ENVIRONMENTAL ENTERIC DYSFUNCTION IN EARLY CHILDHOOD: BRIDGING THE GAP BETWEEN DIET AND STUNTING IN A RANDOMIZED TRIAL OF COMPLEMENTARY FOOD SUPPLEMENTATION IN RURAL BANGLADESH

by

Rebecca Kramer Campbell, MSPH

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Abstract

Background: Stunting continues to burden low- and middle-income countries, with lifelong and intergenerational consequences for health and human capital. Environmental enteric dysfunction (EED), a subclinical abnormality of the intestinal wall, may explain the intractability of stunting and provide avenues for more effective intervention. ***Objective:*** This PhD thesis aims to evaluate novel biomarkers of EED and to characterize its epidemiology and the pathways linking it to diet and to stunting. ***Methods:*** In a substudy nested within a cluster-randomized controlled trial of complementary food supplements (CFSs) in Bangladesh, 539 18-month-old children were enrolled after completing one year of trial interventions. EED was assessed using lactulose:mannitol (L:M) in urine and a panel of intestinal and systemic health biomarkers in stool (myeloperoxidase, neopterin, α-1 antitrypsin) and serum (endotoxin core antibody IgG, glucagon-like peptide-2, C-reactive protein, α-1 acid glycoprotein). EED scores were developed from principal component analysis (PCA) factor loadings. Associations between EED scores and L:M ratio and between EED supplementary feeding, dietary intakes and anthropometric indicators were assessed with regression models. ***Results:*** L:M ratio was elevated (>0.07) in 39.0% of children. PCA-generated inflammation (IS) and permeability (PS) scores together explained only 2.3% of L:M ratio variability. Mean L:M ratio, IS and PS did not differ by CFS group. Dairy consumption was associated with 41% reduction in odds of elevated L:M ratio (p=0.02); no other food group – EED marker associations were observed. Energy and zinc intakes from complementary foods

and CFSs were inversely associated with L:M ratio ($p \leq 0.01$), while higher iron intake was associated with higher L:M ratio ($p=0.04$). Prevalence of stunting and wasting was 45% and 15%, respectively, at 18 months and 41% and 21% at 24 months. L:M ratio was not associated with any anthropometric indicators at 18 or 24 months. Greater PS values, indicative of worse intestinal health, were associated with lower LAZ and WAZ at 18 months (p-values<0.01), while IS was not associated with concurrent anthropometry. Higher IS values, also indicative of worse intestinal health, were associated with smaller gains in WAZ and WLZ from 18 to 24 months ($p<0.03$), while PS was not associated with prospective weight gain, and no EED marker was associated with prospective linear growth. ***Conclusions:*** Elevated L:M ratio was common in this population and associated with weight gain, though not with linear growth, and was not impacted by CFSs. Divergence between L:M ratio and biomarker scores and in their associations with diet and anthropometry highlights the urgent need for an improved gold standard diagnostic test. Dietary micronutrient links suggest an avenue for future interventions but require further study.

Advisor: Dr. Parul Christian

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Abbreviations and Acronyms

AAT	α-1 antitrypsin
AGP	α-1 acid glycoprotein
AUC	Area under the ROC curve
CFS	Complementary food supplement
CHN	Center for Human Nutrition (JHSPH)
CP	Chickpea CFS
CRP	C-reactive protein
CV	Coefficient of variation
DDS	Dietary diversity score
EED	Environmental enteric dysfunction
ELISA	Enzyme-linked immunosorbent assay
EndoCAb	Endotoxin core antibody
F/V	Fruits and vegetables
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GLP-2	Glucagon-like peptide-2
HPIC	High pressure ion chromatography
icddr,b	International Centre for Diarrhoeal Disease Research, Bangladesh
IgA	Immunoglobulin A
IGF	Insulin-like growth factor
IGFBP	IGF binding protein
IgG	Immunoglobulin G
IS	Inflammation score
IUGR	Intrauterine growth restriction
JHSPH	Johns Hopkins Bloomberg School of Public Health
Kcal	Kilocalorie
L:M	Lactulose:mannitol ratio
LAZ/HAZ	Length/height-for-age z-score
LC-PUFA	Long-chain polyunsaturated fatty acid
LM	Lactulose mannitol solution
LMIC	Low- and middle-income country
LSI	Living standards index
MDD	Minimum dietary diversity
MPO	Myeloperoxidase
NEO	Neopterin
PCA	Principal component analysis
PD	Plumpy'doz CFS
PLS	Partial least squares regression

PS	Permeability score
PUFA	Polyunsaturated fatty acid
RCT	Randomized controlled trial
RL	Rice-lentil CFS
ROC	Receiver operating characteristic
SES	Socioeconomic status
SSC	Secondary school completion exam
Th1	Type 1 T helper cell
UNICEF	United Nations Children's Fund
USDA	United States Department of Agriculture
Vit.	Vitamin
WASH	Water, sanitation and hygiene
WAZ	Weight-for-age z-score
WFP	World Food Programme
WHO	World Health Organization
WLZ/WHZ	Weight-for-length/height z-score
WSB++	Wheat-soy blend *plus plus*

Chapter 1: Introduction and Specific Aims

Stunting, defined as length-for-age z-score (LAZ) < -2 relative to the WHO growth standard median,[1] persists at high rates in many low- and middle-income countries (LMICs) worldwide with severe consequences for health and human capital. The most recent estimates put the prevalence of stunting at 23.8% of children under age five years, or 159 million children worldwide.[2] The burden of stunting is distributed unevenly, with nearly half of stunted children living in Asia.[2] The period from conception to 24 months of age (the "first 1,000 days") is considered a critical window for determining adult health and stature. Within that time, nutritional and environmental insults are particularly influential during the six to 24 month complementary feeding period.[3] Inadequate diets, inappropriate feeding practices and frequent infections are known to contribute to the development and persistence of stunting during that time,[4] but nutritional interventions have often proved to be only partially effective in protecting linear growth.[5] The biological barriers to the success of these interventions and the heterogeneity of their impacts in apparently similar children are not well understood, though an emerging literature suggests impaired intestinal structure and function may play a critical role.[6,7]

Environmental enteric dysfunction (EED), a condition characterized by altered morphology, impaired barrier function and increased immune activity in the lining of the small intestine absent acute gastrointestinal illness, is thought to be highly prevalent in LMICs where the burden of stunting is highest.[6,8] Research from The Gambia has

suggested that EED may account for more than 40% of observed stunting in children under age five,[9] while subsequent studies in other settings have found high burden of EED but weaker associations with stunting.[10-12] EED is hypothesized to result from repeated low-level exposures to pathogens and other environmental toxins,[6] and is thought to inhibit growth through malabsorption of nutrients and chronic systemic inflammation.[13,14] Both malabsorption and inflammation increase nutritional demands and strain marginally adequate diets, while nutrient deficiencies may themselves cause enteropathy and inhibit epithelial repair,[15] suggesting a potential cyclical relationship.

Current studies of EED are limited by a lack of validated and practical assessment techniques for field settings. The most common diagnostic tool for settings where intestinal biopsies are not feasible is the lactulose:mannitol (L:M) test, in which a dual sugar solution is administered orally and excretion of each sugar in the urine is assayed. A ratio of the two is thought to indicate the absorptive capacity and permeability of the wall of the small intestine.[16] The L:M test is unwieldy, finicky and expensive, all of which limits expanded EED assessments in settings of prevalent stunting. Alternate markers of EED measured in serum and stool samples have been proposed to complement or replace the L:M test, but none has been formally validated for this purpose.[11,14,17-19]

Much remains to be understood about EED, including its prevalence and distribution and the extent to which it explains stunting in various settings. Further, hypothesized pathways linking dietary intakes, nutritional status, malabsorption, systemic

inflammation and stunting require testing in community-based studies in otherwise healthy children at high risk of stunting.

A complementary food supplementation trial in rural northwestern Bangladesh provided a unique opportunity to study EED, diet and growth in a setting of prevalent stunting. The trial was a cluster-randomized controlled trial of four formulations of complementary food supplements (CFS) and periodic child feeding counseling for mothers versus counseling only for preventing stunting and improving linear growth. A total of 5,449 children were randomized at age six months to one of the five study arms and supplemented for one year until age 18 months. The tested supplements were broadly balanced in their macro- and micronutrient contents, but they differed in their main ingredient and source: two were developed and produced in-country, one made of chickpea flour (CP) and one of rice and lentil flours (RL), one was Plumpy'doz (PD), a commercially distributed peanut-based product (Nutriset, Maulany, France), and the fourth was fortified wheat-soy blend (WSB++). The trial demonstrated benefits of the CFSs for linear growth and prevention of stunting.[20] All groups had declines in LAZ over six to 18 months, but children who received PD, RL or CP had less LAZ decline relative to the counseling-only group. Further, the prevalence of stunting was 5-6% lower in the groups receiving PD and CP relative to the un-supplemented group.

A study designed to characterize EED in this setting and elucidate links between diet, EED and growth was nested within the CFS trial. In a subset of the trial participants at age 18 months, collection of serum, stool and urine (following lactulose and mannitol dosing) samples allowed for the assessment of a comprehensive panel of EED

biomarkers. Nesting this EED assessment within the larger supplementation trial allowed for the biomarker data to be combined with a rich dataset of child and household characteristics, including repeated assessments of diet, breastfeeding and anthropometry over ages six to 24 months.

Goal

The goal of this PhD thesis was to develop and implement, in rural Bangladesh, a panel of intestinal and systemic health biomarkers for the assessment of EED, and to use the biomarker panel to characterize the burden and risk factors for EED in this setting, and the linkages among diet and supplementation, EED and growth (Figure 1.1).

Specific Aims

Aim 1: To develop a composite score of intestinal and systemic health biomarkers that approximates the lactulose:mannitol (L:M) test, and to describe the epidemiology of EED in a cohort of 18-month-old children.

> **Hypothesis 1a:** A data reduction analytic technique will identify a subset of serum and stool biomarkers that closely approximates L:M ratio.
>
> **Hypothesis 1b:** EED, as assessed by elevated L:M ratio and a score of EED biomarkers, will affect a large proportion of the children sampled. Children from

households of lower socioeconomic status and with poorer sanitation infrastructure will be at highest risk of EED.

Aim 2: To evaluate the effect of year-long daily complementary food supplementation on risk of EED at 18 months, and to evaluate associations between dietary intake and EED.

Hypothesis 2a: Children assigned to receive complementary food supplements will have reduced risk of EED at age 18 months relative to those assigned to the counseling-only arm.

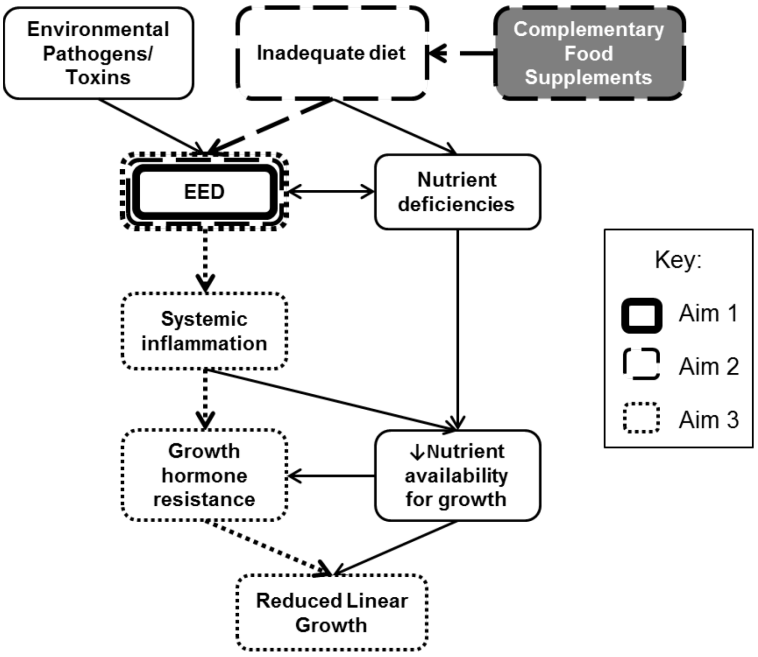
Hypothesis 2b: Better quality diets, measured in terms of dietary diversity and macro- and micro-nutrient intakes, will be protective against EED.

Aim 3: To assess relationships between EED and concurrent and prospective anthropometric measures.

Hypothesis 3a: Children with EED will be shorter and thinner, and at increased risk of stunting and wasting at age 18 months, relative to those without EED.

Hypothesis 3b: Children with EED at age 18 months will have smaller gains in length and weight over the subsequent six months, relative to children without EED.

Figure 1.1. Conceptual framework of environmental enteric dysfunction (EED) with the specific aims of this investigation highlighted[1]



[1]The figure depicts hypothesized relationships among risk factors for EED and pathways to impaired growth. Text box border and arrow line styles indicate the relationships examined in each of the specific aims of this study (specified in the key above). Conceptual frameworks in Panter-Brick et al.[21] and Prendergast et al.[19] were referenced in the development of this framework.

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Chapter 2: Review of the Literature

Overview

Stunting continues to burden a large percentage of children growing up in low- and middle-income countries (LMICs) worldwide, indicative of deprived environments that constrain growth and development and inhibit the accrual of human capital. Some causes of stunting are well established, some have been identified only recently and some are as of yet unknown, as even the most comprehensive and fastidiously implemented interventions have failed to fully rectify growth deficits. A growing literature suggests that persistent subclinical inflammation may be responsible for suboptimal growth in the absence of clinical illness. In many children living in LMICs, this may take the form of environmental enteric dysfunction (EED), a subclinical condition of the gut that limits growth both by diverting nutrients to the inflammatory response and by limiting absorption through perturbed small intestine structure and function. The burden, physiology and epidemiology of stunting, the state of knowledge regarding EED and the links between them will be reviewed in the following pages.

Stunting

In healthy children, linear growth and accrual of lean tissue are primary physiological goals, with the periods from birth to 24 months of age and the pubertal growth spurt featuring the fastest rates of growth and highest proportion of required

nutrients devoted to growth. Over short time periods, acute challenges to survival may eclipse linear growth as a priority; children with acute illnesses tend to experience a temporary decline in their rate of growth that is corrected with more rapid "catch-up growth" upon recovery. In settings where challenges to a child's wellbeing are repeated or chronic, stunting, defined as height-for-age more than two standard deviations below the international growth reference[1] median, may occur, especially if the growth constraining conditions prevent sufficient net catch-up growth between birth and age two years. High rates of early childhood stunting, as are observed in LMICs throughout Asia, Sub-Saharan Africa and Latin America, are indicative of persistent deprivation and inadequate conditions for healthy growth and development. Rectifying these deficits requires improved nutrition and sanitation, along with other yet-to-be determined interventions.

Burden

Stunting persists as a major public health problem in LMICs worldwide, despite decades of improvements in rates of acute malnutrition. Based on the 2015 UNICEF-WHO-World Bank joint child malnutrition estimates, approximately 159 million children (23.8%) worldwide are stunted, more than half of whom live in Asia.[2] In South Asia, the prevalence of stunting has declined in the past decades but, at 37.2%, it remains much higher than in LMICs overall.

Severe stunting (length/height-for-age z-score < -3) in early childhood is associated with a four times greater risk of mortality before age five.[3] Childhood stunting

that is not rectified by two years of age tends to persist into adulthood, though that is somewhat regionally variable according to the timing and duration of the pubertal growth spurt.[4] Observational and intervention studies have demonstrated that adults of short stature have poorer school performance and lower total school achievement, lower IQs, decreased physical work capacities and lower earnings than adults of normal stature.[4] The mechanisms whereby short stature affects long term health are twofold: some functional limitations, such as reduced physical work capacity, result directly from smaller stature, whereas others, such as IQ, are thought to result from the same underlying processes and deprivations that cause stunting.[5] Short adult stature in women is also associated with poor pregnancy outcomes, including maternal mortality, intrauterine growth restriction (IUGR) and increased risk of offspring mortality and short stature.[5] Stunting is not just a matter of being short; stunted children lack the resources necessary for healthy growth and development and manifest lifelong consequences of that deprivation.

Physiologic aspects of stunting in early childhood

Normal growth regulation

Growth is a primary function in childhood. Normal growth is periodic in nature[6] and is tightly regulated in response to a host of environmental and physiological conditions.[7] Growth of long bones, the primary site of statural growth, is regulated by the endocrine and paracrine functions of the growth hormone (GH) – insulin-like growth factor-1 (IGF-1) axis.[8] Growth hormone releasing hormone (GHRH) produced by the hypothalamus causes the pituitary to produce growth hormone, while somatostatin, also

released by the hypothalamus, inhibits the release of growth hormone from the pituitary.[9] Under the control of GHRH and somatostatin, circulating levels of GH are pulsatile, with spikes every two to three hours and otherwise low to undetectable levels.[10] Growth hormone has been shown to act on many tissues directly, but one of its main targets is the liver, where growth hormone receptors bind to circulating GH, triggering a cascade that results in the production and release of IGF-1.[11] Nearly all body tissues have receptors for IGF-1, including the growth plates of long bones. In the growth plates, IGF-1 binding to its receptors triggers differentiation and maturation of preosteoblasts, which induces bone matrix deposition and remodeling, one aspect of long bone growth. IGF-1 also acts on IGF receptors in the hypothalamus and anterior pituitary as part of a negative feedback loop that controls the release of GH.[9] Recent studies in mice with genes coding for the specific hormones or their receptors knocked out have also demonstrated roles in growth regulation for IGF-1 produced locally in the growth plate and actions of GH directly on the growth plate, both in the regulation of local IGF-1 production and independent of IGF-1.[12]

IGF-1 has six binding proteins (IGFBP1-6) with various roles in regulating its level of activity in body tissues.[13] IGF-1 circulates bound to one of its binding proteins, which serve to modulate IGF-1 binding to its receptors and half-life in circulation.[14,15] The majority of IGF-1 circulates in the serum bound to IGFBP-3, though the relative abundance of the various binding proteins is responsive to physiological conditions,[13] including nutritional status.[16,17] The binding proteins are structurally similar but effect their different roles in inhibiting or potentiating IGF actions on target tissues based on

post-translational modifications.[15] IGFBP-4 and -6 seem to act only to inhibit IGF actions, but IGFBP-1, -2, -3 and -5 can inhibit or enhance IGF activity depending on the location and setting.[18] Further, there is some research suggesting that the IGFBPs have roles independent of IGF as well.[13]

Nutritional inhibition of growth processes

The growth hormone axis is also responsible for mediating the effects of undernutrition on linear growth. Both animal models and observational studies in humans suggest a central role for IGF-1 and the growth hormone axis in curtailing growth in the face of nutritional deprivation.[7] Human studies linking nutritional status and bone growth have largely taken place in clinical settings in children undergoing treatment for severe acute malnutrition. Studies of children in nutrition rehabilitation programs report extremely low serum IGF-1 levels at baseline and increases that correspond to the degree of weight gain over one week or more of therapeutic feeding,[19,20] but the diversity of functions of IGF-1 make it a non-specific marker of growth activity.

Some studies have measured IGF-1 binding protein concentrations and serum concentrations of bone deposition markers, which are more specific for linear growth. One study in Bangladesh reported positive associations between collagen synthesis markers reflective of bone and muscle deposition and weight-for-height z-score (WHZ) on admission to a severe acute malnutrition rehabilitation program, and positive associations between changes in bone growth marker concentrations and changes in WHZ, height-for-age z-score (HAZ) and lower leg length over 30 and 90 days following

the initiation of therapeutic feeding.[17] That study also found low levels of IGF-1 and IGF Binding Protein-3 (IGFBP3) on admission with improvements following the start of treatment and positive associations between changes in IGF-1 and IGFBP3 and changes in WHZ, HAZ and lower leg length. Studies in children without acute malnutrition have been fewer in number but found similar relationships between nutritional status, markers of bone growth and subsequent linear growth. In a study of prepubertal boys in The Gambia, researchers found that markers of bone formation and turnover were positively associated with gains in weight and height and negatively associated with markers of inflammation status.[21]

Inflammatory suppression of growth

Inflammation also inhibits growth processes through nutrition-dependent and independent pathways. Differentiating among these mechanisms and separating their effects on growth poses a challenge to researchers, but some progress has been made in isolating the direct effects of inflammation on growth suppression. The mechanisms by which inflammation inhibits growth are largely mediated by pro-inflammatory cytokine actions on the GH axis and directly on the growth plates. Extensive in vivo work in mouse models suggests that these growth modulating effects of pro-inflammatory cytokines occur independent of nutritional status;[22] however, this pathway has not been explored in humans.

The direct effects of inflammatory processes on the growth hormone axis are compounded by nutrition-mediated effects. The acute phase response may cause reduced

absorption and low circulating levels of some micronutrients, which is hypothesized to occur in an effort to "starve" the invading pathogen, but may also contribute to growth inhibition during this period. [23] Additionally, nutrient requirements are elevated during infection. Reeds et al. describes the caloric and micronutrient requirements of individual acute phase proteins and of a "typical" acute phase response.[24] The protein and energy requirements of launching the acute phase response are substantial and may exceed nutrient availability in children of marginal nutritional status, leading to a period of muscle catabolism and halted growth.[25]

Etiologies of stunting

Intrauterine growth restriction

Stunting has proven to be a complex and persistent public health problem in LMICs. The timing of the emergence of stunting varies by region, but in South Asia approximately 30% of infants are stunted at birth and length-for-age declines progressively through 24 months of age.[26,27] The burden of small birth size is thought to be due primarily to intrauterine growth restriction caused by poor maternal nutrition and health before and during pregnancy, with a smaller contribution from preterm births.[28] Growth restriction in utero has persistent effects on growth, with approximately 20% of stunting in early childhood attributable to small size at birth.[29] Still, inadequate feeding practices and frequent illness underlie the majority of stunting cases that emerge in infancy.[3] As the classic UNICEF conceptual framework of the multi-level causes of undernutrition so succinctly illustrates, infection and inadequate diet and feeding

practices are the proximal causes of undernutrition, and each exacerbates the effect of the other to create a vicious cycle of illness and undernutrition.[30] These proximal causes of undernutrition are enabled by a larger social, political and economic context that cannot be ignored in explaining the persistence of stunting in LMICs. What remains unexplained in that model is the heterogeneity of responses to deprivations and interventions observed in children within the same community and even the same household. The factors and processes underlying that heterogeneity may be a key to understanding the persistence of stunting despite interventions that improve diet and reduce the burden of acute illness.

Timing of complementary food introduction

Breastfeeding is an important exposure that is closely linked to nutritional status in early childhood in LMICs. Breastfeeding practices have not been associated directly with poorer linear growth,[31] but mixed feeding, i.e. breastfeeding along with other liquid or solid foods, before age six months may affect growth indirectly by increasing susceptibility and exposure to infections.[3,32] The timing of initiation of complementary feeding may be a driving factor in growth faltering. Introduction of non-breastmilk foods too early can lead to the deterioration of a child's nutritional status due to overall poorer nutrient density of the diet and to the introduction of pathogens and food components that the child's immature gut and immune system are not prepared to manage.[33] Conversely, delaying the introduction of complementary foods beyond the point where the child is nutritionally and developmentally ready for them can also impair the child's nutritional status and initiate or exacerbate a cycle of undernutrition and infection by introducing potentially contaminated complementary foods when the child is malnourished and thus

ill-equipped to handle the immune insult.[33] In Bangladesh, both premature and delayed complementary feeding are common; infants are frequently fed non-breastmilk liquids during the first few months of life, but introduction of semi-solid and solid foods is often delayed beyond age six months, leading to nutritional inadequacy and growth faltering throughout infancy.[34]

The proper timing for initiation of complementary feeding is further complicated in situations where maternal undernutrition and preterm birth are common. In those settings, infants may be born with suboptimal body stores of certain micronutrients, such as iron, which may lead to breastfeeding ceasing to be adequate at an earlier age.[35]

Quality of complementary foods

Beyond the timing of complementary feeding initiation, the adequacy and diversity of foods offered can also contribute importantly to the progression of stunting. The relative contributions of feeding practices and diet quality in the etiology of stunting may differ by setting. Interventions targeting child feeding practices have been shown to positively affect child growth; in settings with sufficient food security, education for parents about best practices for child feeding can improve child growth, while in settings where access to nutritious food is a barrier to appropriate complementary feeding, food supplements are necessary to effect gains in child growth.[31,36]

Dietary quality during the complementary feeding period, in particular the presence of animal source foods in the diet, has been associated with better growth.[36-39] Animal milk, especially, has been associated with improved growth in a number of

observational and intervention trials.[40] In a unique ecological study of country-level food availability data, Ghosh et al. found that energy-adjusted availability of high quality protein accounted for 45% of the variance in prevalence of childhood stunting observed across countries.[41] In individual-level human and animal experiments, protein has been demonstrated to be more closely associated with linear growth than is overall caloric intake,[42,43] and recent evidence suggests inadequate supply of certain amino acids may be related to stunting.[44] Polyunsaturated fatty acids (PUFAs) have also garnered some attention in recent years as potentially growth-limiting when complementary feeding diets are monotonous and nutrient-poor. Though the evidence connecting PUFAs to growth is scant at present, researchers continue to investigate a link, potentially related to the role of PUFAs in regulating inflammation.[45]

Micronutrient deficiencies in the complementary feeding period have been associated with poorer health and increased risk of morbidity, but most are not directly related to linear growth. The primary exception is zinc deficiency, which is associated with stunting at a population level.[46] Prophylactic zinc supplementation has been shown to improve linear growth in populations at high risk of zinc deficiency, but the magnitude of gains tends to be small and results have been mixed and somewhat situation-dependent.[47-49] A recent meta-analysis found that supplementation with multiple micronutrients (any formulation containing ≥3 micronutrients) had a positive effect on height-for-age in children less than five years,[48] suggesting that deficiencies of multiple micronutrients may inhibit the observed benefit of supplementation with single micronutrients, including zinc.

Traditional complementary foods are often lacking in nutrients that are necessary for health and development, but few studies have looked at the adequacy of complementary foods with respect to growth-limiting nutrients specifically. The data that do exist include a multi-country study that found that the most widely used complementary foods were inadequate in energy, lipids, and micronutrients with respect to age-specific nutritional requirements.[50] In Bangladesh, typical complementary foods are lacking in energy and micronutrients including vitamins A and D, calcium, iron and B vitamins,[51] and Kabir et al. found that only approximately 40% of Bangladeshi children ages six to 23 months were consuming a diet that met the minimum criteria for adequacy in terms of both diet quality and feeding practices.[52]

Food supplementation trials have demonstrated some success in reducing rates of stunting,[53-56] which supports the presence of growth-inhibiting gaps in the typical diet. Supplementation trial results do not, however, allow for identifying the specific dietary component(s) of the supplement that were beneficial for linear growth.[36] It is clear that widespread inadequacy in complementary foods and feeding practices is a major contributor to growth faltering in LMIC settings. Small magnitudes of benefit in supplementation trials, however, suggest that inadequate diet alone may not be responsible for stunting.[31,57]

Morbidity

Frequent infections contribute to stunting in combination with nutritional limitations on growth. Illness is common in children under age five years due to frequent

pathogenic exposure and the immaturity of children's immune systems. Diarrheal diseases and acute lower respiratory infections account for the largest burden of illness in children in this age group.[58] The relationship between diarrheal disease and growth faltering has been studied most extensively, due in part to the more obvious effects of diarrhea on dietary intake and nutrient absorption that exacerbate the more general inflammation-mediated effects of illness on growth, but respiratory infections and other types of childhood illnesses also impair growth.[3] Infections inhibit linear growth by limiting dietary intake through suppressed appetite and symptoms such as diarrhea and vomiting.[59] In addition, diarrheal diseases may cause malabsorption and losses of protein and micronutrients including zinc, iron and copper, the severity of which varies greatly depending on the etiology of the infection.[25] Systemic inflammation from gastrointestinal illness or other causes is also associated with malabsorption, catabolism, and increased nutritional requirements, all of which are mediated by pro-inflammatory cytokines and contribute to negative nutrient balance during illness independent of nutrient intakes.[25]

In otherwise healthy children, periods of depressed growth during illness are followed by rapid "catch-up growth" and short episodes of illness are not associated with sustained growth deficits.[60] When illnesses are chronic or repeated, or in settings where marginally adequate diets do not allow for rapid growth post-illness, catch-up growth may not occur to a sufficient degree and growth deficits may accumulate.[61-63] Child feeding practices may also exacerbate growth slowing during illness and acceleration during recovery, as it is common to restrict food quantity or variety during illness and increase the food offered during the recovery period.[64] There was in the past some

controversy regarding the long term impact of isolated instances of infection on growth,[65,66] but more recent analyses suggest a permanent growth deficit resulting from repeated episodes of diarrhea in the first two years of life. A pooled analysis of morbidity and growth data from five countries found a multiplicative effect of each episode of diarrhea on the odds of stunting and concluded that 25% of stunting in LMICs can be attributed to children having five or more diarrhea episodes in the first two years of life.[61] The extent to which post-illness catch-up growth allows for zero net effect on growth is controversial and likely highly dependent on the situation and the underlying nutritional status of the child.

Water, sanitation and hygiene (WASH)

The causes of frequent infections in young children are largely related to sanitation conditions and hygiene practices that allow for the transmission of pathogens via the fecal-oral route. Recent global estimates suggest that 39% of the world's population, or 2.6 billion people, lack access to improved sanitation facilities and nearly 900 million people lack access to a reliable source of clean water.[67] Prevention of diarrheal disease requires knowledge about good hygiene practices as well as access to improved sanitation facilities and clean water.[68] The effect of poor sanitation and hygiene on diarrhea incidence and mortality rates are better described than the effects on stunting. Incidence of diarrhea is halved in children living in households with improved sanitation facilities and one third as high in children living in communities with sanitation infrastructure.[68] An analysis of global Demographic and Health Survey data found improved sanitation and water to be associated with a 13% reduction in risk of diarrhea

and 27% reduction in risk of stunting among children ages one to five years.[69] A review of observational and experimental studies of hand washing worldwide estimated proper hand washing practices could reduce the risk of diarrhea by 42-47%.[70]

Bangladesh has devoted particular attention to improving both sanitation and hygiene in the past two decades. In rural areas, access to improved sanitation, defined as sustainable access to a private or shared sanitation facility that effectively prevents human and animal contact with waste, increased by 23% between 1990 and 2004.[68] Still, only 35% of the rural population had access to improved sanitation in 2004,[68] though rates have since continued to rise.[71] Improved hygiene practices have also had benefits for diarrhea burden in Bangladesh: an educational intervention overseen by the NGO BRAC targeting hygiene practices in rural areas demonstrated positive effects on the incidence of diarrhea in children under 5 years.[72]

Interventions to prevent stunting

As described above, major domains to be targeted in anti-stunting interventions are diet, morbidity and WASH. The best estimates suggest that each alone has a consistent but small impact on stunting. In general, nutrition interventions are estimated to avert many more cases of stunting than are interventions targeting morbidities,[31] though those estimates are restricted to the effectiveness of known interventions. New formulations of complementary food supplements and currently ongoing trials of nutrition and WASH together hold some promise for improving effectiveness of stunting prevention strategies.

