Environmental Enteropathy and Fibroblast Growth Factor 21 are associated with early childhood growth in Bangladesh

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Abstract

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Chronic undernutrition, marked by stunting (low height for age), is associated with increased child morbidity and mortality worldwide. Current intervention strategies are often ineffective at preventing or reversing stunting; while some children grow in response to nutrition programs, many do not. This dissertation aims to improve understanding of two major challenges in the prevention and treatment of chronic undernutrition in previously accrued cohorts of young Bangladeshi children.

CHAPTER 1: Environmental enteropathy (EE), a subclinical intestinal disorder characterized by mucosal inflammation, reduced barrier integrity, and malabsorption, appears to be associated with increased risk of stunting in children in low and middle income countries. Fecal biomarkers indicative of EE [neopterin (NEO), myeloperoxidase (MPO), and alpha-1-antitrypsin (AAT)] have previously been negatively associated with 6-month linear growth.

Associations between fecal markers (NEO, MPO and AAT) and short-term linear growth were examined in a birth cohort of 246 children in Bangladesh. Marker concentrations were

categorized in stool samples based on their distribution (<1st quartile, IQR, >3rd quartile), and a 10-point composite EE score was calculated. Piecewise linear mixed-effects models were used to examine the association between markers measured quarterly (in months 3-21, 3-9, and 12-21) and 3-month change in length for age z-score (ΔLAZ).

Children with high MPO levels at quarterly time points lost significantly more LAZ per 3-month period during the second year of life than those with low MPO (ΔLAZ= -0.100; 95% CI: -0.167, -0.032). AAT and NEO were not associated with subsequent growth, however composite EE score was negatively associated with subsequent 3-month growth.

In this birth cohort of children from an urban setting in Bangladesh, elevated MPO levels, but not NEO or AAT levels, were associated with decreases in short-term linear growth during the second year of life, supporting previous data suggesting the relevance of MPO as a marker of EE.

CHAPTER 2: Undernutrition as manifest by poor child growth is prevalent in resource limited countries and is associated with child morbidity and mortality. Current nutritional intervention strategies are often insufficient to improve child growth; some children respond while others do not. There is an unmet need to identify children who are likely to respond to supplemental nutrition as well as those in need of additional interventions beyond nutritional rehabilitation. Fibroblast growth factor 21 (FGF21) is an endocrine signal of protein restriction that regulates metabolism and growth during periods of reduced protein intake. This study sought to determine the association between plasma FGF21 levels and growth in children receiving nutritional supplementation.

120 underweight children aged 6–13 months were enrolled from an urban slum in Dhaka, Bangladesh. Children received 300 kcal feeding supplements daily for five months and were followed for seven additional months. FGF21 was measured in stored plasma at enrolment and month five. Linear mixed-effects models were used to examine the association between baseline FGF21 and age-standardized length-for-age Z score (LAZ), weight-for-age Z score (WAZ), and head circumference-for-age Z score (ZHC) during and after nutritional supplementation.

At enrolment, the median FGF21 concentration was 241.4 pg/ml (interquartile-range: 111.7, 451.3), and 9 (7.5%) of children had high FGF21. High baseline FGF21 levels were associated with increases in WAZ (0.10 per month, 95%CI: 0.03, 0.16) and ZHC (0.05 per month, 95%CI: 0.01, 0.09) during nutritional supplementation, while low FGF21 levels were associated with decreases in LAZ (-0.09 per month, 95%CI: -0.10, -0.07) and ZHC (-0.04 per month, 95%CI: -0.06, -0.03). Following supplementation, children with low FGF21 continued to experience decreases in LAZ, ZHC, and WAZ. On average, WAZ, LAZ and ZHC did not change during the post-supplementation period among those with high baseline FGF21.

FGF21 may be a useful tool to both identify undernourished children likely to benefit from nutritional supplementation and to identify children in whom additional interventions may be necessary to improve growth.

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Introduction

Chronic undernutrition, marked by stunting, underlies substantial global child morbidity and mortality (1). Stunting is associated with a 5-fold increase in mortality risk among children under five years of age (2, 3); largely attributable to deaths from pneumonia, diarrhea and respiratory illness (4-6). In South Asia and sub-Saharan Africa it is estimated that greater than a third of children under the age of five are stunted (7, 8). Current nutritional intervention strategies are largely ineffective at preventing or reversing stunting in early life (9). Non-invasive tools to identify children at highest risk for stunting and to identify those who are likely to grow in response to available interventions are lacking. We sought to 1) understand the role of intestinal inflammation in predicting poor linear growth in a Bangladeshi birth cohort, and 2) determine whether an endocrine hormone may identify a subgroup of underweight infants who grow while receiving nutritional supplementation.

Defining stunting/chronic undernutrition and links to negative health outcomes

Linear growth faltering, or stunting is a marker for chronic undernutrition. Stature is measured as recumbent length for children under 24 months of age, and height when a child is older than months. A child is considered "stunted" when their height is more than two standard deviations (SD) below the mean height expected from a well-nourished child of that age and sex (length-for-age z-score (LAZ)<-2) (10). Stunting is an indicator which provides an approximation of the prevalence of undernutrition in a population, as only around 2.5% of children in a healthy population would be expected to have heights more than 2 SD below the mean. Because height is downwardly inflexible, poor linear growth is reflective of the cumulative impacts of sustained deprivation and/or unmet metabolic needs, and can be considered a marker of "chronic

undernutrition." Other important population-based anthropometric metrics of undernutrition include underweight (weight-for-age z-score (WAZ) <-2) and wasting (weight-for-length z-score (WLZ) < -2). These measures may be more indicative of acute states of deprivation than stunting, and such children can benefit from provision of food with increased nutrient content and composition. However wasting is valuable for identifying children in need of immediate medical attention; severe wasting (also called severe-acute-malnutrition (WLZ< -3)), is an important warning sign of highly increased infection vulnerability and risk of mortality (11).

Stunting in early childhood has consequences beyond physical development. Stunting is a marker of impaired immune protection and immune system development, sub-optimal cognitive development, and reduced economic potential. In particular, stunting prior to two years of age is associated with decreased cognition, poor school readiness and performance, and reduced economic productivity later in life (8, 12-16). In addition, women who were stunted in childhood have increased risk of maternal mortality and morbidity, and increased risk of delivering small for gestational age infants (8, 15, 17, 18). The children born to such women have a higher risk of death in first 5 years and are more likely to be stunted themselves (17). Therefore, prevention and reversal of child stunting in developing settings may yield multiple sustained population health benefits.

Global timing and distribution of stunting

The first 1000 days after conception appears to be critical for child growth and children who become stunted during this critical period are unlikely to make up this height growth later in life (19, 20). The degree to which poor prenatal growth is responsible for postnatal stunting varies geographically; the estimated mean LAZ among newborns in South Asia (-0.75) is more than a half z-score lower than that of newborns in Europe (-0.23). While infants in Sub-Saharan Africa

begin life on average with higher LAZ than those in South Asia, their average height declines rapidly in the first year and with continual declines in the second year closely resembling that of South Asian children. These differences suggest geographical variation in the relative importance and contributions of causal factors for stunting across the first 1,000 days of life.

Current intervention strategies to treat or prevent stunting are largely ineffective

The development of linear growth faltering is multifactorial; many children in low and middle income countries become stunted despite adequate nutritional intake. Furthermore, nutritional interventions alone often do not effectively normalize growth in stunted children under 2 years of age (9). It is estimated that scaling up implementation of the 10 best evidence-based nutrition interventions to 90% coverage in the 34 highest burden countries would reduce stunting in children under age five by an average of 20.3% (21). That these interventions would fail to prevent or reverse roughly 80% of stunting highlights the need to improve understanding of the underlying determinants of poor child growth in order to more effectively intervene.

Known contributors to stunting include intrauterine growth restriction, low birth weight, inappropriate infant feeding practices, micro and macronutrient deficiencies, and recurrent and chronic infections. A range of functional changes in the small intestine, collectively termed environmental enteropathy (EE), are prevalent among children in resource-limited settings (22-24) and appear to influence risk of stunting (25-27). The complex physiologic mechanisms involved in EE may provide clues far in advance of significant growth or cognitive decline, and therefore markers of EE are being sought in order to investigate intervention strategies. Underlying EE may be one of the factors which makes linear growth faltering especially impervious to nutritional interventions.

When we consider poor child growth broadly, there is there is an unmet need to identify children who are likely to grow in response to supplemental nutrition, in contrast to those needing additional interventions (such as for EE). One strategy is to examine signals which disturb the function of the growth-hormone axis that is essential for normal child growth and development. Disruption of the growth-hormone axis may be partially responsible for the attenuation of child growth in cases of inadequate nutrition and may also be related to inflammation and infection (28-30). Therefore, hormones which regulate the growth-hormone axis in response to nutrient deprivation in particular, may be useful to identify children in whom poor growth could be improved with provision of nutritional supplementation, as well as those children whose poor growth requires additional intervention(s).

Dissertation objectives

This dissertation aims to improve understanding of two major challenges in the prevention and treatment of chronic undernutrition in previously accrued cohorts of young Bangladeshi children. First by examining candidate markers of EE, and second by determining whether a hormone, Fibroblast Growth Factor 21 (FGF21), may be useful in identifying a subset of underweight children most likely to grow while receiving nutritional supplementation.

