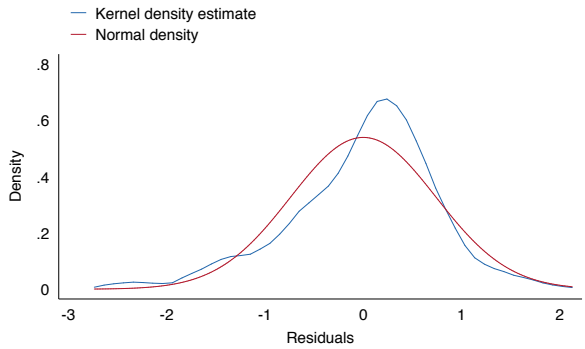


Table 30 (outcome: log faecal myeloperoxidase)

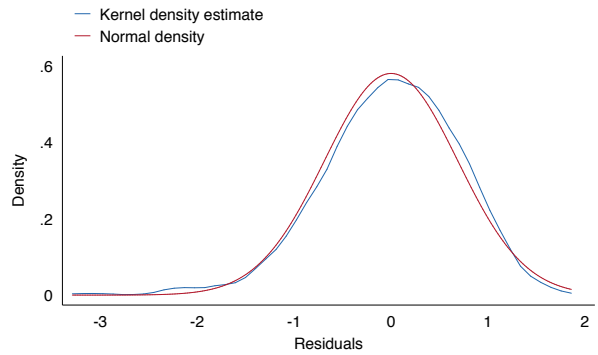


Table 31 (outcome: log faecal neopterin)

Supplementary figure 11: Distribution of predicted residuals by age for models described in Tables 28-31.

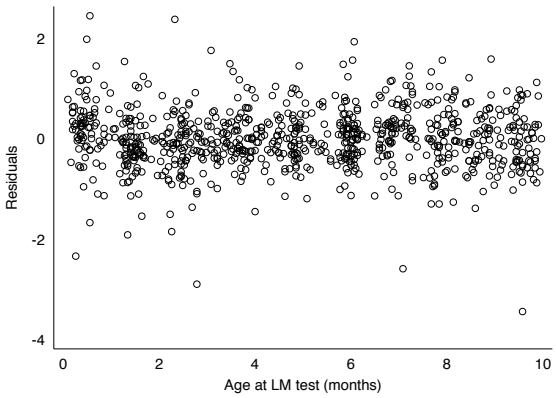


Table 28 (outcome: log LM ratio). Linear regression n=837 coefficient=0.00 p=1.00

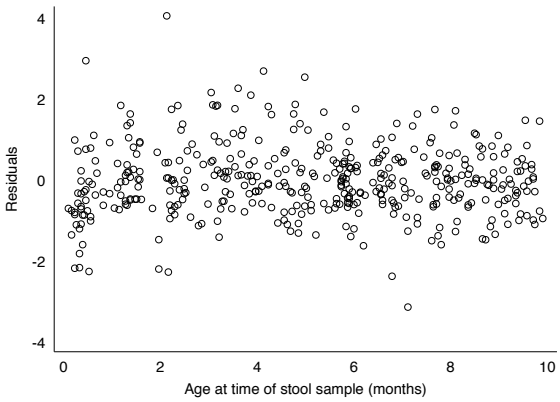


Table 29 (outcome: log faecal alpha-1-antitrypsin). Linear regression n=431 coefficient=0.00 p=1.00

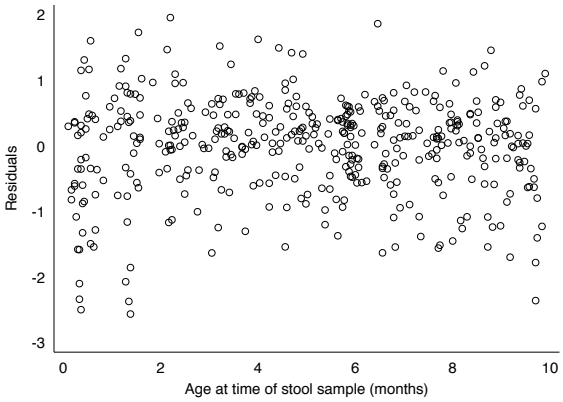


Table 30 (outcome: log faecal myeloperoxidase). Linear regression: n=434 coefficient=0.00 p=1.00

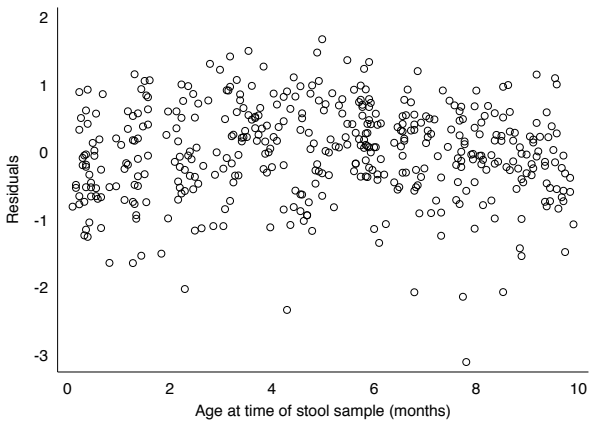


Table 31 (outcome: log faecal neopterin). Linear regression: n=434 coefficient=0.00 p=1.00

Appendix 54: Association of putative exposures with EED in the cohort study: sensitivity models with reversible definition for exclusive breastfeeding status

Supplementary table 21: Association of putative exposures with EED (log LM ratio).
Factors associated with EED (log LM ratio) amongst 99 cohort participants. Multivariable mixed-effects linear regression with inclusion of participant as a random effect. n=837. There is no evidence for modification by season of the association between reversible exclusive breastfeeding status and log LM ratio (likelihood ratio test for interaction: p=0.30).