Complementary food supplements (CFSs) that supply energy, macronutrients and micronutrients to promote growth during the complementary feeding period have been the focus of many field trials in recent years, based on accumulating evidence that, in addition to lacking critical nutrients, complementary feeding diets are often not sufficiently energy dense. Trials of CFSs have consistently demonstrated improvements to the nutrient adequacy of the diet,[73,74] and benefits for recovery from moderate malnutrition[75-77] and, in some but not all trials, improved rates of growth and/or reduced risk of stunting.[53,54,56,75,78] A trial conducted in rural northwest Bangladesh, designed to fill gaps in the CFS literature and within which this study of EED was conducted, tested four CFS formulations and found reductions in the decline in LAZ over ages six to 18 months in children assigned to Plumpy'doz and two locally developed and produced CFSs, chickpea and rice-lentil, and reduced prevalence of stunting in the groups receiving Plumpy'doz and the chickpea CFS.[79]

Studies estimating the effect of one or more WASH interventions on growth have largely concluded that the effect is positive but small in magnitude. In a meta-analysis, Bhutta et al. found that hygiene interventions targeting hand washing could decrease the number of diarrhea episodes in infants and young children by approximately 30%, but would reduce the prevalence of stunting by only 2.4%, even at 99% coverage.[31] A recent Cochrane review concluded that based on the results of five randomized-controlled trials, WASH interventions may have a small positive effect on height-for-age in children under age five years (mean difference 0.08, 95% CI 0.00-0.16), but no corresponding effect was

observed on weight-for-age (MD 0.05, -0.01-0.12) or weight-for-height (MD 0.02, -0.07-0.11).[80]

Given the established links between hygiene and sanitation conditions and diarrhea burden and between repeated diarrhea episodes and stunting, the estimated morbidity-mediated effect of poor sanitation and hygiene on child growth may be low in part because studies have not fully considered nutrition – infection interactions and the prevalence and growth-inhibiting consequences of subclinical infections. Two large-scale nutrition-WASH studies (one in Bangladesh) are currently testing the individual and combined effects of these interventions on childhood stunting. The potential contribution of subclinical infections such as EED to stunting is discussed in more detail in a subsequent section.

Stunting summary

Stunting emerges from situations of persistent deprivation at multiple levels: poor diet quality and frequent illness at the individual level; food insecurity and suboptimal childcare and hygiene practices at the household level; and community- and country-level factors such as inadequate sanitation infrastructure, marginalization of certain populations and poor governance. The presence of non-stunted children in these environments suggest that it is possible to create conditions conducive to healthy growth within adverse contexts, yet intervention trials targeting known causes of stunting suggest that there are significant factors yet to be explained in the etiology of stunting. While rates of weight gain have proven to be quite amenable to intervention, stunting tends to persist,

suggesting deep-seeded and complex mechanisms regulating the rate of linear growth. The interactions of inflammation and undernutrition compound the extent of the impact estimated from nutrient intakes or duration and severity of illness alone, suggesting that nutritient supplements may fall short of meeting true requirements and periods of infection may have broader implications for growth signaling and utilization of nutrients. Additionally, the mediating physiological factors that translate nutritional and environmental conditions to the rate of linear growth are not fully understood, leading to barriers in the efficient and accurate evaluation of intervention studies.

Environmental Enteric Dysfunction

An emerging literature suggests that a subclinical intestinal pathology called environmental enteric dysfunction (EED) may be the missing link between diet quality and environmental exposures that explains the magnitude of the observed global rates of stunting. If the burden of inflammatory processes in LMIC children is truly far higher than previously estimated based on acute illness alone, the direct and nutrition-mediated effects on growth could explain the burden and persistence of stunting. The true prevalence and distribution of EED is still debated, however, and it is not clear how to best intervene if EED is, in fact, underlying the observed burden of stunting.

Normal Gut Physiology

The healthy small intestine is responsible for digestion, absorption, barrier function and local immune responses, a diverse and critical set of interrelated functions

essential for healthy growth. Digestion of foods consumed occurs throughout the stomach and small and large intestines, but the majority takes place in the small intestine.[81] The length and structure of the small intestine allows for maximal absorptive capacity, with repeated folds covered in protruding villi themselves covered in a "brush border" of microvilli serving to increase the absorptive capacity through expansion of the surface area.[81] The brush border is also a site of enzyme secretion and digestive activity that further facilitates nutrient absorption. In addition to absorbing nutrients required for maintenance and growth, the small intestine also resorbs vast quantities of water and digestive enzymes excreted to aid in digestion and resorbed for repeated use.[81]

Immune capabilities develop throughout infancy, primed by immune components in breast milk and habitual exposure to benign and pathogenic foreign particles.[82,83] The wall of the small intestine has its own local immune system that identifies and clears pathogens.[83,84] It also has the capability to present pathogens into the lymph system to initiate a systemic immune response when needed.[83] This triage capacity serves to modulate the severity of immune response, as the gut becomes habituated to its perpetual contact with relatively harmless foreign matter. Emerging evidence suggests that the intestinal microbiota play a critical role in priming the immune system to respond appropriately to pathogenic exposures.[85] When the barrier function of the small intestine is compromised, pathogens may circumvent this local immune system and enter the bloodstream directly, triggering more frequent systemic immune responses.[83]

EED Physiology

In the transition from theory to application, a detailed case definition of EED was never developed. Initially, this condition of growth-inhibiting enteropathy was described as a residual state of intestinal inflammation and increased permeability following an episode of diarrheal illness.[86,87] More recently, EED, as the condition has come to be termed, has been treated as the presence of said inflammation and permeability in the absence of acute diarrheal illness, irrespective of recent morbidity history.[88,89] Still, some researchers focusing on intestinal permeability during the recovery from acute or persistent diarrhea continue to use the term "EED", further confusing the case definition of the condition.[90] While a precise definition for diagnostic purposes does not exist at present, researchers generally agree that the condition is characterized by crypt hyperplasia and partial villous atrophy, as well as leakiness in the tight junctions between enterocytes.[89,91] In the healthy gut wall, villi protrude into the intestinal lumen, increasing the surface area available for absorption, while the enterocytes of properly functioning crypt regions produce enzymes necessary for nutrient digestion and absorption. Crypt hyperplasia and villous atrophy cause a reduction in the surface area of the small intestine available for secretion of digestive enzymes and absorption of nutrients,[92] which may reduce total nutrient absorption to below physiological requirements. At the same time, the tight junctions between enterocytes maintain the barrier between the digestive tract and the bloodstream, preventing microbial translocation and allowing trans-cellular mechanisms to regulate nutrient absorption. When leaky tight junctions allow for translocation of pathogens into the bloodstream, a systemic immune response may

follow, which is hypothesized to explain the elevated acute phase proteins commonly observed in children with EED but no acute illness.[93] Persistent low-grade systemic inflammation of this nature could be another important mechanism whereby EED inhibits growth.[94]

Biomarkers of EED

One of the challenges of studying EED in apparently healthy, non-hospitalized populations is the lack of validated markers appropriate for field use. While the morphological changes that characterize EED can only be diagnosed by intestinal biopsy, ethical considerations about conducting biopsies in population-based studies in LMIC settings, especially in children, all but prohibit their use for this purpose.[95] Alternative non-invasive measures of the functional aspects of EED have been widely used in epidemiologic studies. These may be organized based on the biospecimen required, i.e. serum, urine or stool, or according to the functional domain that they measure, for example, intestinal permeability, microbial translocation, absorptive capacity, inflammation and enterocyte repair and proliferation.

In the most commonly used method, the dual sugar test, researchers administer a solution of known quantities of two sugars, one non-absorbed disaccharide (commonly lactulose) and one transcellularly absorbed monosaccharide (commonly mannitol) and then measure the ratio of the two present in urine collected over the next several hours.[96] Greater lactulose recovered in the urine is considered indicative of increased intestinal

permeability, while the recovery of mannitol indicates the size and absorptive capacity of the intestine.

Serum endotoxin and endotoxin core-specific IgG antibodies (EndoCAb IgG) are also employed as markers of intestinal microbial translocation. EndoCAb is generally preferred to endotoxin for its longer half-life in circulation following endotoxin exposure,[93] however, the exact time course of its detection in serum is not well described.

Several fecal markers of intestinal inflammation have been proposed, [97,98] some of which are becoming rather widely used. Myeloperoxidase, lactoferrin, calprotectin and neopterin are inflammatory markers drawn from the inflammatory bowel and Celiac disease literatures that may indicate enteric inflammation characteristic of EED.[99] Concern regarding effects of breastfeeding on lactoferrin and calprotectin values have made them somewhat less popular for EED studies in young children, while myeloperoxidase and neopterin may be preferable for that population.[98] α-1 antitrypsin is another fecal marker gaining popularity for assessment of intestinal permeability in studies of EED,[98] though classically it is used to measure protein or serum loss in the stool.[100]

Additional domains of markers have been proposed, and their evaluation is ongoing at present. Of particular note, serum glucagon-like peptide-2 (GLP-2) and fecal regenerating gene 1β protein (REG-1B) are markers of enterocyte proliferation, which may be a complementary measurement domain indicative of prior injury and active repair processes.[95,101,102] Fecal host mRNAs are also generating interest for their ability to

capture a broad spectrum of intestinal characteristics, especially as methodologic advances allow for better isolation of human mRNA in the stool.[103,104]

It is of note that at present, while these markers are increasingly common in the EED literature, none has been validated for this purpose. In addition, normative data about the nature of their variability within and between individuals is largely lacking, especially in pediatric populations, which introduces additional uncertainty in their application. Research to better characterize the more promising of these markers is underway.[105] Still, both serum and fecal markers have advantages in terms of the burden of sample collection and laboratory analysis compared to the lactulose:mannitol test. Additionally, a panel of markers may offer improved insight into the nature of intestinal abnormalities relative to a single indicator.[95]

Burden and distribution

Environmental enteric dysfunction is prevalent in contexts with poor sanitation facilities and hygiene practices, and is thought to result from repeated gastrointestinal exposure to environmental pathogens and toxins.[88,106] These conditions abound in LMICs, as discussed above with respect to causes of stunting. The majority of trials that have reported the proportion of children with impaired gut integrity (generally elevated L:M ratio) compared to an age-matched healthy developed country population have found the prevalence of EED to be upwards of 80%,[90,107-109] though those estimates are highly dependent on the cutoff used for "normal" L:M ratio, which ranges widely in the

literature despite minimal reference to detailed normative data.[110] Reported rates of EED are even higher in children hospitalized with acute malnutrition.[111]

A loosely defined idiopathic subclinical intestinal condition common in tropical settings and distinct from symptomatic tropical sprue has been described for some time.[112] Interest in subclinical enteropathy as a cause of stunting in children surged in the early 1990s, spurred by research from The Gambia suggesting that elevated L:M ratios were highly prevalent and closely related to stunting in that setting. Participating infants had elevated L:M values at 76% of monthly assessments and the authors reported that 40% of observed stunting was explained by L:M.[86] That research group continued to characterize EED using L:M ratio at the MRC Dunn Nutritional Laboratory in The Gambia, describing its burden across age groups in the population,[92,113] histological characteristics of the condition[87,114] and relationships with nutritional status and infection history.[87,92,115] That ongoing work largely supported their initial conclusions, that EED is highly prevalent in the population, associated with inflammation and stunting in early childhood and of ambiguous origin.

At the same time, researchers investigating stunting in other settings began to undertake studies of enteropathy in their populations, producing a flurry of reports on the prevalence and risk factors for the condition, based largely on L:M ratios, in their specific populations. (For example, studies in Sao Paulo, Brazil;[116] Guatemala;[117] Malawi;[118] Bangladesh;[119] and Nepal.[107,120]) While each report of prevalent EED spurred further EED studies, divergent assessment methods, age groups, inclusion criteria and other

study design considerations make aggregating findings across these numerous studies challenging.

Links to growth

The effects of EED on growth are difficult to isolate, as many of the hypothesized causes of the condition are also linked directly to impaired growth, but they are important to consider nonetheless, as the crux of the EED hypothesis is that the effects of growth-limiting exposures, such as inadequate diet and repeated infectious morbidities, are underestimated when the full burden and consequences of EED are not included.[88] Studies in children admitted to treatment programs for acute malnutrition have generally found high rates of impaired gut integrity on admission and improvements in gut integrity markers over the course of recovery.[90,111,121] Non-hospital-based prospective studies of gut integrity and linear growth have been fewer and those that have been published report inconsistent findings.[93,109,120] Still, evidence supporting a relationship with growth has been accumulating in recent years. One preliminary report from the MAL-ED multi-country cohort provides longitudinal data attributing poorer six-month growth in children age three to15 months to a score of fecal intestinal inflammation and permeability markers.[98] A study from urban Bangladesh found increased risk of stunting at age 12 months with each unit increase in EndoCAb measured at age six months,[122] and another in Malawi found an inverse association between urinary lactulose recovery and rate of height-for-age z-score change in slightly older children over a three month follow-up period.[109] Additionally a post-hoc case-control analysis of blood samples from stunted and non-stunted HIV-negative 18-month-old children in Zimbabwe found evidence of

persistent inflammation from six weeks to 12 months of age, confirming that inflammation in otherwise healthy children can be chronic in this setting and is associated with stunting.[63]

Environmental and infective causes

The evidence connecting EED with WASH exposures is surprisingly speculative at present, largely relying on hypothesized links between the WASH-stunting and WASH-diarrhea literatures.[88] The idea emerged from a 1993 article by Solomons et al. connecting early childhood stunting to observations in the veterinary literature attributing growth failure in chicks to unhygienic environments.[123] This connection was revived in a 2009 Lancet commentary by Jean Humphrey,[88] which may be considered the origin of much of the current attention to EED as a cause of stunting. Remarkably little direct evidence has been gathered to support or refute that hypothesis. One recent study in Bangladesh found lower L:M ratios in children living in households classified as "clean" based on water source, sanitation facilities and hygiene practices compared to children in "dirty" households, and inverse relationships between L:M ratio and HAZ.[124] The study was observational and not all key associations reached statistical significance, but the results support the hypothesized link from WASH exposures to stunting via EED. More studies are necessary, however, to confirm and expand upon those findings.

At present, a single or defined set of pathogens has not been identified as causal in EED,[89] though recent history of diarrhea may be associated with increased risk.[111] Studies of various diarrheal etiologies have found that invasive bacterial pathogens cause greater

impairments in gut integrity than secretory diarrheal infections do.[125] Studies have not, however, found definitive associations between infection with *Giardia lamblia* or helminthes and impaired gut integrity. The relationship between *Giardia* infection, specifically, and gut integrity has been investigated in several observational and randomized intervention studies with mixed results.[97,107,126,127] The same is generally true of helminths; in most intervention trials, anthelminthic treatment did not alter the burden of EED in children,[126,127] but in one recent study in Malawi, albendazole was protective against increases in L:M ratio over a short time period (one month).[128]

Another proposed etiology of EED is exposure to environmental toxins and food contaminants.[129] Aflatoxin, typically found on food crops such as maize, peanuts, oilseeds, tree nuts, and spices, has been the subject of the majority of research historically.[130,131] Aflatoxin exposure in the food supply is pervasive in many LMIC settings (including Bangladesh),[132,133] and is associated in a dose-response manner with impaired growth in an extensive animal literature and in a growing number of observational studies in human children.[131] Aflatoxin and other mycotoxin exposures may also have intestine-specific toxic effects that lead to alterations in villous and crypt structure and small intestine absorptive and immune functions similar to those observed in EED.[134] At present, this data come strictly from animal studies, largely in chickens, so much remains to be understood about the presence and distribution of mycotoxin-related enteropathy in children, but the existing evidence suggests it may be an important avenue for future research into EED and growth.

Diet quality and nutritional causes

The relationship between EED and nutritional status is difficult to study in humans, as it is of a bidirectional, potentially cyclical nature. Additionally, because nutrients can interact directly with the intestinal epithelia, a demonstrated benefit of supplementation with a specific nutrient on gut health is not necessarily evidence of an underlying deficiency. For that reason, much of the stronger evidence supporting nutrient deficiencies as causal factors in EED comes from the animal literature.

Human studies and mouse models support roles for zinc, vitamin A, and certain amino acids in maintaining intestinal health, while evidence for a relationship with polyunsaturated fatty acids (PUFAs) is more equivocal. As Jacobi et al. describe in a review of nutritional regulation of intestinal structure and function in neonates, dietary sources of typically non-essential amino acids may become necessary in the context of rapid growth in infancy and early childhood, especially if inflammation and immune activation further increase nutrient requirements during that period.[135] Glutamine has been one particular focus of research linking individual amino acids with gut integrity. In animal models, insufficient glutamine is associated with reduced turnover of gut mucosal cells in times of physiological stress.[136] In a randomized controlled trial of supplemental glutamine added to a therapeutic feeding regimen in children with acute malnutrition, the L:M ratio in children receiving glutamine decreased (improved) from 0.31 to 0.10, while those receiving a glycine supplemented diet had no change in L:M ratio.[121] The possibility that some long-chain polyunsaturated fatty acids (LC-PUFAs) are conditionally essential as well is more controversial.[137] LC-PUFAs are associated with

villous height, crypt depth and rate of intestinal repair in piglets,[135] but a large RCT of supplemental LC-PUFAs in infants found no effect on L:M ratio.[45]

In the realm of micronutrients, vitamin A and zinc in particular have been the focus of much study. Vitamin A deficiency in murine models is associated with shortened villi in the small intestine and supplementation with vitamin A is associated with improved recovery in models of intestinal illnesses including inflammatory bowel disease, radiation and intestinal resection, among others.[136] Expanding on the role of vitamin A deficiency in intestinal structure, Duggan et al. demonstrated a small effect of vitamin A deficiency alone on small intestinal villi in mice, and also found a synergistic negative effect of vitamin A deficiency and rotavirus infection on villous height.[138] In humans, one study observed that L:M ratio fluctuated with the seasonal variation in consumption of vitamin A-rich mangoes in The Gambia.[115] The results of supplementation trials, however, have been mixed. In one study in Brazil, supplemental vitamin A was associated with lower rates of *Giardia* infection but no changes in L:M ratio or fecal lactoferrin,[139] while in a trial in India where two groups of infants – one asymptomatic and one hospitalized with diarrhea – were supplemented with vitamin A, significant improvements in L:M ratio were observed in both groups.[115] Differences may be attributable to the age of the supplemented children, the underlying vitamin A status of the two populations, or different etiologies of poor gut integrity, and more research is needed to fully understand the role of vitamin A in EED.

Zinc is known to have a role in the maintenance of the enterocytes of the small intestine, which may be enhanced by supplementation independent of underlying zinc

status, especially in the presence of inflammation.[140,141] Further, supplemental zinc is known to be beneficial during bouts of diarrhea, while benefits of prophylactic supplementation are less clear,[142] which suggests that improvements in EED markers following zinc supplementation are not necessarily indicative of underlying deficiency. At the same time, even mild zinc deficiency is known to be associated with impairments in immune function and epithelial maintenance,[142] two features of EED, and zinc deficiency is prevalent in most of the same settings where EED is observed.[143] To further complicate matters, however, malabsorption secondary to EED could inhibit zinc absorption, such that observed associations between EED and zinc deficiency do not have a clear directionality.[140] Two studies in humans contribute additional information. In Malawi, endogenous fecal zinc was positively correlated with L:M ratio and net zinc retention was negatively correlated with L:M ratio, suggesting a connection between zinc homeostasis and intestinal permeability.[108] In a study in Bangladesh, two weeks of zinc supplementation decreased lactulose recovery in children with acute diarrhea, though the effect was dependent on the etiology of the diarrhea.[125]

Recent studies have also found some improvements in EED-related markers with multiple micronutrient supplementation. A study assessing small intestine morphology by biopsy in Zambian adults negative for HIV found 24% increase in mean villous height and 27.6% greater mean villous area following six weeks of multiple micronutrient supplementation compared to those receiving a placebo.[144] Malawian children supplemented with a multiple micronutrient powder with or without a fish oil capsule had

greater improvements in gut integrity as assessed by L:M ratio over 12 and 24 weeks than those receiving placebos.[145]

While associations between specific nutrients and gut integrity have been elucidated in recent observational and experimental studies, the relative contribution of dietary inadequacy to the observed burden of EED and the extent to which supplementation with a specific nutrient or specially formulated food could rectify or protect against EED is unknown.

EED Summary

EED is a poorly defined, widely studied set of morphologic and functional changes to the small intestine. Maintaining adequate nutritional status and supporting growth in childhood rely on the proper functioning of the intestinal wall, both in its absorptive and barrier capacities. The EED literature suggests that those functions are widely impaired in LMIC settings and may explain a large portion of the stunting observed there. Observational and intervention studies aimed at determining the causes and risk factors for EED and preventive or treatment strategies have abounded, but progress is limited by the lack of a unified case definition and validated diagnostic tools appropriate for field settings. While those methodologic constraints inhibit drawing conclusions across studies, accumulating evidence suggests environmental and nutritional causes, perhaps required in combination, underlie the loosely defined EED syndrome. The strength of reported associations between intestinal health markers and growth varies

widely between studies, and at present it is not clear the extent to which that link exists and in what settings, if any, EED may explain a large portion of observed stunting.

Summary

Stunting remains a major public health problem, with implications for the health of individuals and their offspring and for the development of human capital. Inadequate diets, repeated morbidity episodes, environmental toxins and inadequate health care and other factors are known to constrain growth throughout the fetal, infancy and early childhood periods. Environmental and nutritional factors that impair the functioning of the intestine may exacerbate the effects of known risk factors on the development and persistence of stunting. EED may be a missing link that explains the intractability of stunting and holds the key to developing more effective interventions for the prevention of stunting. Many questions remain, however, and a critical first step in advancing this field of study will be the development of improved diagnostic measures appropriate for use in field settings. At present, the relationship between EED and stunting, and in particular, the contributions of diet quality and nutritional status, are not well described. Research elucidating those issues may provide valuable insight into the burden of stunting in LMICs and strategies for more effectively supporting healthy early childhood growth.

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Chapter 3: Study Design and Methodology

Overview

A community-based randomized controlled trial of complementary food supplements (CFS) provided a unique opportunity to conduct an investigation of environmental enteric dysfunction, nutritional inputs and growth in a rural South Asian setting. The trial took place at a long-established research site (The JiVitA Project) in rural northwest Bangladesh, an area considered generally representative of the Gangetic flood plain region of South Asia in terms of population SES, public health concerns and health-related beliefs and practices. A subset of children enrolled in the CFS trial participated in a one-time assessment of environmental enteric dysfunction. Biospecimens collected from participating children were analyzed at JHSPH (blood, stool) and icddr,b (urine) for markers of gut function and inflammation. Biomarker data were combined with longitudinal assessments of anthropometry, diet and breastfeeding, morbidity and other child and household characteristics from the parent trial to allow for this investigation.

Setting

JiVitA Study Area – history and organization

The JiVitA study site is located in a rural, densely populated region of northwest Bangladesh, comprised of parts of the Gaibandha and Rangpur districts. In that region,

residents' livelihoods are primarily agricultural and typical household landholdings are small. The study site has been host to maternal and infant nutrition and health studies since 2001, including three large randomized controlled micronutrient supplementation trials.[1-3] The 450 km^2 study area contains a population of approximately 650,000, with households and landmarks fully GPS-mapped and residents enumerated.

JiVitA-4 Trial

The JiVitA-4 trial (Trial #NCT01562379, clinicaltrials.gov) was a cluster-randomized controlled trial of four formulations of CFSs for preventing stunting and improving linear growth in young children, implemented from September 2012 through October 2014.[4] In this setting, stunting is highly prevalent (>40% of children under age 5 years), with the most rapid decline in LAZ generally coinciding with the initiation of complementary feeding. Typical complementary foods in the area are known to be frequently inadequate in their quantity, diversity, and micronutrient content.[5-7] Blanket distribution of complementary food supplements specially formulated to rectify deficits in usual complementary foods for the prevention of stunting has been somewhat successful in African settings,[8,9] but the efficacy and acceptability of various types of CFSs in a South Asian setting was unknown. In order to evaluate this question, the JiVitA-4 trial tested three different CFSs in comparison to Plumpy'doz, which was considered the standard lipid-based supplement, or no CFS. The CFSs differed by their main ingredient – chickpea flour (Chickpea, CP), rice and lentil flours (Rice-Lentil, RL), wheat and soy flours (fortified Wheat-Soy Blend, WSB++) or ground peanuts (Plumpy'doz, PD)– and the location of their development and production – CP and RL

were designed and produced in-country while WSB++ and PD were imported. All groups also received child feeding counseling for mothers, including the group that did not receive a food supplement. The primary outcome of the trial was stunting (LAZ< -2) at age 18 months after one year of supplementation following enrollment at 6 mo of age. The trial was conducted by investigators at JHU and JiVitA in partnership with the World Food Programme (WFP) and International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) and funded by the United States Department of Agriculture (USDA).

The main supplementation trial results were published in 2015.[4] The trial demonstrated a 5-6% reduction in the prevalence of stunting in the groups receiving the Chickpea and Plumpy'doz CFSs relative to the unsupplemented control group, and a reduction in the rate of decline in LAZ over ages 6-18 months in the groups receiving Chickpea, Rice-lentil and Plumpy'doz relative to the control. The study design and methods are described in detail in that publication.[4] To summarize key points briefly, a total of 5,536 children were enrolled at age 6 months and assigned to one of the five study arms based on their geographic sector of residence. All identified children living in the study area were eligible to participate if they reached age 6 months during the enrollment period (September 2012 – April 2013) and did not reach their 7 month birthday prior to enrolling and initiating their assigned treatment. In all groups, participating households were visited by child feeding counselors every 1-2 months for one year to deliver a curriculum of nutrition, child feeding and household hygiene messages. Participants in the four groups receiving CFSs additionally were supplemented

with a daily CFS for one year, to age 18 months, to assess the effects of the CFSs on length, risk of stunting and a number of secondary outcomes. In all arms of the study, repeated measures of diet, breastfeeding and anthropometry, as well as weekly monitoring of supplementation adherence (in groups receiving supplements) and morbidity, were conducted over the yearlong supplementation period (see Table 3.1 for assessment timing). An additional assessment of anthropometry, diet, breastfeeding, morbidity and other characteristics occurred at age 24 months, 6 months after the end of the intervention, to allow for evaluations of the extent to which observed trial impacts persisted. Supplementation adherence, as well as diet, morbidity, household socioeconomic status and anthropometry data collected within the main trial were utilized within the EED study.

JiVitA-4 Substudy

In a subset of the JiVitA-4 study area selected to be geographically contiguous and balanced by number of participants per treatment arm, additional assessments were done to determine the effects of the supplements on body composition, biochemical nutritional status and cognitive development. All enrolled children living within the designated area were considered eligible and approached for an additional parental consent. Consenting participants (n=821) had body composition assessed by Bioelectrical Impedance Analysis at ages 6, 12 and 18 months, blood drawn at the conclusion of supplementation (age 18 months) to be tested for micronutrient status, and cognitive development at 18 months assessed with the Bayley-III Scales of Child Development modified for the local language and cultural context.

Blood was collected at designated centers within the study area by JiVitA substudy technicians with prior experience drawing blood in infants and young children. Blood was collected by venipuncture using a butterfly needle attached to a 3 mL syringe and then transferred to a blood collection tube free of anti-coagulants. The blood was allowed to clot undisturbed in a dark space for 30 minutes and then stored and transported in a cold box to the JiVitA project headquarters laboratory. Once received by the lab, it was centrifuged and the serum transferred to two labeled 1.5 mL cryovials, which were stored in liquid nitrogen until shipment to the Center for Human Nutrition lab at JHSPH (Baltimore, MD). Upon receipt in Baltimore, all samples were transferred to -80°C freezers to await analysis.

Hemoglobin concentrations and blood type were assessed immediately upon blood collection using the blood remaining in the butterfly needle tubing. Results were reported to the mother verbally and on a "health report card" presented to mothers for their own record. All children found to be iron deficient received a one-month course of supplemental iron syrup.

Field Methods

An assessment of environmental enteric dysfunction (EED) was undertaken within the JiVitA-4 substudy, capitalizing on the planned blood collection. The EED assessment consisted of urine collection following oral lactulose and mannitol solution dosing, along with collection of a single stool sample. These were analyzed for a panel of

proposed EED markers. The serum collected for micronutrient analysis was also analyzed for markers of EED and systemic health. This comprehensive approach to EED assessment was undertaken with the aim of investigating in a single study the relative merits of a panel of proposed EED markers in comparison to the widely used L:M test. Carefully designed cold chain procedures, field laboratory facilities for processing samples and a highly coordinated field management team allowed for collection of high quality biospecimens carefully scheduled to be collected simultaneously with the other 18 month study assessments.

Eligibility and Consent

Children were recruited for participation in the EED assessments by trained interviewers at the time of their regularly scheduled 18 month follow-up assessment. A standard consent statement was prepared and field workers were thoroughly trained in the purpose and procedures of the study so that they could respond to mothers' questions fully and accurately. Following the 18 month interview, the interviewer read the standard statement to explain the purpose and procedures of the EED study, answered any questions, and then obtained a signature from the consenting mother or primary caregiver. If a mother or other family member had concerns or further questions about the purpose and procedures of the EED assessments, the interviewer contacted her supervisor, who visited the family to provide further information and reassurance. If the family was still unwilling to participate, they were still considered eligible for all other aspects of the main trial and substudy, but did not undergo any EED assessments.

Sample Size

A sample size of 100 children per study arm was chosen to be both feasible and informative for testing the hypotheses of interest. At a power of 80% and type I error of 5% and using the standard deviation found in prior pilot work in the same cohort (σ=0.644), this sample size was predicted to allow for the detection of a minimum difference of 0.46 (0.72*σ) in mean L:M ratio between each supplementation group and the control group, assuming equal variance across groups and adjusting for multiple comparisons.

With the intention of enrolling approximately two-thirds of the substudy participants (500 of 821) and allowing for a 10% rate of refusal and "not-met", eligible households were approached for consent beginning six weeks after the start of the 18 month follow-up visits in mid-September 2013, such that a sufficient number of eligible children could be recruited over the duration of the 18 month substudy assessments. As the consent rate was considerably higher than predicted, gut study activities were discontinued in the field in early April 2014 when the intended sample size was reached.

Stool Collection

Stool collection took place in the household according to collection instructions relayed to mothers by trained field workers. Cold boxes and frozen ice packs were delivered to participating households in the evening along with stool collection kits consisting of a sealed sterile collection cup, sterilized collection mat and spoon, and polythene bag for collecting soiled materials. The following day, generally early in the

morning, mothers helped the participating child to defecate on the provided sterilized mat. The mother then transferred the stool to the collection cup with the provided spoon, sealed the cup and placed the cup in the cold box surrounded by the ice packs. A field worker returned to the house later that morning to retrieve the cold box with stool sample and brought the sample, along with all waste, to the project lab. Field workers charged with delivering and collecting cold boxes traversed the study area by motorcycle to minimize the time between sample production/collection and processing. Cold boxes and ice pack configurations were pre-tested to ensure that cold packs would keep samples at acceptable temperatures (<4° C) for the period before they were returned to the lab. Lab technicians confirmed that samples and ice packs were cold on receipt.

To ensure samples were uncontaminated and properly refrigerated, field workers accepted only samples that had been collected on the day of pick-up (not the previous night), transferred to the collection cup and stored in the cold box within 30 minutes of defecation and collected from the given mat, not the ground or another surface. If these conditions were not met, field workers discarded the sample and collection kit and provided the household with a new kit and cold box to repeat the collection. If the child did not defecate on the designated collection day, field workers replaced the household's cold packs in the evening and instructed the mother to repeat the procedures on the following morning. These stool collection procedures were repeated so long as mothers were willing until an acceptable sample was collected or until more than seven days passed from the time of the blood collection and lactulose:mannitol test.

Upon receipt by the project laboratory, stool samples were stirred to homogenize and then measured in 1 g aliquots into three 1.5 mL cryovials. These were labeled with unique sample ID numbers and stored in liquid nitrogen tanks pending shipment to Baltimore. Upon receipt by the CHN lab in Baltimore, samples were transferred to -80° freezers pending analysis.