EE: Infants in South Asia are born smaller on average than in other regions, and it is important to quantify the degree to which EE plays a role in the persistence and worsening of linear growth deficits among children in this setting. Bangladesh has one of the highest prevalences of stunting in the world (31) and EE has been associated with poor immunologic responses to routine immunizations among Bangladeshi infants (32). The first chapter of this dissertation sought to identify critical periods during which three candidate fecal markers of EE were associated with the attenuation of linear growth in a birth cohort of Bangladeshi children. We were interested in

whether intestinal inflammation plays an etiologic role in growth faltering, and therefore linear growth following EE marker assessment was the appropriate outcome. We used piecewise linear mixed effects models to incorporate EE marker data from multiple time points, allow for natural variation in growth rates at different ages, include time-varying covariates (diarrhea in the period), and account for correlated data.

Fibroblast growth factor 21 (FGF21): FGF21 is an endocrine signal of protein restriction that regulates metabolism and growth during periods of reduced protein intake. FGF21 may be useful in predicting children's growth responsiveness to nutritional intervention, as high FGF21 could identify children whose poor growth is in part attributable to macronutrient deprivation. This hypothesis is based upon the following observations: blood levels of FGF21 increased in adults following prolonged fasting and chronic protein deprivation, elevated FGF21 levels led to growth hormone resistance in the liver and bone plate in murine and in-vitro models, and blood levels have been negatively associated with early child growth in two settings. It is not known whether EE influences circulating FGF21 levels in undernourished children.

The second chapter of this dissertation examines the association between circulating levels of FGF21 and subsequent growth in a cohort of underweight infants receiving nutritional supplementation in urban Bangladesh. An interaction term in linear mixed effects models of monthly anthropometry was used to test whether growth patterns differed between children with high or low baseline hormone levels. As we anticipated that any differences in growth would most likely occur during supplementation, the supplemented and non-supplemented periods were modeled separately. A cross-sectional comparison of mean EE marker levels in children with high versus low baseline FGF21 levels was also conducted.

CHAPTER 1: Fecal markers of environmental enteropathy and subsequent growth in Bangladeshi children

Introduction

Chronic malnutrition contributes substantially to global child morbidity and mortality. Over a third of children under the age of five years in South Asia and sub-Saharan Africa are stunted (length-for-age z-score (LAZ) <-2) (7). Stunting is associated with a 5-fold increased risk of mortality among children under age five (2, 3); largely as a result of increased mortality due to diarrhea, pneumonia and other respiratory illness (4-6). Stunting is also associated with decreased cognitive development, school readiness and performance, and reduced economic productivity later in life (8, 12-16). In addition, women who are stunted in childhood have increased risk of maternal mortality and morbidity and increased risk of delivering small for gestational age infants (8, 15, 17, 18). Children born to women who are stunted are more likely to be stunted themselves and have a higher risk of death in the first 5 years of life (17).

Environmental enteropathy, also known as environmental enteric dysfunction, a subclinical intestinal disorder observed among children living in settings of poor hygiene and sanitation, is highly prevalent among children in resource limited settings and is associated with reduced linear growth (22). EE is marked by mucosal and systemic inflammation, reduced intestinal barrier integrity, bacterial translocation, and reduced intestinal absorptive capacity (23, 33, 34). The intestinal histology of children with EE often includes villus atrophy, villus crypt proliferation, and lymphocyte infiltration of the lamina propria (22).

Obtaining specimens to examine histology requires invasive endoscopy and biopsy procedures. As a result, less invasive biomarkers of EE are of interest for defining children at risk (35-38).

Candidate EE fecal markers include those measuring intestinal inflammation (neopterin (NEO) and myeloperoxidase (MPO)) and those evaluating intestinal permeability (alpha-1-antitrypsin (AAT)). NEO is a molecule produced and released by macrophages and dendritic cells upon stimulation by activated T-lymphocytes and NEO concentration in stool is used as a marker of intestinal Th1 immune activation (39). MPO is a lysosomal protein contained within primary granules that are released into the gut lumen by activated neutrophils and other phagocytes in acute inflammation (40, 41). Higher concentrations of MPO in the stool suggest lymphocytic infiltration of the lamina propria, one of the histological findings associated with EE (22). AAT is a protease inhibitor abundant in serum which appears to protect cells from inflammatory proteases secreted by neutrophils and macrophages. AAT is a large, polar, molecule that does not cross the luminal barrier unless there is significantly aberrant permeability. As a result, clearance of AAT is a useful marker of intestinal permeability and it has been used as a marker of protein-losing enteropathy (42-44). All three fecal markers (NEO, MPO, and AAT) have been shown to be negatively associated with subsequent 6-month linear growth in children under 12 months of age in multiple settings in the multi-site Malnutrition and Enteric Diseases (MAL-ED) birth cohort (36, 37). In addition, a disease activity score (composite EE score) which combined these markers was more predictive of long-term growth faltering than any individual marker (37).

The first two years of life represent a critical period of growth and is also one in which children are frequently exposed to multiple enteric pathogens, particularly as they wean from breast milk (45). As the normal rate of linear growth varies considerably in the first two years of life, examination of the relationship between fecal markers and growth over short intervals is important for identifying period(s) during which these markers may be most predictive. While previous studies, including the large multi-site MAL-ED study have reported an association

between fecal markers and 6-month growth, the relationship of these markers with growth over shorter periods has not been described (37). In addition, there may be specific differences in the performance of these markers based on the population or geography in which they are tested. The present study describes the longitudinal patterns of AAT, MPO, and NEO among a cohort of children in Bangladesh with high rates of stunting to determine the contribution of EE to linear growth failure.

Methods

Study protocols were reviewed and approved by several Institutional Review Boards and regional health authorities as part of the multisite MAL-ED birth cohort, including the Ethical Review Committee of International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) (46).

Study population: The study utilized data from the Bangladesh MAL-ED site, a 2-year prospective observational study. Details of the parent study have been published previously (46, 47). Briefly, healthy newborns living in the Bauniabadh area of section 11 of Mirpur, an urban slum in Dhaka, Bangladesh were recruited by field workers within the first 17 days of life between February 2010 and February 2012 (46). Enrolled children were visited every other day for two years by field research assistants who interviewed their parents about morbidity and breastfeeding behavior. Exclusion criteria for the cohort study were "maternal age of <16 years, not a singleton pregnancy, another child already enrolled in the MAL-ED study, severe disease requiring hospitalization prior to recruitment, and severe acute or chronic conditions diagnosed by a physician (e.g. neonatal disease, renal disease, chronic heart failure, liver disease, cystic fibrosis, congenital conditions)" (46).

Data collection: Assessment of household/maternal information was done at enrollment, and caregivers reported morbidity and breastfeeding behavior every other day. Child anthropometry was assessed at monthly intervals, children were weighed using metric pediatric balances with a certified accuracy of 100 g and length was measured using a marked platform with a sliding footboard (48). Stool samples were collected without fixative by field health care workers during home visits and frozen at −70°C pending processing (36). Stool was collected at monthly intervals in the first year and quarterly intervals in the second year. Caregiver-reported diarrhea was defined as ≥3 loose stools/day. NEO, MPO, and AAT concentrations were measured in stool samples from months 3, 6, 9, 12, 15, 18, and 21 (quarterly) to ensure consistent time periods over the study period and to avoid inclusion of overlapping periods of growth. Stool samples were excluded from children who had acute diarrhea or diarrhea symptoms within 7 days prior to collection and from those who had dual sugar permeability testing within one day prior to collection. These factors alter stool water content to a variable extent, making the interpretation of fecal biomarker concentrations unreliable in the absence of obtaining dry weights. Obtaining dry weights was considered unfeasible given the number of specimens processed (36).

Laboratory Methods: All laboratory procedures were conducted in laboratories at icddr,b in Dhaka, Bangladesh. AAT, NEO, and MPO were measured in stool samples using commercially available enzyme-linked immunosorbent assays (ELISA) (36, 37). ELISAs were run per instructions on the package insert, except that for MPO (Alpco, Salem, NH) the initial dilutions run were 1:500 and NEO (GenWay Biotech, San Diego, CA) was diluted 1:1000 in 0.9% saline. AAT (Biovendor, Candler, NC) was run according to the package insert at a dilution of 1:500. Samples out of range of the standard curve for any of the assays were run at higher or lower concentration (as appropriate) (37).

Statistical Methods: All analyses were performed using STATA version 12.1 IC (College Station, Texas, USA). World Health Organization Anthro software for PC was used to calculate Z-scores from raw anthropometric data. Length-for-age data from regular intervals (3, 6, 9, 12, 15, 18, 21, and 24 months of age) were included in the analyses. All fecal marker results from +/- 15 days of the child's indicated age were included. Fecal marker concentrations were categorized based on distribution of all measurements: low (in 1st quartile), medium (in the interquartile range), or high (in 4th quartile). At each time point, the composite EE score (0-10) was calculated from the 3 fecal markers, as described in a previous study (37). Categories were assigned values as 0 (low), 1 (medium), or 2 (high). The formula for the composite EE score is:

$$EE\ score = 2 \times (AAT\ category) + 2 \times (MPO\ category) + 1 \times (NEO\ category).$$

To test the association between fecal markers and short-term linear growth, the individual fecal marker or the composite EE score was the primary exposure and the subsequent 3-month change in LAZ was the outcome. Growth periods of 3 months were chosen in order to account for differences in age-dependent growth rates and to examine potential determinants of growth velocity unrelated to age. This allowed for the inclusion of 7 non-overlapping 3-months growth periods which followed stool sample collection. The relationship between EE markers and growth was also examined separately in the first and second year, given the contribution of low birthweight to stunting and the fact that most stunting occurs in the first year of life. Diarrhea was included as a covariate in all models in order to view associations between fecal markers and growth beyond that explained by diarrhea. Diarrhea was parameterized as the proportion of days that a child experienced diarrhea in the 3-month period after stool collection. Exclusive breastfeeding was not included as a covariate due to collinearity with age.