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Age (months)			4.8 (0.1-10.0)	0.05	0.03 to 0.07	<0.001
Sex		Male	447 (53)	-	-	0.16
		Female	390 (47)	0.11	-0.05 to 0.27	
Log peak breath hydrogen during next day's breath hydrogen test (ppm minutes)			1.4 (0.0-4.1)	-0.01	-0.06 to 0.05	0.80
Exclusive breastfeeding during past week?		No	335 (40)	-	-	0.27
		Yes	502 (60)	0.08	-0.06 to 0.22	
Season		Dry	401 (48)	-	-	0.06
		Rainy	436 (52)	0.08	0.00 to 0.17	
WASH principal component analysis first component score			-0.1 (-2.9 to 3.4)	0.01	-0.05 to 0.08	0.63
During 30 days preceding LM test	Antibiotics	No	626 (75)	-	-	0.09
		Yes	211 (25)	-0.11	-0.23 to 0.02	
	Diarrhoea	No	802 (96)	-	-	0.36
		Yes	35 (4)	0.10	-0.12 to 0.33	
	Breathing difficulty	No	505 (60)	-	-	0.38
		Yes	332 (40)	-0.05	-0.16 to 0.06	
	Fever (slide negative)	No	649 (78)	-	-	0.81
		Yes	188 (22)	-0.02	-0.11 to 0.14	
	Fever (slide positive)	No	812 (97)	-	-	0.78
		Yes	25 (3)	-0.04	-0.32 to 0.24	
	Iron supplementation	No	792 (95)	-	-	0.73
		Yes	45 (5)	-0.04	-0.24 to 0.17	

1. For categorical variables: n (% of total). For continuous variables: median (range).

Supplementary table 22: Association of putative exposures with EED (log faecal alpha-1-antitrypsin). Factors associated with EED (log faecal alpha-1-antitrypsin) amongst 97 cohort participants. Multivariable mixed-effects linear regression with inclusion of participant as a random effect. n=431. There is no evidence for modification by season of the association between reversible exclusive breastfeeding status and log faecal alpha-1-antitrypsin (likelihood ratio test for interaction: p=0.62).

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Age (months)			5.2 (2.5 to 7.5)	-0.04	-0.09 to 0.00	0.07
Sex		Male	235 (55)	-	-	0.76
		Female	196 (45)	-0.03	-0.22 to 0.16	
Log peak breath hydrogen during next day's breath hydrogen test (ppm minutes)			1.4 (0.7 to 2.1)	0.06	-0.04 to 0.16	0.21
Exclusive breastfeeding during past week?		Yes	149 (35)	-	-	0.76
		No	282 (65)	-0.04	-0.31 to 0.23	
Season		Dry	218 (50)	-	-	0.47
		Rainy	213 (50)	0.07	-0.11 to 0.24	
WASH principal component analysis first component score			-0.1 (-1.1 to 0.9)	-0.06	-0.13 to 0.01	0.11
During 30 days preceding stool sample	Antibiotics	No	320 (75)	-	-	0.78
		Yes	111 (25)	-0.03	-0.27 to 0.20	
	Diarrhoea	No	413 (96)	-	-	0.86
		Yes	18 (4)	0.04	-0.40 to 0.48	
	Breathing difficulty	No	256 (59)	-	-	0.10
		Yes	175 (41)	0.17	-0.03 to 0.38	
	Fever (slide negative)	No	332 (77)	-	-	0.07
		Yes	99 (23)	-0.23	-0.48 to 0.01	
	Fever (slide positive)	No	419 (97)	-	-	0.83
		Yes	12 (3)	-0.06	-0.62 to 0.50	
	Iron supplementation	No	416 (97)	-	-	0.78
		Yes	15 (3)	0.07	-0.40 to 0.54	

1. For categorical variables: n (% of total). For continuous variables: median (range).

Supplementary table 23: Association of putative exposures with EED (log faecal myeloperoxidase).
Factors associated with EED (log faecal myeloperoxidase) amongst 97 cohort participants. Multivariable mixed-effects linear regression with inclusion of participant as a random effect. n=434. There is no evidence for modification by season of the association between reversible exclusive breastfeeding status and log faecal myeloperoxidase (likelihood ratio test for interaction: p=0.16).