L:M Test

The lactulose:mannitol (L:M) test is a measure of the urinary recovery of two sugars administered in an oral solution. Doses of lactulose and mannitol solution were prepared for children according to their most recent measured weights (generally from within the past 72 hours). Doses were prepared daily by a laboratory technician under highly supervised hygienic conditions in the project headquarters lab. The solution was composed of 50 mg mannitol powder (Sigma-Aldrich, St. Louis, MO, USA) and 255 mg lactulose in 0.375 mL lactulose syrup (Square Pharmaceuticals, Dhaka, Bangladesh) per mL of solution with the remainder Mum brand locally procured bottled water. The doses were measured into labeled sterile dosing cups based on the child's weight: 2 mL solution per kg body weight up to a maximum of 20 mL. Dosing cups were labeled with the child's name and unique ID, dose volume and dose preparation date and stored in a refrigerator reserved for doses and dose-making materials only. Substudy technicians transported the doses to the field clinics in cold bags each morning. Any dose remaining in the refrigerator for more than 72 hours or carried to the field but not used was discarded.

Local community field workers escorted children and their mothers to one of two substudy clinics and ensured a two hour fast (excluding breastmilk) prior to dosing. Children with high fevers or current loose, watery or bloody stools as reported by the mother had L:M tests and other substudy assessments postponed. Upon arrival at the field office, children were encouraged to urinate and then were given their L:M dose by a substudy technician. After a further 30 minutes of fasting, the children were given biscuits, milk and water, but were not allowed to consume fruit, fruit juice or candy for the duration of the urine collection period. All urine from the child was collected in a "potty" receptacle assigned to that child over a two hour collection period. The two hour collection period was designated based on evidence that lactulose and mannitol recovered in urine more than two hours post-dosing is likely indicative of colonic absorption,[10] in addition to feasibility constraints for a longer collection period. The potty was lined with a polythene bag and each time the child urinated the technician transferred the urine from the bag into a collection container and replaced the polythene bag. Any urine lost on the ground or contaminated with stool was noted on the urine collection form. At the end of the two hour urine collection period, or when the child had produced at least 20 g of urine a minimum of one hour after dosing, up to a maximum urine collection period of three hours, the collected urine was mixed with chlorhexidine (a disinfectant) according to the total volume collected (Table 3.2) and three aliquots were prepared. Aliquots were stored and transported back to the project headquarters lab each day in cold bags with ice packs. Once received by the lab they were stored in liquid nitrogen pending transfer to icddr,b for analysis.

Biomarker Panel

A panel of biomarkers to be tested in the serum, stool and urine specimens was designed to comprehensively assess the EED syndrome and to be inclusive of a range of accepted and novel markers described in the literature. The focus of the biomarker panel was the integrity and functioning of the walls of the small intestine. The lactulose:mannitol (L:M) test, currently accepted as a quasi-gold standard feasible for field use, formed a central component of the assessment, against which other biomarkers, alone and in sets, could be evaluated. Additional markers of systemic inflammation and endocrine regulation were included as contextual markers of systemic health.

Enteric Structure and Function

The health of the small intestine, including its structural integrity, absorptive capacity and inflammation status, was the primary target of the biomarker assessment. Intestinal health was measured with the lactulose:mannitol (L:M) urine test, serum concentrations of immunoglobulin G endotoxin core antibody (EndoCAb IgG) and glucagon-like peptide-2 (GLP-2), and stool myeloperoxidase (MPO), α-1 antitrypsin (AAT) and neopterin (NEO). The dual sugar test of intestinal permeability, of which L:M is the currently preferred method, has been widely used in clinical and epidemiologic applications as a marker of gut integrity,[11,12] while serum EndoCAb and the stool markers are becoming more common in epidemiological studies,[13-16] and GLP-2 is a novel marker for this application.

In the L:M test, the recovery of the two sugars in the urine is compared to quantity of each in the oral dose, based on an assumption that neither lactulose nor mannitol is found in the regular diet. The smaller sugar, mannitol, is absorbed transcellularly by the enterocytes of the small intestine at a rate reflective of the overall absorptive function of the small intestine.[17] In EED, the reduced surface area of the small intestine limits absorption and is associated with a corresponding lower level of mannitol recovery in the urine.[18,19] Lactulose is not normally absorbed and is only recovered in the urine when the tight junctions between enterocytes are leaky and allow large molecules to translocate into the bloodstream.[11] When the lactulose: mannitol ratio is elevated, it suggests increased leakiness and decreased absorptive capacity indicative of EED.[19]

Serum concentration of endotoxin core antibody (EndoCAb) have also been employed as markers of intestinal microbial translocation, though to date only one gut integrity study has used EndoCAb alone without also reporting L:M ratio values,[13] and no formal validation of serum EndoCAb as a marker of impaired gut integrity has been published. Those studies that have measured both L:M ratio and EndoCAb have largely found strong associations between the two,[20] but much research remains to be done on the interpretation and reliability of this relatively untested marker.

Glucagon-like peptide-2 (GLP-2) is a peptide hormone measured in serum. Its function is to induce the crypt cells of the intestinal wall to proliferate.[21] Elevated serum concentrations of GLP-2 have been observed after injury to the intestine.[21] In EED, elevated GLP-2 may indicate repair following damage.

The fecal markers measure intestinal immune activation and permeability. They have potential advantages over L:M and the serum markers in that they are methodologically simple in terms of sample collection and lab analysis, and they may also allow for more direct assessment of intestinal conditions. Caution must be used in the design and interpretation of studies using fecal markers, however, as the concentration of samples is not tightly regulated as in blood, and may influence the detection of analytes in those media. Some studies address this issue by freeze drying a portion of the stool sample to allow for relating detected concentrations to dry weight of stool.[22,23] MPO is a measure of neutrophil activation in the intestinal epithelia,[24] selected from among other similar markers because it is thought to be unaffected by breastfeeding status.[25] AAT circulates bound to proteins in the bloodstream and is detectable in stool in the case of protein losing enteropathy, when excess intestinal permeability allows protein loss into the intestinal lumen.[26] NEO is a product of Th1 cells and, thus, is elevated in stool in the presence of Th1-mediated intestinal inflammation.[22] One recent multi-country study observed an association between a score composed of these three fecal markers and subsequent linear growth.[15] One other study found negative associations between NEO and subsequent gains in height and weight.[22] While these newer markers of EED are promising based on feasibility and associations with growth, no study to date has fully investigated their joint relationships, potential redundancy, and strength of association with L:M.[27] For these reasons we assessed EED using L:M ratio along with a comprehensive panel of promising biomarkers.

Inflammation

Systemic inflammation may contribute to poorer growth in children and has been observed in connection with EED.[28,29] Additionally, repeated symptomatic illness, especially diarrhea, is one hypothesized casual factor in the development of EED. The serum proteins C-reactive protein (CRP) and α-1 acid glycoprotein (AGP) are elevated during the acute phase response, indicating a current systemic immune response.[30]

Endocrine activity

In childhood, growth is regulated by the growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis. IGF-1 is not specific to linear growth, as nearly every type of tissue has receptors for it, but it is generally at low concentrations during periods of acute malnutrition or infection and elevated during periods of rapid growth, and thus may be considered indicative of current growth processes.[31]

Laboratory Methods

Urine was analyzed for concentrations of lactulose and mannitol by High Pressure Ion Chromatography (HPIC, Dionex, Thermo Fisher Scientific, Sunnyvale, CA) in the lab of Dr. Rubhana Raqib at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) in Dhaka, Bangladesh. Standard solutions with known dilutions of lactulose and mannitol and uniform quantities of a control sugar, melibiose, were used for quality control purposes.

Analyte concentrations in stool were measured in the Johns Hopkins Center for Human Nutrition (CHN) Lab with ELISA kits from ALPCO Diagnostics (Salem, NH, USA) for MPO and NEO and BioVendor (BioVendor, LLC, Asheville, NC, USA) for AAT. Stool samples were diluted 1:50 by weight in wash buffer, agitated to mix thoroughly and then centrifuged for 10 minutes, supernatant extracted and centrifuged again in an Eppendorf centrifuge for 5 minutes and the supernatant aliquoted and frozen for subsequent analysis. At the time of analysis, samples were thawed and diluted with wash buffer at a ratio of 1:10, 1:500, and 1:2.5 for MPO, AAT and NEO, respectively. Standards and controls provided with the assay kits were run in duplicate on each plate along with a pair of participant-derived control samples to monitor reliability (see CV's in Table 3.3 below). Additionally, duplicate sample extractions from 8 samples were tested for MPO concentration to assess the extent of intra-sample variability in stool protein concentrations.

The serum assays were also conducted in the CHN lab. CRP and IGF-1 were measured by chemiluminescent immunoassay (Immulite, USA) and AGP was measured by radial immunodiffusion (Kent Laboratories, USA). EndoCAb IgG and GLP-2 were measured by ELISA using commercial assays (EndoCAb: Hycult Biotech, Plymouth Meeting, PA; GLP-2: EMD Millipore, St. Charles, Missouri). In serum assays, quality control measures analogous to those used in the stool assays were implemented including commercial and participant-derived control samples run on each plate and intra- and inter-plate variability monitored (Table 3.3).

Analytic Methods

Data Management

All field data were collected on standardized forms by trained interviewers or substudy technicians. Forms were transmitted via established protocols to the JiVitA project data management center (Gaibandha, Bangladesh) where they were entered in duplicate into form-specific data entry screens by trained data entry operators. Values outside of predefined ranges for plausible values were returned to the field via a standard field query system to ensure accuracy of data. Anthropometry data were transmitted daily via mobile phone for immediate quality control review, and values outside of plausible ranges were flagged for re-measuring on the following day. Computerized data were backed up regularly on site and to remote servers. Baltimore-based data management personnel monitored and cleaned the final data to produce standard final data sets for analysis. Paper forms were stored in orderly and climate controlled conditions in Gaibandha for a minimum of seven years after data collection. Confidentiality and security of written and electronic data are protected throughout the data collection, entry and storage processes through extensive training of personnel, on-site facility design and security and data encryption technologies.

The lactulose:mannitol (L:M) recovery ratio was calculated from the concentrations of each sugar in urine determined by HPIC by multiplying each concentration by the total weight of urine collected and dividing by the weight of each sugar in the initial dose to determine the percent recovery of each. For each child, the

ratio of percent lactulose recovery to percent mannitol recovery was reported, henceforth referred to as the "L:M ratio".

Biomarker values were explored relative to aspects of sample collection and assay implementation using visual displays of the data. Values of L:M ratio and lactulose and mannitol recovery separately were explored relative to the dose administered, the frequency and timing of urination during the urine collection period, and whether any urine was lost during urine collection. Overall, these procedural aspects did not appear to influence L:M ratio values, though when children urinated only once and urination occurred during the first hour of urine collection, mannitol recovery values were very low and, thus, L:M ratio values very high. Based on visual inspection of mannitol recovery versus timing of urination, a minimum mannitol recovery of 0.13% was set and L:M ratio values for mannitol recovery below that minimum were omitted from subsequent analyses. Plate-to-plate variability in serum and stool assays were also investigated based on replicates of control samples and central tendencies and spreads of the sample values, but no adjustment to the data based on that investigation was required.

Variable Creation

Socioeconomic status (SES) data assessed via a series of questions about household means and assets and the physical quality of the house structure were collapsed into a set of indices (e.g. living standards index, wealth index) that summarize various aspects of household SES, according to a PCA method previously developed for this study site.[32] For the purpose of this study, the living standards index (LSI) was used

to indicate household SES. Measures of supplement adherence were derived for daily supplement consumption summarized into an overall percent of total intended dose consumed during the six through 11 month period, 12 through 17 month period and for six through 17 months overall. Indices for SES and adherence were generated for the parent trial[4] and were integrated unaltered into the present analysis.

The dietary intake data were also handled according to methods used in previous analyses of the same data. A seven-group dietary diversity score was calculated according to guidelines published by the World Health Organization.[33] The food groups were (1) Grains and starchy staples, (2) Legumes and nuts, (3) Dairy, (4) Flesh foods, (5) Eggs, (6) Vitamin A-rich fruits and vegetables, and (7) Other fruits and vegetables. Foods were assigned to food groups according to the detailed WHO guidelines. Mixed dishes that contained foods from more than one food group were counted towards all of the food groups represented in its ingredients. "Junk foods" like cookies, cake and candy were not counted towards any food group. Dietary diversity score (DSS), with possible values ranging from 0 to 7, was calculated as the number of food groups consumed according to the past 24 hour recall data. Minimum dietary diversity (MDD) was defined as DDS ≥ 4, as per WHO guidelines.[33]

Exploratory Data Analysis

Analysis of the biomarker and field data began with exploratory data analysis, consisting of graphical and numerical displays of the central tendencies, spreads, and normality of the measured variables and relevant covariates from the JiVitA-4 dataset,

including child demographics, household SES and sanitation/hygiene measures, and child anthropometry. As many of the biomarker variables were not normally distributed, transformations were explored to improve normality and enable the use of parametric statistical methods in subsequent modeling.

Length and weight values measured at each time point were converted to age- and sex-specific z-scores based on the WHO Multicentre Growth Study reference data.[34] Absolute length and weight, length-for-age z-score (LAZ), weight-for-age z-score (WAZ) and weight-for-length z-scores (WLZ) were all explored for central tendencies and normality, and for trends over time. For each time point, stunting, underweight and wasting were defined as LAZ, WAZ and WLZ, respectively, < -2 relative to the reference median.

Most of the biomarkers used had accepted cutoffs to define elevated values, which we adopted, though not all of the cutoffs were specific to juvenile populations. In all of the subsequent analyses, L:M ratio greater than 0.07 was considered elevated based on previous studies.[35-37] For MPO, AAT and NEO, normative cutoffs for healthy adult populations were used (2000 ng/mL, 270 μg/mL and 70 nmol/L, respectively), as has been done in EED studies previously.[15,38] Cutoffs for elevated CRP and AGP values were 5 mg/L and 1 g/L, respectively, which are widely accepted values for children.[39] Normative values were not available for EndoCAb, GLP-2 and IGF-1, so they were reported and analyzed as continuous values only.

Outliers in distributions of log-transformed biomarker variables and length and weight and their z-scores were investigated. Visual examination of histograms and scatterplots with lowess smoothed trend lines were used to identify observations with extreme values that were responsible for inconsistent trends in the tails of bivariate distributions. Individual outlying observations were dropped from the analysis, but the other data for children with single outlying biomarkers values were retained. As stated previously, mannitol recovery and L:M ratio values for children with mannitol recovery <0.13% (n=26) were coded to missing. Lactulose recovery values >0.55% (n=4) were omitted. For the stool markers, MPO values <400ng/mL (n=16), AAT values <49µg/mL (n=11) or >5000µg/mL (n=3) and NEO values <180nmol/L (n=5) or >4000nmol/L (n=8) were omitted. For serum markers, GLP-2 values <0.67ng/mL (n=9) and EndoCAb <0.4mu/mL (n=3) were excluded. For anthropometric data, values that corresponded to z-score <-6 (max. n = 1 per time point) were excluded from all analyses.

Paper 1: Development of EED Score

The biomarkers being examined represented multiple interrelated and overlapping aspects of EED. Paper 1 sought to explore the relationships among the biomarkers, including with L:M ratio, to determine the set of markers that provided the most informative and efficient definition of EED, and the extent to which those markers approximated the L:M ratio. Additionally, the paper included descriptive analyses of the burden of EED in the study sample and the child and household sociodemographic and health characteristics that accounted for its distribution.

After exploring data for central tendencies and outliers and performing transformations, as described above, pairwise correlation coefficients between log-transformed biomarkers were explored. Two data reduction techniques, principal component analysis (PCA) and partial least squares regression (PLS), were used to create EED scores. PCA generates orthogonal linear combinations of variables ("factors") according to their joint variability,[40] which can then be used to generate scores, one per factor, for each participant's biomarker values by summing the product of each biomarker value and its respective loading for that factor. This method has been used previously to produce a score of EED biomarkers.[15] PLS analysis allows for the specification of one or more dependent variables along with the set of independent variables. The method generates linear combinations of independent variables, similar to those from PCA, but in PLS each factor maximizes the covariance between the dependent and independent variables and is orthogonal to all of the previously specified factors. The PLS factor(s) were also converted to scores for each participant by summing the products of the biomarker values, their factor loadings, and the absolute values of their factor weights. PLS was used as an auxiliary approach to PCA, as it may better identify groupings of candidate biomarkers that explain variability in the outcomes of interest, for example L:M ratio or anthropometric measures, as per the pre-specified hypotheses.

For the PCA analysis, MPO, AAT, NEO, GLP-2 and EndoCAb IgG were included as independent variables in all models. Continuous, log-transformed biomarker values were used and the correlation matrix option was selected to make the PCA procedure scale invariant. Varimax orthogonal rotation was used to generate independent

factors. Sensitivity analyses were also conducted for different treatments of the variables, including as continuous and quintiles of the untransformed distribution. Models were also tested with L:M ratio, lactulose recovery and/or mannitol recovery separately, CRP and/or AGP, and IGF-1 as additional independent variables. Each model was evaluated based on the number of variables that loaded on each factor and the strength of the loadings, as well as the total variability explained by 1- and 2-factor solutions. L:M ratio was not included in the final PCA procedure to accommodate the aim of evaluating the resultant scores relative to this commonly used indicator for EED. The number of retained components in the final model was determined using a combination of criteria, including the Kaiser criteria (Eigenvalues>1), scree plots and based on the factor loadings and number of variables loading on each factor.[41]

Two PLS models were developed, one with dependent variable continuous log-L:M and one with dependent variable continuous log-IGF-1. Models with dependent variable 18 month length and LAZ were also explored but ultimately not included in the final analysis. The analytic approach was very similar to that used for PCA: continuous log-transformed MPO, AAT, NEO, GLP-2 and EndoCAb IgG were included as independent variables in all models, with additional models developed with CRP, AGP and/or IGF-1 included as additional independent variables. Models with dependent variables continuous log-transformed lactulose and mannitol, in separate and combined models, were also explored. Only the PLS model with dependent variable log-L:M was ultimately included in subsequent analyses for paper 1.

After PCA and PLS models were finalized, scores for individual participants were calculated based on the factor loadings for PCA and loadings and weights for PLS. In the case of the PCA analysis, which generated a two factor solution, the two factor scores were each given a descriptive name, "Inflammation Score" (IS) for factor 1 and "Permeability Score" (PS) for factor 2, based on the biomarkers that loaded most strongly on them. Since the PLS analysis generated a one factor solution, it was subsequently referred to as the "PLS EED Score". Scores were standardized around their means and standard deviations and oriented such that they were positively correlated with L:M ratio, i.e. so that a higher value for each score was indicative of worse intestinal health. Scores were also shifted to have lower bound 0.

Receiver operating characteristic (ROC) analysis was used to determine optimal cutoffs for each EED score to differentiate those with and without EED. Dichotomous L:M ratio ($\leq$ vs. $>$ 0.07) was used as the reference test. The area under the curve (AUC) was used as a metric to compare the predictive ability of each score, and the sensitivities and specificities at the optimal cut-points, based on the Youden index[42,43] were compared.

The extent to which the EED scores and the individual biomarkers explained the variability in L:M ratio was also examined in regression models with continuous L:M as the dependent variable and continuous scores or biomarkers as the independent variables. Univariate models were developed for each biomarker, as were multivariable models with the complete biomarker panel included in one model. Multivariable models adjusted for child sociodemographic characteristics were also developed.

To evaluate associations between markers and child and household characteristics, simple linear regression models were developed for dependent variables L:M ratio, each PCA score and the PLS score and indicator variables for one variable from the set of child and household characteristics – sex, LSI, toilet type, inflammation (normal vs. elevated AGP and CRP) and stunting and wasting status.

This process and the details of the resultant scores form the basis of the first paper, which is presented as Chapter 4. Additionally, the EED scores developed according to these procedures were utilized in the subsequent explorations of EED associations with diet and CFS receipt (Chapter 5) and with anthropometry and growth (Chapter 6).

Paper 2: Diet Effects on EED

The associations between dietary intakes and EED, including the effect of CFSs on EED, were the focus of the second paper. Diet and breastfeeding were characterized using 24-hour recall data obtained during the 18 month interview. Dietary intake was summarized with food groups, dietary diversity score (DDS) and minimum dietary diversity (MDD, DDS≥4). Macronutrient and micronutrient intakes were also calculated based on reported foods and their quantities consumed in the 24-hour diet recall questionnaire. CFS effects on EED were assessed using assigned supplementation group and maternal reported daily compliance. Breastfeeding continuation and frequency were also examined in relation to EED.

EED was measured with the L:M ratio and the EED scores generated as described above. As was done previously with this data, L:M ratio was log-transformed prior to analysis and EED biomarkers were collapsed into an "inflammation score" (IS) and "permeability score" (PS) based on factors derived from PCA, and the "PLS EED Score" (PES) was calculated based on factor loadings and weights from PLS. L:M ratio was dichotomized using the cutoff of 0.07 and the EED scores were dichotomized around their medians. The distributions of the EED scores were also divided into quartiles and analyzed in logistic regression models as quartile four versus quartile one to confirm that models comparing the halves of the distribution did not mask trends in more extreme values. Log-transformed CRP and AGP were also examined as outcomes in separate models, but were not ultimately included in the manuscript due to lack of agreement with findings for EED.

Food group and nutrient intake summary statistics were explored and normality checked. Food groups, DDS and MDD were calculated as described above. Past 24-hour breastfeeding frequency was categorized based on maternal report in the 18 month interview into: 0 times (not currently breastfeeding), 1-10 times, 11-20 times and 21+ times. Nutrient intakes included nutrients from home foods and CFSs, based on assigned group and the percent of the daily portion reportedly consumed on the day corresponding to the diet recall (in those assigned to receive CFSs), while CFSs were not included in food group and dietary diversity calculations. Nutrient intakes did not include nutrients from breastmilk.

Factors that could potentially confound the observed associations between CFS assignment and EED, a particular concern due to the EED assessments taking place in a 10% subsample of the randomized trial participants only, were explored in simple linear, logistic and ordinal logistic regressions with standard errors adjusted for clustering by sector, the unit of randomization. Factors that differed by CFS group at baseline were retained as potential confounders in regressions of EED on CFSs.

To assess the effect of CFSs on EED, logistic regression models were developed for dependent variables elevated vs. normal L:M and high vs. low (i.e. above or below the median value) IS and PS. The PLS generated with dependent variable log-transformed L:M and dichotomized around its median value was also included in the investigation of CFS group and EED, but those results were not reported as they did not differ qualitatively from the models of the PCA-generated scores. Independent variables were indicator variables for CFS group (CP, PD, RL, WSB++ and CFC-only, the reference group). Models were also developed with independent variable any supplementation (any of the groups receiving CFSs) versus none. Analyses were done on an intention-to-treat basis. Models adjusted for potential confounders were also developed, adjusting for child sex and exact age at the 18 month interview and the household's LSI. Models adjusted for average compliance to daily CFS portion in the groups receiving CFSs were also explored, as were models adjusted for baseline (6 month) stunting status. All regression models had standard errors allowing for non-independence of observations within sectors (the unit of randomization), using the "vce(cluster *clustvar*)" option, with the sector identifier specified as the "*clustvar*".

Relationships between EED and dietary intakes were investigated with logistic regression models. The same dependent variables as above, elevated vs. normal L:M and high vs. low IS, PS and PES, were examined. Independent variables of interest were individual food group intakes, in univariate models and combined in one multivariable model, continuous DDS, dichotomous MDD, and breastfeeding frequency. Models were adjusted for child sex, exact age and LSI and the standard errors corrected for clustering by sector.

Linear regression models were developed for associations between continuous EED markers and total energy, macronutrient and micronutrient intakes. Total energy intake was divided by 100 prior to inclusion in models to improve the interpretability of the regression coefficients. Intakes of protein and fat were included in the models as the percent of total energy intake contributed by each. The percent of energy from carbohydrates was omitted from models due to collinearity with protein and fat contributions. Macronutrient and micronutrient intakes were included as independent variables in separate models for each nutrient and combined, with one model for macronutrients and one for micronutrients. All models were adjusted for child sex, exact age and LSI and the standard errors corrected for clustering by sector. Models of micronutrient intakes were also adjusted for total energy intake.

Paper 3: EED and Growth

Paper three examines associations between EED and anthropometric measures. As in prior analyses, EED was measured as continuous log-transformed L:M ratio and

continuous standardized values of the PCA-generated EED scores – the "inflammation score" (IS) and the "permeability score" (PS) and the PLS EED score, though models for the PLS score were not retained in the final analysis, as they did not differ qualitatively from models of IS and PS. Variables for quintiles of the distributions of L:M ratio, IS and PS were also developed. Anthropometric measures at 18 and 24 months were converted to length-for-age, weight-for-age and weight-for-length z-scores using the WHO Multicentre Growth Reference data,[34] and LAZ, WAZ and WLZ < -2 were classified as stunting, underweight and wasting, respectively. Anthropometric measures and their z-scores at 18 months, concurrent with the EED assessment, and the change in anthropometric measures and z-scores from 18 to 24 months, the six months following the EED assessment, were the main outcomes. The changes in anthropometric measures were calculated to account for the exact number of days between the anthropometric assessments. Risk of stunting, underweight and wasting at 18 and 24 months were also examined.

The growth data required extensive exploratory analysis prior to modeling. Individual growth trajectories were examined graphically, as were baseline characteristics predictive of divergent growth patterns. Then, linear and logistic regression models were developed to quantify relationships between EED and cross-sectional anthropometry at 18 months, prospective growth from 18 to 24 months, and stunting, underweight and wasting status at 18 and 24 months.

For cross-sectional associations between anthropometry and EED, linear regression models were developed with dependent variables length (cm), weight (kg),

LAZ, WAZ and WLZ at 18 months, separately for each EED marker – L:M ratio, IS and PS – as the independent variable. Unadjusted models were developed for initial examination of bivariate associations, and then models adjusted for child sex and age, household LSI and assigned supplementation group were developed. Models adjusted for baseline (6 mo) anthropometry were also explored, but were not ultimately presented, as associations between sequential anthropometric measures were quite strong and require further characterization prior to inclusion in these analyses.

To examine associations between EED and prospective growth, linear regression models similar to those described above were developed. Models were developed with dependent variables Δlength (cm), Δweight (kg), ΔLAZ, ΔWAZ and ΔWLZ, separately for independent variables L:M ratio, IS and PS. Unadjusted models and those adjusted for child sex and age at 18 month assessment, supplementation group and household LSI were explored. Models adjusted for baseline anthropometry were explored, but ultimately not presented, for the reasons described above and because no relationships were observed between baseline measures and magnitude of growth from 18 to 24 months.

Relationships between EED and dichotomous anthropometric indicators were also explored. Logistic regression models were developed for dependent variables odds of stunting, underweight and wasting at 18 and 24 months, separately for independent variables continuous L:M ratio, IS and PS, unadjusted and adjusted for the same child and household covariates as above. Models of 24 month anthropometric status were also adjusted for 18 month status, to more closely mirror the continuous models that capture change from 18 to 24 months. Interaction terms between 18 month stunting status and

EED marker were also included in models and tested for statistical significance to assess whether 18 month status modified the effect of EED on risk of stunting at 24 months. The same was done for interactions with underweight and wasting status at 18 months for models of the same at 24 months.

All analyses related to supplementation group were done on an intention-to-treat basis. The PLS function was implemented in Stata using the PLSSAS command, which calls the PLS procedure in SAS (SAS, version 9.4, SAS Institute Inc., Cary, NC). All other analyses were conducted using Stata, version 14.1 (StataCorp, USA).

Tables and Figures

Table 3.1. Schedule of JiVitA-4 trial interviews and assessments

Method	Data Category	Child's Age in Months					
		6	**9**	**12**	**15**	**18**	**24**
Anthropometry	Length, weight, arm/head/chest circumferences	X	X	X	X	X	X
Interview	Diet	X	X	X	X	X	X
	Breastfeeding						
	Immunization History						
Interview	Socioeconomic status (SES)	X					
Interview	Supplement adherence		Twice weekly				
Interview	Morbidity	X	Twice weekly				X

Table 3.2. Reference table for chlorhexidine volume added to collected urine sample by total weight of urine

Urine weight (g)	Chlorhexidine (µL)
<20	10
20-49	20
>250	30

Table 3.3. Coefficients of variation (CV) for each biomarker included in the assessment of environmental enteric dysfunction (EED)

Biomarker	Intra-assay CV (%)	Inter-assay CV (%)
Lactulose	-	3.61
Mannitol	-	9.35
Myeloperoxidase (MPO)	5.4	11.1
α-1 antitrypsin (AAT)	9	20.3
Neopterin (NEO)	11.3	23.5
Endotoxin core antibody (EndoCAb) IgG	29.1	19.2
Glucagon-like peptide-2 (GLP-2)	8.1	15.4
C-reactive protein (CRP)	-	8.6
α-1 acid glycoprotein (AGP)	-	16.0
Insulin-like growth factor -1 (IGF-1)	-	8.1

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Chapter 4: Defining environmental enteric dysfunction in children enrolled in a randomized controlled trial of complementary food supplementation in rural Bangladesh

Abstract

Background: Environmental enteric dysfunction (EED) may impair growth and development in Bangladesh and other low- and middle-income countries where the prevalence of childhood stunting is high, but methodologic challenges limit its study. ***Objective:*** We aimed to relate a panel of blood and stool biomarkers to the commonly considered gold standard urine-based lactulose:mannitol ratio (L:M) test and describe the burden and distribution of EED in rural Bangladesh. ***Methods:*** In 539 18-month-old children enrolled in a substudy of a randomized supplementation trial, serum, stool and urine (following dosing with the dual sugars) were collected. Lactulose and mannitol recovery in urine were determined by high pressure ion chromatography. Stool was analyzed for intestinal inflammation markers myeloperoxidase (MPO) and neopterin (NEO) and intestinal permeability marker α-1 antitrypsin (AAT). Serum was analyzed for permeability marker endotoxin core antibody (EndoCAb) IgG, enterocyte proliferation marker glucagon-like peptide-2 (GLP-2) and systemic inflammation markers C-reactive protein (CRP) and α-1 acid glycoprotein (AGP). Principal component analysis (PCA) and partial least squares regression (PLS) were used to derive orthogonal factors (linear combinations) of biomarkers that were converted to EED scores for each participant.

Associations between each score and the L:M ratio, and differences in mean L:M ratio and EED scores by child health and sociodemographic characteristics, were evaluated with regression models. ***Results:*** The prevalence of EED based on L:M ratio (>0.07) was 39.0%. CRP (>5 mg/L) and AGP (>1 g/L) were elevated in 20.4% and 57.0% of participants, respectively. Correlations between intestinal biomarkers were low, ranging from 0.01 between MPO and NEO to 0.33 between MPO and AAT. In multivariable regression models, the biomarker panel explained 6.0% of variability in L:M ratio; PCA-generated scores explained 2.3% whereas the PLS-derived score with L:M ratio as the dependent variable explained 5.2%. EED indicators were not consistently associated with child or household risk factors. ***Conclusions:*** EED is common in this setting of prevalent stunting, but a panel of serum and stool biomarkers were poor predictors of the L:M ratio. The biomarker panel may have measured a broader spectrum of morphologic and functional changes characteristic of EED than L:M ratio alone measures, contributing to low agreement. An improved measure appropriate for field settings is necessary to definitively diagnose EED.

Introduction

Environmental enteric dysfunction (EED), a subclinical perturbation in the structure and function of the wall of the small intestine, has received abundant attention in recent years as a potential cause of persistent stunting in children living in low- and middle-income countries (LMIC) around the world.[1-4] EED is characterized by villous

atrophy, crypt hyperplasia, leaky tight junctions and enteric immune cell proliferation,[1,5] thought to result from chronic exposure to environmental pathogens and toxins.[1,3] Observational studies have reported nearly universal EED in low resource settings,[6-9] and some studies have found strong inverse associations between EED and length or rate of linear growth,[9-14] suggesting this may be a critical and underappreciated contributor to stunting.