Piecewise linear mixed effects models with first-order autoregressive residual structures were used to test the association between markers and short-term linear growth. The equations below depict the parameterization of the models for short-term growth and association with fecal markers for the full 21-month period (stool from months 3-21), early period (months 3-9), and second year (months 12-21) respectively. In the equations, the b_j term is a random intercept (unique for each study child), test=med indicates the marker was in the interquartile range, and test=high indicates that the marker was in the 4th quartile. LAZ at time of stool collection was included as a covariate in all models as it is highly correlated with subsequent growth. In models assessing the association between short-term growth and composite EE score, $\beta_{1 \text{ and }} \beta_2$ below were replaced by a single grouped linear term for composite EE score.

$LAZ_{(i+3),j} - LAZ_{ij} = b_j + \beta_0 + \mathbf{\beta_1(test= med)} + \mathbf{\beta_2(test= high)} + \beta_3(\text{Diarrhea}) + \beta_4(LAZ_{ij}) +$

Full $\beta_5(\text{month= 6}) + \beta_6(\text{month= 9}) + \beta_7(\text{month= 12}) + \beta_8(\text{month= 15}) + \beta_9(\text{month= 18}) + \beta_{10}(\text{month= 21})$

Months 3-9 $\beta_5(\text{month= 6}) + \beta_6(\text{month= 9})$

Months 12-21 $\beta_5(\text{month= 15}) + \beta_6(\text{month= 18}) + \beta_7(\text{month= 21})$

For comparison with the multi-site study findings, the relationships between fecal markers and subsequent 6-month growth were also examined with the same modelling approach and residuals structure as in a previous paper of the multi-site MAL-ED study (37).

Results

Overall, 265 children were enrolled A total of 246 children provided stool samples which were unassociated with diarrhea or previous intestinal permeability testing. Summary statistics are provided for these 246 children and their mothers (Table 1). Gender was equally represented in this cohort. Of the 1,195 stool samples provided, 1,142 (n= 237) were accompanied by subsequent 3-month growth data, and 1,127 of these were tested for all 3 fecal markers (Figure 1). Of the 246 children whose samples were included in this study, 36 (14.6%) were lost to

follow up prior to 24 months of age, with 14 (5.7%) classified as lost to follow up prior to 12 months of age.

At birth, nearly 16% of children were stunted (median LAZ, WAZ, and WHZ were -1, -1.3, and -0.9 respectively). Median LAZ declined by 3 months of age to -1.2, however WAZ and WHZ improved to -0.9, and 0.1 respectively. 78% of the children stunted at birth were stunted at 24 months of age, compared to 44% of those not stunted at birth. Median LAZ was relatively stable in the first 6 months then declined steadily between months 6 and 18, before stabilizing at -2.0 (Figure 2).

Maternal characteristics

Enrolled mothers had a median age of 25 years, with a median height and weight of 149 cm and 49 kg respectively. More than 10% of mothers had a body mass index (BMI) less than 18.5 kg/m^2. Most mothers were married and monogamous (86.2%), and had married in their late teens. More than 80% of mothers had received some schooling, with most completing at least 5 years. The majority of mothers had been pregnant previously, having become pregnant for the first time at a median age of 18.

Fecal marker distribution and categorization

The distribution of AAT, MPO and NEO from the 1,195 samples (N= 246) is presented in Table 2. The marker category distribution and composite EE score distribution by month of age is presented in Figure 3. Approximately 63, 68%, and 95% of samples were above values considered normal in non-tropical settings for AAT (< 0.27 mg/g), MPO (< 2,000 ng/ml), and NEO (< 70 nmol/L), respectively (49-51).

Fecal markers and short-term linear growth

Fecal levels of MPO in the full period (months 3-21) and the early period (months 3-9) were not associated with subsequent 3-month linear growth (Table 3). However, in the model restricted to the second year of life, children with high MPO levels at month 12, 15, 18, or 21 lost an average of 0.100 more LAZ in the subsequent 3-month period than children with low levels (95% CI: -0.167, -0.032) after adjustment for LAZ at time of marker assessment and diarrhea experienced. The crude estimate without adjustment for diarrhea was of similar magnitude (crude estimate: -0.094, 95% CI: -0.162, -0.025). A sensitivity analysis which omitted quarterly MPO data in a stepwise fashion from the year 2 model did not substantially alter the magnitude of the estimate (-0.08 to -0.12 LAZ per 3 month period).

Fecal levels of AAT and NEO in months 3-21 were not associated with subsequent 3-month linear growth, nor were they associated with linear growth when marker data from months 3-9 and months 12-21 were examined separately (Table 3).

The composite EE score was negatively associated with subsequent 3-month LAZ change in the full period and when models were restricted to stool data from months 12-21, but not in months 3-9. A single unit increase in composite EE score in months 3-21 was associated with a loss of 0.009 LAZ per 3-month period (95% CI: -0.018, 0.000), after adjustment for LAZ at time of marker assessment and diarrhea experienced. In the second year, each unit of composite EE score was associated with a loss of 0.013 LAZ per 3-month period (95% CI: -0.023, -0.004). Therefore, a child with the highest composite EE score (10) lost 0.13 LAZ more than a child with a composite EE score of zero over the subsequent 3 month period. This corresponds to 0.36 cm less length gained between children in the highest versus the lowest composite EE score. When all models were re-parameterized with marker concentrations categorized into tertiles, the results did not change substantially (data not shown). When the significance level was adjusted

for multiple comparisons (using either the Benjamini-Hochberg or Bonferroni method), none of the marker coefficients remained significant. In mixed-effects models of 6-month growth, none of the fecal markers tested, nor the composite EE score, were significantly associated with subsequent growth (Supplemental Table 1).

Discussion

High fecal MPO levels in Bangladeshi children were associated with decreases in 3-month linear growth in the second year of life. However, neither AAT nor NEO were associated with subsequent growth during any observed period in this analysis. In this study, the composite EE score was associated with decreases in short-term linear growth. Prior MAL-ED multi-site analyses also demonstrated an association between MPO and growth (37). In addition, a previous study in Bangladesh observed an association between MPO (but not AAT or NEO) at age 12 weeks, and change in LAZ over the first year of age among 700 infants living in the same area of Mirpur (32). However, our observations contrast with prior multi-site analyses of MAL-ED data (which included some of the data presented here) which have reported an association between all three of these fecal markers and subsequent 6-month growth. Prior analyses have suggested that the composite EE score predicts such growth better than any single marker. In this study, which was restricted to individuals in the Bangladesh MAL-ED site, none of the fecal markers tested, nor the composite EE score, were significantly associated with subsequent 6-month growth (Supplemental Table 1).

The etiologies of and risk factors for stunting differ in different populations. In this setting in an urban slum in Bangladesh, major contributors to stunting include low birth weight [(LBW) as a result of intrauterine growth restriction and/or prematurity], inappropriate infant and young child feeding practices, food insecurity, recurrent infections, and EE. Consistent with other reports

from South Asia, prenatal growth deficits were common in this cohort. 27.6% of children had low birthweight (LBW; <2500 g), even though children with very low birthweight (VLBW; <1500 g) were excluded (52). Nearly 17% of children had stunting at birth, and the mean birth LAZ was −1.0, well below the mean of −0.5 among newborns in developing settings (20). These prenatal growth deficits may be related in part to poor maternal nutrition and the small average size of mothers, 13% of whom had a BMI under 18.5 kg/m^2. Prenatal factors may play a relatively more important role in Bangladesh as compared to other settings; 78% of the children stunted at birth were stunted at 24 months of age, compared to 44% of those who were not stunted at birth. Nevertheless, it appears that EE, as indicated by fecal MPO levels, contributed to growth faltering in this setting between ages 12 and 21 months.

Overall, AAT, MPO, and NEO levels among the children in this cohort from Mirpur were highly elevated in comparison to populations in high income countries, suggesting widespread intestinal inflammation and increased intestinal permeability. Such levels, however, were lower than those reported in the multi-site MAL-ED study (Supplemental Table 2) (37). MPO levels in particular were much lower; the 3rd quartile (7,430.1 ng/ml) was less half of that observed in the previous study (20,526.3 ng/ml) and over 3,000 ng/ml lower than the multi-site median (11,118.9 ng/ml). The present study includes marker data from children in a wider age range (3-21 months) than the previous multi-site study (3-9 months), and because most marker levels declined during the second year (see median marker levels by age in Supplemental Table 3), this may have reduced the predictive ability of marker categories in the first year. That MPO was predictive of linear growth despite lower levels observed in this setting suggests that the inflammation measured by MPO may significantly contribute to growth shortfalls in Bangladesh at levels lower than previously observed.