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Age (months)			5.2 (2.5 to 7.5)	0.07	0.02 to 0.11	0.002
Sex		Male	237 (55)	-	-	0.55
		Female	197 (45)	0.06	-0.14 to 0.27	
Log peak breath hydrogen during next day's breath hydrogen test (ppm minutes)			1.4 (0.7 to 2.1)	-0.01	-0.10 to 0.08	0.87
Exclusive breastfeeding during past week?		Yes	150 (35)	-	-	0.007
		No	284 (65)	0.34	0.09 to 0.59	
Season		Dry	219 (50)	-	-	0.27
		Rainy	215 (50)	0.09	-0.07 to 0.25	
WASH principal component analysis first component score			0.1 (-1.1 to 0.9)	0.04	-0.03 to 0.12	0.27
During 30 days preceding stool sample	Antibiotics	No	323 (74)	-	-	0.99
		Yes	111 (26)	0.00	-0.21 to 0.22	
	Diarrhoea	No	415 (96)	-	-	0.65
		Yes	19 (4)	-0.09	-0.48 to 0.30	
	Breathing difficulty	No	258 (60)	-	-	0.26
		Yes	176 (40)	-0.11	-0.08 to 0.30	
	Fever (slide negative)	No	335 (71)	-	-	0.29
		Yes	99 (23)	-0.12	-0.35 to 0.10	
	Fever (slide positive)	No	422 (97)	-	-	0.12
		Yes	12 (3)	0.41	-0.10 to 0.93	
	Iron supplementation	No	419 (97)	-	-	0.64
		Yes	15 (3)	0.10	-0.32 to 0.53	

1. For categorical variables: n (% of total). For continuous variables: median (range).

Supplementary table 24: Association of putative exposures with EED (log faecal neopterin).
Factors associated with EED (log faecal neopterin) amongst 97 cohort participants. Multivariable mixed-effects linear regression with inclusion of participant as a random effect. n=434. There is no evidence for modification by season of the association between reversible exclusive breastfeeding status and log faecal neopterin (likelihood ratio test for interaction: p=0.11).

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Age (months)			5.2 (2.6 to 7.5)	-0.01	-0.04 to 0.03	0.71
Sex		Male	237 (55)	-	-	0.21
		Female	197 (45)	0.10	-0.06 to 0.26	
Log peak breath hydrogen during next day's breath hydrogen test (ppm minutes)			1.4 (0.7 to 2.1)	-0.07	-0.15 to 0.02	0.12
Exclusive breastfeeding during past week?		No	151 (35)	-	-	0.22
		Yes	283 (65)	0.14	-0.08 to 0.36	
Season		Dry	218 (50)	-	-	0.98
		Rainy	216 (50)	0.00	-0.19 to 0.19	
WASH principal component analysis first component score			0.1 (-1.1 to 0.9)	0.00	-0.06 to 0.06	0.96
During 30 days preceding stool sample	Antibiotics	No	323 (75)	-	-	1.00
		Yes	111 (25)	0.00	-0.19 to 0.19	
	Diarrhoea	No	415 (96)	-	-	0.65
		Yes	19 (4)	0.08	-0.27 to 0.43	
	Breathing difficulty	No	257 (60)	-	-	0.56
		Yes	177 (40)	0.05	-0.12 to 0.22	
	Fever (slide negative)	No	337 (78)	-	-	0.58
		Yes	97 (22)	-0.06	-0.26 to 0.15	
	Fever (slide positive)	No	422 (97)	-	-	0.05
		Yes	12 (3)	0.46	0.00 to 0.92	
	Iron supplementation	No	419 (97)	-	-	0.55
		Yes	15 (3)	0.12	-0.27 to 0.50	

1. For categorical variables: n (% of total). For continuous variables: median (range).

Appendix 55: Association of putative exposures with linear growth in the cohort study: sensitivity model defining EED using percentage lactulose excretion instead of LM ratio

Supplementary table 25: Association of putative exposures with linear growth.
Factors associated with linear growth amongst infants in Junju, Kilifi. Multivariable mixed-effects linear regression with inclusion of participant (groups=97) as a random effect. n=671. Outcome is difference in LAZ between the month prior to and the month after the index LM test. Diarrhoea, fever and breathing difficulty occurred during the 30 days preceding the index LM test.

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Previous (baseline) LAZ			-0.8 (-7.0 to 3.5)	-0.87	-0.95 to -0.80	<0.001
Age (months)			4.9 (0.8-9.0)	-0.10	-0.13 to -0.06	<0.001
Sex		Female	312 (46)	-	-	0.004
		Male	359 (54)	0.47	0.15 to 0.79	
Log percentage lactulose excretion			-3.3 (-6.9 to -0.6)	0.00	-0.05 to 0.05	0.93
Log peak breath hydrogen during next day's breath hydrogen test (ppm)			1.1 (0.0-4.0)	-0.04	-0.11 to 0.03	0.30
Exclusive breastfeeding		Yes	202 (30)	-	-	0.98
		No	469 (70)	0.00	-0.17 to 0.17	
During last 30 days	Diarrhoea	No	638 (95)	-	-	0.21
		Yes	33 (5)	0.16	-0.09 to 0.42	
	Breathing difficulty	No	386 (58)	-	-	0.04
		Yes	285 (42)	0.13	0.01 to 0.25	
	Fever (slide negative)	No	512 (76)	-	-	0.05
		Yes	159 (24)	-0.15	-0.30 to 0.00	
	Fever (slide positive)	No	648 (97)	-	-	0.44
		Yes	23 (3)	-0.13	-0.44 to 0.19	
Weight at enrolment			3.1 (2.1 to 4.2)	1.22	0.84 to 1.59	<0.001

1. For categorical variables: n (% of total). For continuous variables: median (range).

Appendix 56: Association of markers of EED and SIBO with percentage lactulose excretion

Supplementary table 26: Association of markers of EED and SIBO with log percentage lactulose excretion. Five separate mixed linear regression models each with outcome log percentage lactulose excretion and exposure one of: faecal alpha-1-antitrypsin, faecal neopterin faecal myeloperoxidase, peak breath hydrogen, breath hydrogen area under the curve. All factors are log-transformed to achieve normal distribution. To adjust for possible clustering of data and associations arising from multiple observations per participant, participant is included as a random effect in all three models.