EED may inhibit growth through two main pathways: malabsorption and systemic immune activation.[1,3] Villous atrophy and crypt hyperplasia reduce the absorptive capacity of the small intestine by reducing the surface area for secreting digestive enzyme and absorbing nutrients. This may cause inadequate nutrient availability and stunting, especially as EED commonly occurs in settings where children also face marginally adequate dietary intakes. Leaky tight junctions, another characteristic of EED, may allow for translocation of enteric pathogens into the blood stream, leading to systemic inflammation that sequesters nutrients and redirects them toward launching an immune response. Elevated acute phase proteins have been observed in children with no acute illness but with evidence of EED[11] and in LMIC children more broadly, and could be an important mechanism whereby EED contributes to growth faltering.[1,11,15,16]

Assessing the burden of EED and quantifying its contribution to stunting in the most affected regions continues to be inhibited by the available assessment tools. The true gold standard diagnostic test is an intestinal biopsy, which is not feasible in field settings and in otherwise well children,[2,17] especially in the absence of a proven therapy for identified cases. In place of biopsies, the dual sugar absorption test is widely used as a

quasi-gold standard, but no formal validation exists of the test for this application.[18,19] The dual sugar test, typically lactulose and mannitol (L:M), is also difficult to implement in field settings, as it requires fasting subjects, collection of uncontaminated urine samples over an extended period (usually 2-5 hours), and a cold chain for sugar solution doses and urine samples. Studies have also found it to perform unreliably, with high variability and inconsistent associations with other markers and predictors of intestinal health.[19] Further, the L:M test measures only intestinal permeability and malabsorption, which does not capture the full EED syndrome (Figure 4.1). Other markers of EED in serum and stool have been proposed for use in addition to or in place of the L:M test,[2,4,13] but, at present, very limited data are available for the performance of each.

The lack of validated, feasible and reliable biomarkers presents a major limitation in the study of EED, its causes, consequences and best strategies for intervention. This study aimed to assess a comprehensive panel of EED biomarkers in 18-month-old children in rural Bangladesh, a setting with very high prevalence of stunting, to determine the most efficient and informative subset of markers for diagnosing EED. A secondary aim was to describe the burden and distribution of EED in early childhood in this setting.

Methods

Setting This study was undertaken in the Gaibandha and Rangpur districts of northwestern Bangladesh, an area that is rural and densely populated, with predominantly agricultural livelihoods and small household landholdings. Assessment of EED was

nested as a substudy within a community-based cluster randomized controlled trial of complementary food supplements (CFS) for children ages 6-18 months of age, which demonstrated benefits for linear growth and prevention of stunting.[20] The CFS trial enrolled 6-month-old children between August 2012 and April 2013, and follow-up continued through May 2014. Participants were assigned to one of five study arms: child feeding counseling only or counseling plus one of four CFS formulations. CFSs were distributed to the household for daily consumption as a snack in addition to usual breastfeeding and complementary foods for a full year from age 6 to 18 months. Daily adherence to supplementation and morbidity symptoms were assessed in biweekly home visits, while interviews including child anthropometry were conducted at enrollment (age 6 months) and ages 9, 12, 15, 18 and 24 months.

Participants In a geographically designated substudy area, a blood sample for serum was collected at the end of supplementation (age 18 months) to measure biochemical nutritional status. All enrolled children living within this area were eligible for the substudy and approached for an additional written parental consent. EED assessment eligibility required enrollment in the parent study and date of birth between March 14, 2012 and September 13, 2013.

Sample Size A target sample size of 500, 100 per study arm, was set based on the ability to detect a difference in L:M ratio by supplementation group. At a power of 80% and type I error of 5% and using the standard deviation in L:M ratio from a pilot study in the same cohort (σ=0.644), this sample size was predicted to allow for the detection of a minimum difference of 0.46 (0.72*σ) in mean L:M ratio between each supplementation

group and the control group, assuming equal variance in all supplementation groups and adjusting for multiple comparisons between each supplemented group and the control.

Biomarker Panel The assessment of EED involved administering the L:M test of intestinal permeability and collecting serum, urine, and stool samples, which were then analyzed to produce a panel of intestinal health biomarkers.

The L:M test requires administering orally a solution containing known quantities of lactulose and mannitol, and then measuring the recovery of each in urine collected over a set collection period. Lactulose is not absorbed transcellularly, so its detection in urine indicates excess intestinal permeability, while mannitol is absorbed in proportion to absorptive surface area in the intestine, such that the ratio of the recovery of the two in urine captures the permeability of the small intestine, scaled for its overall size or absorptive capacity.[18,19]

In addition to the L:M test, serum and stool biomarkers of EED were selected based on the existing literature as follows (Figure 4.1). Myeloperoxidase (MPO), α-1 antitrypsin (AAT) and neopterin (NEO) are measured in stool and are widely used to assess disease activity in inflammatory bowel and celiac diseases.[21] MPO and NEO are markers of immune cell activity in the walls of the small intestine.[21,22] AAT circulates in the bloodstream bound to protein; measured in stool, it is indicative of protein-losing enteropathy, a type of excess intestinal permeability.[23] These stool markers have been used infrequently in assessments of EED, but are gaining popularity.[2] One study from 1996-7 used NEO along with L:M ratio and found an inverse association between NEO

and subsequent height and weight gains, but no association between L:M ratio and NEO.[22] A more recent study using a score created from MPO, AAT and NEO found associations with linear growth, but did not compare the individual markers or the score to L:M ratio.[13]

Endotoxin core antibody (EndoCAb) is produced in response to endotoxins in circulation, which are generally of intestinal origin in the absence of another acute illness, and remains in circulation after the endotoxins themselves are cleared.[11] EndoCAb measured in serum is an indicator of intestinal permeability that has been one of the more common alternates to L:M ratio in EED studies. Several studies have demonstrated associations between EndoCAb and L:M ratio[11] and EndoCAb and growth,[11,14] while others have failed to find associations.[24,25]

Serum glucagon-like peptide-2 (GLP-2), which, to our knowledge, has not been used previously in EED assessment, is a peptide hormone that induces the crypt cells of the intestinal wall to proliferate and that may be elevated following damage to the small intestine, as in EED.[26]

C-reactive protein (CRP) and α-1 acid glycoprotein (AGP), acute phase proteins, and insulin-like growth factor-1 (IGF-1), a growth-regulating hormone, were also evaluated to provide information about systemic health.

Field Methods Participants received 2 mL of L:M solution – composed of 50 mg mannitol powder (Sigma-Aldrich, St. Louis, MO, USA) and 255 mg lactulose in 0.375 mL lactulose syrup (Square Pharmaceuticals, Dhaka, Bangladesh) per mL – per kg body

weight up to a maximum of 20 mL. Children were fasted, aside from breastmilk, for 2 hours prior to and 30 minutes following dosing, and were not allowed to consume fructose (fruit, fruit juice or candy) for the duration of the urine collection period. Urine was collected for two hours, or until the child produced a minimum of 20 g of urine at least one hour after dosing, up to a maximum of three hours of total urine collection time. Urine was mixed with chlorhexidine (a disinfectant) and aliquoted for transport and storage.

Blood was collected by venipuncture into blood collection tubes free of anti-coagulants, allowed to clot and then transported in a cold box to the project field laboratory, where it was centrifuged and the serum transferred to cryovials and stored in liquid nitrogen pending shipment to the Johns Hopkins Center for Human Nutrition (CHN) laboratory (Baltimore, MD).

Stool collection took place in the household. Cold boxes, ice packs and stool collection kits (sterilized cup, spoon, and mat) were delivered to participating households and stool samples were retrieved promptly by field workers and returned in cold boxes to the field laboratory. Samples that were too old (defecation occurred on the previous day), not collected from the supplied sterilized mat, or not stored in the cold box within 30 minutes of defecation were discarded and rescheduled. Upon receipt by the field laboratory, stool samples were stirred to homogenize, aliquoted into cryovials and stored in liquid nitrogen pending shipment to JHSPH. All biospecimen collections were rescheduled for children with high fever or diarrhea/dysentery as reported by the mother.

Laboratory Methods Urine was analyzed for concentrations of lactulose and mannitol by high pressure ion chromatography (Dionex, Thermo Fisher Scientific, Sunnyvale, CA, USA) by a collaborating laboratory at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) in Dhaka, Bangladesh. Standard solutions with known dilutions of lactulose and mannitol and uniform quantities of a control sugar, melibiose, were used for quality control purposes. From the resultant sample concentrations of each sugar, L:M recovery ratio was calculated by multiplying each concentration by the total weight of urine collected and dividing by the weight of each sugar in the initial dose to determine the percent recovery of each. The final test result for each child was expressed as the ratio of percent lactulose recovery to percent mannitol recovery, henceforth referred to as "L:M ratio".

Stool concentrations of MPO, AAT and NEO were assessed in the CHN lab using commercial ELISA kits (MPO and NEO: ALPCO Diagnostics, Salem, NH; AAT: BioVendor, LLC, Asheville, NC). Participant samples were diluted 1:50 by weight in wash buffer, centrifuged and then the supernatant centrifuged again in an Eppendorf centrifuge. Resulting supernatant was then aliquoted and frozen. At the time of analysis, aliquots were thawed and diluted with wash buffer at a ratio of 1:10, 1:500, and 1:2.5 for MPO, AAT and NEO, respectively.

Serum assays were also conducted in the CHN lab. EndoCAb IgG and GLP-2 were measured with commercial ELISA kits (EndoCAb IgG: Hycult Biotech, Plymouth Meeting, PA, USA; GLP-2: EMD Millipore, St. Charles, Missouri, USA). CRP and IGF-1 were assessed with chemiluminescent immunoassay (Immulite 2000, Siemens

Diagnostics, USA) and AGP by radial immunodiffusion (Kent Laboratories, USA). In serum and stool assays, standards and controls provided with the assay kits along with at least two participant-derived control samples were run in duplicate on each plate to monitor plate performance and reliability.

Covariate Assessment Extensive child and household data were collected within the main supplementation trial. Socioeconomic status (SES) was assessed via a questionnaire administered during the enrollment interview that included details about household membership, assets and possessions, the quality of the physical house structure and sanitation facilities. Demographic and education details for all household members were also recorded in that questionnaire, from which the education of the child's mother was determined. Anthropometric measurements were taken at enrollment (age 6 months) and at ages 9, 12, 15, 18 and 24 months by trained and standardized anthropometrists. Daily morbidity symptom information was elicited from the mother in twice-weekly field worker visits to the household over the duration of the supplementation period.

Statistical Analysis Exploratory data analysis was undertaken to describe the distributions and central tendencies of the measured biomarkers and their values with respect to accepted cutoffs. L:M ratio greater than 0.07 was considered elevated and indicating EED.[7,27,28] For MPO, AAT and NEO, normative cutoffs for healthy adult populations were used (2000 ng/mL, 270 μg/mL and 70 nmol/L, respectively), as has been done previously.[13,29] Cutoffs for elevated CRP and AGP values were 5 mg/L and 1 g/L, respectively, which are widely accepted values for children.[30] Biomarker values were log transformed and central tendencies were reported as geometric means and 95%

confidence intervals. Log-transformed biomarkers were examined individually in histograms and in pairs in scatterplots with lowess smoothed trend lines. Outlying values were identified based on extremeness and deviation from bivariate trends and dropped from the analysis.

SES indicators were used to create a living standards index (LSI) and dichotomized around the internal median value of the index, as has been done previously for this cohort.[31] Maternal education was categorized as none, 1-9 years, SSC (secondary school completion exam) passed, or 11+ years. Sanitation facilities were characterized as improved (water sealed or slab) versus not. Morbidity episodes were defined as any reported instance of diarrhea, dysentery or pneumonia (concurrent cough, fever and difficulty breathing) separated by at least two asymptomatic days. Sex- and age-specific length and weight z-scores were calculated based on international standards.[32] Stunting and wasting were defined as length-for-age z-score and weight-for-length z-score <-2 relative to their respective reference medians.

To evaluate agreement and redundancy within the biomarker panel, Pearson correlation coefficients between log-transformed L:M ratio (log-L:M) and log-transformed intestinal health biomarkers and between pairs of log-transformed biomarkers were examined. Two empirical data reduction techniques were used to describe the joint variability within the biomarker panel with the aim of creating an EED score: principal component analysis (PCA) and partial least squares regression (PLS). PCA generates orthogonal linear combinations of variables according to their joint variability,[33] and has been used previously to produce a score of EED biomarkers.[13]

MPO, AAT, NEO, GLP-2 and EndoCAb IgG were included as independent variables in all PCA models. Additional models were tested with L:M ratio, lactulose recovery and/or mannitol recovery separately, CRP and/or AGP, and IGF-1 included as independent variables. Each model was evaluated based on the number of variables that loaded on each factor and the strength of the loadings, as well as the total variability explained by 1- and 2-factor solutions. L:M ratio was not included in the final PCA procedure based on the aim of evaluating the resultant scores relative to the L:M ratio. Biomarkers were included in the PCA model as continuous, log transformed values. The correlation matrix option was used to make the PCA procedure scale invariant. Varimax orthogonal rotation was used to generate independent factors. The number of retained components in the final model was selected based on the number of variables loading on each factor and the total variability explained.

Partial least squares (PLS) regression analysis allows for the specification of one or more dependent variables along with a set of covariates. PLS generates linear combinations of explanatory variables such that the first factor maximizes the covariance between the explanatory and outcome variables, and subsequent factors are orthogonal to the previous factor(s) and maximize the remaining covariance.[34] The PLSSAS command, which calls the PLS procedure in SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) from within Stata, was used. The same biomarkers were used in the PLS models: MPO, AAT, NEO, GLP-2 and EndoCAb IgG, with dependent variable L:M ratio. Additional models were developed that included CRP, AGP and/or IGF-1 in the set of independent variables.

Once the PCA and PLS models were finalized, the resultant factors were converted to EED scores. Scores were calculated for each child with complete biomarker data, separately for each retained factor in the final PCA model, by summing the product of the individual biomarker values and their respective factor loadings. Scores were calculated for each child for each retained factor in the final PLS model by summing the product of the individual biomarker values, their respective factor loadings and the absolute values of their factor weights. Scores were named for clarity in subsequent analyses based on interpretation of their highest loading biomarkers. PCA and PLS-derived scores were standardized around their means and standard deviations, oriented such that higher values are indicative of more extensive EED (i.e. positive correlation with L:M ratio) and then shifted to have lower bound zero. Associations between scores and continuous L:M ratio were evaluated in linear regression models. Receiver operating characteristic (ROC) curves were used to evaluate the ability of the PCA and PLS-generated scores to predict EED status as defined by L:M ratio. The area under the ROC curve (AUC) is an indicator of the ability of the test to distinguish a child with versus without EED as defined by L:M ratio, with possible values ranging from 0.5 (no discriminatory power) to 1.0 (perfect discriminatory power).[35] The optimal cutoff point for each factor was determined using the Youden index,[36,37] and maximum sensitivity and specificity compared.

To examine relationships between L:M ratio and the intestinal health biomarkers, simple and multivariable linear regression models were developed with continuous log-L:M as the dependent variable and the intestinal health biomarkers as independent

variables, singly and together. Multivariable models were also developed that adjusted for child and household characteristics. Models of the same form were developed for lactulose recovery and mannitol recovery as dependent variables in separate models. To assess differences in EED marker values by child and household characteristics, regression models for L:M ratio, PCA scores and PLS score each as a continuous dependent variable were developed separately for each child/household characteristic, with indicator variables for child sociodemographic characteristics – sex, LSI, toilet type (improved/not), inflammation – normal/elevated AGP and CRP, and stunting and wasting status.

Protocols were approved by the Institutional Review Boards of the Johns Hopkins Bloomberg School of Public Health and icddr,b. Written consent from the mother or primary caregiver was required for participation. All analyses were conducted in Stata (StataCorp, College Station, TX, USA), version 14.

Results

Of all eligible children (n=566), parents of 27 children (4.8%) refused the EED assessment, yielding a consenting sample size of 539 children. Of those, serum, stool and urine samples were collected from 509 (94.4%), 515 (95.6%) and 434 (80.5%) children, respectively. The mean (SD) age of children was 18.3 (0.3) mo (Table 4.1). Rates of stunting and wasting were high at 45.0% and 14.6%, respectively. Morbidities were common: 71.2% and 77.9% of participants reported at least one episode of diarrhea and

dysentery, respectively, and 44.9% reported pneumonia in the previous year. Evidence of inflammation was also widespread: 20.4% of children had elevated CRP (>5 mg/L), while 57.0% had elevated AGP (>1 g/L) (Table 4.2). Children enrolled in the EED assessment were largely similar to those enrolled in the main trial only based on a number of baseline characteristics, though some indicators of socioeconomic status appeared to be somewhat higher in the EED assessment children (Appendix Table A2.1), most likely as a result of the geographic designation of the substudy area.

The geometric mean of the lactulose:mannitol (L:M) ratio was 0.06 (95% CI 0.05, 0.06) and L:M ratio was elevated (>0.07) in 39.0% of children (Table 4.2). Stool markers of enteric inflammation were also elevated, although the prevalence differed by marker; it was 84.3% for MPO, 55.5% for AAT and 100% for NEO. Correlations between continuous log-transformed L:M ratio and log-transformed stool biomarkers were modest: 0.15 for MPO and AAT and 0.11 for NEO (p-values<0.05, Table 4.3). Mean MPO was higher in children with elevated L:M relative to those with normal L:M (p=0.02), while mean AAT and NEO trended toward higher concentrations (p=0.03 and 0.10, respectively) in those with elevated L:M (Table 4.2). Neither EndoCAb IgG, a marker of microbial translocation, nor GLP-2, a marker of epithelial repair, was correlated with continuous L:M ratio. Mean EndoCAb IgG was somewhat lower in children with elevated versus normal L:M (p=0.10), while GLP-2 did not differ by L:M status. Both CRP and AGP, markers of systemic inflammation, were positively correlated with L:M ratio (r=0.12 and r=0.07, respectively) and mean values of both were higher in

children with elevated L:M, but neither reached statistical significance (Tables 4.2 and 4.3).

Correlations between log-transformed biomarkers ranged from 0 to 0.54. The highest correlation coefficient between intestinal health biomarkers was between MPO and AAT ($r=0.33$, $p<0.01$) and most other correlations were 0.10 to 0.15 or below (Table 4.3). MPO, AAT, NEO and CRP were positively associated with L:M ratio in simple linear regression models, and all but CRP remained significant in multivariable models with all biomarkers included as independent variables (Table 4.4). The biomarker panel explained 6% of the observed variability in L:M ratio, while the fully adjusted model including child sex, baseline stunting status and household LSI had $R^2=0.16$. Minimal attenuation of regression coefficients in the multivariable model suggested low collinearity between biomarkers. AAT and, marginally, NEO and CRP were associated with lactulose recovery, while none of the included biomarkers was associated with mannitol (Appendix Table A2.2).

Principal component analysis (PCA) generated a 2-factor solution (Table 4.5). The first factor had highest loadings for AAT (0.69) and MPO (0.59) and moderate loading in the negative direction for GLP-2 (-0.37), and explained 27.5% of the total biomarker variability. This factor, when converted to a score according to the biomarker loadings, was named the "inflammation score" or "IS". The second factor had high positive loading for EndoCAb IgG (0.73), moderate negative loading for GLP-2 (-0.51) and moderate positive loading for NEO (0.46); it independently explained 21.8% of the total biomarker variability. The second factor, when converted to a score, was named the

"permeability score" or "PS". Together the PCA-generated factors explained only 2.3% of variability in L:M ratio. The PCA was repeated to include CRP and AGP in the set of biomarkers, but both systemic inflammation markers loaded strongly on their own factor only and the percent of total biomarker variance explained declined to 23.6% and 20.1% for factors 1 and 2, respectively, suggesting minimal shared variability between the markers of systemic and intestinal inflammation (Appendix Table A2.3).

Partial least squares (PLS) regression with dependent variable log-L:M produced a 1-factor solution with high loadings and weights for MPO and AAT (loading (weight): 0.71 (0.65) and 0.62 (0.61), respectively), moderate loading/weight for NEO (0.32 (0.48)), and negligible loadings and weights for GLP-2 and EndoCAb IgG (Table 4.5). The PLS-generated factor explained 23.8% of variability in the set of biomarkers and 5.2% of variability in L:M ratio. Adding CRP and AGP suggested lack of shared variability as in the PCA approach and was not retained for subsequent analyses (Appendix Table A2.3). The final PLS factor, when converted to a score, was termed the "PLS EED score" or "PES".

The AUC was 0.56 and 0.52 for the inflammation score and permeability score, respectively (Figure 4.2). The AUC for the PLS EED score was only slightly better at 0.57. At the optimal cutoffs for each score, sensitivity and specificity were 0.70 and 0.41 for the PCA inflammation score, 0.20 and 0.88 for the PCA permeability score, and 0.72 and 0.44 for the PLS EED score. A linear combination of the two PCA scores based on coefficients from a logistic regression analysis with elevated vs. normal L:M ratio as the dependent variable was used to estimate the discriminatory ability of the two scores

together. The AUC for the joint score was only 0.56, with sensitivity 0.54 and specificity 0.59 at the optimal cut point, suggesting no additional predictive power from combining the two scores relative to the inflammation score alone and a slight improvement in specificity but large loss in sensitivity.

When exploring EED scores by levels of child characteristics, the geometric means of the L:M ratio were higher in females ($p=0.05$) and in those of low SES ($p<0.001$). Children with elevated CRP had higher geometric mean L:M ratio compared with those with normal CRP (0.07 vs. 0.06, $p=0.03$), while L:M ratio did not differ by AGP status or by stunting or wasting status (Table 4.6). The PCA-generated inflammation score did not differ by any of the examined child/household characteristics, while the permeability score had slightly higher mean value in children from households with unimproved toilets relative to those with improved toilets (3.16 vs. 2.92, $p=0.06$), significantly higher mean value in those who were stunted at 18 mo vs. not (3.11 vs. 2.85, $p=0.01$), and lower mean value in those with high CRP vs. normal CRP (2.75 vs. 3.02, $p=0.02$) (Table 4.6). The permeability score did not differ by AGP status or wasting status, or by child sex or household SES. The PLS EED score had higher mean value in children with elevated AGP vs. normal (2.92 vs. 2.70, $p=0.02$), but marginally lower mean value in wasted children relative to non-wasted children (2.63 and 2.86, $p=0.09$). The PLS EED score did not differ by household SES or toilet type or by child sex or CRP or stunting status. Similar trends were observed when examining individual biomarkers by levels of child characteristics (Appendix Table A2.4).

Discussion

In 18-month-old children enrolled in a randomized controlled supplementation trial in rural northwest Bangladesh, environmental enteric dysfunction (EED) as measured by elevated L:M was observed in over 40% of participants. Contrary to expectations, a panel of serum and stool intestinal health biomarkers demonstrated low agreement internally and with L:M ratio. Biomarker scores developed with principal component analysis (PCA) and partial least squares regression (PLS) suggested stable groupings of the biomarkers based on their common variability, but none of the scores even closely approximated EED as reflected by an elevated L:M ratio. Taken together, the results suggest the biomarker panel suffered from high variability and lower than expected associations with L:M ratio, raising questions in part about the ability of either L:M or a carefully selected set of serum and stool biomarkers to reliably identify cases of EED.

The observed prevalence of EED based on elevated L:M ratio is in line with findings from other studies in similar settings and age groups. For example, prevalence of elevated L:M ratio, using the same 0.07 cutoff, was estimated to be 71% in the non-malnourished reference population in an urban Bangladesh cohort ages 6-24 months,[7] while in urban Nepal, 92% of children ages 0-60 months had elevated L:M values, using a cutoff of 0.12.[6] Studies in The Gambia have also consistently reported rates of elevated L:M near 100% using the 0.12 cutoff in children under age 5 years,[5] while some studies in South Africa and Latin America have found much lower rates of EED.[19] Thus, while

objectively a high prevalence, our observed EED burden is somewhat lower than in several other studies in South Asia, though values may be expected to rise through age 2 years before gradually returning to normal values over the course of childhood.[38] It should be noted that the handling of the L:M data, for example whether the reported value was adjusted based on the administered dose, whether the reported mean is an arithmetic or geometric mean,[39] and the choice of cutoff for abnormal L:M value may all affect the observed prevalence of EED.[19] Aside from those methodologic considerations, a somewhat lower burden of EED in this rural Bangladesh context is still plausible, as access to improved sanitation facilities and relatively clean tubewell drinking water are common, in contrast to many other LMIC contexts. Still, some sanitation and hygiene limitations persist, which have been associated with EED in a prior study in several districts of Bangladesh,[40] and the burden of diarrhea and the extent of pathogen and toxin exposures in this setting are all quite high.[41-43] It should also be noted that the parent trial could have had some positive impact on the risk of EED, by improving nutritional status, and, even in the control arm, through child feeding counseling messages regarding hand washing, soap use and food handling hygiene, as well as appropriate complementary foods and feeding practices.

The PCA and PLS approaches were employed as data reduction techniques to capture joint variability within the panel of biomarkers, as a strategy to identify the most informative biomarkers and their optimal combination in a score or set of scores. PCA has been used extensively in related fields, for example for creating indices of SES[31,44] and dietary patterns,[45-47] and has also been used previously for developing a score of

EED.[13] PLS has the advantage of allowing for specifying a dependent variable or dependent variables of interest,[34] which addresses one potential limitation of a PCA-derived solution, namely that low agreement between the PCA score(s) and L:M ratio could be due to a divergence between the combination of markers most related to each other (i.e. explaining the most common variance) and the combination most related to L:M ratio. In the present data, while the PLS score demonstrated somewhat better agreement with L:M ratio than the PCA scores did, both were poor predictors of L:M ratio, suggesting that little is gained by using the less well known PLS method.

The groupings of biomarkers revealed in the PCA and PLS processes are still perhaps illustrative despite low joint variability and low agreement with L:M ratio. The PCA produced a two factor solution, where MPO and AAT loaded most strongly in the positive direction on the first factor, EndoCAb and NEO loaded positively on the second factor, and GLP-2 loaded negatively at approximately half strength on both factors. The PLS generated a one factor solution, with strong loadings and weights for MPO and AAT and weaker loading and weight for NEO, all in the positive direction. The PLS score is notably similar to the EED score generated in the multi-country MAL-ED study.[13] The PCA results are somewhat more complex to interpret. MPO and AAT are clearly the most closely related of the biomarkers, despite MPO and NEO both being indicative of enteric inflammatory processes, while AAT and EndoCAb are related to intestinal permeability. It may be that the split between MPO/AAT and NEO/EndoCAb is related to different relevant time frames for the different markers, but further research on the individual markers is required to confirm that hypothesis. GLP-2 has not, to our

knowledge, been used previously in combination with these biomarkers or for the study of EED, but it is consistent with our understanding of it as a marker of enterocyte proliferation and repair that the resolution or absence of the processes captured in each PCA-based score is associated with increased proliferative activity, whether normal turnover and growth in the absence of illness or replacement of damaged cells following an inflammatory insult.

It is also worth highlighting that while inflammation was common in this sample, neither AGP nor CRP was closely related with L:M ratio or the intestinal health biomarkers or scores. This is in contrast to hypotheses linking EED to growth by way of systemic inflammation,[3,16] and suggests that other causes of systemic inflammation may be more prominent in this population. The primary cause(s) of elevated CRP and AGP in this sample requires further investigation in and of itself, and the lack of association between EED and the acute phase proteins must be considered when linking EED to growth in this setting, as we aim to do in a subsequent analysis of this data.

The biomarker panel and resultant scores may have differed from L:M ratio because of differences in their underlying biology, but a lack of precision in the measurement of the set of biomarkers, indicated by large standard deviations relative to mean values, likely also contributed. Some recent studies have questioned the validity of the L:M test, in particular suggesting that mannitol may be an inappropriate sugar for this purpose,[2] based on prior evidence that it can be found in the urine of un-dosed, fasting patients,[48] and that an alternate analytic method to HPIC is necessary to accurately determine lactulose concentration.[49] Furthermore, the L:M ratio is thought to measure

only two main aspects of the intestinal pathology characteristic of EED, increased permeability and reduced absorptive capacity, while the panel of serum and stool biomarkers was selected deliberately to include markers covering a broader spectrum of EED effects. Thus, some portion of the low agreement between the markers and L:M ratio may be due, by design to some extent, to their capturing different underlying processes. Unfortunately, without a better gold standard diagnostic test for EED, it is not possible to determine the extent to which other markers are providing additional information about EED status where they diverge from L:M ratio.

The present study had several strengths including the broad panel of candidate biomarkers that were assessed in combination with L:M ratio. Further, we were able to obtain and analyze biospecimens from an unusually large sample of children. The setting of the study nested within a large randomized trial also allowed for enrollment of participants from an enumerated source population, which, along with the very high participation rate, suggests a low risk of bias and good generalizability to rural Bangladesh and perhaps even more broadly to other rural South Asian populations.

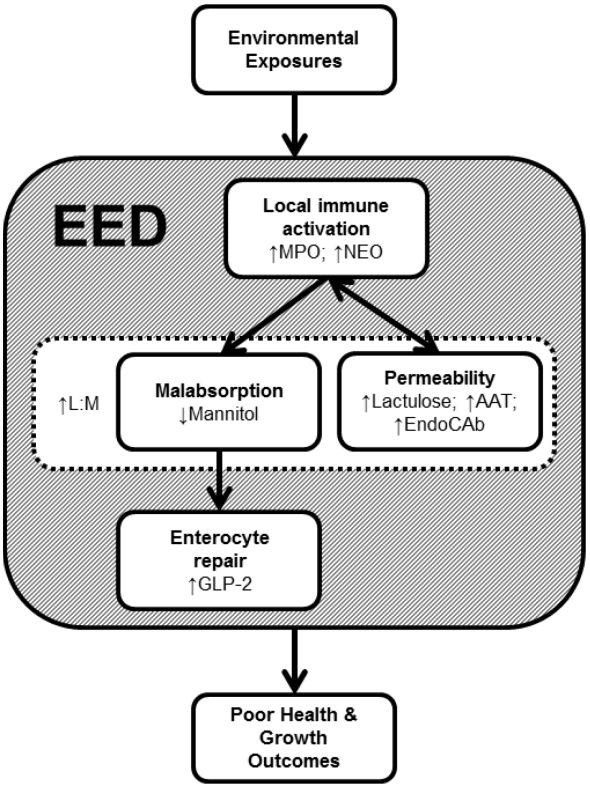
Some weaknesses include that biospecimens were collected at one time point only, a tradeoff we accepted for a larger sample size, with the idea that more observations at a single time point would provide more data about the interrelationships and agreement among the set of biomarkers. We were also limited in the lack of appropriate normative data for several of the serum and stool biomarkers, as much remains unknown about their characteristics and performance under various normal conditions, a gap in knowledge that carefully conducted clinical studies may be most efficient in addressing. The main

weakness that must be mentioned is the lack of a reliable gold standard measure of EED, a current limitation in this area of study more generally. In the absence of a true gold standard appropriate for this type of field setting, it is not possible to fully characterize other biomarkers in terms of their diagnostic properties.

In a large study characterizing EED in 18-month-old children participating in a randomized controlled feeding trial in rural Bangladesh, EED prevalence exceeded 40%. A panel of biomarkers thought to capture different aspects of the EED syndrome revealed two distinct processes or aspects when analyzed for common variability using PCA and PLS, but low agreement between EED scores and L:M ratio inhibited their interpretation. Because the L:M ratio is of unknown accuracy and likely poor precision for EED, the EED scores may capture meaningful aspects of EED not measured by L:M ratio, or they may be too variable or non-specific to be good alternate diagnostic tools. Developing a better gold standard appropriate for field use should be a research priority moving forward; without that, it is not possible to characterize the true burden and distribution of EED or develop a practical and reliable diagnostic test for more widespread field use.