Because of the complex interplay among the many contributors to chronic malnutrition, nutritional interventions do not effectively normalize linear growth and reduce morbidity in stunted children less than 2 years of age. A systematic review of complementary feeding interventions targeting malnutrition among children 6-24 months of age reported that while several interventions strategies effectively improve the weight growth of children, the majority of interventions have modest effects on linear growth (9). It remains critical to improve the identification of children at risk of linear growth failure, so that corrective approaches may be tested and validated. EE is an appealing target in this regard, as the complex physiologic mechanisms by which unhygienic environmental exposures impede healthy growth may provide clues far in advance of significant growth or cognitive decline. Since none of the candidate EE markers included here provide a comprehensive measure of the persistent physiological dysfunction thought to underlie EE's relationship with chronic malnutrition, the composite EE score was created in an attempt to more accurately reflect intestinal dysfunction by incorporating uncorrelated fecal marker data (36, 37). However, while the composite EE score was associated with decreases in 3-month linear growth in the current study, the magnitude of effect for an 8-10 unit difference in composite EE score was similar to that for high MPO. In this setting, fecal MPO appears to be the most important contributor to the score's association with subsequent growth. The association between fecal MPO and short-term growth should be examined in other settings to determine the generalizability of these findings and to further gauge the potential usefulness of MPO as a screening tool for linear growth failure.

Our study had several important limitations. There was loss-to-follow-up among the included children; nearly 15% of children were lost to follow-up prior to 24 months of age. In addition, a lower number of stools were fully tested at each quarterly time point from months 3-9 than in the

second year. This may have been due in part to the higher incidence of diarrhea episodes in the early period. The higher amount of missing marker data in the first year reduced the power to detect associations with short-term growth in that period. About twenty percent (173) of the 817 stool samples included from children between 3 and 9 months were also included in the previous multi-site study, constituting 164 AAT results, 152 MPO results, and 127 NEO results. These samples had a higher median MPO concentration (8,800 ng/ml) than the full set of samples from months 3-9 in the present study (5,444 ng/ml), and may have influenced the analysis of MPO and short term growth. Median NEO (1470 nmol/L) and AAT (0.49 mg/g) concentrations in these samples did not differ substantially from the full set of samples from months 3-9. AAT as a marker of intestinal permeability may be less sensitive to small gaps in mucosal integrity due to its large size, than smaller molecules such as urinary lactulose. While no fecal marker p-values remain significant when adjusted for multiple comparisons using the Benjamini-Hochberg or Bonferroni method; the use of unadjusted p-values is acceptable in exploratory analyses. Our comparisons to the multisite study must be read with some caution, as these analyses may not be sufficiently powered to detect differences in effects, and no formal statistical comparison was run. While inflammatory marker data was available from children at time points closer to birth, we chose to use equally spaced marker data with non-overlapping growth periods. Fecal EE markers from samples collected at birth may not reflect postnatal environmental insults, and their inclusion may attenuate estimates of the association between EE and linear growth.

Despite these limitations, this study was well-designed, benefited from high-quality laboratory facilities, skilled staff, and utilized appropriate statistical methods. Enrolled children were recruited from a single setting using a well-defined recruitment protocol and rigorous inclusion and exclusion criteria established by the MAL-ED consortium, facilitating comparability of

results with those from other MAL-ED sites. The high population density, poor sanitation, and low socioeconomic status of the Bauniabadh area are representative of a typical urban slum in Dhaka and are similar to others in South Asia (46). A unique strength in this study is that children were visited several times each week to collect highly detailed surveillance information on the incidence of diarrhea, respiratory disease, and other morbidity events. The detailed morbidity data facilitated precise adjustment for diarrhea in analyses and enabled the exclusion of stool samples whose close proximity to diarrhea symptoms or intestinal permeability testing would have potentially diluted marker concentrations (36). The use of piecewise linear mixed effects models to examine associations between fecal markers and growth allowed for natural variation in growth rates at different ages, enabled the inclusion of time-varying covariates and adequately accounted for correlated data coming from the same individuals. This approach also had good interpretability and comparability with the previous study (37).

In summary, in this analysis of children in Mirpur, Bangladesh, only high MPO levels were associated with decreases in short-term linear growth only in the second year of life. The composite EE score was negatively associated with subsequent 3-month LAZ change, mostly driven by fecal MPO levels. In Bangladeshi children, fecal MPO appears predictive of linear growth at levels lower than in the multi-site MAL-ED analysis. Our findings suggest that the overall attributable impact of EE on linear growth outcomes may differ in various settings and populations, suggesting that further efforts to improve the interpretation of fecal markers may need to be site or population-specific.

Figures (Chapter 1)

Figure 1: One thousand one hundred and ninety-five samples from 246 children were evaluated for fecal levels of neopterin (NEO), myeloperoxidase (MPO), and alpha-anti-trypsin (AAT). Samples used in the growth analysis were restricted to stools from children with no history of diarrhea in the last 7 days or history of lactulose administration on the day of or before stool collection and for which complete anthropometric data were available.

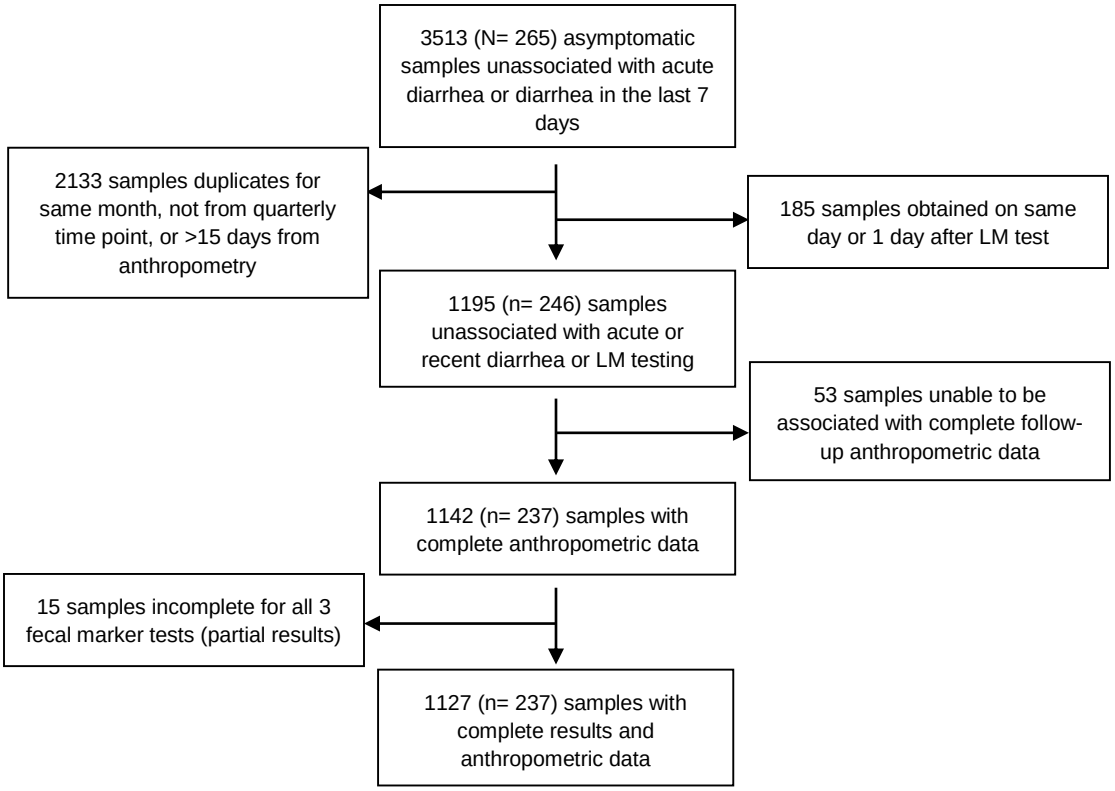


Figure 2: Quarterly LAZ among children contributing 1 or more stools unassociated with acute or recent diarrhea or LM testing (N= 246, median value displayed)