Factor	n	Participants	Median (IQR)	Coefficient	95% CI	p
Log faecal alpha-1-antitrypsin (mg/g)	419	97	-0.6 (-1.1 to -0.1)	-0.04	-0.15 to 0.08	0.56
Log faecal myeloperoxidase (ng/g)	420	97	9.1 (9.7 to 10.1)	0.07	-0.05 to 0.19	0.25
Log faecal neopterin (nmol/L)	420	97	7.9 (7.4 to 8.4)	0.14	0.00 to 0.29	0.06
Log peak breath hydrogen (ppm)	837	99	1.4 (0.7 to 2.1)	-0.10	-0.19 to 0.00	0.03
Log breath hydrogen area under the curve (ppm minutes)	837	99	5.4 (4.9 to 6.2)	-0.10	-0.18 to 0.00	0.04

Supplementary table 27: Association of markers of EED and SIBO with log percentage lactulose excretion. Five separate mixed linear regression models each with outcome log percentage lactulose excretion and exposure one of: faecal alpha-1-antitrypsin, faecal neopterin faecal myeloperoxidase, peak breath hydrogen, breath hydrogen area under the curve. All factors are log-transformed to achieve normal distribution. To adjust for possible clustering of data and associations arising from multiple observations per participant, participant is included as a random effect in all three models. Age (continuous months) and sex are included as fixed effects.

Factor	n	Participants	Median (IQR)	Coefficient	95% CI	p
Log faecal alpha-1-antitrypsin (mg/g)	419	97	-0.6 (-1.1 to -0.1)	-0.03	-0.15 to 0.09	0.58
Log faecal myeloperoxidase (ng/g)	420	97	9.1 (9.7 to 10.1)	0.08	-0.05 to 0.21	0.24
Log faecal neopterin (nmol/L)	420	97	7.9 (7.4 to 8.4)	0.14	0.00 to 0.29	0.05
Log peak breath hydrogen (ppm)	837	99	1.4 (0.7 to 2.1)	-0.10	-0.19 to 0.00	0.05
Log breath hydrogen area under the curve (ppm minutes)	837	99	5.4 (4.9 to 6.2)	-0.09	-0.18 to 0.00	0.07

Appendix 57: Association of putative exposures with EED in the cohort study: sensitivity model defining EED using percentage lactulose excretion instead of LM ratio

Supplementary table 28: Association of putative exposures with EED.
Factors associated with EED (log percentage lactulose excretion) amongst 99 cohort participants. Multivariable mixed-effects linear regression with inclusion of participant (n=99) as a random effect. n=837.

Factor		Category	Distribution[1]	Coefficient	95% CI	p
Age (months)			4.8 (0.1-10.0)	-0.05	-0.09 to 0.00	0.02
Sex		Male	447 (53)	-	-	0.09
		Female	390 (47)	-0.19	-0.42 to 0.03	
Log peak breath hydrogen during next day's breath hydrogen test (ppm minutes)			1.4 (0.0-4.1)	-0.07	-0.16 to 0.02	0.13
Season		Dry	401 (48)	-	-	<0.001
		Rainy	436 (52)	0.31	0.16 to 0.47	
Exclusive breastfeeding status		Yes	284 (34)	-	-	0.09
		No	553 (66)	0.22	-0.04 to 0.48	
WASH principal component analysis first component score			-0.1 (-2.9 to 3.4)	0.03	-0.06 to 0.12	0.55
During preceding 30 days	Antibiotics	No	626 (75)	-	-	0.39
		Yes	211 (25)	-0.09	-0.31 to 0.12	
	Diarrhoea	No	802 (96)	-	-	0.37
		Yes	35 (4)	0.18	-0.22 to 0.58	
	Breathing difficulty	No	505 (60)	-	-	0.31
		Yes	332 (40)	0.10	-0.09 to 0.29	
	Fever (slide negative)	No	649 (78)	-	-	0.28
		Yes	188 (22)	0.12	-0.10 to 0.35	
	Fever (slide positive)	No	812 (97)	-	-	0.57
		Yes	25 (3)	0.15	-0.35 to 0.64	
	Iron supplementation	No	792 (95)	-	-	0.65
		Yes	45 (5)	-0.08	-0.44 to 0.27	

1. For categorical variables: n (% of total). For continuous variables: median (range).

Appendix 58: Characterisation of gut microbiota using 16S sequencing: methods and interim results

Methods

Total nucleic acid (TNA) extraction[†††]