Tables and Figures

Figure 4.1. Conceptual framework of the panel of environmental enteric dysfunction (EED) biomarkers



Conceptual framework is based on Panter-Brick et al.[12] and Prendergast et al.[2] Abbreviations and biomarker definitions: AAT, α-1 antitrypsin, a stool marker of protein losing enteropathy;[23] EED, environmental enteric dysfunction; EndoCAb, endotoxin core antibody immunoglobulin G, an intestinal permeability marker indicating systemic exposure to enteric pathogens;[11,14] GLP-2, glucagon-like peptide-2, a serum marker of enterocyte proliferation and repair;[26] L:M, lactulose:mannitol ratio, a marker of intestinal permeability and absorptive capacity measured in urine following oral dosing with a solution of the two sugars;[18,50] MPO, myeloperoxidase, a stool marker of enteric neutrophil activity;[21] NEO, neopterin, a stool marker of enteric T_h1-mediated inflammation.[22]

Table 4.1. Characteristics of 18-month-old children participating in the environmental enteric dysfunction (EED) assessment (n=539)

Characteristic	n	Mean (SD)/%
Age (mo)		18.3 (0.3)
Sex (% female)	269	49.9
Stunting (% LAZ<-2)	239	45.0
Wasting (%WLZ<-2)	77	14.6
Household assets		
Any land	396	73.7
Cattle	268	49.9
Goats	154	28.7
Irrigation pump	93	17.3
Electricity	165	30.7
Improved toilet[1]	443	82.2
Living standards index (LSI)		0.11 (1.05)
Mother's education		
None	116	21.6
1-9 years	348	64.8
SSC passed	33	6.1
11+ years	40	7.4
Morbidity history[2]		
Diarrhea	384	71.2
Dysentery	420	77.9
Pneumonia	242	44.9

[1]Water sealed or slab toilet. [2]Any episode of morbidity in the past year (between 6 and 18 months of age). Abbreviations: EED, environmental enteric dysfunction; LAZ, length-for-age z-score; LSI, living standards index; SSC, secondary school completion exam; WLZ, weight-for-length z-score.

Table 4.2. Summary statistics for biomarkers of environmental enteric dysfunction (EED) and systemic health and associations with L:M ratio in 18-month-old children

Biomarker	n	GM (95% CI)[1]	Elevated, n (%)[2]	L:M Status		
				Normal, GM (95% CI)[1]	Elevated, GM (95% CI)[1]	P-value[3]
L:M ratio	446	0.06 (0.05, 0.06)	174 (39.0)			
MPO (ng/mL)	498	4460.3 (4145.0, 4799.5)	420 (84.3)	4186.6 (3775.6, 4642.3)	5081.0 (4490.8, 5748.7)	0.019
AAT (μg/mL)	501	326.9 (303.1, 352.5)	278 (55.5)	314.2 (282.3, 349.7)	378.4 (332.4, 430.8)	0.031
NEO (nmol/L)	502	767.4 (716.5, 821.8)	502 (100)	744.8 (676.1, 820.5)	846.4 (752.6, 951.9)	0.102
GLP-2 (ng/mL)	490	3.0 (2.9, 3.1)		2.9 (2.7, 3.1)	3.0 (2.8, 3.3)	0.432
EndoCAb (mu/mL)	501	45.4 (41.7, 49.4)		47.3 (42.2, 53.1)	40.4 (34.8, 47.0)	0.096
CRP (mg/L)	505	1.20 (1.03, 1.38)	103 (20.4)	1.12 (0.92, 1.37)	1.27 (0.98, 1.64)	0.451
AGP (mg/dL)	505	105.7 (102.8, 108.8)	288 (57.0)	102.7 (98.8, 106.7)	106.3 (101.5, 111.3)	0.264

[1]Values are geometric mean (95% confidence interval). [2]Cutoffs for normal vs. elevated biomarker values: L:M>0.07[7]; MPO>2000 ng/mL[13,29]; AAT>270 μg/mL[13,29]; NEO>70 nmol/L[13]; CRP>5 mg/L[30]; AGP>100 mg/dl[30]. [3]P-values from simple linear regression model with dependent variable log-transformed biomarker and indicator variable for elevated L:M. Abbreviations: AGP, α-1 acid glycoprotein; AAT, α-1 antitrypsin; CRP, C-reactive protein; EED, environmental enteric dysfunction; EndoCAb, endotoxin core antibody immunoglobulin G; GM, geometric mean; GLP-2, glucagon-like peptide-2; L:M, lactulose:mannitol ratio; MPO, myeloperoxidase; NEO, neopterin.

Table 4.3. Correlations between biomarkers of intestinal and systemic health in 18-month-old environmental enteric dysfunction (EED) study participants[1,2]

Biomarker	L:M	MPO	AAT	NEO	GLP-2	EndoCAb	CRP	AGP
L:M	1.000							
MPO	0.147***	1.000						
AAT	0.151***	0.329***	1.000					
NEO	0.114**	0.011	-0.123***	1.000				
GLP-2	0.025	-0.049	-0.121**	0.023	1.000			
EndoCAb	-0.054	-0.065	-0.042	0.080*	-0.055	1.000		
CRP	0.116**	0.179***	0.123***	0.057	-0.079*	0.022	1.000	
AGP	0.074	0.104**	0.064	0.107**	0.042	-0.000	0.535***	1.000

[1]Cell values are Pearson correlation coefficients between log-transformed biomarkers..[2]Missing values for pairwise correlations range from 34 to 120. Abbreviations: AGP, α-1 acid glycoprotein; AAT, α-1 antitrypsin; CRP, C-reactive protein; EED, environmental enteric dysfunction; EndoCAb, endotoxin core antibody immunoglobulin G; GLP-2, glucagon-like peptide-2; L:M, lactulose:mannitol ratio; MPO, myeloperoxidase; NEO, neopterin. . *, p≤0.10; **, p≤0.05; ***, p≤0.01.

Table 4.4. Associations between L:M ratio and intestinal and systemic health biomarkers in 18-month-old environmental enteric dysfunction (EED) study participants

Biomarker[1]	Univariable			Multivariable[2]		Multivariable[3]	
	β (SE)	P-value	R^2	β (SE)	P-value	β (SE)	P-value
MPO (ng/mL)	0.14 (0.05)	0.002	0.022	0.10 (0.05)	0.049	0.12 (0.05)	0.014
AAT (μg/mL)	0.14 (0.04)	0.002	0.023	0.12 (0.05)	0.020	0.10 (0.05)	0.042
NEO (nmol/L)	0.12 (0.05)	0.018	0.013	0.13 (0.05)	0.012	0.12 (0.05)	0.021
GLP-2 (ng/mL)	0.04 (0.07)	0.606	0.001	0.03 (0.08)	0.732	0.01 (0.08)	0.895
EndoCAb (mu/mL)	-0.04 (0.04)	0.268	0.003	-0.02 (0.04)	0.722	-0.02 (0.04)	0.639
CRP (mg/L)	0.05 (0.02)	0.016	0.013	0.04 (0.03)	0.142	0.04 (0.03)	0.126
AGP (mg/dl)	0.19 (0.12)	0.128	0.005	-0.06 (0.15)	0.709	-0.08 (0.15)	0.615
R^2				0.060		0.164	

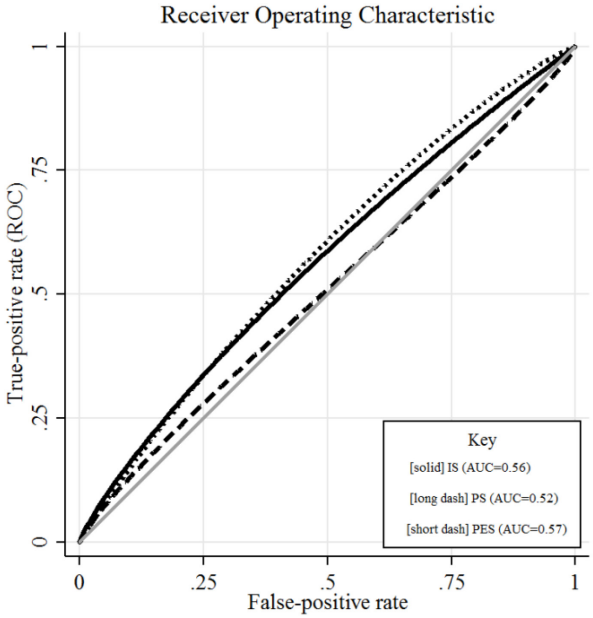
[1]Biomarkers were log-transformed prior to analysis. [2]Multivariable model includes all listed biomarkers as independent variables in one model. [3]Multivariable model adjusted for child/household characteristics: child sex, stunting status at age 6 mo, household living standards index (LSI), maternal education level, child age, assigned supplementation group, breastfeeding continuation to 18 months (yes/no), number of household members, maternal occupation (working outside of the home vs. not), maternal age, household land ownership, ownership of cattle, goats/sheep and chickens. Abbreviations: AGP, α-1 acid glycoprotein; AAT, α-1 antitrypsin; CRP, C-reactive protein; EED, environmental enteric dysfunction; EndoCAb, endotoxin core antibody immunoglobulin G; GLP-2, glucagon-like peptide-2; L:M, lactulose:mannitol ratio; MPO, myeloperoxidase; NEO, neopterin.

Table 4.5. Environmental enteric dysfunction (EED) score development: principal component analysis (PCA) and partial least squares regression (PLS) model outputs

Biomarker[1]	PCA		PLS[2]	
	Factor 1 Loading	Factor 2 Loading	Factor 1 Loading	Factor 1 Weight
MPO (ng/mL)	0.592	-0.036	0.711	0.654
AAT (μg/mL)	0.690	-0.038	0.619	0.605
NEO (nmol/L)	-0.185	0.458	0.319	0.476
GLP-2 (ng/mL)	-0.366	-0.505	0.013	0.037
EndoCAb (mu/mL)	-0.072	0.730	-0.096	-0.083
% of X variance[3]	27.50	21.78	23.77	
% of Y variance[4]	2.26		5.21	

[1]Biomarkers were log-transformed and outliers removed prior to analysis. [2]Partial least squares regression with dependent variable log-transformed L:M ratio. [3]Percent of total variance in independent variables (biomarkers) explained by each factor. [4]Percent of variance in L:M ratio explained by each score model (generated by PLS command; for PCA, based on R^2 from regression of log-transformed L:M ratio on PCA factors 1 and 2). Abbreviations: AAT, α-1 antitrypsin; EED, environmental enteric dysfunction; EndoCAb, endotoxin core antibody immunoglobulin G; GLP-2, glucagon-like peptide-2; L:M, lactulose:mannitol ratio; MPO, myeloperoxidase; NEO, neopterin; PCA, principal component analysis; PLS, partial least squares regression.

Figure 4.2. Receiver operating characteristic (ROC) curves for environmental enteric dysfunction (EED) scores vs. dichotomous L:M ratio[1]



[1]PCA permeability score was reversed (values multiplied by -1) to produce a score with AUC between 0.5 and 1.0, i.e. with higher values predictive of increased likelihood of abnormal L:M. Abbreviations: AUC, area under the curve; EED, environmental enteric dysfunction; IS, inflammation score (from PCA); L:M, lactulose:mannitol ratio; PCA, principal component analysis; PES, PLS EED score; PS, permeability score (from PCA); PLS, partial least squares regression; ROC, receiver operating characteristic.

Table 4.6. Mean L:M ratio and environmental enteric dysfunction (EED) scores by domains of child and household characteristics in 18-month-old study participants

Domain/ Characteristic	L:M ratio, GM (95% CI)[1]	Inflammation Score, Mean (SD)	Permeability Score, Mean (SD)[2]	PLS EED Score, Mean (SD)
Sociodemographic Characteristics				
Sex				
Male	0.05 (0.05, 0.06)	2.49 (1.05)	2.94 (1.02)	2.80 (1.04)
Female	0.06 (0.06, 0.07)	2.49 (0.94)	2.99 (0.98)	2.84 (0.96)
p-value[3]	0.050	0.985	0.592	0.648
LSI				
Low	0.07 (0.06, 0.07)	2.46 (0.98)	2.98 (1.01)	2.81 (0.92)
High	0.05 (0.05, 0.06)	2.53 (1.02)	2.95 (1.00)	2.84 (1.07)
p-value	0.000	0.509	0.734	0.783
Improved toilet[4]				
No	0.06 (0.05, 0.07)	2.54 (1.04)	3.16 (0.92)	2.81 (0.90)
Yes	0.06 (0.05, 0.06)	2.48 (0.99)	2.92 (1.01)	2.82 (1.02)
p-value	0.554	0.662	0.058	0.896
Systemic Inflammation				
Elevated CRP[5]				
No	0.06 (0.05, 0.06)	2.46 (0.98)	3.02 (0.98)	2.78 (0.98)
Yes	0.07 (0.06, 0.08)	2.63 (1.08)	2.75 (1.04)	2.97 (1.07)
p-value	0.026	0.159	0.023	0.123
Elevated AGP[5]				
No	0.06 (0.05, 0.06)	2.45 (0.96)	2.96 (0.97)	2.70 (0.98)
Yes	0.06 (0.05, 0.07)	2.53 (1.03)	2.97 (1.02)	2.92 (1.01)
p-value	0.364	0.455	0.928	0.024
Anthropometry				
Stunted[6]				
No	0.06 (0.05, 0.06)	2.49 (0.97)	2.85 (0.99)	2.82 (1.03)
Yes	0.06 (0.05, 0.07)	2.50 (1.04)	3.11 (0.99)	2.83 (0.97)
p-value	0.849	0.893	0.006	0.880
Wasted[6]				
No	0.06 (0.05, 0.06)	2.52 (1.01)	2.93 (1.01)	2.86 (1.01)
Yes	0.06 (0.05, 0.08)	2.34 (0.96)	3.15 (0.92)	2.63 (0.95)
p-value	0.624	0.168	0.116	0.089

[1]Values are geometric mean (95% confidence interval). [2]Permeability score is reversed relative to loadings reported in Table 4.5 (values multiplied by -1) to produce a score with higher values indicative of worse intestinal health. [3]P-values are from simple linear regression models with dependent variable log-transformed L:M ratio or standardized EED score and indicator variable for child or household characteristic. [4]Water sealed or slab toilet. [5]CRP>5 mg/L, AGP>100 mg/dL.[30] [6]Stunted: LAZ<-2; wasted: WLZ<-2.
Abbreviations: AGP, α-1 acid glycoprotein; CRP, C-reactive protein; EED, environmental enteric dysfunction; GM, geometric mean; LAZ, length-for-age z-score; L:M, lactulose:mannitol ratio; LSI, living standards index; PCA, principal component analysis; PLS, partial least squares regression; SSC, secondary school completion exam; WLZ, weight-for-length z-score.

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Chapter 5: Environmental enteric dysfunction (EED) is associated with diet quality in children enrolled in a randomized trial of complementary food supplements in rural Bangladesh

Abstract

Background Environmental enteric dysfunction (EED) is thought to be highly prevalent and a major cause of stunting in low- and middle-income country children, but the effects of diet quality and complementary food supplements (CFS) on EED are unknown. ***Objective*** To determine, within a randomized controlled trial, the impact of CFSs and total dietary intakes on EED. ***Methods*** Participants were randomized to receive one of four CFSs plus nutrition counseling or counseling only for one year from 6-18 mo of age. In a subset of participants at age 18 months, EED was assessed with urinary lactulose:mannitol (L:M) ratio and two scores, called the "inflammation score" (IS) and "permeability score" (PS), were derived from serum and stool biomarkers using principal component analysis. Diet and breastfeeding were assessed with a semi-structured 24-hr recall. Dietary intakes were summarized by food group and with a seven-item dietary diversity score. Total nutrient intakes including CFSs but not breastmilk were calculated. Relationships between continuous and dichotomous L:M ratio and EED scores and dietary variables were assessed with linear and logistic regression models. ***Results*** CFSs were not associated with odds of EED by elevated L:M ratio (>0.07) or high IS or PS (>median value). Consuming dairy foods was protective against elevated L:M ratio (OR

0.59, 95% CI: 0.38, 0.92), but no other food group was associated with elevated L:M, nor were food groups associated with odds of high IS or PS. Lower breastfeeding frequency (1-10 times vs. 11-20 times) was marginally protective against high IS (OR: 0.65, 95% CI: 0.42, 1.00) but not against elevated L:M or high PS. Energy intake was inversely associated with L:M ratio ($p<0.01$), but not with IS or PS. Protein intake was marginally inversely associated with IS ($p=0.08$), but not with L:M or PS, while fat intake was not associated with any of the EED markers. Zinc and riboflavin were associated with lower, and iron and vitamin B6 associated with higher, L:M ratio, while phosphorous was associated with lower IS, vitamin C with higher IS and calcium with higher PS. ***Conclusions*** CFSs were not protective against EED as measured by L:M ratio or biomarker scores, but total dietary intakes were associated with EED markers. Better quality diets, including supply of certain micronutrients, may protect against EED and may improve growth through an EED-mediated pathway. The suggestion of a harmful effect of higher iron intake on L:M ratio requires further investigation.

Introduction

Environmental enteric dysfunction (EED) is an inflammatory condition of the small intestine that is pervasive in low- and middle-income country (LMIC) populations and is thought to be responsible in part for the prevalence and persistence of stunting (length-for-age <-2 z-scores below the reference median) in those settings.[1] EED is characterized by partial villous atrophy, crypt hyperplasia, increased permeability in the

tight junctions between enterocytes and T cell mediated immune cell proliferation in the intestinal wall.[2,3] It is thought to be caused by repeated low-level exposure to environmental pathogens and toxins,[1,2,4] though the specific pathogens or toxins responsible have not been identified, and may differ by setting.[5] EED emerges with the introduction of non-breastmilk foods and liquids, which, in many settings, coincides with a large increase in pathogenic exposures and a sharp decline in the nutritional adequacy of the infant's diet.[6] The start of complementary feeding and the emergence of EED also coincide with the timing of the most pronounced decline in length-for-age z-score during early childhood.[7]

In many LMIC settings, the primary complementary food is a thin porridge made from the local starchy staple, which is typically bulky and lacking the micronutrient density and diversity to meet young children's specific nutritional needs.[8] In some settings complementary foods can be enhanced by adding nutrient-rich foods available in the home, such as meat, eggs, legumes and dark green leafy vegetables.[9,10] Where seasonal or ongoing food insecurity inhibits sufficient enhancement of the complementary feeding diet, complementary food supplements (CFS) have been found to be effective and necessary to satisfy nutrient requirements, prevent wasting, and improve linear growth.[11] To our knowledge, however, few CFS trials have examined the impact on EED.

Existing evidence regarding dietary intake and EED suggests they interact and each may compound the effects of the other. In some cases, inadequate dietary intakes may be a causal factor in the development of EED. This is corroborated by evidence from

animal models suggesting severe deficiencies may produce EED-like intestinal morphology.[12,13] Diets that are less severely deficient may still exacerbate existing EED and prevent or prolong recovery. In particular zinc, vitamin A and certain amino acids are required for enterocyte repair and may be in short supply in the typical complementary feeding diet.[14,15] EED may also exacerbate marginally adequate dietary intakes via malabsorption secondary to villous atrophy and crypt hyperplasia.[16] Further, if systemic inflammation is present, nutrient requirements may be elevated relative to normal and more likely to exceed availability.[17] The relationship between dietary intakes and EED is corroborated by evidence from micronutrient supplementation trials, which have demonstrated benefits of supplementation with vitamin A,[18] zinc,[19,20] multiple micronutrients,[21] and the amino acid glutamine[22] on EED, while supplementation with polyunsaturated fatty acids has not produced observable benefits for EED.[23]

While it is clear based on observational and interventional studies that shortfalls in the complementary feeding diet are widespread and associated with growth failure, the extent to which poor diet quality causes or perpetuates EED in the same settings is not clear. Within a randomized controlled trial of complementary food supplements for children ages 6-18 months in rural Bangladesh, we assessed at age 18 months dietary intake and a panel of EED biomarkers with the aim of characterizing relationships between diet and EED, including the effect of CFSs on EED.

Methods

Setting The study took place in the Rangpur and Gaibandha districts of northwest Bangladesh within a study site that has hosted several previous randomized trials of maternal and child nutrition interventions.[24-26] The region is rural but densely populated. Livelihoods are primarily agricultural and landholdings are generally small, with a large proportion of the population employed as day laborers to supplement sub-subsistent agricultural production.

JiVitA-4 trial A complementary food supplementation trial, known as the "JiVitA-4 trial", enrolled participants between September 2012 and April 2013, with follow-up continuing through May 2014. The trial is described in detail and main findings presented elsewhere.[27] Briefly, children were enrolled at age six months and randomized based on their geographic sector of residence to receive one of four CFSs plus child feeding counseling for mothers or child feeding counseling only for one year through age 18 months. Two of the CFSs were developed and produced in-country, one made of chickpeas (CP) and one of rice and lentils (RL). The third was fortified wheat-soy blend (WSB++), which is widely used by the World Food Programme in emergency and non-emergency food aid settings, and which was reformulated prior to the trial to better meet the specific nutritional requirements of the complementary feeding period. The fourth CFS was Plumpy'doz (Nutriset, Maulany, France), a commercially distributed peanut-based product. The CFSs were broadly equivalent in their micronutrient content, though the main ingredients and relative macronutrient contents differed. Each daily

portion of the CFSs were designed to provide approximately 75% of children's required micronutrients, but only 20-30% of total energy requirements, with the aim of enhancing children's diets to meet their macro- and micro-nutrient needs without displacing breastmilk or usual complementary foods (See Christian et al. 2015 for details of CFS contents). The main trial outcomes were length-for-age z-score and risk of stunting at age 18 months. CFS benefits for growth and prevention of stunting were observed.[27]

Child anthropometry, morbidity history, past 24-hour diet and breastfeeding recall and household demographic and socioeconomic characteristics were assessed at enrollment. Anthropometry, diet and breastfeeding assessments were repeated quarterly throughout the supplementation period. Local field workers distributed supplements weekly and visited households twice weekly to monitor adherence to daily supplementation.

EED Assessment In a sub-region of the study area, participants were invited to enroll in a substudy involving more comprehensive assessments of CFS effects, including a blood draw at age 18 months to measure micronutrient status. In approximately 500 of those substudy participants born within a designated date range and balanced by supplementation group, the lactulose mannitol urine test was conducted and a stool sample collected to allow for assessment of a panel of EED biomarkers.

The blood, urine and stool collection procedures and the EED biomarker panel are described in detail in Chapter 4. Briefly, blood samples were collected by venipuncture into blood collection tubes free of anticoagulants. The samples were allowed to clot

undisturbed for 30 minutes and then stored in a cold bag and transported to the field laboratory on the same evening. Blood was centrifuged and the serum transferred to a cryovial and stored in liquid nitrogen pending shipment to the JHSPH Center for Human Nutrition laboratory (Baltimore, MD).

Urine collection following dosing with lactulose and mannitol solution was conducted on the same day as the blood collection. Participants were fasted for two hours, ingested a dose of lactulose and mannitol solution, and then had all excreted urine collected over the subsequent two hours. The lactulose mannitol solution contained 50 mg mannitol powder (Sigma-Aldrich, St. Louis, MO, USA) and 255 mg lactulose in 0.375 mL lactulose syrup (Square Pharmaceuticals, Dhaka, Bangladesh) per mL. Children received 2 mL per kg body weight up to a maximum of 20 mL. Urine samples were analyzed by a collaborating laboratory at icddr,b (Dhaka, Bangladesh) to determine the recovery of lactulose and mannitol via High Pressure Ion Chromatography (Dionex, Thermo Fisher Scientific, Sunnyvale, CA). The lactulose mannitol recovery ratio (L:M) is reported as a ratio of the proportion of each recovered relative to the quantity administered.

A single stool sample was collected from each participant, generally on the morning of or morning following the urine and serum collections. Stool was collected on a sterilized mat, transferred to a sterile sample collection cup, and stored in the household in a Styrofoam cold box filled with ice packs for 2-5 hours pending return to the field laboratory, where it was aliquoted and stored in liquid nitrogen for storage and shipping to the Center for Human Nutrition lab.

The EED biomarker panel consisted of urinary L:M recovery ratio and serum and stool protein markers of inflammation and intestinal health. Stool was analyzed for myeloperoxidase (MPO) and neopterin (NEO), markers of immune cell activity in the wall of the small intestine,[28,29] and α-1 antitrypsin (AAT), a marker of protein losing enteropathy. Serum samples were analyzed for immunoglobulin G endotoxin core antibody (EndoCAb IgG), a marker of pathogenic exposure and excess permeability, and glucagon-like peptide-2 (GLP-2), a marker of intestinal repair.

Data reduction techniques used to summarize the EED biomarker panel into scores of EED activity are described in detail in Chapter 4. For this analysis, the two PCA-generated scores, called the "inflammation score" (IS) and the "permeability score" (PS), were used as markers of EED.

Diet Assessment Diet was assessed at ages 6, 9, 12, 15 and 18 months using a semi-structured past 24-hour recall administered by a trained interviewer. The respondent was the child's mother or primary caregiver. The questionnaire contained 29 commonly consumed food items, plus spaces to specify up to six additional foods not listed in the questionnaire. Interviewers reviewed the food list with the respondent, asking for each food, "From yesterday morning to today morning, has the child been fed _____?" If yes, the interviewer asked how much was fed to the child and recorded the quantity and unit of measure. The interviewer carried a set of standard measures (spoons, bowls and cups) for the mother to reference when reporting portion sizes.

Breastfeeding was assessed on the same occasions as diet. Respondents were asked whether the child was currently breastfed at the time of the interview and, if yes, how many times s/he was breastfed in the 24 hours from yesterday morning to the present morning. Reponses were recorded as 1-10 times, 11-20 times or 21 or more times. For the present study, only diet and breastfeeding data from the 18 month interview were used.

A mixed methods recipe development process was conducted to understand food perceptions and preparation methods in order to convert food-based dietary intake data to nutrient intake data. This process is described in detail elsewhere (Hurley et al., in preparation). The resultant recipes allowed all foods reported in the diet questionnaires, including "other" foods specified by respondents, to be matched with entries in the Bangladesh Food Composition Tables[30] and other food composition tables,[31-34] and thereby converted to nutrient intakes using data on quantity consumed.

A dietary diversity score (DDS) was developed for each child using a seven food group model as per WHO/UNICEF guidelines,[35] such that the DDS ranged from 0 to 7. The food groups were (1) Grains and starchy staples, (2) Legumes and nuts, (3) Dairy, (4) Meat and fish, (5) Eggs, (6) Vitamin A-rich fruits and vegetables (F/V) and (7) Other F/V. Foods were assigned to categories as per the WHO guidelines. Mixed dishes were counted toward all of the food groups represented by their ingredients. CFSs were not counted toward food group intakes, nor included in the calculation of DDS. Minimum dietary diversity was defined as DDS ≥ 4, as per the WHO/UNICEF guidelines.[35]

Nutrient intakes were defined as the sum of that consumed from home foods based on the diet recall questionnaire and from the child's assigned CFS and reported adherence. The contribution of CFSs to nutrient intakes was calculated for each child by multiplying the nutrient contents of the child's assigned CFS by his/her reported fraction of the daily serving consumed on the day prior to the diet interview (i.e. the day corresponding to the 24-hour diet recall). Breastfeeding status and frequency measures were not included in estimates of total nutrient intakes.

Statistical Methods The biomarkers of EED were log-transformed and extreme outliers were removed prior to analysis, as described in Chapter 4. In the present analysis, L:M and the PCA-generated IS and PS were used as indicators of EED. L:M was dichotomized as elevated (>0.07) versus normal (≤0.07).[36] IS and PS were standardized about their means and standard deviations for analysis as continuous variables, and were dichotomized as high versus low around their internal median values. Characteristics of the households' socioeconomic status, including asset ownership and the physical structure of the house, were condensed in an index, termed the "living standards index" (LSI), and dichotomized around the internal median value, as has been described previously for this population.[37] Breastfeeding status and frequency in the past 24 hours were combined into a single categorical variable with levels: 0 times (not currently breastfeeding), 1-10 times, 11-20 times, and 21+ times. Anthropometric measures were converted to z-scores relative to the WHO growth reference[38] and children with length-for-age, weight-for-age and weight-for-length z-scores < -2 was classified as stunted, underweight and wasted, respectively.

The distributions and central tendencies of nutrient intake values were explored for normality and outliers. Energy, protein, fat and carbohydrates were examined, as was a panel of micronutrients for which food composition table data and CFS contents were available. Energy intake was divided by 100 to improve the interpretability of regression coefficients (i.e. coefficients were expressed as change in dependent variable per 100 kcal difference in energy intake). Protein, fat and carbohydrate intakes were expressed as the percent of total kilocalorie intake supplied by each. Micronutrient intake values were standardized about their mean and standard deviation prior to inclusion in regression models.

Because EED biomarker data were measured in only a subset of the randomized trial participants, the extent to which the CFS groups were balanced on key child and household characteristics was assessed using simple linear, logistic and ordinal logistic regression analyses (for continuous, dichotomous and categorical outcomes, respectively) with standard errors adjusted for non-independence of observations within sectors, the unit of randomization, using the "vce(cluster *clustervar*)" command. This allowed for identification of potential confounders to be adjusted for in models of food supplementation effects on EED.

Logistic regression models were developed to quantify relationships between the dichotomous EED indicators (elevated vs. normal L:M ratio, high vs. low IS, high vs. low PS) and dietary intake markers. First, relationships between CFS group assignment and EED markers were evaluated with multivariable logistic regression models with each EED marker as the dependent variable and indicator variables for the CFS groups,

adjusted for potential confounders: child sex, exact age at the 18 month interview, LSI and baseline stunting status, and with standard errors adjusted for clustering of observations within sectors.

Relationships between dietary characteristics and dichotomous EED markers were then assessed with multivariable logistic regression models of the same form. Separate models were developed for the independent variables food groups, MDD and breastfeeding frequency. Models were generated for each food group separately and with all food groups included in one model. All models were adjusted for child sex, exact age at 18 month assessment, LSI and baseline stunting status, and standard errors allowed for clustering of observations within sectors.

Associations between markers of EED and macro- and micronutrient intakes were examined in multivariable linear regression models. Models were developed for dependent variables continuous L:M ratio, IS and PS, separately for energy intake and macronutrient intakes. Macronutrient intakes were included in one model with independent variables percent of total energy from protein and from fat, while percent of energy from carbohydrates was omitted due to collinearity with the other macronutrients. Linear regression models with micronutrient intakes as independent variables were of the same form, with models developed for each micronutrient separately and for all micronutrients combined in one model. All models were adjusted for sex, age, LSI, CFS group and baseline stunting status, and standard errors were adjusted for clustering by sector. Models with micronutrient intakes as the independent variable(s) were also adjusted for total energy intake.

All analyses were conducted in Stata version 14.1 (StataCorp, College Station, TX). CFS group assignment was analyzed on an intention-to-treat basis. The study protocols were approved by the Institutional Review Boards of the Johns Hopkins Bloomberg School of Public Health and the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b). Written consent was required from the primary caregiver of each child for participation.

Results

In total 539 children were enrolled in the EED study, all of whom also had complete 18 month diet interviews available. Of the participating children, 49.9% were female and 45.0% and 14.6% were stunted and wasted, respectively, at age 18 months (Table 5.1). The mean L:M ratio was 0.06 (95% CI: 0.05, 0.06) and 39.0% of participating children had abnormal L:M values (>0.07). The standardized inflammation and permeability scores had means (SE) 256 (1.0) and 3.0 (1.0), respectively. Mean adherence to supplementation (% of daily portion consumed) was 85.5 (SD 22.3) over ages 12 through 17 months and did not differ by supplementation group (p=0.5) (results not shown).