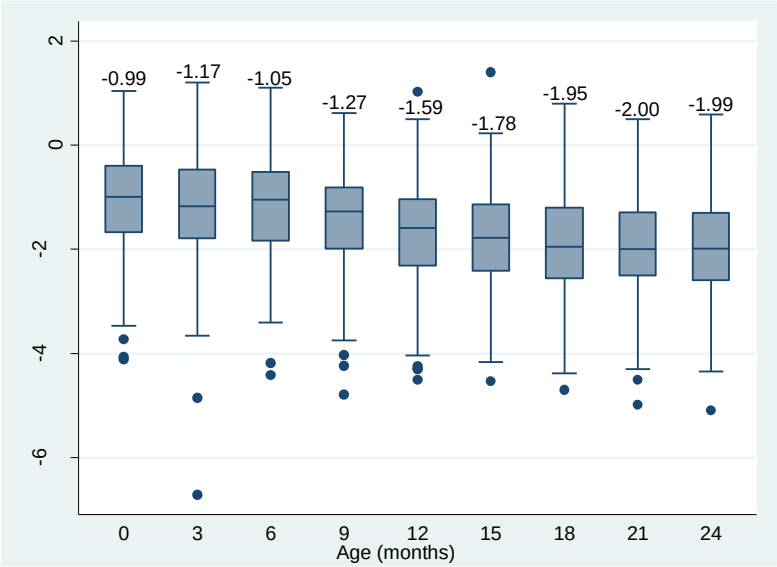
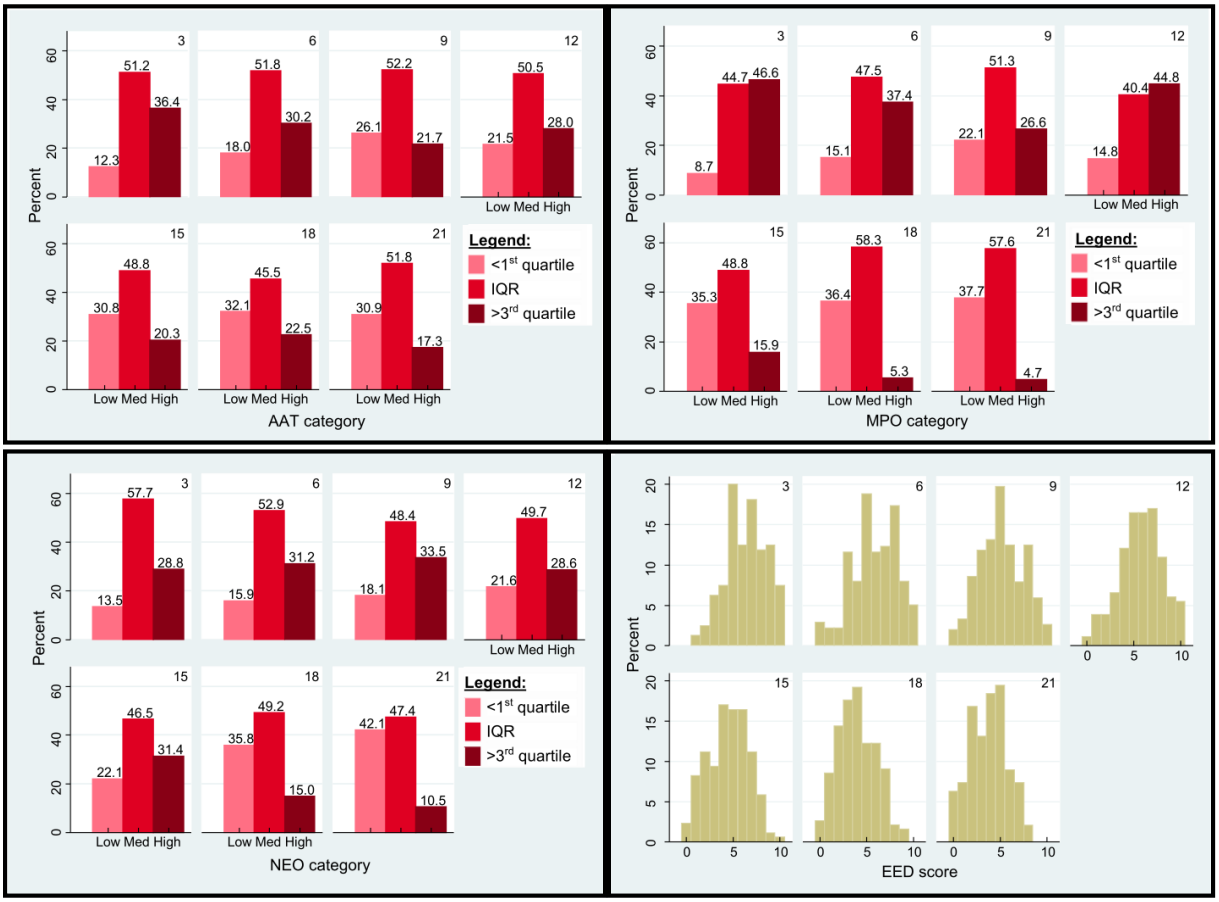


Figure 3: Fecal marker category distribution by month of age (N= 246 children)



Tables (Chapter 1)

Table 1: Characteristics of participants (N= 246 unless otherwise noted)

			n (%) or median (IQR)
Infant		Female	124 (50.4)
		Birth weight (kg)	2.75 (2.47, 3.06)
		Birth length (cm)	48.1 (47.0, 49.5)
	Anthropometry at birth	LAZ	-0.99 (-1.68, -0.40)
		Stunting	41 (16.7)
		WAZ	-1.30 (-1.88, -0.61)
		Low birthweight (<2500 g)	68 (27.6)
		WHZ	-0.93 (-1.65, -0.31)
	Anthropometry (3 months)	LAZ	-1.17 (-1.80, -0.47)
		Stunting	38 (15.6)
		WAZ	-0.89 (-1.65, -0.39)
		WHZ	0.07 (-0.72, 0.72)
	Anthropometry (24 months)^	LAZ	-1.99 (-2.60, -1.30)
		Stunting	104 (49.5)
		WAZ	-1.62 (-2.31, -0.99)
		WHZ	-0.87 (-1.35, -0.10)
	Breastfeeding	Exclusively breastfed until (day)	102.5 (58, 150)
	Diarrhea (days)	Days in year 1[+]	11 (5, 22)
		Days in year 2^	8 (4, 15)
Mother		Age (years)	25 (21, 28)
	Anthropometry at baseline	Height (cm)	149 (146, 153)
		Weight (kg)	48.5 (43.1, 55.8)
		BMI	21.8 (19.6, 24.6)
		BMI<18.5	32 (13.0)
	Marital status	Married- only wife	212 (86.2)
		Married- polygamous	34 (13.8)
		Age at first marriage	17 (16, 19)
	Maternal education	Never attended school	46 (18.7)
		Schooling completed (years)	5 (2, 7)
	Pregnancy information	Age at first pregnancy	18 (17, 20)
		Lifetime gravidity	2 (1, 3)
		Number of live births	2 (1,2)

[+]232 children with 12 months follow up

^210 children with 24 months follow up

Table 2: Fecal marker distribution in stool collected from 3-21 months of age (N= 246 children)

	AAT (mg/g)	MPO (ng/ml)	NEO (nmol/L)	EE score (0-10)
1st quartile	0.1900	1594.9	366.2	3
Median	0.3800	3354.9	1017.6	5
3rd quartile	0.7175	7430.1	2210.8	7
n (samples)	1194	1185	1190	1179

Table 3: Fecal markers and subsequent 3-month change in LAZ

		Months 3-21 (n= 237) Coefficient (95% CI)	**Months 3-9 (n= 221^) Coefficient (95% CI)**	**Months 12-21 (n= 221) Coefficient (95% CI)**
AAT	Low	Ref	Ref	Ref
	Medium	-0.005 (-0.053, 0.043)	0.003 (-0.102, 0.107)	-0.013 (-0.061, 0.034)
	High	-0.042 (-0.098, 0.014)	-0.037 (-0.153, 0.078)	-0.042 (-0.100, 0.017)
	Diarrhea proportion	-0.408* (-0.765, -0.052)	-0.184 (-0.815, 0.447)	-0.698† (-1.113, -0.283)
	LAZ	-0.065‡ (-0.085, -0.045)	-0.099‡ (-0.140, -0.057)	-0.047‡ (-0.067, -0.027)
	Constant	-0.143‡ (-0.215, -0.071)	-0.193† (-0.313, -0.074)	-0.198‡ (-0.263, -0.132)
	N (samples)	1141	436	705
MPO	Low	Ref	Ref	Ref
	Medium	0.003 (-0.045, 0.051)	0.062 (-0.051, 0.175)	-0.017 (-0.063, 0.030)
	High	-0.047 (-0.107, 0.013)	0.030 (-0.089, 0.149)	-0.100† (-0.167, -0.032)
	Diarrhea proportion	-0.418* (-0.775, -0.062)	-0.207 (-0.839, 0.425)	-0.704† (-1.116, -0.292)
	LAZ	-0.066‡ (-0.086, -0.046)	-0.103‡ (-0.145, -0.061)	-0.047‡ (-0.067, -0.028)
	Constant	-0.148‡ (-0.222, -0.073)	-0.258‡ (-0.388, -0.129)	-0.165‡ (-0.233, -0.096)
	N (samples)	1133	432	701
NEO	Low	Ref	Ref	Ref
	Medium	0.006 (-0.042, 0.055)	0.065 (-0.047, 0.177)	-0.013 (-0.061, 0.034)
	High	-0.019 (-0.076, 0.038)	0.019 (-0.101, 0.140)	-0.024 (-0.084, 0.035)
	Diarrhea proportion	-0.402* (-0.758, -0.046)	-0.199 (-0.830, 0.432)	-0.672† (-1.085, -0.258)
	LAZ	-0.064‡ (-0.084, -0.045)	-0.101‡ (-0.143, -0.059)	-0.045‡ (-0.065, -0.026)
	Constant	-0.161‡ (-0.233, -0.089)	-0.253‡ (-0.379, -0.127)	-0.200‡ (-0.265, -0.135)
	N (samples)	1137	434	703
EE score	Score (0-10)	-0.009* (-0.018, 0.000)	-0.002 (-0.019, 0.015)	-0.013† (-0.023, -0.004)
	Diarrhea proportion	-0.433* (-0.790, -0.076)	-0.209 (-0.844, 0.425)	-0.740‡ (-1.155, -0.324)
	LAZ	-0.064‡ (-0.084, -0.044)	-0.100‡ (-0.143, -0.058)	-0.048‡ (-0.068, -0.028)
	Constant	-0.107* (-0.190, -0.023)	-0.199† (-0.338, -0.059)	-0.136† (-0.215, -0.058)
	N (samples)	1127	428	699

* $p < 0.05$

† $p < 0.01$

‡ $p < 0.001$

^ n= 219 for MPO & composite EE score

Supplemental Material (Chapter 1)

Supplemental Table 1: Fecal markers in asymptomatic stool and subsequent 6-month change in LAZ