Of the 443 stool samples that preceded a successful LM test by 10 of fewer days, volume was adequate for 410 to undergo total nucleic acid extraction. From each thawed stool sample, 200mg was aseptically weighed into a 2ml screw-cap microcentrifuge tube pre-loaded with 370mg acid-washed 212-300 µM glass beads (Sigma G1277-500G). TNA extraction was then carried out using Qiamp Fast DNA Stool Mini Kit (Qiagen 51604). The manufacturer's protocol was modified in two ways to enhance cell lysis: firstly, by mechanical disruption through bead beating and secondly through an additional incubation of the lysate (95°C, five minutes) after addition of 1ml Inhibitex buffer. The samples were centrifuged at 15,000g for 90 seconds to pellet the stool particles and then the supernatant taken through further steps of inhibitor removal, purification and elution of TNA using spin columns as per the kit protocol. DNA yield was measured using Qubit 2.0 Fluorimeter, first by making 1/10 dilutions from each TNA extract (1µl TNA : 9µl nuclease-free water) and then 2µl of each dilution added to a corresponding tube containing 198µl buffer / dye mix (1µl dye : 199µl buffer) as per the Qubit dsDNA HS Assay Kit protocol.

[†††] Text prepared by Wilson Gumbi (KEMRI Wellcome Trust Research Programme)

16S sequencing[‡‡‡]

The TNA extracts were used as template for 16S PCR targeting the V3-V4 region of the 16S rRNA gene. An amplicon size of around 550 base pairs was amplified using the 341F and 785R primer set with Illumina adapter overhangs. The amplification was carried out in 25µl reactions comprising of 12.5µl Q5® Hot Start High-Fidelity 2x Master Mix (NEB), 5µl each of the 1µM forward and reverse 16S Amplicon PCR primers and 2.5µl of the template. The reaction was carried out using the ABI Veriti thermocyclers (Applied Biosystems) under the following conditions: 95°C for three minutes, 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, then a final extension at 72°C for five minutes and a final holding stage at 4°C. The amplified products were then verified on 1% agarose gel before purifying using Agencourt AMPure XP beads (Beckman Coulter) for library preparation.

Library preparation was done according to the Illumina 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System. The libraries were prepared by ligating Illumina Nextera XT dual indices and sequencing adapters (Illumina) as per manufacturer's instructions. Libraries were again purified using the AMPure XP beads (Beckman Coulter) and quantified using Qubit 3.0 fluorometer (Life Technologies). Size verification was determined by the DNA 1000 Assay kit using the 2100 Bioanalyzer (Agilent) for a size approximately 630 base pairs. The indexed libraries were normalised and pooled at equimolar concentrations and spiked with 8% PhiX DNA. The spiked library was sequenced on an Illumina MiSeq Platform with an

[‡‡‡] Text prepared by Joyce Ngoi and Zaydah de Laurent (KEMRI Wellcome Trust Research Programme)

output of 2× 300 base pair paired reads using the MiSeq® Reagent Kit v3 (600 cycle). 400 TNA aliquots passed quality control and proceeded to bioinformatics analysis.

16S bioinformatics[§§§]

Following adapter trimming using cutadapt version 1.18,[****] the DADA2 pipeline[††††] in QIIME 2[‡‡‡‡] was used to denoise raw sequences, merge paired reads, then remove chimeras. Forward reads were truncated to 270 base pairs and reverse reads to 200 base pairs to trim low-quality bases towards the end of each read. Sequences from each run were processed separately and the resulting feature tables then merged. Subsequent steps were performed in the programming language R. Taxonomic assignment was carried out using the RDP naïve Bayesian classifier trained on the Silva rRNA database (version 132). Sequences were included in the analysis of they were 390–440 base pairs in length, bacterial, and present at >0.1% relative abundance in at least one sample. Samples with <30,000 quality-filtered sequences were excluded from certain final analyses.

[§§§] Text prepared by Edward Parker (London School of Hygiene & Tropical Medicine)
[****] Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 2011;17(1):10-2
[††††] Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-3
[‡‡‡‡] Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37(8):852-7

Results

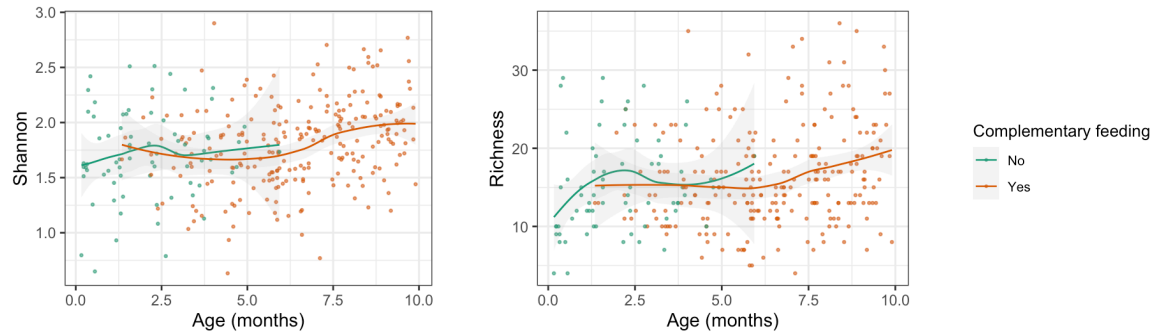
Shannon index and richness both increased with age (Supplementary figure 12). Adjusted for age, there was weak evidence to support higher gut microbial alpha diversity during exclusive breastfeeding (Supplementary table 29, Supplementary figure 12).