In logistic regression models of dichotomous EED markers on assigned CFS groups adjusted for potential confounders, no group-wise differences in EED were observed (Table 5.2). Odds of elevated L:M, IS and PS did not differ by CFS group.

Among study participants, mean DDS was 3.7 (SD: 1.3), and 55.5% of participants consumed 4 or more food groups, the threshold for minimum dietary

diversity (MDD). Within the seven food groups used to define the DDS, children consumed grains most frequently (98.1% of respondents), followed by non-vitamin A-rich fruits and vegetables (73.3%) and meat (62.2%), and then vitamin A-rich fruits and vegetables (35.8%), legumes (35.6%), eggs (35.3%) and dairy (31.4%) (Figure 5.1). Continued breastfeeding exceeded 95% at age 18 months (Table 5.3). At the nutrient level, mean 24-hour energy intake from complementary foods and CFSs but not breastmilk was 692.9 kcal (SD: 337.0), which is 77.5% of the estimated total requirement for 18-month-old children, but exceeds, by 145 kcal, the estimated requirement from complementary foods assuming continued breastfeeding and typical breastmilk consumption.[39] Protein provided mean 11.1% (SD 2.8), fat 28.1% (SD: 10.1) and carbohydrates approximately 60.8% (SD 11.6) of total kcals from home plus supplementary foods (Table 5.3).

In regression models of dichotomous EED markers on food groups consumed, past 24-hour dairy consumption was protective against elevated L:M ratio (OR: 0.59, 95% CI: 0.38, 0.92), while none of the other food groups was significantly associated with elevated L:M, and no food group was associated with high IS or PS (Table 5.3). MDD was not associated with risk of EED based on L:M ratio, IS or PS. Past 24-hour breastfeeding frequency was not significantly associated with risk of elevated L:M ratio or high PS, but lower breastfeeding frequency was protective against high IS (OR: 0.65, 95% CI: 0.42, 1.00, in children breastfed 1-10 times compared to those breastfed 11-20 times).

Total past 24-hour energy intake from home foods and CFSs was inversely associated with L:M ratio (Table 5.4). Each 100 kcal increase in energy intake was associated with a 0.03 (SE: 0.01, $p<0.01$) reduction in log-L:M ratio. Energy intake was not associated with IS or PS. L:M ratio was not associated with macronutrient intakes. IS trended toward an inverse association with the percent of calories consumed from protein ($p=0.08$), while no association was observed between PS and macronutrient intakes.

In regressions with micronutrient intakes as independent variables in a single model, adjusted for energy intake, intakes of zinc (β: -0.81, SE: 0.32, $p=0.01$) and riboflavin (β: -0.33, SE: 0.15, $p=0.03$) were associated with lower log-L:M ratio, while intakes of iron (β: 0.29, SE: 0.14, $p=0.04$) and vitamin B6 (β: 0.66, SE: 0.22, $p<0.01$) were associated with increased log-L:M ratio (Table 5.5). Phosphorous was associated with reduced and vitamin C with elevated IS: a one SD increase in phosphorous was associated with a 0.88 SD (SE: 0.42, $p=0.04$) reduction in IS, while a one SD increase in vitamin C was associated 0.14 (SE: 0.07, $p=0.05$) greater IS. Only calcium intake was associated with PS. A one SD increase in Ca was associated with a 0.27 SD (SE: 0.13, $p=0.04$) increase in PS.

Discussion

In 18-month-old children enrolled for one year in a randomized controlled trial of complementary food supplementation in rural Bangladesh, markers of EED were not associated with assigned supplementation group, despite high daily adherence to CFSs.

Consuming dairy foods was associated with reduced risk of elevated L:M ratio, while food group intakes were not associated with risk of high IS or PS, and MDD was not associated with any EED marker. Higher energy intakes were inversely associated with L:M ratio but not with IS or PS, while protein intakes trended towards an inverse association with IS but were not related to L:M ratio or PS, and fat intakes were not related to any of the EED markers. Some relationships between EED markers and micronutrient intakes were observed. Most notably, zinc intake was associated with lower L:M ratio, while iron intake was associated with higher L:M ratio, holding intakes of energy and other micronutrients constant. These findings suggest that inadequate dietary intakes may contribute to EED in this setting of prevalent stunting and wasting, but the CFSs tested in this supplementation trial were not protective against EED.

The lack of association between supplementation and EED runs counter to expectations. The micronutrient contents of the CFS, which were broadly balanced across the different CFS formulations, may have been expected to have protective effects against EED relative to the un-supplemented control group. Results from prior studies of micronutrient supplementation are mixed, with benefits reported from vitamin A and zinc supplementation, alone or in combination,[18-20,40] but not multiple micronutrient supplementation.[21] Based on evidence from animal studies suggesting long-chain fatty acids may be critical for tight junction maintenance in the small intestine,[15] the CFSs with higher fat contents, i.e. Chickpea, Rice-Lentil and Plumpy'doz, might have been expected to benefit intestinal health markers more than WSB++ or no CFS, an effect we did not observe, though our findings are in line with another trial of PUFA-rich supplements on

EED that also found null effects.[23] While in this trial children receiving CFSs had improved adequacy of nutrient intakes,[41] the micronutrient contents of the CFSs may not have been sufficient to protect against EED, or the participants' underlying nutritional statuses may have differed from those in the other trials that reported benefits of supplementation. It remains possible that in a subgroup of participants with more inadequate home food intake or availability, the CFSs had a protective effect on risk of EED, a hypothesis the current investigation was not powered to examine.

The protective effect of lower breastfeeding frequency (1-10 times versus 11-20 times) against elevated IS was a surprising finding. The effect may be confounded by child size, as this is a period of transitioning away from breastfeeding, and larger, healthier children may breastfeed less often and have transitioned more to solid foods. It is also possible that the effect is more acute, i.e. during an underlying illness indicated by higher IS, children breastfed relatively more often than normal. Though breastmilk could displace critical nutrients required from complementary foods and increase risk of EED, without data about the volume of breastmilk consumed, it would be unwarranted to conclude that higher breastfeeding frequency carries an increased risk of EED.

Few associations were observed between food group intakes and EED markers, possibly because the food groups do not differentiate among children's diets according to characteristics most relevant to EED risk, especially when drawing on one day of recalled diet only. Still, the observation that consumption of dairy foods was protective against elevated L:M ratio is potentially important. Animal milk is known to provide many benefits to growing children, attributed to its energy, PUFA, and micronutrient content,

as well as to growth factors and potentially other unknown properties,[42] all of which may also be beneficial to the maintenance and repair of the intestinal epithelium.

Energy intake was inversely associated with L:M ratio but not with IS or PS, and the percent of kilocalories from protein trended towards an inverse association with IS. This effect may also be confounded by child size, with larger, healthier children consuming more energy and at lower risk of EED. Recent attention to the quality of dietary protein suggests that diets of children in LMICs may supply inadequate quantities of certain amino acids despite apparently adequate total protein intakes.[43] Further investigation into the amino acid content of children's diets in this study may reveal inadequacies not captured by total protein intake only, and perhaps also associations between amino acid intakes and EED risk.

The finding that zinc intake was associated with lower L:M ratio is consistent with other evidence that zinc deficiency can cause enteropathy and that supplemental zinc may be protective against EED.[44-46] Zinc deficiency is typically highly prevalent in settings of prevalent stunting, and there is broad consensus that children in Bangladesh are at very high risk of zinc deficiency.[47,48] It seems reasonable in that context, then, that relatively better zinc intakes are protective against EED. If inadequate zinc intakes are a main driver of EED in this setting and if CFSs contained inadequate quantities or bioavailability of supplemental zinc, that could explain the lack of benefit for EED observed from the CFSs. Examination of indicators of micronutrient status in this sample may further elucidate relationships between nutritional status and EED, including whether underlying status modifies the effect of nutrient intakes on EED.

The positive association between iron intake and L:M ratio is also notable, as there is accumulating evidence in the literature that supplemental iron may increase intestinal inflammation and the incidence and severity of diarrhea.[49,50] This finding is unexpected in the Bangladeshi context, however, because the groundwater is known to be rich in iron such that variations in dietary iron may not greatly influence total iron intake or status.[51,52] Effects of iron supplementation on EED specifically have not, to our knowledge, been describe at present but, if confirmed, that relationship could be responsible for reducing observed benefits of multiple micronutrient and food supplements, including CFSs, for nutritional status and growth. Further, differential effects of supplemental iron versus dietary iron on EED must be examined, as our data suggest an effect of total dietary iron, while existing evidence focuses on iron supplementation only. Links between other micronutrients (B vitamins, phosphorous, calcium, vitamin C) and EED are not, to our knowledge, readily explained based on existing evidence, but may require further investigation, especially if corroborated by future studies.

This investigation of diet and EED is a unique addition to the literature, in that the effects of dietary intake and complementary food supplementation on EED in otherwise healthy children has not, to our knowledge, been studied previously. This study also benefited from a large sample size and randomized allocation to dietary supplementation with multiple CFS formulations. Additionally, the assessment of EED included a robust panel of candidate biomarkers, which could be examined individually and with composite scores developed in a previous investigation of this data.

The study was limited by some of the assessment methods and temporality. Diet was assessed based on maternal report and recall rather than direct observations or diaries. Assessing diet in a quick interview was essential, however, to enable the inclusion of such a large sample, and the interviewer-administered format obviated the need for respondent literacy. Additionally, data on dietary intakes in the past 24 hours only were collected, which may not be representative of the participants' usual dietary intakes. The tradeoff, however, is that past 24-hour recall may be more accurate than a recall over a longer time period. Aggregated over the population studied, 24-hour recalls likely give an accurate picture of typical diets, though observed relationships between diet and EED may have been attenuated if individuals' past 24-hour intakes were not representative of their usual intakes. Additionally, the assessment of EED was limited in that EED biomarkers were measured at a single time point only, also a product of the tradeoff between intensity of assessments and feasible sample size. Further, the assessment of EED was limited more generally by imperfect and un-validated diagnostic methods, a more complete discussion of which can be found in Chapter 4. This study did have the advantage of including a broad panel of EED and systemic health biomarkers including the quasi-gold standard L:M test, which lends confidence to the findings regarding dietary predictors of EED in this study.

This study begins to address a central question in understanding and preventing EED, namely the effect of diet quality and specific dietary components on EED. The results suggest that better quality diets are, for the most part, associated with reduced risk of EED, though CFSs were not protective against EED. The role of specific

micronutrients in EED requires further study, especially zinc deficiency as an underlying cause and benefits of zinc supplementation for preventing and treating EED. In addition, the potential for higher iron intakes and supplemental iron to induce or exacerbate EED requires urgent further investigation.

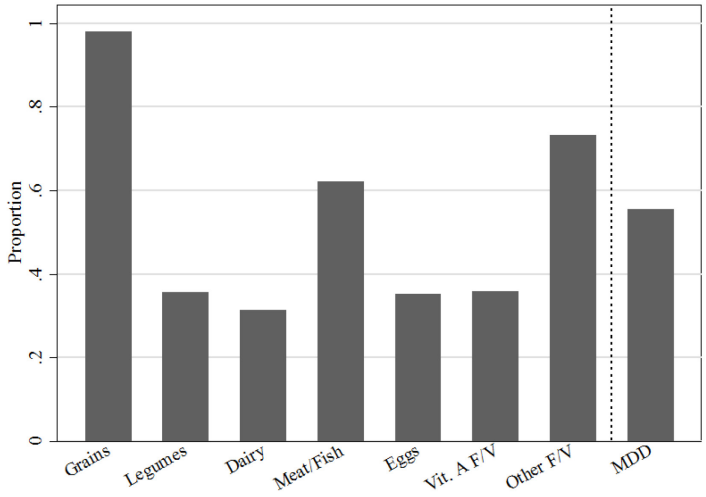
Tables and Figures

Table 5.1. Sociodemographic characteristics and environmental enteric dysfunction (EED) marker values in EED assessment participants at age 18 months (N=539)

Characteristic	n (%)[1]
Sex, female	269 (49.9)
Length, cm, mean (SD)	76.2 (3.0)
Stunted (LAZ<-2)	239 (45.0)
Weight, kg, mean (SD)	8.8 (1.1)
Underweight (WAZ<-2)	197 (37.1)
Wasted (WLZ<-2)	77 (14.6)
Maternal education	
None	116 (21.6)
1-9 years	348 (64.8)
SSC passed	33 (6.1)
11+ years	40 (7.4)
Living standards index (LSI), high	271 (50.5)
L:M, geometric mean (95% CI)	0.058 (0.054, 0.063)
L:M, elevated (>0.07)	174 (39.0)
IS, mean (SD)	2.5 (1.0)
PS, mean (SD)	3.0 (1.0)

[1]Values are n (%) unless otherwise specified in the row label. Abbreviations: EED, environmental enteric dysfunction; IS, PCA-derived inflammation score; LAZ, length-for-age z-score; L:M, lactulose:mannitol ratio; LSI, living standards index; PS, PCA-derived permeability score; SSC, secondary school completion exam; WLZ, weight-for-length z-score.

Figure 5.1. Proportion of 18-month-old children consuming each of seven food groups and reaching minimum dietary diversity (MDD) following one year of enrollment in a randomized controlled complementary food supplementation trial



Note: bars indicate the proportion of participants consuming each food group and, for the MDD column, the proportion reaching MDD (dietary diversity score ≥ 4). Abbreviations: F/V, fruits and vegetables; MDD, minimum dietary diversity; Vit. A, vitamin A.

Table 5.2. Associations between markers of environmental enteric dysfunction (EED) and assigned supplementation group at age 18 months following one year of enrollment in a randomized controlled complementary food supplementation trial[1]

CFS Group	Elevated L:M[1]	High IS[1]	High PS[1]
	OR (95% CI)[2]	OR (95% CI)[2]	OR (95% CI)[2]
Counseling only	1.00	1.00	1.00
Chickpea	0.92 (0.45, 1.85)	1.06 (0.60, 1.86)	0.81 (0.40, 1.63)
Plumpy'doz	1.17 (0.60, 2.28)	1.39 (0.71, 2.73)	0.90 (0.52, 1.55)
Rice-lentil	0.77 (0.37, 1.60)	1.87 (0.99, 3.53)	1.05 (0.52, 2.13)
Wheat-soy blend *plus plus*	1.00 (0.51, 1.94)	1.46 (0.82, 2.61)	0.94 (0.53, 1.68)

[1]Dichotomous EED markers based on cutoff >0.07 for L:M ratio and >median value for IS and PS. [2]Values are odds ratio (95% confidence interval) from logistic regressions with indicator variable for each CFS group, adjusted for sex, exact age, living standards index (LSI) and baseline stunting status, and with standard errors allowing for clustering by sector. Abbreviations: CFS, complementary food supplement; EED, environmental enteric dysfunction; IS, inflammation score; L:M, lactulose:mannitol ratio; PS, permeability score.

Table 5.3. Associations between environmental enteric dysfunction (EED) markers and dietary intake in 18-month-old children following one year of enrollment in a randomized controlled complementary food supplementation trial

Past 24-hr dietary intake		Elevated L:M[1]	High IS[1]	High PS[1]
Indicator	**Reported intake, n (%)[2]**	**OR (95% CI)[3]**	**OR (95% CI)[3]**	**OR (95% CI)[3]**
Grains	529 (98.1)	1.18 (0.23, 6.16)	1.07 (0.23, 5.10)	3.16 (0.57, 17.50)
Legumes	192 (35.6)	1.30 (0.83, 2.05)	1.04 (0.68, 1.61)	0.82 (0.54, 1.23)
Dairy	169 (31.4)	0.59 (0.38, 0.92)**	0.88 (0.59, 1.33)	1.20 (0.77, 1.89)
Meat	335 (62.2)	1.11 (0.74, 1.66)	1.02 (0.67, 1.55)	1.29 (0.79, 2.10)
Eggs	190 (35.3)	0.88 (0.59, 1.31)	0.72 (0.46, 1.13)	1.07 (0.75, 1.54)
Vitamin A-rich F/V	193 (35.8)	0.83 (0.56, 1.22)	1.42 (0.92, 2.19)	0.90 (0.58, 1.40)
Other F/V	395 (73.3)	0.93 (0.61, 1.41)	0.76 (0.48, 1.21)	0.87 (0.58, 1.32)
MDD	299 (55.5)	0.99 (0.66, 1.48)	0.78 (0.54, 1.13)	0.95 (0.60, 1.50)
Breastfeeding frequency				
0	14 (2.6)	0.22 (0.02, 2.01)	0.82 (0.22, 3.03)	0.90 (0.24, 3.32)
1-10	137 (25.4)	0.71 (0.44, 1.14)	0.65 (0.42, 1.00)**	0.84 (0.54, 1.30)
11-20	338 (62.7)	1.00	1.00	1.00
21+	50 (9.3)	0.99 (0.56, 1.76)	1.58 (0.81, 3.08)	0.91 (0.46, 1.80)

[1]Dichotomous EED markers based on cutoff >0.07 for L:M ratio and >median value for IS and PS. [2]Number (percent) of participants consuming each food group, reaching MDD and in each breastfeeding frequency category based on maternal report in past 24 hour diet recall. [3]Logistic regression models with dependent variables dichotomous L:M, IS and PS, developed separately for each domain of diet characteristics (food groups, MDD, breastfeeding), adjusted for child sex, exact age at 18 mo interview, household LSI and baseline stunting status and with standard errors allowing for clustering by sector. Abbreviations: EED, environmental enteric dysfunction; F/V, fruits and vegetables; IS, inflammation score; L:M, lactulose:mannitol ratio; MDD, minimum dietary diversity (dietary diversity score ≥ 4); PS, permeability score. **, p≤0.05; ***, p≤0.01.

Table 5.4 Associations between environmental enteric dysfunction (EED) markers and reported past 24-hour macronutrient intakes from home foods and CFSs at age 18 months

Nutrient	Recommended intake	Reported intake, mean (SD)	L:M	IS	PS
			β (SE)[1]	β (SE)[1]	β (SE)[1]
Energy (kcal)[2]	894[3]	692.9 (337.0)	-0.03 (0.01)***	0.00 (0.02)	-0.00 (0.01)
Protein (% kcal)	5-20[4]	11.1 (2.8)	-0.01 (0.02)	-0.03 (0.02)*	0.01 (0.02)
Fat (% kcal)	30-40[4]	28.1 (10.1)	0.00 (0.00)	0.00 (0.00)	-0.00 (0.01)
Carb. (% kcal)	45-65[4]	60.8 (11.6)	-	-	-

[1]Linear regression models with dependent variable continuous L:M ratio, IS or PS and separate models for (1) energy and (2) macronutrients as independent variables. All models were adjusted for child sex, age, household LSI, assigned supplementation group and baseline stunting status, and standard errors allowing for clustering by sector. [2]Energy coefficients are presented as change in dependent variable per 100 kcal change in energy intake. [3]FAO.[39,53] [4]Acceptable macronutrient distribution ranges (AMDR), IOM.[54] Abbreviations: CFS, complementary food supplement; EED, environmental enteric dysfunction; IS, inflammation score; L:M, lactulose:mannitol ratio; PS, permeability score. *p≤0.10; **p≤0.05; ***p≤0.01.

Table 5.5. Associations between environmental enteric dysfunction (EED) markers and reported past 24-hour micronutrient intakes from home foods and CFSs at age 18 months

Micronutrient[1]	Recommended intake	Reported intake, mean (SD)	L:M	IS	PS
			β (SE)[2]	β (SE)[2]	β (SE)[2]
Ca (mg)	500[3]	295.5 (175.1)	0.03 (0.09)	0.21 (0.11)	0.27 (0.13)**
Fe (mg)	5.8[3,5]	5.4 (2.9)	0.29 (0.14)**	0.13 (0.24)	0.13 (0.24)
Mg (mg)	60[3]	116.4 (61.5)	-0.18 (0.13)	-0.29 (0.17)	0.07 (0.14)
P (mg)	460[4]	338.0 (173.2)	0.39 (0.26)	-0.88 (0.42)**	0.23 (0.45)
K (mg)	3000[4]	620.1 (340.3)	-0.24 (0.12)	0.04 (0.22)	-0.13 (0.18)
Zn (mg)	4.1[3,6]	4.0 (1.9)	-0.81 (0.32)**	0.24 (0.67)	-0.53 (0.60)
Vit. A (μg)	400[3]	276.7 (225.0)	0.13 (0.12)	0.14 (0.16)	0.19 (0.20)
Vit. D (μg)	5[3]	2.6 (2.6)	0.25 (0.14)	-0.03 (0.26)	0.35 (0.22)
Vit. E (mg)	5[3]	3.8 (2.9)	-0.07 (0.11)	-0.13 (0.18)	0.06 (0.14)
Thiamine (mg)	0.5[3]	0.4 (0.2)	0.18 (0.16)	0.26 (0.25)	0.24 (0.24)
Riboflavin (mg)	0.5[3]	0.5 (0.4)	-0.33 (0.15)**	0.21 (0.25)	-0.22 (0.28)
Niacin (mg)	6[3]	6.8 (3.6)	0.03 (0.07)	0.09 (0.09)	-0.01 (0.07)
Vit. B6 (mg)	0.5[3]	0.5 (0.3)	0.66 (0.22)***	-0.20 (0.46)	-0.04 (0.37)
Folate (μg)	150[3]	101.7 (60.6)	-0.27 (0.15)	-0.30 (0.26)	-0.43 (0.29)
Vit. C (mg)	30[3]	12.6 (17.2)	-0.05 (0.03)	0.14 (0.07)**	0.00 (0.07)

[1]Micronutrient intakes were standardized around their means and standard deviations prior to analysis. [2]Linear regression models with dependent variable L:M ratio, IS or PS and the micronutrients as independent variables in a single model adjusted for child sex, age, household LSI, assigned supplementation group and baseline stunting status, and standard errors allowing for clustering by sector. [3]RNI.[55] [4]AI.[54] [5]Assuming moderate bioavailability of dietary iron. [6]Assuming 10% bioavailability of dietary zinc. **p≤0.05; ***p≤0.01.

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Chapter 6: Markers of environmental enteric dysfunction predict weight gain but not length gain in children enrolled in a randomized trial of complementary food supplements in rural Bangladesh

Abstract

Background Environmental enteric dysfunction (EED), a disorder characterized by a constellation of structural and functional changes in the wall of the small intestine, is common in low- and middle-income countries (LMICs) and may be linked to stunting. In rural Bangladesh, stunting prevalence exceeds 40% in children <5 years of age, but the contribution of EED to stunting there is not known. ***Objective*** To determine the extent to which EED explains attained length and weight and prospective growth. ***Methods*** In 539 children enrolled in a randomized controlled trial of complementary food supplementation in rural Bangladesh, EED was assessed at age 18 mo with the lactulose:mannitol (L:M) ratio in urine and a panel of serum and stool intestinal health biomarkers collapsed into inflammation (IS) and permeability (PS) scores. Length, weight, length-for-age (LAZ), weight-for-age (WAZ) and weight-for-length (WLZ) z-scores at 18 mo and prospective change in length, weight, LAZ, WAZ and WLZ over 18-24 mo were regressed on EED markers adjusting for confounders. ***Results*** Stunting (45.0%), wasting (14.6%) and EED (39.0% based on elevated L:M ratio) were common at age 18 mo in participating children. L:M ratio was not associated with any of the anthropometric indicators at 18 mo or with changes from 18 to 24 mo. Length, weight,

LAZ and WAZ at 18 mo were inversely associated with PS ($p<0.01$ for all), while 18 mo WLZ was not associated with PS. Odds of 18 mo stunting and underweight were 1.30 (95% CI: 1.07, 1.58) and 1.27 (1.02, 1.58) times greater, respectively, per SD increase in PS. IS was not associated with 18 mo anthropometry. Change in weight, WAZ and WLZ, but not length or LAZ, from 18 to 24 months was inversely associated with IS ($P<0.05$), while neither linear nor ponderal growth was associated with PS. Odds of underweight and wasting at 24 mo, adjusted for 18 mo status, were 1.40 (1.04, 1.87) and 1.33 (1.05, 1.68) times greater, respectively, with each SD increase in IS. ***Conclusions*** EED scores were associated with concurrent length and prospective ponderal but not linear growth, which adds to a growing body of literature suggesting that links between EED and linear growth are not universal. Further research is needed to better characterize the individual- and population-level characteristics that explain variation in these relationships.

Introduction

Resolving stunting (length/height-for-age <-2 z-scores below the reference median[1]) continues to elude public health practitioners, as its risk factors are tightly aligned with poverty but its prevention and remedy are apparently far more complex.[2] Stunting is associated with an increased risk of early childhood morbidity and mortality.[3] It also often persists into adulthood, accompanied by increased risk of cognitive deficits, poorer school achievement and work performance, metabolic dysfunction and, in women, adverse pregnancy outcomes, with lifelong and intergenerational consequences for

health, human capital and economic development.[4,5] Although stunting prevalence has declined by nearly 40% from 1990 to 2014, an estimated 159 million children under five years of age worldwide are stunted. Moreover, considerable heterogeneity exists in stunting rates and trends between and within countries.[6] In Bangladesh, rates of stunting have been reduced over the past decade, but national estimates continue to place the prevalence at over 40%, and levels are even higher among rural populations and those of lowest socioeconomic status.[7,8]

Inadequate dietary intakes and repeated episodes of morbidities such as diarrhea and pneumonia are known causes of stunting,[3,9] recognized as major contributors to the sharp decline in length-for-age z-score (LAZ) that occurs during infancy and early childhood.[10,11] Estimates suggest, however, that the combined effects of diet and morbidity explain at most about 36% of the observed burden of stunting.[12] The contribution of other known risk factors, for example, intrauterine growth restriction, are similarly inadequate to explain the magnitude of observed stunting.[13]

A pervasive subclinical inflammation of the small intestine, termed "environmental enteric dysfunction" or EED, may be a missing piece to the puzzle explaining the burden and persistence of stunting in low- and middle-income countries (LMICs). EED is characterized by villous atrophy, crypt hyperplasia and leaky tight junctions in the enterocytes of the small intestine.[14,15] It is thought to result from chronic exposure to environmental pathogens and toxins.[15,16] EED may lead to impaired growth through both malabsorption of nutrients and chronic systemic inflammation, which may limit nutrients available for growth through anorexia, by sequestering nutrients or

diverting them to inflammatory processes and by exacerbating enterocyte damage.[17,18] Some observational studies from diverse settings have found high prevalence of EED and associations between markers of EED and lower LAZ or reduced rate of growth, suggesting this may be an important, previously overlooked contributor to stunting.[19-21] It is not known, however, the extent to which EED contributes to the burden of stunting observed in rural Bangladesh. This setting is of particular interest because the prevalence of stunting is so high, while the environmental factors typically linked to EED, such as lack of access to improved water and sanitation facilities, are relatively better in Bangladesh compared to many other LMICs,[22] making the role of EED and its causes there a topic of debate.

The present study aimed within a community-based randomized controlled trial of complementary food supplements in rural northwest Bangladesh to determine the extent to which EED was related to anthropometry and prospective growth.

Methods

Setting Participants were enrolled from a randomized controlled trial of complementary food supplements set in the Gaibandha and Rangpur districts of rural northwest Bangladesh at the "JiVitA Project" research site. The JiVitA site comprises approximately 450 km^2 of mapped and enumerated households in an area broadly representative of the Gangetic flood plain region of South Asia. The setting is rural and densely populated with largely agrarian livelihoods and small household landholdings.

The site has been host to several previous randomized nutrition and health trials,[23-25] as well as numerous observational studies of maternal and child health and nutrition.[26-29]

The parent trial tested multiple formulations of complementary food supplements (CFS), energy dense and micronutrient fortified food products fed in addition to usual breastfeeding and complementary foods to enhance the nutritional content of complementary feeding diets. The CFS trial was a five arm, un-blinded, cluster randomized controlled trial that took place during 2012-2014.[30] All children living within the study area who reached age 6 months during the enrollment period (August 2012 – April 2013) were eligible to participate with parental consent, after which they were assigned to one of the five study arms according to their geographic sector of residence. All study arms received child feeding counseling for mothers. In addition, four study arms received one of four CFS formulations: chickpea (CP) or rice-lentil (RL), both locally developed and produced, Plumpy'doz (PD), a commercially distributed peanut-based product produced by Nutriset (Maulany, France) or fortified wheat-soy blend (WSB++), which is widely used by the World Food Programme for food aid purposes in emergency and non-emergency settings. CFSs were distributed to households to be consumed as a daily snack in addition to regular complementary foods and breastfeeding over six to 18 months of age. The main trial outcomes were length-for-age and stunting at age 18 months.

Child and household characteristics, including anthropometry, were assessed at enrollment and anthropometry was repeated at ages 9, 12, 15 and 18 months. Additionally, a post-supplementation assessment including anthropometric measurements

was conducted at age 24 months. Interviewers were trained and standardized in anthropometric techniques prior to the start of the study. Quality control procedures included random rechecking of measurements by expert anthropometrists, and interviewers were retrained and re-standardized as needed throughout the study period.

EED Assessment In a subset of the CFS trial participants, additional assessments were conducted to measure markers of environmental enteric dysfunction (EED) at age 18 months following one year of trial participation. A substudy area was designated within the parent trial for more intensive assessments of study effects. The area included 99 (of 596) sectors selected to be geographically contiguous, accessible by road and to contain an approximately equal number of children enrolled in each study arm, with total substudy enrollment targeted at 750 children. All children enrolled in the main trial and living within the substudy area were eligible for the substudy with parental consent.

Substudy participants were additionally enrolled in the assessment of EED, with parental consent, beginning in mid-September 2013. In those children, urine following lactulose:mannitol dosing, serum and stool samples were collected. The full details of the sample collection and laboratory analyses are described in Chapter 4. Briefly, children received an oral dose of dual sugar solution following a two hour fast. Urine was collected over the subsequent two hours, weighed, mixed with disinfectant and stored in liquid nitrogen pending shipment to a collaborating laboratory at icddr,b (Dhaka, Bangladesh). Blood was collected into tubes free of anticoagulants, allowed to clot and then centrifuged and the serum transferred to cryovials and stored in liquid nitrogen for shipment to JHSPH. A single stool sample for each participant was collected in the

household using sterilized collection materials and stored in cold boxes until processing and storage in liquid nitrogen for shipment to JHSPH.

Urine was analyzed at icddr,b by high pressure ion chromatography (Dionex, Thermo Fisher Scientific, Sunnyvale, CA) for concentrations of lactulose and mannitol. Concentrations were related back to the dose volume to determine the percent recovery of each, expressed as the ratio of lactulose recovery to mannitol recovery (L:M ratio). L:M ratio greater than 0.07 was considered elevated.[31-33] Stool samples were analyzed for myeloperoxidase (MPO) and neopterin (NEO), markers of immune activity in the wall of the small intestine,[34,35] and α-1 antitrypsin (AAT), a marker of protein-losing enteropathy,[36] using commercially available ELISA kits (MPO and NEO: ALPCO Diagnostics, Salem, NH; AAT: BioVendor, LLC, Asheville, NC). Serum was analyzed for immunoglobulin G endotoxin core antibody (EndoCAb IgG), a marker of intestinal permeability,[37] and glucagon-like peptide-2 (GLP-2), a growth factor thought to be indicative of enterocyte proliferation and repair.[38] Both were assessed with commercial ELISA kits (EndoCAb IgG: Hycult Biotech, Plymouth Meeting, PA, USA; GLP-2: EMD Millipore, St. Charles, Missouri, USA). Standards and controls provided in the kits, along with participant sample-derived control samples, were run in duplicate on each plate and coefficients of variation monitored.