		Months 3-18 (n=232) Coefficient (95% CI)
AAT	Low	Ref
(940)	Medium	0.031 (-0.036, 0.097)
	High	0.018 (-0.060, 0.097)
	Diarrhea incidence	-0.015 (-0.033, 0.003)
	Constant	-0.342 (-0.439, -0.244)
MPO	Low	Ref
(932)	Medium	0.045 (-0.023, 0.113)
	High	-0.015 (-0.095, 0.065)
	Diarrhea incidence	-0.015 (-0.033, 0.003)
	Constant	-0.331‡ (-0.433, -0.230)
NEO	Low	Ref
(937)	Medium	0.033 (-0.035, 0.101)
	High	0.003 (-0.075, 0.080)
	Diarrhea incidence	-0.016 (-0.034, 0.002)
	Constant	-0.338‡ (-0.435, -0.241)
EE score	Score (0-10)	0.000 (-0.013, 0.012)
(929)	Diarrhea incidence	-0.015 (-0.033, 0.003)
	Constant	-0.316‡ (-0.429, -0.204)

* $p < 0.05$
† $p < 0.01$
‡ $p < 0.001$

Supplemental Table 2: Fecal marker distribution from stool in Mirpur and multi-site MAL-ED cohort

	AAT (mg/g)		MPO (ng/ml)		NEO (nmol/L)	
	Multi-site	**Mirpur**	**Multi-site**	**Mirpur**	**Multi-site**	**Mirpur**
1st quartile	0.21	0.19	5650.5	1594.9	1171.4	366.2
Median	0.44	0.38	11118.9	3354.9	1846.7	1017.6
3rd quartile	0.86	0.72	20526.3	7430.1	2997.9	2210.8
N (samples)	1,169	1194	916	1185	954	1190
n (children)	537	246	537	246	537	246

Supplemental Table 3: Fecal marker distribution by age

Age in months	**AAT (mg/g)**		**MPO (ng/ml)**		**NEO (nmol/L)**	
	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)
3	162	0.51 (0.28, 0.93)	161	6817.5 (3509.4, 14864.4)	163	1444.2 (717.0, 2456.4)
6	139	0.42 (0.23, 0.92)	139	4956.6 (2745.9, 10745.3)	138	1522.8 (634.7, 2662.6)
9	157	0.38 (0.19, 0.65)	154	4133.1 (1785.9, 7726.0)	155	1662.7 (705.7, 2979.0)
12	186	0.38 (0.22, 0.78)	183	6561.8 (2953.8, 14886.0)	185	1230.1 (529.1, 2356.6)
15	172	0.32 (0.17, 0.59)	170	3225.6 (1219.9, 5520.5)	172	1281.9 (437.0, 2689.2)
18	187	0.36 (0.16, 0.70)	187	2022.1 (1341.7, 3312.2)	187	645.5 (268.3,1351.8)
21	191	0.33 (0.17, 0.59)	191	1859.0 (1140.6, 2603.2)	190	456.3 (213.3, 812.0)
Total	1194	0.38 (0.19, 0.72)	1185	3354.9 (1594.9, 7430.1)	1190	1017.6 (366.2, 2210.8)

CHAPTER 2: Fibroblast Growth Factor 21 predicts growth responsiveness to nutritional supplementation among underweight children in Bangladesh

Introduction

Poor growth as a result of inadequate nutrition and frequent infections contributes substantially to child morbidity and mortality in resource-limited countries (1, 7). Poor linear growth, marked by stunting (length-for-age z-score (LAZ) < −2), is reflective of the cumulative impacts of chronic deprivation and repeated infectious insults. Poor weight gain, marked by underweight (weight-for-age z-score (WAZ) <-2) and wasting (weight-for-length z-score (WLZ) < -2) appear to be indicative of acute states of deprivation or illness (10). Nutritional interventions alone often do not effectively reverse poor childhood growth, particularly stunting (9). There is an unmet need to identify children who are likely to grow in response to supplemental nutrition as well as those in need of additional interventions beyond nutritional rehabilitation.

Hormonal responses to nutrient intake may correlate with a child's growth pattern and may be useful as markers of growth responsiveness to intervention. Growth hormone (GH) is the principal endocrine regulator of growth and is secreted nightly by somatotroph cells in the anterior pituitary (53). GH regulates growth in part through binding to receptors in the liver which control expression of insulin-like-growth factor 1 (IGF-1), one of the hormones responsible for stimulating growth in local tissues and systemically. GH resistance, defined by elevated GH levels and low levels of IGF1, is exhibited in states of undernutrition, including chronic caloric insufficiency, protein deficiency, and isolated micronutrient deficiencies (zinc, Vitamin A, magnesium) (28). GH resistance may mediate the relationship between these nutritional deficiencies and poor linear and ponderal growth in children. Because GH resistance

is not nutrient-specific, IGF-1 and GH cannot be used to identify children who are likely to grow in response to supplemental nutrition. An endocrine hormone called Fibroblast Growth Factor 21 may be more informative, as it appears to play a role in the pathway by which chronic caloric/protein deprivation in particular triggers GH resistance and inhibits skeletal growth (28, 54-59).

Fibroblast growth factors (FGF) are a family of protein growth factors that regulate diverse biological processes including growth and development. FGF21 is an endocrine hormone produced primarily by the liver and adipocytes that regulates glucose and lipid metabolism, and is a signal of protein restriction that regulates metabolism and growth during periods of reduced protein intake (54, 55, 60). In humans, FGF21 levels rise following sustained fasting, and in response to protein deprivation (60, 61). Chronic exposure to FGF21 results in reduced expression of hepatic GH receptors, inhibition of GH signaling, and disruption of GH action at the bone growth plate (28, 54-59). Limited pediatric data suggest that serum and plasma levels of FGF21 are negatively associated with linear growth in healthy and preterm children under 1 years of age (59, 62). Collectively, these data suggest that FGF21 may be a useful marker to identify undernourished children whose growth is impeded by insufficient macronutrient (particularly protein) intake. Therefore high levels of FGF21 may identify those children who are more likely to grow in response to nutritional supplementation than others whose poor growth is attributable to other factors such as environmental enteropathy (EE). EE, also known as environmental enteric dysfunction, is subclinical intestinal disorder prevalent among children in resource-limited settings, and appears to be an important cause of linear growth faltering (22).

The present study examines the association between circulating levels of FGF21 and subsequent growth in a cohort of underweight children receiving nutritional supplementation in urban Bangladesh.

Ethics

Study protocols for the multisite MAL-ED "Case-Control" study were reviewed and approved by Institutional Review Boards (IRB) at all collaborating institutions, and governmental and regional health authorities, including the Ethical Review Committee (ERC) of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) (46, 47). The quantification of FGF21 in previously collected samples was approved by the ERC. The University of Washington IRB approved the use of coded data and samples previously gathered in icddr,b's ERC-approved study.

Methods

Study population: The study utilized data from a sub-sample of 120 children enrolled in the MAL-ED "Case Control" study, a 1-year prospective interventional study. In the parent study, 500 moderately to severely underweight (WAZ<-2) children aged 6–24 months living in the Bauniabadh area of section 11 of Mirpur, an urban slum in Dhaka, Bangladesh were recruited by field workers from February 2010–February 2012 (46). Children were identified either through an initial community-wide WAZ screening of children aged 6-24 months or by active surveillance which continued through the study period.

The present analysis was restricted to the youngest 120 children who were moderately underweight at enrolment ($-3 \leq WAZ < -2$), and had at least 200µl of stored plasma available from enrolment and month five of follow-up (Figure 1). The youngest children were sampled in order

to limit potential age-related heterogeneity of FGF21 assay results, and were between 6-13 months of age at enrolment. Exclusion criteria included, “illnesses that might impact nutritional status or response to treatment (e.g. severe diarrhea or pneumonia at the time of enrolment, persistent diarrhea, cleft lip or palate, blindness, tuberculosis, jaundice, renal or cardiac disease, cerebral palsy, or chromosomal disorders including trisomy 21).” Details of the parent study have been published previously (46, 47).

Participants received 300 kcal of locally produced “Pushti packet” food supplements six days a week for five months, or until the child achieved “graduation” (WAZ ≥ -1). Only one child in this sample achieved graduation after four months of follow up. Consumption of supplements was directly observed by study staff on-site at the study clinic. Each 150 kcal Pushti packet contains 20 g roasted rice powder, 10 g roasted lentil powder, 3 g soybean oil, and 5 g molasses (63). All children also received high-dose vitamin A capsules at six month intervals, multiple micronutrient powder, and routine immunizations, and their caretakers received counselling on how to improve the child’s nutritional status (46). All children who developed severe acute malnutrition (SAM; WLZ<-3 and/or bipedal edema) were admitted to the icddr,b Dhaka hospital and treated following current World Health Organization (WHO) guidelines (11). Children with SAM were not released for community-based care until WLZ exceeded -2. Ten children (8.3%) met the criteria for SAM at enrolment or at least once in the five month supplementation period. Five children (4.2%) developed SAM in the seven month post-supplementation period.

Data collection: Household/maternal characteristics were assessed at enrolment, and caregivers reported morbidity and breastfeeding behavior every other day. Child anthropometry was assessed at monthly intervals. Children were weighed using pediatric balances with a certified accuracy of 100 g, length was measured using a marked platform with a sliding footboard, and

head circumference was measured using a non-stretch synthetic measuring tape with 0.1 cm increments (48).