Supplementary table 29: Association between two measures of gut microbial alpha diversity (Shannon index and richness (number of sequence variants detectable at ≥0.1% relative abundance)) and exclusive breastfeeding status. Four separate mixed effects linear regression models each with participant as random effect. Each model is adjusted for age at time of stool sample. Analysis is restricted to the 311 stool samples (from 93 participants) with minimum read depth 30,000 sequences.

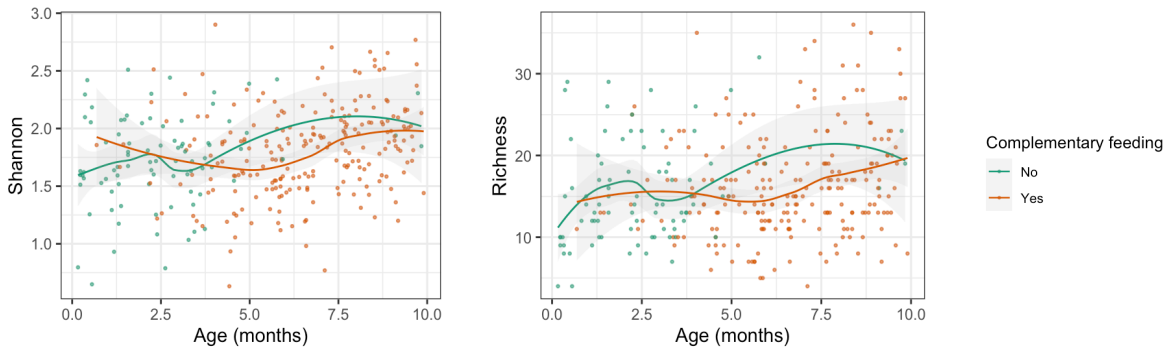
Outcome	Factor	Category	Stool samples	Coefficient	95% confidence interval	p
Shannon index	Exclusive breastfeeding since birth	Yes	71	-	-	0.08
		No	240	-0.12	-0.26 to 0.01	
	Exclusive breastfeeding since last weekly home visit	Yes	85	-	-	0.08
		No	226	-0.11	-0.24 to 0.01	
Richness	Exclusive breastfeeding since birth	Yes	71	-	-	0.19
		No	240	-1.48	-3.74 to 0.78	
	Exclusive breastfeeding since last weekly home visit	Yes	85	-	-	0.09
		No	226	-1.79	-3.87 to 0.29	

Supplementary figure 12: Gut microbial alpha diversity by age for all 400 sequenced faecal samples (96 participants). Shannon index and richness (number of sequence variants detectable at ≥0.1% relative abundance) by age, split by exclusive breastfeeding status since birth (A) and since last weekly home visit (B) and by season (C). Lines and shaded grey areas indicate locally smoothed means and their 95% confidence intervals derived by local regression.