Statistical Methods Distributions of EED biomarkers and anthropometric measures were examined prior to analysis. EED biomarkers were log-transformed and extreme outliers removed, as described in Chapter 4. Length-for-age (LAZ), weight-for-age (WAZ) and weight-for-length (WLZ) z-scores relative to the WHO Multicentre

Growth Reference Study growth standards[1] and prevalence of stunting (LAZ<-2), underweight (WAZ<-2) and wasting (WLZ<-2) at ages 18 and 24 months were calculated. Extreme outlying values (|z-score|>6) were omitted from the analysis. Changes in length, weight, LAZ, WAZ and WLZ between the 18- and 24-month assessments were calculated accounting for the exact number of days between the assessments.

Two orthogonal scores of EED – an "inflammation score" (IS) and a "permeability score" (PS) – derived with principal component analysis (PCA) on log-transformed EED biomarkers (MPO, AAT, NEO, EndoCAb IgG, and GLP-2), described in detail in Chapter 4, were used as indicators of EED along with L:M ratio. The PCA-derived EED scores were standardized and then oriented and scaled to each have minimum value zero and higher values indicative of worse intestinal health. Log-transformed L:M ratio was standardized around its mean and standard deviation to simplify interpretation of regression coefficients. L:M ratio and EED scores were also classified into quintiles of their respective distributions for additional analyses and clarity of graphical displays.

Models of length, LAZ, weight, WAZ, WLZ, stunting and wasting at 18 months were developed to determine cross-sectional associations with markers of EED. Multivariable linear regression models were developed with length, LAZ, weight, WAZ and WLZ at 18 months of age as dependent variables, separately for independent variables L:M ratio, IS and PS, with all models adjusted for child sex, exact age at the 18 month assessment, household LSI and assigned supplementation group. Associations

between EED at 18 months and prospective change in anthropometric measures from 18 to 24 months were examined with multivariable linear regressions with Δlength (cm), Δweight (kg), ΔLAZ, ΔWAZ and ΔWLZ as dependent variables and separate models with L:M ratio, IS and PS as independent variables, adjusted for child sex, age at 18 month assessment, household LSI and assigned supplementation group. In all models, standard errors were adjusted for non-independence of observations within sectors, the unit of randomization, using the "vce(cluster *clustervar*)" command.

To examine associations between EED markers and dichotomous outcomes of stunting, underweight and wasting, multivariable logistic regression models were developed with 18-month stunting, underweight and wasting as dependent variables, separately for independent variables continuous L:M ratio, IS and PS, adjusted for the same child and household covariates as in the linear models. Multivariable logistic regression models of the same form were also developed for stunting, underweight and wasting status at 24 months as dependent variables, with the same covariates as above. Models of stunting status at 24 months were adjusted for 18-month stunting status, and models of 24-month underweight and wasting were adjusted for 18-month underweight and wasting status, respectively. Interaction terms between 18 month stunting status and EED markers were also tested, to determine whether the relationship between EED and 24-month stunting status differed by 18-month stunting status. The same models were run with underweight and wasting at 24 months as the outcome. Logistic regression models were repeated with indicator variables for quintiles of the distributions of L:M ratio, IS and PS as independent variables.

All protocols were approved by the institutional review boards of the Johns Hopkins Bloomberg School of Public Health (Baltimore, MD) and the International Center for Diarrhoeal Disease Research (icddr,b, Dhaka, Bangladesh). Written parental consents were required for participation in the CFS trial and in the EED biomarker assessment. All analyses were conducted using Stata, version 14.1 (StataCorp, College Station, TX).

Results

Of 566 eligible children, parental consent for 539 was obtained for the EED assessment. Among these, height and weight data were available for 531 (98.5%) and 506 (93.9%) at ages 18 and 24 months, respectively. Stunting was common, with prevalence 45.0% at 18 months and 40.5% at 24 months (Table 6.1). Underweight and wasting were also common, with higher prevalence of both at 24 months relative to 18 months; 43.5% and 20.9% were underweight and wasted, respectively, at 24 months.

L:M test results were available for 446 (82.7%) of consented participants, while complete data for EED scores were available for 437 (81.1%). Most missing biomarker data were due to sample collection failure in the field, though some were also removed from analyses because of implausible values. Geometric mean of L:M ratio was 0.058 (95% CI 0.054, 0.063), and 39.0% of the children (n=174) had elevated L:M values (>0.07) indicative of EED. The mean values of the PCA-generated IS and PS were 2.49

(SD 1.0) and 2.96 (SD 1.0), respectively, with higher values of each indicative of poorer intestinal health. (See Table 4.5 for factor loadings.)

In cross-sectional models, L:M ratio was not associated with any of the anthropometric measures, nor was IS associated with anthropometric measures at age 18 months. Length, LAZ, weight and WAZ, but not WLZ, at 18 months were inversely associated with PS in adjusted models (Table 6.2). Each standard deviation increase in PS was associated with difference in length of -0.38 cm (95% CI: -0.67, -0.10), LAZ of -0.14 (95% CI: -0.24, -0.04), weight of -0.13 kg (-0.22, -0.04) and WAZ of -0.12 (-0.21, -0.03).

Changes in anthropometric measures from 18 to 24 months were also not associated with L:M ratio. Changes in weight, WAZ and WLZ from 18 to 24 months were inversely associated with IS, while changes in length and LAZ were not associated with IS (Table 6.2). Each standard deviation increase in IS was associated with a 0.06 kg (95% CI: -0.10, -0.01) reduction in weight change from 18 to 24 months, and with 0.05 (-0.09, -0.01) and 0.07 (-0.13, -0.01) z-score lower changes in WAZ and WLZ, respectively, from 18 to 24 months. PS was not associated with any markers of change in anthropometry from 18 to 24 months.

Neither L:M ratio nor IS was associated with the odds of stunting, underweight or wasting at age 18 months (Table 6.3). PS was associated with increased risk of stunting and underweight at 18 months, but not with risk of wasting. Each standard deviation increase in PS was associated with a 1.30 (95% CI: 1.07, 1.58) times increase in odds of stunting and 1.27 (1.02, 1.58) times increase in odds of underweight at 18 months.

In models of stunting, underweight and wasting at 24 months, interactions between EED markers and 18-month status were not statistically significant for any of the EED marker – anthropometry combinations, and were not retained in the final models. L:M ratio was not associated with odds of 24-month stunting, underweight or wasting. Greater IS was associated with increased risk of 24-month underweight and wasting after adjusting for 18-month underweight or wasting status, but IS was not associated with risk of stunting at 24 months. The odds of wasting was 4.06 (95% CI: 1.37, 12.0), 3.17 (1.10, 9.08) and 3.47 (1.28, 9.38) times greater in the 3rd, 4th and 5th quintiles of IS relative to the 1st quintile (Figure 6.1).The odds of underweight was higher in the 4th quintile of IS relative to the 1st quintile (OR: 3.66, 95% CI: 1.54, 8.69), while comparisons between other quintiles did not reach statistical significance. PS was not associated with any of 24 month stunting, underweight or wasting (Table 6.3).

Discussion

In a subsample of children enrolled in a randomized controlled trial of complementary food supplementation in rural Bangladesh, prevalence of stunting, underweight and wasting were high, with stunting prevalence of 45.0% at 18 months and wasting prevalence increasing from 14.6% at 18 months to 20.9% at 24 months. Prevalence of EED as measured by L:M ratio neared 40%. Elevated L:M ratio was not, however, associated with either length or weight or their z-scores at 18 months, nor was it associated with the change in anthropometric measures prospectively from 18 to 24

months. The PCA-generated intestinal permeability score (PS) was associated with length, LAZ and risk of stunting at age 18 months whereas the intestinal inflammation score (IS) was predictive of change in weight, WAZ and WLZ from 18 to 24 months and wasting status at 24 months, controlling for 18-month wasting.

The high burden of stunting observed is in line with other recent estimates in the same area. The 2014 Demographic and Health Survey report estimated 46.3% of children age 18 to 23 months nationally to be stunted.[39] The growth trajectories we observed contrast with more widely reported trends, however. Typically, LAZ is thought to decline through 24 months of age before stabilizing and rebounding slightly, while WLZ begins to improve at or before 18 months.[40] Our results suggest that children were becoming increasingly thin over 18 to 24 months, even as their linear growth stabilized relative to the growth standard. For that reason, WAZ and WLZ may be more susceptible to environmental and nutritional constraints like EED than LAZ is in this setting and age group. It should also be considered that the relative decline in WLZ over 18 to 24 months could be a statistical artifact: WLZ is not pinned to age and fatness naturally decreases from a peak at approximately six months of age, so stunted children may seem to be becoming increasingly wasted, when in fact they have normal ponderal growth for their age, but their height is that of a much younger, "chubbier" child. To illustrate, the mean height in our sample at age 18 months was 76.2 cm, the height achieved by the median reference population child at age 13 months. Still, the associations between EED and ponderal growth over 18 to 24 months were also observed in models with weight as an absolute measure rather than in z-scores.

In cross-sectional models, the PS, a PCA-derived composite score with high loading by EndoCAb IgG, a permeability marker, moderate loading by NEO, an enteric inflammation marker, and moderate negative loading by GLP-2, an enterocyte repair marker, was associated with length, LAZ, weight, WAZ and risk of stunting and underweight. In contrast to many of the other markers included in our panel, EndoCAb is related to prior endotoxin exposure rather than current permeability, *per se*, which could explain why the score on which that marker loads strongly is more closely associated to concurrent anthropometry (i.e., prior growth) relative to markers thought to be more responsive to present intestinal conditions.[37] These findings are consistent with the literature describing associations between EED and length/height,[41,42] though much of that literature describes associations between length/height and EED as measured by L:M ratio, while we did not observe that relationship. Additionally, logistic regression models of the odds of stunting on EED markers suggested PS may be a factor explaining the burden of stunting in this setting, that is, that the PS-LAZ association is pertinent to the portion of the length distribution surrounding the cutoff for stunting. Based on our data alone, it is not clear that the direction of causation runs from EED to LAZ, as is proposed in our (Figure 2.1) and other conceptualizations of EED and stunting.[15,16,43] It is possible that poor growth precedes EED, with undernutrition or another cause common to both conditions. Longitudinal growth models could clarify the extent to which EED is associated with recent alterations in growth trajectory versus longstanding differences between children, however more information about the dynamics of EED in this

population are necessary to fully parse out the directionality of the EED-growth relationship.

Prospective change in weight, WAZ and WLZ were inversely associated with IS, a PCA-derived composite score of biomarkers of intestinal health with high positive loadings for MPO, an enteric inflammation marker, and AAT, a permeability marker, and moderate negative loading for GLP-2 (enterocyte repair). The magnitude of the effects of IS on WAZ and WLZ were quite small, less than one tenth of a z-score difference in rate of growth per standard deviation change in IS, but the finding that EED as measured by higher quintiles of IS predicts a 3-4 times greater risk of wasting at 24 months suggests EED could be an important contributor to observed declines in ponderal growth relative to normative growth data over this time period.

None of the EED markers was associated with prospective linear growth. This is in contrast to studies that have reported lower LAZ gains in children with EED.[20,37,44,45] Of note, the majority of those studies included children under age 18 months only. Rate of linear growth slows markedly in the second year of life, potentially making it less responsive to insults such as EED. Additionally, rate of growth may be uncoupled from prior health status during that period, with historically healthier, better nourished children growing at a slower rate relative to those with prior growth constraints such as periods of diarrheal or other morbidity.[46] EED may inhibit weight gain but not length gain in our sample because of the time period observed, during which ponderal growth may be relatively more susceptible to EED and other growth-limiting conditions than linear growth is.

Interpretation of divergent trends among L:M ratio, IS and PS presents challenges, as the extent to which IS and PS capture aspects of EED is not clear given their limited association with L:M ratio. Still, MPO and AAT, which load strongly on the IS, and EndoCAb and NEO, which load strongly on the PS, have been associated with rate of growth in prior studies of EED,[20,37,47] which suggests consistency in these findings, though doesn't resolve the interpretation issues around L:M and the other EED markers.

The hypothesized pathway from complementary food supplementation to growth via improved EED was not observed in this study. In the parent trial, CSFs were shown to be beneficial for linear growth and for preventing stunting,[30] but the present investigation of EED suggested no associations between CFSs and EED (see Chapter 5), and minimal links between EED and stunting, in total suggesting that food supplementation affected growth via pathways not mediated by EED. This may be due to a lack of benefit of the particular CFS formulations or the quantities given for EED, or because EED was not a major factor in limiting growth in this setting, both topics to be investigated further in future studies.

The strengths of the present study include the large sample size, comprehensive panel of EED and systemic health biomarkers and repeated assessments of anthropometry conducted with the utmost attention to anthropometrist reliability in a large study with anthropometric measures as the main outcome. Weaknesses include the temporality of the EED assessment with respect to the series of anthropometric assessments and with respect to the most sensitive period of early childhood growth. The timing of the EED

assessment was driven by a primary hypothesis focused on the effects of the supplementation trial on EED markers, which necessitated a relatively later assessment of EED, and supported prioritizing a larger sample size over repeated EED assessments. The lack of a true gold standard of EED inhibits the interpretation of the biomarkers where they diverge from agreement with L:M ratio, which may be considered a limitation in this field of study more generally at the time the study was conducted.

In this rural Bangladesh cohort where stunting and EED were common, some markers of intestinal health, but not L:M ratio, were associated with length, LAZ, weight and WAZ at 18 months and with change in weight, WAZ and WLZ from 18 to 24 months. The findings suggest that some aspects of EED likely contribute to constraining growth in this setting, though the directionality of the relationships between EED and linear and ponderal growth up to and beyond 18 months of age requires further investigation. The nature of linear and ponderal growth during this period also requires further study, as the window in which EED and other environmental and nutritional exposures exert the most influence over linear growth may have already closed by 18 months in this population.

Tables and Figures

Table 6.1. Anthropometric characteristics of environmental enteric dysfunction (EED) assessment participants in rural Bangladesh (n=539)

	Time Point (Age)	
	18 months	**24 months**
n	539	513
	Mean (SD)/ n (%)	***Mean (SD)/ n (%)***
Length, cm	76.2 (3.0)	81.2 (3.2)
Weight, kg	8.8 (1.1)	9.6 (1.1)
LAZ	-1.9 (1.0)	-1.8 (1.0)
WAZ	-1.7 (1.0)	-1.8 (1.0)
WLZ	-1.1 (1.0)	-1.2 (0.9)
LAZ<-2 (%)	239 (45.0)	205 (40.5)
WAZ<-2 (%)	197 (37.1)	221 (43.5)
WLZ<-2 (%)	77 (14.6)	106 (20.9)

Abbreviations: LAZ, length-for-age z-score; WAZ, weight-for-age z-score; WLZ, weight-for-length z-score.

Table 6.2. Associations between markers of environmental enteric dysfunction (EED) and anthropometry at 18 months and change in anthropometry from 18 to 24 months

EED Marker	Length (cm)	Weight (kg)	LAZ	WAZ	WLZ
	β (SE)[1]	**β (SE)[1]**	**β (SE)[1]**	**β (SE)[1]**	**β (SE)[1]**
	At 18 months				
L:M	-0.06 (0.11)	-0.04 (0.04)	-0.02 (0.04)	-0.04 (0.04)	-0.04 (0.04)
IS	-0.05 (0.14)	-0.00 (0.04)	-0.02 (0.05)	0.00 (0.04)	0.02 (0.04)
PS	-0.38 (0.14)***	-0.13 (0.05)***	-0.14 (0.05)***	-0.12 (0.05)***	-0.07 (0.04)
	Change from 18 to 24 months				
L:M	-0.10 (0.07)	-0.02 (0.02)	-0.03 (0.02)	-0.01 (0.02)	0.02 (0.03)
IS	-0.00 (0.07)	-0.06 (0.02)**	0.00 (0.03)	-0.05 (0.02)***	-0.07 (0.03)**
PS	0.07 (0.06)	0.03 (0.02)	0.04 (0.02)	0.04 (0.02)	0.03 (0.04)

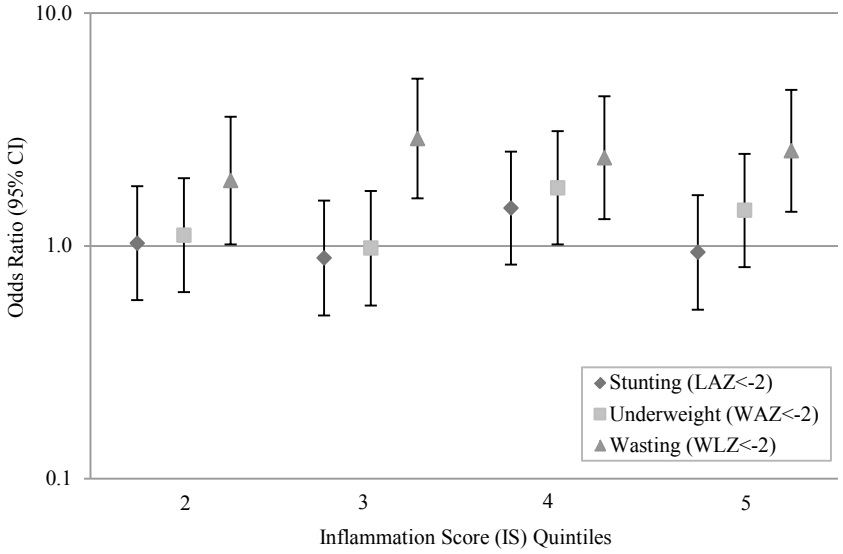
[1]Values are coefficient (standard error) from linear regression models with each anthropometric measure, z-score or change in measure or z-score as the dependent variable and standardized EED marker (L:M ratio, IS or PS) as the independent variable, adjusted for sex, age, LSI and supplementation group, and with standard errors adjusted for clustering by sector. Abbreviations: EED, environmental enteric dysfunction; IS, inflammation score; LAZ, length-for-age z-score; L:M, lactulose:mannitol ratio; PS, permeability score; WAZ, weight-for-age z-score; WLZ, weight-for-length z-score. **, p≤0.05; ***, p≤0.01.

Table 6.3. Risk of stunting, underweight and wasting at 18 and 24 months in relation to markers of environmental enteric dysfunction (EED) measured at 18 months

EED Marker	Stunting (LAZ<-2) OR (95% CI)[1]	Underweight (WAZ<-2) OR (95% CI)[1]	Wasting (WLZ<-2) OR (95% CI)[1]
		At 18 months	
L:M	0.97 (0.81, 1.18)	1.13 (0.95, 1.36)	1.02 (0.81, 1.29)
IS	1.01 (0.84, 1.22)	0.98 (0.81, 1.19)	0.84 (0.65, 1.09)
PS	1.30 (1.07, 1.58)***	1.27 (1.02, 1.58)**	1.25 (0.97, 1.62)
		At 24 months[2]	
L:M	1.12 (0.84, 1.48)	1.16 (0.86, 1.56)	1.13 (0.84, 1.52)
IS	1.13 (0.84, 1.52)	1.40 (1.04, 1.87)**	1.33 (1.05, 1.68)**
PS	0.91 (0.68, 1.21)	1.36 (0.98, 1.87)*	1.09 (0.80, 1.48)

[1]Values are odds ratio (95% confidence interval) from logistic regression models with each dichotomous anthropometric indicator (stunting, underweight, wasting) as the dependent variable and standardized EED marker (L:M ratio, IS or PS) as the independent variable, adjusted for sex, age, LSI and supplementation group, and with standard errors allowing for clustering by sector. [2]Models with dependent variable 24 month stunting, underweight or wasting status were adjusted for 18 month status. Abbreviations: EED, environmental enteric dysfunction; IS, inflammation score; LAZ, length-for-age z-score; L:M, lactulose:mannitol ratio; PS, permeability score; WAZ, weight-for-age z-score; WLZ, weight-for-length z-score. **, p≤0.05; ***, p≤0.01.

Figure 6.1. Risk of stunting, underweight and wasting at age 24 months by quintile of the inflammation score (IS) measured at age 18 months[1]



[1]Odds ratios produced with "margins" command following multivariable logistic regression models with dependent variable 24 month stunting, wasting or underweight status and indicator variables for quintiles of IS, adjusted for child sex, age, household LSI, supplementation group and 18 month stunting, wasting or underweight status, with standard errors adjusted for clustering by sector. Abbreviations: IS, inflammation score; LAZ, length-for-age z-score; WAZ, weight-for-age z-score; WLZ, weight-for-length z-score.

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Chapter 7: Conclusions

Stunting affects a huge number of children around the world, with the burden distributed unevenly across low- and middle-income countries (LMICs) and concentrated in the poorest and most marginalized households.[1] Environmental enteric dysfunction (EED), a subclinical condition of the small intestine, is thought to be highly prevalent in LMICs and responsible for a large proportion of observed stunting.[2-4] The specific causes of EED, the pathways through which it inhibits growth and the extent to which it explains the observed prevalence of stunting across settings worldwide are not well understood at present, nor have effective intervention strategies to prevent or treat EED been identified. Observational and experimental studies are needed in a diversity of settings where risk of stunting is high to advance understanding of EED beyond anecdotes and analogies. Additionally, validated and standardized assessment methods appropriate for field use are urgently needed to allow for this necessary proliferation of community-based studies and to enable comparison of resultant findings. It is not yet clear that EED is the missing link between deprived environments and intractable stunting, but the existing evidence suggests that it very well may be.

Within a randomized controlled trial of complementary food supplementation in rural northwest Bangladesh that reduced the rate of decline in LAZ over the supplementation period in three of the CFS groups (CP, RL, and PD) and reduced prevalence of stunting in children receiving CP and PD,[5] we took advantage of repeated assessments of anthropometry and diet, a planned serum blood draw and the randomized

allocation to food supplementation to conduct a novel study of EED, diet and growth. We aimed to develop a comprehensive and efficient set of biomarkers to diagnose EED, to describe the burden and risk factors for EED in this setting, and to characterize relationships between diet and EED and EED and growth.

Summary of findings

The EED assessment enrolled 539 children, an approximate 10% subsample of the supplementation trial participants. Serum, stool and urine samples were available for 509 (94.4%), 515 (95.6%) and 434 (80.5%) of the enrolled children, respectively. The prevalence of stunting was 45.0% and 40.5% at 18 and 24 months, respectively, while the prevalence of underweight and wasting rose throughout the observation period to 43.5% and 20.9%, respectively, at 24 months. The main results and programmatic implications of the study are summarized below.

The L:M ratio, a test for EED, was elevated in nearly 40% of children at 18 months of age. Novel serum and stool biomarkers of intestinal and systemic health that we assessed did not closely approximate the L:M ratio; however, we were able to construct two separate scores of intestinal inflammation and permeability using these markers (Chapter 4).

The lactulose:mannitol (L:M) ratio is widely used as a "gold standard" measure of EED in settings where intestinal biopsies are not feasible, but its performance has proven inconsistent.[6,7] Several biomarkers in serum and stool have been proposed as alternates to

L:M ratio,[8-11] but no study has implemented a comprehensive panel of these biomarkers along with L:M ratio. An extensive biomarker panel was evaluated in this study to thoroughly characterize EED in this setting, and to advance understanding of the relative performance, agreement and redundancy of a set of candidate markers.

In this sample, elevated L:M ratio values, indicative of EED, were observed in 39.0% of participants, while the stool markers myeloperoxidase (MPO), α-1 antitrypsin (AAT) and neopterin (NEO) were elevated in 84.3%, 55.5% and 100%, respectively. Evidence of systemic inflammation was also common: the acute phase proteins C-reactive protein (CRP) and α-1 acid glycoprotein (AGP) were elevated in 20.4% and 57.0% of participants, respectively. Correlations between serum and stool biomarkers and between those and L:M ratio, however, were low, ranging from 0 (endotoxin core antibody IgG (EndoCAb IgG) and AGP) to 0.54 (CRP and AGP), and the complete panel of biomarkers explained only 6% of variability in L:M ratio.

Two data reduction techniques, principal component analysis (PCA) and partial least squares regression (PLS) were used to generate scores likely reflecting EED based on common variability within the biomarker panel. PCA produced a two factor solution. Factor 1, which we named the "inflammation score" or "IS", had loadings 0.59 for MPO, 0.69 for AAT and -0.37 for glucagon-like peptide-2 (GLP-2). Factor 2, the "permeability score" or "PS", had loadings 0.73 for EndoCAb IgG, 0.46 for NEO and -0.51 for GLP-2. PLS with L:M ratio specified as the dependent variable produced a one factor solution, which we refer to as the "PLS EED Score" or "PES", with high loadings and weights for MPO and AAT and moderate loading and weight for NEO. Receiver operating

characteristic (ROC) analysis revealed that the scores had effectively no discriminatory power to differentiate those with or without elevated L:M ratio. Child and household characteristics were not associated with either L:M ratio or the EED scores.

Dietary intakes of some macro- and micro-nutrients, but not complementary food supplementation, were associated with biomarkers of intestinal health (Chapter 5).

EED and inadequate complementary feeding diets are known to widely coexist, but little is known about the extent to which inadequate diets contribute to the development or persistence of EED. Complementary food supplements (CFSs) can improve micronutrient intakes and fill the dietary gap, which in turn promotes linear and ponderal growth in the complementary feeding period.[5,12-14] However, the potential for CFSs to prevent or ameliorate EED has not, to our knowledge, been described. In the present study, we observed no impact of the four CFS formulations on risk of elevated L:M ratio or high IS or PS, despite the CFS trial demonstrating high levels of adherence and benefits for linear growth and prevention of stunting.[5]

Associations were observed, however, between EED markers and indicators of total dietary intakes. Using past 24-hour dietary recall we examined the proportion of children consuming seven different food groups. Dairy consumption was associated with a reduced risk (OR: 0.59, 95% CI: 0.38, 0.92) of elevated L:M, but no other food group intake was associated with elevated L:M, IS or PS. Reaching minimum dietary diversity (defined as consuming 4 or more of the 7 food groups) was not associated with reduced risk of EED based on L:M ratio, IS or PS.

Energy intake from home foods and CFSs combined was inversely associated with L:M ratio ($p<0.01$), but not with IS or PS. The percent of calories consumed from protein trended toward an inverse association with IS ($p=0.08$), while no association was observed between protein intake and L:M ratio or PS. The percent of calories from fat was not associated with any of the EED markers.

Of the micronutrients examined, total intakes from home foods plus CFSs of zinc (β: -0.81, 95% CI: -1.45, -0.17) and riboflavin (β: -0.33, -0.63, -0.03) were associated with lower L:M ratio, while intakes of iron (β: 0.29, 0.01, 0.57) and vitamin B6 (β: 0.66, 0.23, 1.09) were associated with higher L:M ratio. Phosphorous intake was associated with reduced IS (β: -0.88, -1.71, -0.04) and vitamin C with elevated IS (β: 0.14, 0.00, 0.28), while only calcium intake was associated with increased PS (β: 0.27, 0.02, 0.53).

Permeability and inflammation biomarker scores, but not L:M ratio, were associated with length at 18 months and with weight gain from 18 to 24 months (Chapter 6).

We had available to us repeated measures of length and weight in the first two years of life, with the EED assessment timed at 18 months at the end of supplementation. This allowed for examination of associations between EED and concurrent anthropometric measures (length, weight, LAZ, WAZ, WLZ, stunting, wasting and underweight) at 18 months, and between EED and prospective changes in anthropometric measures over the subsequent six months.

EED as measured by elevated L:M ratio was not associated with any concurrent or prospective change in anthropometric measures. PS was inversely associated with

length, weight, LAZ and WAZ at 18 months ($p<0.01$ for all), and also associated with increased risk of stunting and underweight (ORs: 1.30, 95% CI: 1.07, 1.58 and 1.27, 1.02, 1.58 for stunting and underweight, respectively, per SD increase in PS). However, PS was associated with neither linear nor ponderal growth over the subsequent six months to 24 months of age. Conversely, IS was not associated with any concurrent anthropometric measures at 18 months, but was inversely associated with change in weight, WAZ and WLZ, but not length or LAZ, from 18 to 24 months ($p<0.05$ for all). Odds of underweight and wasting at 24 months, adjusted for 18 month status, were 1.40 (1.04, 1.87) and 1.33 (1.05, 1.68) times greater, respectively, with each SD increase in IS. None of the EED markers was predictive of risk of stunting at 24 months.

Strengths and limitations

The study had numerous strengths that lend support to the findings. The randomized design of the supplementation trial and temporality of assessments allowed for the evaluation of a causal relationship between CFSs and EED. Additionally, the recruitment of EED study participants from those enrolled in the parent RCT and the high enrollment rate suggest a low risk of selection bias and good generalizability to rural Bangladesh and, perhaps, rural South Asia more broadly. The comprehensive panel of EED biomarkers was a unique feature of this investigation, which allowed for a thorough characterization of EED in this setting, and for a novel investigation of the interrelationships between and relative performance of several candidate biomarkers for

the assessment of EED. In particular, the inclusion of the L:M test, a widely accepted biomarker for EED, along with the panel of novel biomarkers is unique in the literature and allowed for more critical evaluation of candidate biomarkers. The large sample size of over 500 children is also a strength of this investigation. Field and laboratory methods were carefully designed and monitored to minimize those as sources of variability in the biomarker values. Additionally, L:M ratio was determined in urine by a laboratory at icddr,b with extensive experience with this technique.

Assessment methodology and timing did present some limitations for the analysis and interpretation of the data. The assessment of EED at only one time point precluded an examination of the dynamics of EED within this population, and limited some of the conclusions that could be drawn about relationships between EED and growth. Additionally, the timing of the EED assessment at 18 months coincided with a period when linear growth velocity naturally declines and relatively higher growth rates may be indicative of poorer previous growth, confounding associations between health characteristics and rate of growth. The timing of the assessment was driven by the aim of evaluating the impact of the CFS trial on EED, and the ability to assess the effect of a randomly allocated dietary intervention on EED remains a unique strength of our study. Additionally, as we sought to evaluate relationships among several biomarkers of EED, we prioritized a larger sample size at a single time point over repeated assessments in a smaller sample. Lastly, the state of EED assessment more generally was a limitation in this study, as our ability to evaluate the set of candidate biomarkers and to define and

characterize EED in this setting was constrained by the shortcomings of the L:M test and by the lack of precision observed in many of the serum and stool biomarker assays.

Implications

This multipart study set out first to identify a comprehensive, efficient and feasible panel of biomarkers for use in field studies of EED. L:M ratio was included as the gold standard against which to evaluate the candidate biomarkers, as it is widely used but unwieldy for field applications. In practice, we found L:M ratio was weakly and inconsistently associated with other biomarkers and with a host of child and household factors expected to predict EED. Our experience adds to an accumulating body of evidence suggesting that L:M ratio is not a suitable gold standard measure of EED, and that a better diagnostic test is essential to allow for further progress in this field of study.

Examination of dietary intakes and food supplementation in relation to EED revealed no protective effect of one year of complementary food supplementation on risk of EED at 18 months. Still, we did observe relationships between total dietary intakes, from usual complementary foods and CFSs, and EED. The protective effect of dairy consumption against elevated L:M requires further investigation, as components of animal milk may help maintain and repair enterocytes, but dairy consumption was uncommon in this sample, so confounding by another dietary or other factor remains possible. The observed protective effect of total zinc intake and detrimental effect of total iron intake are both notable and consistent with prior literature. It is possible that these

contrasting effects explain the lack of overall impact of the multiple micronutrient fortified CFSs on risk of EED. The findings also underscore the extent to which known but underappreciated factors, namely enteric effects of zinc deficiency and pro-inflammatory consequences of excess low-bioavailable iron intake, may be primary drivers of EED. The relative contributions of nutritional and environmental factors and their interactions in the development and persistence of EED continues to require further investigation.