Blood samples were gathered in Li-heparin tubes for the purpose of micronutrient testing (zinc, retinol, vitamin D, and ferritin) at enrolment and after the 5 months of nutritional supplementation, and were centrifuged at 3000 rpm for 10 minutes and surplus plasma was stored at –70°C for future analyses. EE was assessed in children at enrolment using alpha-1-antitrypsin (AAT), a serum protein whose fecal excretion is elevated in states of increased intestinal permeability. Stool samples were collected by caretakers and stored in cold packs within an hour without fixative prior to collection by field workers during a home visit. Stool samples were frozen at −70°C pending processing (36). Stool samples were excluded from children who had diarrhea symptoms within 7 days prior to collection and from those who had dual sugar permeability testing within one day prior to collection. These factors alter stool water content to a variable extent, making the interpretation of AAT concentrations unreliable without measuring dry weights.

Laboratory Methods: Procedures were conducted in the icddr,b laboratories in Dhaka, Bangladesh. FGF21 was quantified in plasma samples using commercially available enzyme-linked immunosorbent assay (ELISA- R&D systems, Minneapolis, MN, USA) per package insert instructions; sulfuric acid (9.8%) stop solution was produced locally. Each plasma sample was tested in duplicate, read directly at 450 nm using Gen5 software (BioTek, Winooski, VT, USA), and results were expressed as the mean value. A wavelength correction was not performed, as neither a 540 nm or 570 nm filter were available. ELISA results which exceeded the absorbance of the highest standard (2,000 pg/ml) by more than 20% were assigned the value of 2462 pg/ml. ELISA results which fell below the absorbance of the lowest standard (31.3 pg/ml) by more than

20% were assigned the value of 25.4 pg/ml. ELISA results from two of the 120 enrolment samples exceeded the absorbance of the highest standard (in both wells), and results from four samples fell below the absorbance of the lowest standard. At month five of follow up, ELISA results from two of the 120 samples exceeded the absorbance of the highest standard, and results for six samples fell below the absorbance of the lowest standard.

AAT was measured in stool samples using commercially available ELISA kits (BioVendor, Candler, NC, USA), and was run per package insert instructions at a dilution of 1:500 (36, 37). Samples out of range of the standard curve for the AAT assay were run at higher or lower concentration (as appropriate) (37).

Statistical Methods: Analyses were performed using STATA version 12.1 IC (College Station, TX, USA). WHO Anthro software was used to calculate Z-scores from raw anthropometric data. Height observations were dropped from the dataset if deemed implausible (≥1 cm lost from the previous month, or bounded by values lower by ≥1cm). Implausible height data were omitted from one child at 2 time points (enrolment and month 1), and from seven children at single time points during the intervention period (1.3% of measurements in that period). LAZ, WAZ, and head-circumference-for-age z-score (ZHC) data from monthly intervals were included in the analyses. Examination of the baseline FGF21 concentrations revealed a bimodal distribution (Figure 2). Therefore, children were dichotomized by FGF21 above (high) or below (low) 1468 pg/ml, which was the midway point between the maximum of the lower peak (1222 pg/ml) and the minimum of the upper peak (1714 pg/ml).

To determine whether children with high FGF21 concentrations were more responsive to nutritional supplementation, linear mixed effects models with first-order autoregressive residual structures were used to test if the association between month of follow up (the exposure) and the

outcomes of LAZ, WAZ, and ZHC differed by baseline FGF21 status. To examine whether any differences in the average growth velocity of children with high versus low baseline FGF21 were sustained after cessation of supplementation, the relationship was examined separately for the 5 month supplementation period and the post-supplementation period (months 5-12). Children were excluded from a model if they contributed less than two anthropometric observations during the time period being assessed. Each model contained a random intercept term, fixed terms for high baseline FGF21 and month of follow up, and an interaction term for the effect of high baseline FGF21 on monthly change in z-score. The interaction term was used to assess whether monthly change in z-score differed by FGF21 status. If an interaction term was significant, the models for WAZ, ZHC, and LAZ were stratified by FGF21 status. For models of the post-supplementation period, the month variable pertained to time since cessation of supplementation (month of follow up – 5). Lowess curves were used to describe and compare the 12-month weight, height, and head circumference trajectories of individuals who had high versus low plasma FGF21 at baseline.

Linear mixed effects models with first-order autoregressive residual structures were used to test whether change in FGF21 status was associated with monthly change in WAZ, LAZ, or ZHC during the supplementation period. Children were categorized into groups based on change in FGF21 over the 5 months of supplementation: 1) dropping from the high into the low FGF21 category, 2) remaining in the low category, and 3) moving from low baseline FGF21 into the high FGF21 category. Each model contained a random intercept term, fixed terms for change in FGF21 status (described above) and month of follow up, and 2 interaction terms for the effect of change in FGF21 status on monthly change in z-score. If the interaction terms were significant,

the data for WAZ, ZHC, and LAZ were stratified by FGF21 group. Lowess curves were used to describe the 5-month weight, height, and head circumference trajectories of the groups.

To examine whether EE differed between children with and without high FGF21 levels, a student's t-test with unequal variances was used to compare the mean baseline AAT levels in children with and without high levels of FGF21 at enrolment.

Results

One hundred and twenty moderately undernourished children with baseline and month five blood samples were included from the parent study (Figure 1). At enrolment, age ranged from 182 to 385 days and 56% of subjects were female (Table 1).

Maternal demographics: Enrolled mothers had a median age of 23 years, and most mothers were married and monogamous (85%) and had married in their mid-late teens (Table 1). More than 80% of mothers had received some schooling, with most completing at least 4 years. The majority of mothers had been pregnant previously with a median age of first pregnancy of 18 years. The median monthly household income was 7,000 Bangladeshi Taka (IQR: 5,000-9,000), which is equivalent to $90 USD.

Child anthropometry: Valid anthropometry data were available from 3-5 monthly time points during the five months of supplementation, including the child who ceased supplementation at month 4 due to WAZ graduation. 115 children had valid anthropometry data available from 2-8 monthly time points in the 7 months following supplementation.

At enrolment, nearly 60% of children were stunted and just over 30% were wasted (10). Median LAZ, WAZ, and ZHC were –2.2, –2.4, and -1.9 respectively. Median LAZ and ZHC declined to –2.7 and -2.2 respectively after 12 months of follow up, while WAZ remained relatively

unchanged at –2.5. After 12 months of follow up, 88% of the 100 children still being followed were stunted, and 22% were wasted.

FGF21 distribution, categorization, and cofactors: The median FGF21 concentration among the 120 underweight children was 241 pg/ml at enrolment and 139 pg/ml at the month 5 follow up visit (Table 1). Nine (7.5%) of the 120 children had high FGF21 (>1468 pg/ml) at enrolment, while three different children (2.5%) had high FGF21 at the end of supplementation. On average, children in the high baseline FGF21 group had poorer growth status and lower plasma zinc and retinol levels at enrolment than those in the low baseline FGF21 group (Supplementary Table 1).

Baseline FGF21 and weight gain during and following nutritional supplementation: High FGF21 plasma levels at enrolment were associated with increased weight gain both during and after receipt of nutritional supplementation based upon the significant interaction terms between month of follow up and high baseline FGF21 in period-specific models (p=0.001 and 0.037 respectively). Adjustment for baseline WAZ and relevant micronutrient levels did not alter the significance of the interaction term in any of the models, and were therefore not retained. During the five months of nutritional supplementation, children with high baseline FGF21 levels gained an average of 0.10 WAZ per month of follow up (95% CI: 0.027, 0.162), while WAZ did not improve among those with low levels (Figure 3 and Table 2). This difference was the equivalent of an additional 80 grams gained per month among those with high baseline FGF21 in an age-adjusted model (Supplemental Table 2). Differences in weight gain were sustained in the post-supplementation period (Figure 3 and Table 2); children with high baseline FGF21 levels maintained their WAZ over the seven months, while those with low baseline FGF21 lost an average of 0.01 WAZ per month (95% CI: -0.023, -0.002). This was the equivalent of an

additional 50 grams gained per month among those with high baseline FGF21 in an age-adjusted model (Supplemental Table 2).

Baseline FGF21 and linear growth during and following nutritional supplementation: Monthly linear growth during the 5 months of nutritional supplementation differed significantly by baseline FGF21 status based upon the significant interaction term in the period-specific model (p= 0.001). Adjustment for baseline LAZ and relevant micronutrient levels did not alter the significance of the interaction term in any of the models, and were therefore not retained. During the five months of nutritional supplementation, children with high baseline FGF21 levels maintained their stature, while those with low levels lost an average of 0.08 LAZ per month (95% CI: -0.102, -0.065, p<0.001) (Figure 3 and Table 2). This was the equivalent of 0.3 cm more growth per month among those with high baseline FGF21 in an age-adjusted model (Supplemental Table 2). Adjustment for baseline anthropometry and micronutrient levels did not alter the significance of the interaction term in any of the models, and were therefore not retained. Monthly linear growth in the 7-month post supplementation period did not differ significantly by baseline FGF21 status as the interaction term was not significant. However, children with high baseline FGF21 appear to have maintained their LAZ (Table 2), while those with low baseline FGF21 lost an average of 0.03 LAZ per month (-0.035, -0.014).