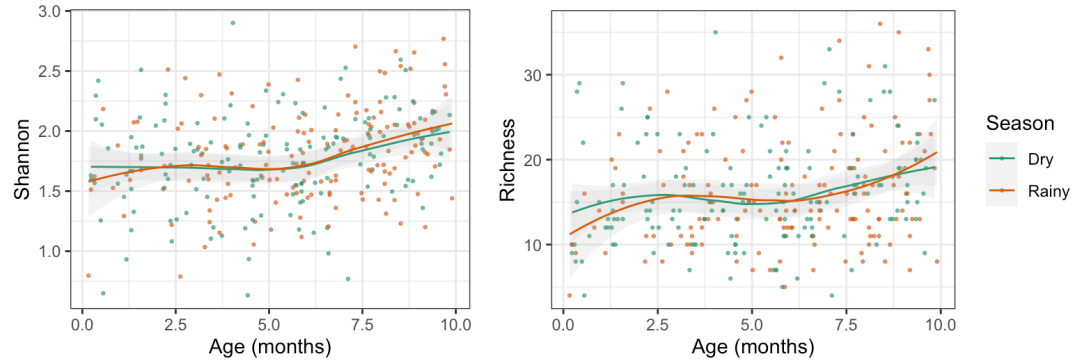
A



B

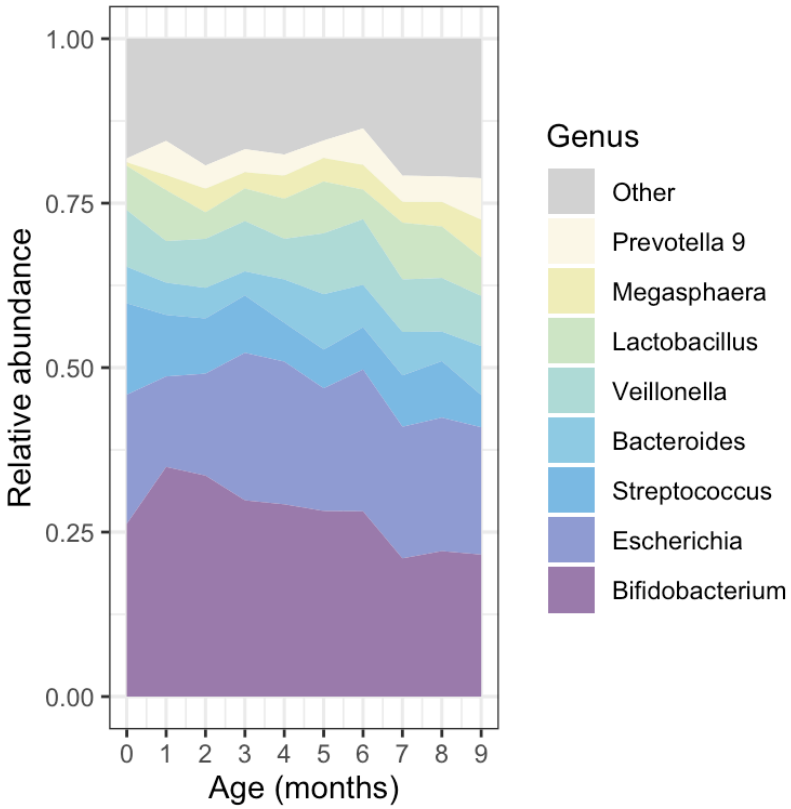


C



Bifidobacterium genus relative abundance peaked at one month then declined, with other rarer genera accounting for a greater proportion beyond six months (Supplementary figure 14).

Supplementary figure 14: Genus relative abundance by age for all 400 sequenced faecal samples (96 participants).

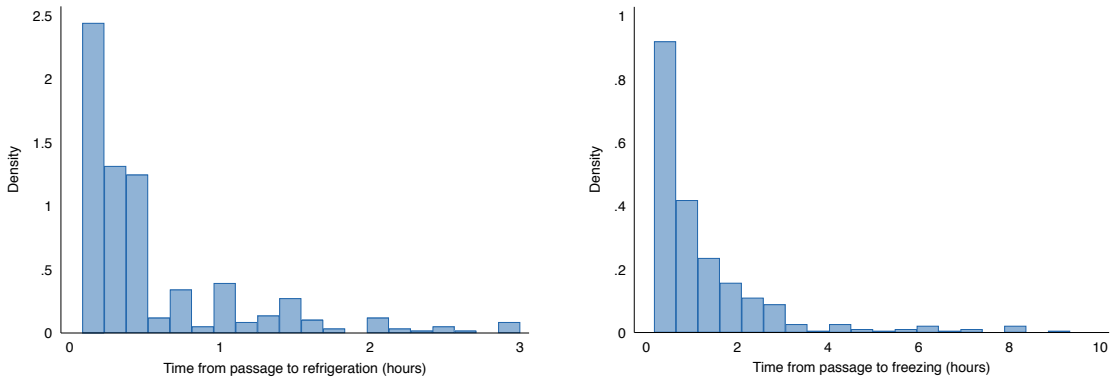


Strong evidence existed for a higher proportion of variability in beta diversity to be attributed to sequencing run but not cold chain time (Supplementary Figure 15). Run was therefore included as a permutational constraint in subsequent PERMANOVA analyses, where strong evidence for attribution recurred across age categories only for sex and recent febrile illness, whilst strong evidence was seen for season only amongst 4-6 month-olds in the unweighted

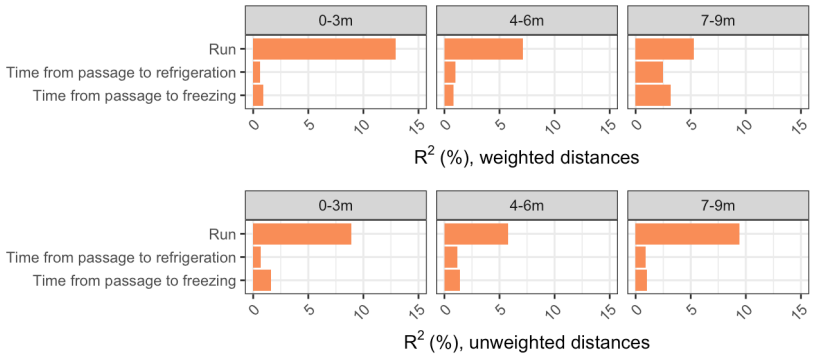
analysis and for recent exclusive breastfeeding status only amongst 4-6 month-olds in the unweighted analysis (Supplementary figure 16).

Supplementary figure 15: Frequency distribution of time from stool passage to coolbox refrigeration (2-8°C) and from passage to freezing (-193°C) amongst all 400 sequenced stool samples from 96 participants (A). Median time from passage to freezing was 0.7 hours. Derived weighted and unweighted R^2 values from age-stratified PERMANOVA analysis of attribution of variability in beta diversity by technical and laboratory factors (B). Analysis was restricted to samples with minimum read depth 30,000 sequences and one sample per participant was randomly selected within each age category nested cross-section (0-3 months n=63; 4-6 months n=70; 7-9 months n=69).