As expected, stunting was highly prevalent in our sample, while moderate wasting was surprisingly common as well. The dynamics of growth were unlike what is typically described in the literature, however, with stunting declining and wasting increasing over 18 to 24 months. The associations we found between EED markers and rate of weight, WAZ and WLZ change but not length or LAZ change are consistent with those larger trends in growth, but have implications for future studies of EED. For one, relationships between EED and growth may be observed more clearly earlier in the complementary feeding period when the rate of normal linear growth is greater. Later in childhood, effects of EED on health may be better assessed with other outcomes, such as cognitive development, morbidity burden and biomarkers of nutritional and metabolic status or physiologic stress. Further, the implications for longer-term stature and health of EED and other exposures limiting ponderal growth during the complementary feeding period are not, to our knowledge, well described in the literature and may require further examination. Finally, these findings challenge assumptions regarding the plasticity of

linear growth during the second year of life, which may need to be reassessed, as those assumptions are currently central to strategies for combatting stunting.

We found that the pathway of effect that we set out to define, from CFSs to growth via reduced EED, did not emerge in our data. Still, findings linking EED to dietary intake and to patterns of growth enhance the literature and advance thinking about the causes and consequences of EED, as do the null findings that challenge current EED assessment methodologies and excessive attribution to EED of stunting that emerges in the second year of life.

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Appendix 1: Data collection forms

Appendix 1A: Consent statement

Appendix 1B: Data collection forms

1. Substudy Child 18 Month Gut Examination (SC18MoGE)
2. Substudy Child 18 Month Stool Examination (SC18MoSE)

The JiVitA-4 Project
and
Johns Hopkins Bloomberg School of Public Health, Baltimore, USA

Sub-study Gut Health - Informed Consent Document
(*Completed by Team Leader Interviewer*)

Study Title: Evaluation of Complementary Food Supplements For Reducing Childhood Undernutrition

Principal Investigator: Parul Christian (JHU), Tahmeed Ahmed (ICDDR,B)
IRB#: 00003703
Version Date: v1, July 18, 2013

Introduction:
Salaam alaikuum. I am a worker of the JiVitA Project being implemented by Johns Hopkins University (*USA, PI: Parul Christian*) as a project under the Ministry of Health and Family Welfare, Government of Bangladesh. I am visiting you as you and your child are already participating in the JiVitA study involving food supplements or nutrition education and in a special health checkup study.

Procedures:
I am here today as your child is about 18 months of age. As you may remember, you gave permission to bring your child, when she/he reached 18 months, to a nearby location (name of JiVitA local office) for some special tests. In addition to those tests, we want to study the health of your child's "gut". To do this we will collect his/her urine and fresh stool. I am here to explain how to collect a sample of stool after a child has defecated. Another JiVitA worker will come to pick up the stool at your home. Urine from your child will be collected when you visit the JiVitA office. First we will give your child water mixed with special sugar. Urine will be collected over 2 hours after this.

Risks/Discomforts:
Taking stool and urine are safe. The sugar drink poses a small risk of causing loose stools for a short time.

Benefits:
There are no direct personal benefit to your child undergoing these tests.

Protecting data confidentiality:
Any information obtained as a result of your participation in these activities will be kept confidential by JiVitA staff. Your identity or that of your child will not be revealed when the information is used. The JiVitA study will keep your child's samples of urine and stool safe and secure from anyone outside of the project.

Protecting subject privacy during data collection:
The stool sample will be collected in the privacy of your home. The urine samples will also be collected in a private area in the JiVitA office.

Cost of participation in the study:
There is no cost to you for participating in the study. The urine and stool collection will not take much of your time. The urine collection will be done during the same time when you visit our study office for other tests and will take another hour of your time.

Biological specimens:
The urine we collect will be to measure, later in a laboratory, nutrients and other substances that will inform us about your child's health. Some of the urine and stool we collect from your child may be stored for a longer time in our laboratories so that other nutrients and substances can be checked in future. If you permit us to keep the urine and stool for long term storage, you may in the future change your mind and ask that your specimens be removed from storage and destroyed and we will do that.

Voluntariness:
We are asking you to participate in these additional special tests. Your decision to participate is up to you. If you want to stop or refuse any measurements, you are free to do so at any time. Your decision to take part or not will not affect your right to receive JiVitA study food supplements or nutrition education. Please know that joining in these activities does not replace good health care.

Persons to Contact:
If you have any questions or problems about the study, I can answer them now or you may contact our area coordinator (mention area coordinator's name) in the local field office. For questions about your rights as human subjects in this project you may contact Dr. Hasmot Ali or Mr. Abu Ahmed Shamim, senior project officers, through the Gaibandha JiVitA office (tel: 0541-61283) or contact Mr Salam Khan at the local IRB office in Dhaka (tel: +880-2-8860523-32, extension 3206).

Do you agree to allow us to give your child the sugar drink and to collect urine and stool from your child? (*If yes, proceed. If no, do not collect urine/stool and note on form.*)

Do you agree to allow JiVitA to keep urine/stool we collect for up to ten years? (*If yes, proceed. If no, collect urine/stool but note refusal of long-term storage on form.*

If you agree, can you please sign or make your mark below on two copies of this form? You will receive one copy of this form and we will keep the other one.

Thank you.

Signature or Mark of Parent _____ Date _____

Signature of Person Obtaining Consent _____ Date _____

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Substudy Child 18 Month Gut Examination (SC18MoGE)

Week of Examination: ☐☐☐ Date: ☐☐ - ☐☐ - ☐☐ (dd - mm - yy)

SPECIMEN ID:

Worker ID: ☐☐☐☐ _____ Initials

SECTION A: IDENTIFIERS/ADDRESSES

(Note to SST: Confirm name and identifiers of the woman and child on the form. Please draw a '✓' against each checked identifier.)

SST
PLACE
CHILD UID
STICKER HERE

DMC DO NOT ENTER:
SST copy from SS_SST:

HH ID: ☐☐☐☐

If no sticker, SST complete from Substudy Schedule:

Union ☐☐☐ Mauza _____ TL PIN ☐☐ Sector ☐☐☐

Mother UID: ☐☐☐☐☐☐ Mother's name: _____

Child UID: ☐☐☐☐☐☐ Father's name: _____

Sex: ☐ 1=Male 2=Female Child's name: _____

SST complete from Daily Schedule:

1. Child's Weight: ☐☐.☐☐ kg

2. Redo anthropometry? ☐ 0= No 1= Yes (Complete Section E at end of exam)

Form Status: 1= Form completed

☐ 2=Not met until 4 weeks after 18 month birthday
5=Child adopted
6=Refused examination
7=Permanently moved
8=Child died → **STOP**

SECTION B: INTAKE OF FOOD

1. Time last food consumed: ☐☐ : ☐☐ ☐ (Hours : Minutes; 1=AM 2=PM)

   Calculated dosing time (+ 2 hrs): ☐☐ : ☐☐ ☐ (Hours : Minutes; 1=AM 2=PM)

2. Time child was last breastfed: ☐☐ : ☐☐ ☐ (Hours : Minutes; 1=AM 2=PM; 99:99 = Don't know/ Not applicable)

SECTION C: 7-DAY MORBIDITY ASSESSMENT

How many days in the past 7 days has your child had:

1. A high fever (hot to touch)? ☐ (0-7 = Number of days; 9 = Don't know)
2. Loose/watery stools? ☐ (0-7 = Number of days (Go to 2a if >0); 9 = Don't know)
   - 2a. Has child had today? ☐ (0= No; 1= Yes (**STOP** and reschedule visit))
3. Bloody stools? ☐ (0-7 = Number of days (Go to 3a if >0); 9 = Don't know)
   - 3a. Has child had today? ☐ (0= No; 1= Yes (**STOP** and reschedule visit))
4. Cough, cold, or difficulty breathing? ☐ (0-7 = Number of days; 9 = Don't know)

SECTION D: LACTULOSE:MANNITOL TEST

1. Did the child urinate within hour before dosing? ☐ (0= No; 1= Yes)
2. Urine collection pot number: ☐☐
3. Date of dose preparation (on label): ☐☐ - ☐☐ - ☐☐ (dd - mm - yy)
4. Dose volume (on label): ☐☐ mL
5. Weight of Dose (LM solution + Cup + Cap) ☐☐.☐ g
   --remove parafilm
6. Did the child ingest the dose? ☐ (0=No (**STOP**); 1=Yes; 2=Yes, with incident)

Comments:

7. Time of Dosing: ☐☐ : ☐☐ ☐ 1=AM 2=PM
   (Hour : Minutes)

   20 ml Water Given (☑): ☐

   + 2 hrs

   Calculated Collection End Time: ☐☐ : ☐☐ ☐ 1=AM 2=PM
   (Hours : Minutes)

8. Weight of Empty Cup + Cap: ☐☐.☐ g

9. Urine collection log:

Time of Collection (hour: minutes)	Post-collection weight (g)
i ☐☐ : ☐☐ ☐ 1=AM 2=PM	☐☐☐
ii ☐☐ : ☐☐ ☐ 1=AM 2=PM	☐☐☐
iii ☐☐ : ☐☐ ☐ 1=AM 2=PM	☐☐☐
iv ☐☐ : ☐☐ ☐ 1=AM 2=PM	☐☐☐

10. Follow up urine collected? ☐
    0= None collected
    1= Yes
    2= Yes, with incident
    6= Refused

    Chlorhexidine added (☑): ☐

    Urine sample aliquoted (☑)? A☐ 4.5 mL B☐ 2 mL C☐ 2 mL

    Labels placed on urine tubes (☑): A☐ B☐ C☐

Comments:

SECTION E: ANTHROPOMETRY

1. Weight: ☐☐.☐☐ kg
   00.00=Refused
   99.99=Not possible to measure

2. Length (cm)
   a. ☐☐.☐ b. ☐☐.☐ c. ☐☐.☐
   00.0=Refused
   99.9=Not possible to measure

SST NOTE: Enter "1" for Form Status on Page 1

SECTION F: FOR LAB USE ONLY (*DMC please enter*)

1. Sample status on receipt: ☐
   0= Cold packs frozen, sample cold
   1= Cold packs melted, sample cold
   2= Cold packs melted sample warm
   3= Sample frozen
   4= Sample lost

2. Urine samples received (☑)? A☐ B☐ C☐

3. Time samples frozen: ☐☐ : ☐☐ ☐ 1=AM 2=PM
   Hours *Minutes*

Date: ☐☐ ☐☐ ☐☐ Lab Worker ID: ☐☐☐☐ _____
dd mm yy *Initials*

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Substudy Child 18 Month Stool Examination (SC18MoSE)

Week of Collection: ☐☐☐ Date: ☐☐ - ☐☐ - ☐☐
dd *mm* *yy*

Worker ID: ☐☐☐☐ _____
Initials

SPECIMEN ID:

SECTION A: IDENTIFIERS/ADDRESSES

(Note to TL: Confirm name and identifiers of the woman and child on the form. Please draw a '✓' against each checked identifier.)

TL
PLACE
CHILD UID
STICKER HERE

DMC DO NOT ENTER:
TL copy from SS_GAIC

HH ID: ☐☐☐☐

If no sticker, TL complete from DMC List:

Union ☐☐☐ Mauza ☐☐☐☐☐☐☐☐☐☐ TL PIN ☐☐ Sector ☐☐☐

Mother UID: ☐☐☐☐☐☐ Mother's name: ☐☐☐☐☐☐☐☐☐☐

Child UID: ☐☐☐☐☐☐ Father's name: ☐☐☐☐☐☐☐☐☐☐

Sex: ☐ 1=Male 2=Female Child's name: ☐☐☐☐☐☐☐☐☐☐

Form Status: 1= Stool Collected

☐ 2=Not met until one week after clinic visit
6=Refused examination → **STOP**

SECTION B: STOOL SAMPLE COLLECTION

1. Date of defecation: ☐☐ - ☐☐ - ☐☐
dd *mm* *yy*

2. Time of defecation: ☐☐ : ☐☐ ☐ 1=AM 2=PM

3. Time of stool collection by mother: ☐☐ : ☐☐ ☐ 1=AM 2=PM
Hours *Minutes*

99:99 = Don't know

4. Sample pick-up by TL:

5a. Date: ☐☐ - ☐☐ - ☐☐ (*mm* - *dd* - *yy*) 5b. Time: ☐☐ : ☐☐ (*Hours* : *Minutes*) ☐ 1=AM 2=PM

6. Sample status at household: ☐
   1= Cold pack cold, sample cold
   2= Cold pack warm, sample cold
   3= Cold pack warm, sample warm
   4= Sample frozen

TL NOTE: Enter "1" for Form Status on Page 1

SECTION C: FOR LAB USE ONLY (*DMC please enter*)

1. Sample status on receipt: ☐
   0= Cold pack frozen, sample cold
   1= Cold pack melted, sample cold
   2= Cold pack melted sample warm
   3= Sample frozen
   4= Sample lost

2. Stool sample received (☑)? ☐

3. Sample weight (Stool + Cup + Cap): ☐☐.☐☐ g

4. Stool type: ☐
   0= Formed, hard
   1= Formed, soft
   2= Unformed
   3= Liquid

5. Samples aliquoted (☑)? A☐ B☐ C☐

6. Labels placed on cryovials (☑): A☐ B☐ C☐

7. Time samples frozen: ☐☐ : ☐☐ ☐ 1=AM 2=PM (*Hours* : *Minutes*)

Date: ☐☐ - ☐☐ - ☐☐ (dd - mm - yy) Lab Worker ID: ☐☐☐☐ _____ *Initials*

Appendix 2: Supplemental results tables

Table A2.1. Baseline characteristics of children enrolled in the EED study and those in the main supplementation trial only

Child/Household Factor	EED Study (n=539)		Main Study Only (n=4,910)		P-value[1]
	n	Mean (SD)/%	n	Mean (SD)/%	
Sex (female)	269	49.9	2459	50.1	0.939
Stunting (LAZ<-2)	148	27.7	1203	25.0	0.184
Underweight (WAZ <-2)	106	19.8	979	20.3	0.770
Wasting (WLZ<-2)	27	5.0	280	5.8	0.462
Household Characteristics					
Owns land	396	73.7	3403	69.6	0.047
Owns cattle	268	49.9	2498	51.0	0.624
Owns goats	154	28.7	1214	24.8	0.049
Irrigation pump	93	17.3	570	11.6	0.000
Electricity	165	30.7	1532	31.3	0.786
Improved toilet[2]	443	82.2	3745	76.3	0.002
Living Standards Index (LSI)		-0.01 (0.99)		0.11 (1.05)	0.010
Mother's education					0.239
None	116	21.6	1192	24.4	
1-9 yrs.	348	64.8	3153	64.5	
SSC passed	33	6.1	238	4.9	
11+ yrs.	40	7.4	306	6.3	

[1]P-values from Pearson's χ^2 tests for all categorical characteristics; from ANOVA for continuous LSI. [2]Water sealed or slab toilet. Abbreviations: EED, environmental enteric dysfunction; LAZ, length-for-age z-score; LSI, living standards index; SSC, secondary school completion exam; WAZ, weight-for-age z-score; WLZ, weight-for-length z-score.

Table A2.2. Associations between lactulose and mannitol and intestinal and systemic health biomarkers in 18-month-old environmental enteric dysfunction (EED) study participants

Biomarker[1]	Lactulose					Mannitol				
	Univariate			Multivariable[2]		Univariate			Multivariable[2]	
	β (SE)	P-value	R^2	β (SE)	P-value	β (SE)	P-value	R^2	β (SE)	P-value
MPO (ng/mL)	0.11 (0.05)	0.025	0.011	0.01 (0.05)	0.825	-0.05 (0.06)	0.421	0.002	-0.09 (0.07)	0.175
AAT (µg/mL)	0.23 (0.05)	0.000	0.054	0.16 (0.05)	0.002	0.05 (0.05)	0.345	0.002	0.06 (0.06)	0.359
NEO (nmol/L)	0.06 (0.05)	0.215	0.003	0.10 (0.06)	0.059	-0.07 (0.06)	0.254	0.003	-0.03 (0.07)	0.647
GLP-2 (ng/mL)	-0.11 (0.08)	0.132	0.005	-0.07 (0.08)	0.421	-0.19 (0.09)	0.034	0.011	-0.10 (0.10)	0.332
EndoCAb (mu/mL)	0.02 (0.04)	0.565	0.001	-0.00 (0.05)	0.953	0.07 (0.05)	0.157	0.005	0.02 (0.06)	0.693
CRP (mg/L)	0.06 (0.02)	0.012	0.014	0.05 (0.03)	0.077	0.01 (0.03)	0.627	0.001	0.02 (0.04)	0.519
AGP (mg/dL)	0.03 (0.13)	0.795	0.000	-0.22 (0.16)	0.172	-0.12 (0.15)	0.426	0.001	-0.10 (0.20)	0.624
R^2				0.117					0.114	

[1]Biomarkers were log-transformed prior to analysis. [2]Multivariable model includes all listed biomarkers as independent variables in one model, adjusted for child and household characteristics: child sex, 6-month stunting status, household LSI, maternal education level, child age, assigned supplementation group, breastfeeding continuation to 18 months (yes/no), number of household members, maternal occupation (working outside of the home vs. not), maternal age, household land ownership, ownership of cattle, goats/sheep and chickens. Abbreviations: AGP, α-1 acid glycoprotein; AAT, α-1 antitrypsin; CRP, C-reactive protein; EndoCAb, endotoxin core antibody immunoglobulin G; GLP-2, glucagon-like peptide-2; L:M, lactulose:mannitol ratio; MPO, myeloperoxidase; NEO, neopterin.

Table A2.3. Environmental enteric dysfunction (EED) score development: principal component analysis (PCA) and partial least squares regression (PLS) model outputs for intestinal health biomarkers only and with systemic inflammation markers

Biomarker[1]	PCA				PLS[2]			
	Intestinal Health Biomarkers		Intestinal and Systemic Health Biomarkers		Intestinal Health Biomarkers		Intestinal and Systemic Health Biomarkers	
	Factor 1, Loading	Factor 2, Loading	Factor 1, Loading	Factor 2, Loading	Factor 1, Loading	Factor 1, Weight	Factor 1, Loading	Factor 1, Weight
MPO (ng/mL)	0.592	-0.036	0.150	0.535	0.711	0.654	0.556	0.587
AAT (µg/mL)	0.690	-0.038	0.013	0.665	0.619	0.605	0.468	0.544
NEO (nmol/L)	-0.185	0.458	0.288	-0.367	0.319	0.476	0.264	0.426
GLP-2 (ng/mL)	-0.366	-0.505	-0.009	-0.244	0.013	0.037	-0.009	0.032
EndoCAb (mu/mL)	-0.072	0.730	0.104	-0.263	-0.096	-0.083	-0.070	-0.077
CRP (mg/L)			0.660	0.087			0.481	0.428
AGP (mg/dL)			0.669	-0.022			0.409	0.236
% of X variance[3]	27.50	21.78	23.56	20.11	23.77		21.71	
% of Y variance[4]	2.26		3.26		5.21		5.42	

[1]Biomarkers were log-transformed and outliers removed prior to analysis. [2]Partial least squares regressions with dependent variable log-transformed L:M ratio. [3]Percent of total variance in independent variables (biomarkers) explained by each factor. [4]Percent of variance in L:M ratio explained by each score model (generated by PLS command; for PCA, based on R^2 from regression of log-transformed L:M ratio on PCA factors 1 and 2). Abbreviations: AGP, α-1 acid glycoprotein; AAT, α-1 antitrypsin; CRP, C-reactive protein; EED, environmental enteric dysfunction; EndoCAb, endotoxin core antibody immunoglobulin G; GLP-2, glucagon-like peptide-2; L:M, lactulose:mannitol ratio; MPO, myeloperoxidase; NEO, neopterin; PCA, principal component analysis; PLS, partial least squares regression.

Table A2.4. Mean values of individual intestinal and systemic health biomarkers by domains of child and household characteristics in 18-month-old environmental enteric dysfunction (EED) study participants

Domain/ Characteristic	MPO (ng/mL), GM (95% CI)[1]	AAT (µg/mL), GM (95% CI)	NEO (nmol/L), GM (95% CI)	GLP-2 (ng/mL), GM (95% CI)	EndoCAb (mu/mL), GM (95% CI)	CRP (mg/L), GM (95% CI)	AGP (mg/dL), GM (95% CI)
Sociodemographic Characteristics							
Sex							
Male	4460.7 (4016.0, 4954.8)	332.8 (298.4, 371.1)	726.3 (658.5, 801.0)	2.9 (2.8, 3.1)	46.6 (41.3, 52.5)	1.2 (1.0, 1.4)	105.3 (101.3, 109.5)
Female	4459.8 (4024.0, 4942.7)	320.8 (288.9, 356.3)	811.5 (737.0, 893.5)	3.1 (2.9, 3.3)	44.2 (39.2, 49.8)	1.2 (1.0, 1.5)	106.2 (101.9, 110.6)
p-value	0.998	0.634	0.112	0.464	0.544	0.697	0.788
LSI							
Low	4460.9 (4056.7, 4905.5)	319.7 (287.5, 355.4)	767.3 (697.8, 843.6)	3.1 (2.9, 3.4)	45.7 (40.8, 51.3)	1.1 (0.9, 1.4)	106.7 (102.5, 111.2)
High	4470.5 (3993.5, 5004.3)	335.7 (301.3, 374.1)	767.5 (694.2, 848.5)	2.9 (2.7, 3.1)	45.0 (39.7, 51.0)	1.3 (1.0, 1.6)	104.9 (100.8, 109.0)
p-value	0.977	0.524	0.997	0.049	0.851	0.290	0.536
Improved toilet[2]							
No	4561.3 (3910.5, 5320.3)	374.2 (304.0, 460.5)	726.5 (625.0, 844.5)	3.2 (2.9, 3.6)	40.2 (33.9, 47.6)	1.3 (1.0, 1.9)	107.6 (100.9, 114.7)
Yes	4439.4 (4086.6, 4822.8)	317.4 (292.9, 343.9)	776.7 (719.1, 839.0)	2.9 (2.8, 3.1)	46.6 (42.3, 51.3)	1.2 (1.0, 1.4)	105.3 (102.1, 108.7)
p-value	0.784	0.100	0.462	0.109	0.186	0.462	0.573
Systemic Inflammation							
Elevated CRP[3]							
No	4316.8 (3976.4, 4686.2)	325.7 (298.9, 354.9)	750.0 (693.1, 811.5)	3.1 (2.9, 3.2)	44.7 (40.6, 49.2)		
Yes	5176.6 (4360.7, 6145.1)	370.1 (309.7, 442.4)	828.1 (708.1, 968.4)	2.7 (2.5, 3.0)	47.8 (40.0, 57.2)		

p-value	0.051	0.193	0.264	0.043	0.521		
Elevated AGP[3]							
No	3943.0 (3529.3, 4405.3)	319.2 (281.9, 361.3)	682.6 (616.4, 755.8)	2.9 (2.7, 3.1)	46.8 (40.9, 53.6)		
Yes	4948.6 (4484.4, 5460.9)	344.4 (311.8, 380.4)	836.5 (760.4, 920.2)	3.1 (2.9, 3.3)	44.5 (39.9, 49.6)		
p-value	0.003	0.340	0.005	0.275	0.556		
Anthropometry							
Stunted[4]							
Yes	4403.2 (3975.6, 4876.9)	321.5 (291.1, 354.9)	798.2 (727.9, 875.2)	2.9 (2.7, 3.1)	49.7 (44.1, 55.9)	1.2 (1.0, 1.4)	104.4 (100.7, 108.3)
No	4513.5 (4058.9, 5019.1)	331.3 (294.5, 372.8)	725.5 (653.8, 805.0)	3.1 (2.9, 3.3)	40.7 (36.1, 45.9)	1.2 (1.0, 1.5)	107.4 (102.7, 112.3)
p-value	0.742	0.697	0.176	0.133	0.021	0.892	0.334
Wasted[4]							
Yes	4576.0 (4223.4, 4958.0)	329.5 (303.5, 357.8)	782.5 (725.8, 843.6)	2.9 (2.8, 3.1)	45.9 (41.8, 50.5)	1.2 (1.1, 1.5)	105.6 (102.5, 108.8)
No	3838.7 (3175.3, 4640.8)	302.1 (245.9, 371.1)	650.7 (547.1, 774.0)	3.4 (3.0, 3.8)	42.1 (35.0, 50.8)	1.0 (0.7, 1.5)	106.9 (97.9, 116.7)
p-value	0.103	0.427	0.060	0.026	0.483	0.258	0.767

[1]Values are geometric mean (95% confidence interval). P-values are from simple linear regression models with dependent variable log-transformed biomarker and indicator variable for child/household characteristic. [2]Water sealed or slab toilet. [3]Elevated CRP>5 mg/L; elevated AGP>100 mg/dl (Thurnham et al 2015).[4]Stunted, length-for-age z-score<-2; wasted, weight-for-length z-score<-2 (based on WHO Multicentre Growth Reference Study Group 2006). Abbreviations: AGP, α-1 acid glycoprotein; CRP, C-reactive protein; EED, environmental enteric dysfunction; GM, geometric mean; LAZ, length-for-age z-score; L:M, lactulose:mannitol ratio; LSI, living standards index; PCA, principal component analysis; PLS, partial least squares regression; SSC, secondary school completion exam; WLZ, weight-for-length z-score.

Curriculum Vitae

Rebecca Kramer Campbell, MSPH

420 E 70th St #12A, New York, NY 10021 904-716-7833, rfkramer@jhu.edu

EDUCATION

2012-Present **Johns Hopkins Bloomberg School of Public Health**, Baltimore, MD
PhD Candidate
Concentration: Human Nutrition, International Health
Dissertation title: *Environmental enteric dysfunction in early childhood: bridging the gap between diet and stunting in a randomized trial of complementary food supplementation in rural Bangladesh*

2010-2012 **Johns Hopkins Bloomberg School of Public Health**, Baltimore, MD
Master of Science in Public Health
Concentration: Human Nutrition, International Health

2005-2009 **Brandeis University**, Waltham, MA
Bachelor of Arts, Cum Laude, Dean's List
Major: Economics; Minor: Mathematics

2008 **Jawaharlal Nehru University**, New Delhi, India
Centre for Economic Studies and Planning
Study abroad program coordinated by IES Abroad Delhi

2007 **Sea|Mester**, Mediterranean Sea
Coastal Navigation and International Crew Certifications

PROFESSIONAL EXPERIENCE

2015 **Project Manager**
Nutrition in the First 1,000 Days of Life eCourse Project, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

2011-Present **Graduate Research Assistant**
JiVitA Bangladesh Project, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD; Principal Investigator Parul Christian, DrPH

2011 **Graduate Research Assistant**
Nepal Nutrition Intervention Project – Sarlahi, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD; Principal Investigator Keith West, DrPH

2011 **Graduate Research Assistant**
Baltimore Healthy Eating Zones, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD; Principal Investigator Joel Gittelsohn, PhD, MS

PROFESSIONAL ACTIVITES

Teaching

2015 **Grading Assistant**
Food and Nutrition Policy (1st Quarter), Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD; Instructor: Rolf D.W. Klemm, DrPH

03/22/2015 **Guest Lecturer**
Fundamentals of Epidemiology, Krieger School of Arts and Sciences, Johns Hopkins University, Baltimore, MD; Instructor: Darcy Phelan-Emrick, PhD

2015 **Section Instructor**
Fundamentals of Epidemiology (Spring Semester), Krieger School of Arts and Sciences, Johns Hopkins University, Baltimore, MD; Instructor: Darcy Phelan-Emrick, PhD

10/03/2014 **Invited Co-Presenter**
Critical Thinking in Nutrition, Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD; Instructor: Lawrence Cheskin, MD

2014 **Teaching Assistant**
Assessment of Nutritional Status (2nd Quarter), Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD; Instructor: Kerry Schulze, PhD

2014 **Teaching Assistant**
Food and Nutrition Policy (1st Quarter), Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD; Instructor: Rolf D.W. Klemm, DrPH

2013 **Teaching Assistant**
Nutrition in Disease Treatment and Prevention (4th Quarter), Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD; Instructor: Laura Murray-Kolb, PhD

2013 **Teaching Assistant**
Nutrition and Life Stages (3rd Quarter), Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD; Instructor: Parul Christian, DrPH

2009-2010 **CoaHCorps Full-time Tutor**
City on a Hill Public Charter High School, Roxbury, MA

Society Memberships

2011-Present American Society for Nutrition
Global Nutrition Council

Editorial Service (Ad Hoc Reviewer)

Nutrition (2); Ecology of Food and Nutrition (2)

Consultancies

Summer 2015 Division of Data, Research and Policy, Data and Analytics Section, UNICEF, New York, NY; Supervisor: Julia Krasevec

Fall 2014 Division of Data, Research and Policy, Data and Analytics Section, UNICEF, New York, NY; Supervisor: Julia Krasevec

AWARDS AND HONORS

2015 Harry D. Kruse Publication Award in Human Nutrition

2015 George Graham Endowment Travel Award

2014 International Health Departmental Alumni Award

2013-2014 Harry J. Prebluda Fellowship in Nutritional Biochemistry

2013 George Graham Endowment Travel Award

2012-2013 Bacon Chow Memorial Fellowship

2012 Harry D. Kruse Publication Award in Human Nutrition

2012 George Graham Endowment Travel Award

2005-2009 Justice Louis D. Brandeis Scholar

LANGUAGES

Proficient Bangla

Basic French

PUBLICATIONS

Shin A, Surkan PJ, Coutinho AJ, Suratkar SR, **Campbell RK**, et al. Impact of Baltimore Healthy Eating Zones: an environmental intervention to improve diet among African American youth. Health Educ Behav 2015;42:97S-105S.

Campbell RK, Talegawkar SA, Christian P, et al. Evaluation of a novel single-administration food frequency questionnaire for assessing seasonally varied dietary patterns among women in rural Nepal. Ecol Food Nutr: 2015:1-14.

Campbell RK, Talegawkar SA, Christian P, et al. Seasonal dietary intakes and socioeconomic status among women in the Terai of Nepal. J Health Popul Nutr. 2014; 32:198-216

Shamim AA, Hanif AAM, Merrill RD, **Campbell RK**, et al. Preferred delivery method and acceptability of Wheat Soy Blend (WSB++) as a daily complementary food supplement in northwest Bangladesh. Ecol Food Nutr. 2015;54:74-92.

Kramer RF, Coutinho AJ, Vaeth E, et al. Patterns of home food preparation and youth and caregiver psychosocial factors are associated with youth BMI in African American households. J Nutr. 2012;142: 948-954.

PRESENTATIONS

Campbell R, Hurley K, Shamim AA, et al. Children Receiving Complementary Food Supplements (CFS) Have Higher Nutrient Intakes from Home Foods in JiVitA-4 Field Trial, Rural Bangladesh. Asian Congress of Nutrition 2015: Yokohama, Japan.

Campbell R, Hurley K, Shamim AA, et al. Children Receiving Complementary Food Supplements (CFS) Have Higher Nutrient Intakes from Home Foods in JiVitA-4 Trial, Rural Bangladesh. Experimental Biology 2015: Boston, MA.

Kramer RF, Shamim AA, Kumkum MA, et al. Formative study on the acceptance of Wheat Soy Blend Plus Plus (WSB++) as a daily complementary food supplement (CFS) in rural Bangladesh. Experimental Biology 2013: Boston, MA.

Kramer RF, West KP, Talegawkar SA, Christian P. A novel food frequency questionnaire (FFQ) to assess usual seasonal intakes in rural Nepalese women. Experimental Biology 2012: San Diego, CA.

Date of Birth: May 30, 1987

Place of Birth: Pittsburgh, Pennsylvania