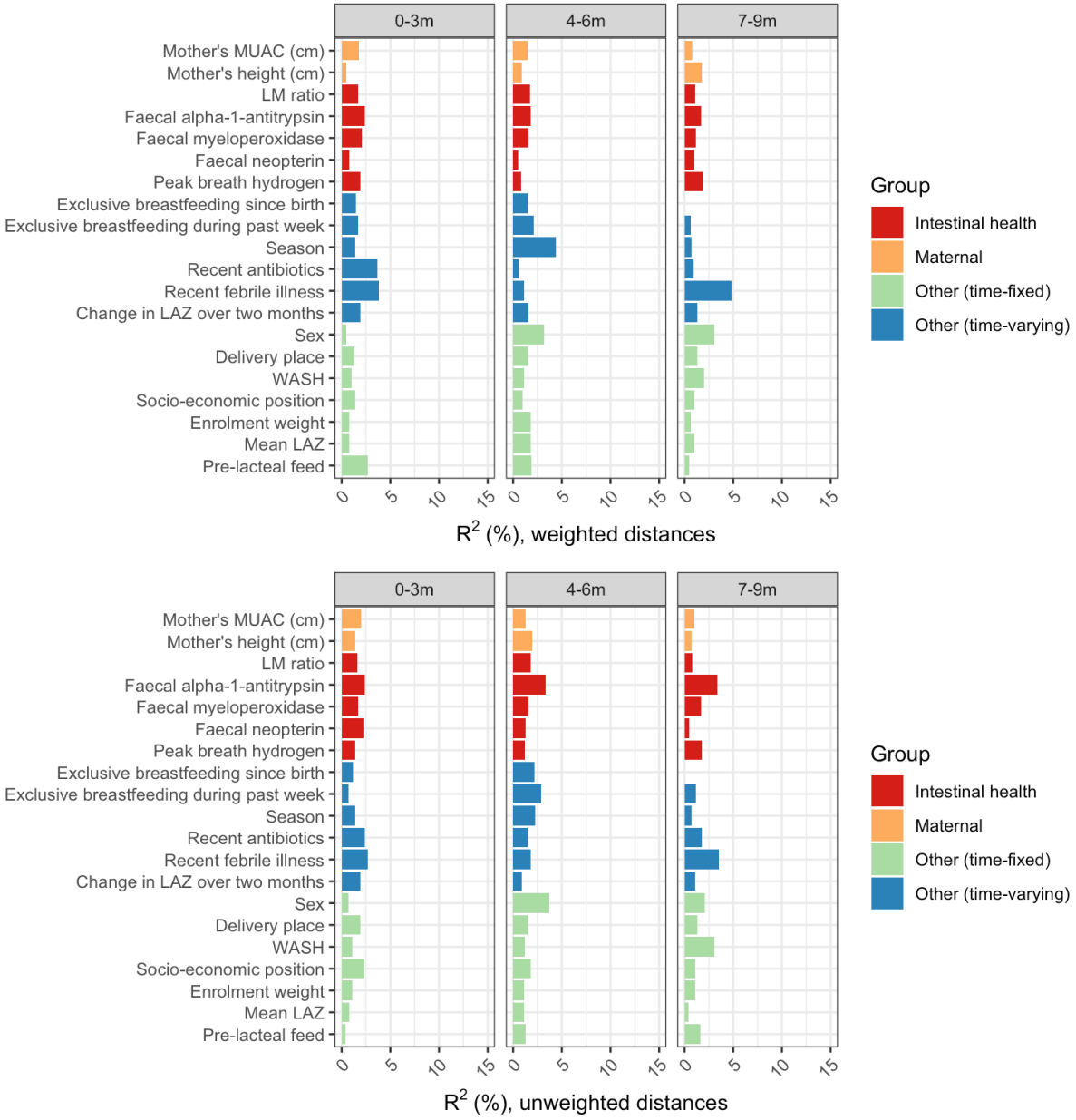
A



B



Supplementary figure 16: Derived weighted and unweighted R^2 values from age-stratified PERMANOVA analysis of attribution of variability in beta diversity by selected factors. Analysis was restricted to samples with minimum read depth 30,000 sequences and one sample per participant was randomly selected within each age category nested cross-section (0-3 months n=63; 4-6 months n=70; 7-9 months n=69). Strong evidence was seen in the weighted analysis for attribution by recent antibiotics (p=0.02) and febrile illness (p=0.01) amongst 0-3 month-olds, by season (p=0.005) and sex (p=0.02) amongst 4-6 month-olds and by febrile illness (p=0.002) and sex (p=0.02) amongst 7-9 month-olds. In the unweighted analysis, strong evidence was seen for attribution by faecal alpha-1-antitrypsin (p=0.01), recent exclusive breastfeeding (p=0.03) and sex (p=0.01) amongst 4-6 month-olds and by faecal alpha-1-antitrypsin (p=0.01), febrile illness (p=0.009) and WASH (p=0.02) amongst 7-9 month-olds



Relative abundance of specific phyla did not differ by exclusive breastfeeding status (Supplementary figure 17A). *Actinobacteria* phylum was more abundant at around five months of age during the rainy season, and *Bacteroidetes* and *Proteobacteria* phyla were more abundant at around three and five months of age respectively during the rainy season (Supplementary figure 17B).

Supplementary figure 17: Relative abundance of dominant bacterial phyla by age for all 400 sequenced faecal samples (96 participants) split by exclusive breastfeeding status since birth (A) and by season (B). Lines and shaded grey areas indicate locally smoothed means and their 95% confidence intervals derived by local regression.