Baseline FGF21 and head circumference growth during and following nutritional supplementation: High FGF21 plasma levels at enrolment were associated with increasing ZHC during nutritional supplementation based upon the significant interaction term in the period-specific model (p=0.004). Adjustment for baseline ZHC and relevant micronutrient levels did not alter the significance of the interaction term in any of the models, and were therefore not retained. During the five months of nutritional supplementation, children with high baseline

FGF21 levels gained an average of 0.05 ZHC per month of follow up (95% CI: 0.009, 0.085; p= 0.016), while those with low levels lost an average of 0.04 ZHC per month (95% CI: -0.059, -0.025; p<0.001) (Figure 3 and Table 2). This was the equivalent of 0.1 cm more increase in head circumference per month among those with high baseline FGF21 in an age-adjusted model (Supplemental Table 2). In the 7-month post supplementation period, there was a trend (p=0.065) for a difference in the monthly change in head circumference by baseline FGF21 status. In this period, children with high baseline FGF21 appear to have maintained their ZHC (Table 2), while those with low baseline FGF21 lost an average of 0.02 ZHC per month (95% CI: -0.028, -0.005).

Change in FGF21 status and growth during supplementation: Over supplementation, FGF21 levels decreased by a median of 1921 pg/ml (IQR: -1981, -1635) in the high baseline FGF21 group. All nine children had month 5 FGF21 below 1468 pg/ml, and these children made up the high-low FGF21 group in statistical models. In the low baseline FGF21 group, the median change in FGF21 levels over the supplementation period was 41 pg/ml (IQR: -192, 36). Among the 111 children who had low baseline FGF21 levels, 3 had month 5 FGF21 levels above 1468 pg/ml and were categorized as being in the low-high FGF21 group. The remaining 108 children from the low baseline FGF21 group were categorized as low-low in statistical models. Children in the low-high FGF21 group experienced significantly more diarrhea episodes during supplementation compared with other children (means of 3.3 and 1.9 episodes respectively, p= 0.0313).

The three children whose FGF21 status increased over supplementation (low-high) had worse growth on average during supplementation than those whose FGF21 decreased or remained low (Figure 4). There were significant interaction terms between month of follow up and change in FGF21 status in models of WAZ (p=0.001 for low-low and p<0.001 for low-high), LAZ

(p=0.001 for low-low and low-high), and ZHC (p=0.005 for low-low and p=0.003 for low-high). The low-high FGF21 group declined an average of 0.23 WAZ, 0.18 LAZ, and 0.13 ZHC per month of follow up, while the mean WAZ of the 108 with unchanged FGF21 status did not improve and they lost an average of 0.08 LAZ and 0.04 ZHC per month (Table 3). Growth in the high-low FGF21 group was described previously for the high baseline FGF21 group; children gained an average of 0.10 WAZ and 0.05 ZHC per month and had unchanged LAZ.

EE and FGF21 status: The median fecal AAT concentration at enrolment was 0.268 mg/g among the 102 children with valid results (IQR: 0.163, 0.673) (Table 1), with a highly skewed distribution (Supplemental Figure 1). The mean baseline AAT concentration was not significantly different among children with high and low baseline FGF21 levels (0.434 and 0.536 mg/g, respectively, p= 0.4733) and similar findings were observed when the values for AAT were log transformed.

Discussion

In this sample of 120 moderately underweight children in Bangladesh, those with high baseline FGF21 demonstrated significant improvements in age-standardized weight and head circumference but did not improve linear growth during five months of nutritional supplementation. In contrast, children with low baseline FGF21 experienced continued declines in age-standardized length and head circumference and did not experience changes in weight during nutritional supplementation. These findings are consistent with the hypothesis that high FGF21 levels may be useful as a biomarker to predict a priori which children are likely to grow in response to nutritional supplementation.

High FGF21 identified a small group of children who gained weight and head circumference z-scores while receiving nutritional supplementation. The cohort was enrolled on the basis of poor weight status, and those identified as having high baseline FGF21 improved their weight during supplementation to a greater degree than those with low FGF21 levels. The differences in head circumference growth observed in the two groups of children are especially important in this cohort, as the median ZHC after supplementation was below the definition for borderline microcephaly (ZHC= -2) (64). Head circumference is a proxy measurement of brain development; small head circumference in infants appears to be associated with increased risk of neurocognitive disorders and decreased cognition later in childhood (65, 66). The absence of improved linear growth among children with high baseline FGF21 is not surprising, as feeding interventions often fail to normalize linear growth (9). That these children did not become more stunted during supplementation is noteworthy given that growth faltering in South Asian children generally worsens at this age (20). Those children with low baseline FGF21 did become more stunted and grew an average of 1.5 cm less over the 5 months of supplementation. Therefore, FGF21 may be useful as a biomarker to predict which children are likely to grow in response to nutritional supplementation while also identifying those children in whom alternative interventions are likely to be required to improve growth. EE as measured by fecal levs of AAT did not differ significantly by baseline FGF21 status, and may also have influenced growth during supplementation. Unfortunately, there are currently no interventions which have been demonstrated to effectively prevent or treat EE. Such interventions are critical.

FGF21 levels are hypothesized to be part of the late adaptive response to starvation, as serum FGF21 increase in healthy human adults after 7-10 days of fasting (61, 67). Protein deprivation in particular appears to drive increases in circulating FGF21, as plasma levels in healthy adults

increased markedly following 28 days of low protein diet, but did not change in controls who were not protein-restricted (60). The children with high FGF21 levels in our study had levels greatly exceeding those reported from healthy adults following a 10 day fast(61), suggesting that their high FGF21 levels may have been driven by sustained caloric and protein deprivation. There are limited published data on FGF21 levels in healthy children, and levels observed in this Bangladeshi cohort were much higher than observed in a previous study of preterm and term Chilean infants (62). The importance of dietary protein for child growth is well documented, the typical infant diet following weaning from exclusive breastfeeding in Bangladesh often provides insufficient protein. High circulating FGF21 levels may be useful in identifying a subset of children whose poor growth status is in large part attributable to sustained caloric/protein deprivation, and who will therefore be more likely to grow in response to nutritional supplementation.

This study is the first to evaluate the association between FGF21 and growth responsiveness during nutritional supplementation in underweight children. Previous pediatric studies have reported that plasma FGF21 is negatively associated with linear growth in preterm infants. For example, a study of very preterm infants reported a negative association between average FGF21 levels over the first 5 postnatal weeks and concurrent change in LAZ (59), suggesting that early elevated FGF21 levels are associated with postnatal growth failure. Another study reported a negative correlation between LAZ change from 6-12 months of age and concurrent FGF21 change in a small sample of preterm infants (62). Consistent with these observations, in the present study there were significant declines in the average LAZ of children whose FGF21 increased over a 5 month period.

This study had several notable strengths. The study leveraged a well-designed and implemented cohort. Enrolled children were recruited from a single setting using a well-defined recruitment protocol and rigorous inclusion and exclusion criteria established by the MAL-ED consortium. The high population density, poor sanitation, and low socioeconomic status of the Bauniabadh area are representative of a typical urban slum in Dhaka and are similar to others in South Asia (46). A unique strength in this study is that children were visited several times each week to collect highly detailed surveillance information on the incidence of diarrhea, respiratory disease, and other morbidity events. The detailed morbidity data enabled the exclusion of stool samples whose close proximity to diarrhea symptoms or intestinal permeability testing would have potentially diluted AAT concentrations (36). In addition, the use of linear mixed effects models allowed for utilization of monthly anthropometry data, facilitated examination of growth rates in the supplemented and non-supplemented periods, and adequately accounted for correlated anthropometric data coming from the same individuals.

This study also had several important limitations. There were relatively few children with high baseline levels of FGF21. While a child's gestational age and size at birth may influence circulating FGF21 levels, these data were not available. Due to limited sample volumes, it was not possible to repeat FGF21 ELISAs using dilution in results which greatly exceeded the absorbance of the highest standard. The intra-assay coefficient of variance (CV) was 7.7% for the 226 samples within the detectable range of the assay (not assigned values). Some of this variability was likely due to the fact that wavelength correction, used to correct for optical imperfections in the ELISA plate, was not performed. The timing of blood draws was not standardized and it is unclear whether diurnal cycle or proximity to meals impacts FGF21 levels in children. However FGF21 levels were relatively unaffected by time of day or standardized

food intake over 24 hours in five adults tested after a 25 hour fast (67). Because all children with high baseline levels had considerable declines in plasma FGF21 over the intervention period, we were unable to determine the association between sustained high FGF21 levels and concurrent growth.

Our evaluation of whether EE is associated with levels of FGF21 was limited by the small number of children with high FGF21 in the sample, and the sole reliance on AAT as a measure of EE. AAT describes elevated intestinal permeability which is just one aspect of the range of functional changes thought to underlie the relationship between EE and poor linear growth (27). AAT may be less sensitive to small gaps in mucosal integrity due to its large size, than smaller molecules such as urinary lactulose. Without simultaneous serum measurements, fecal AAT levels may provide a less reliable estimate of intestinal permeability, given the variability of serum AAT levels (68).

In summary, in a cohort of moderately underweight children, the subset with high initial FGF21 levels were most likely to respond to nutritional supplementation of the type used in this study, demonstrating improvements in ZHC and WAZ. While LAZ remained unchanged in children with high baseline FGF21, LAZ declined considerably in similarly underweight peers with low baseline FGF21. Increases in FGF21 appear to be associated with concurrent declines in children's linear growth, ZHC, and WAZ during nutritional supplementation. Given that FGF21 is increased by chronic macronutrient deprivation and is physiologically tied to growth, FGF21 may identify undernourished children more and less likely to be growth responsive to nutritional supplementation. A strategy which incorporates information from both FGF21 and fecal EE markers may be especially useful for improving the treatment and prevention of poor growth in children, and warrants investigation.

Figures (Chapter 2)

Figure 1: Study flow diagram, bold border denotes children featured in analyses

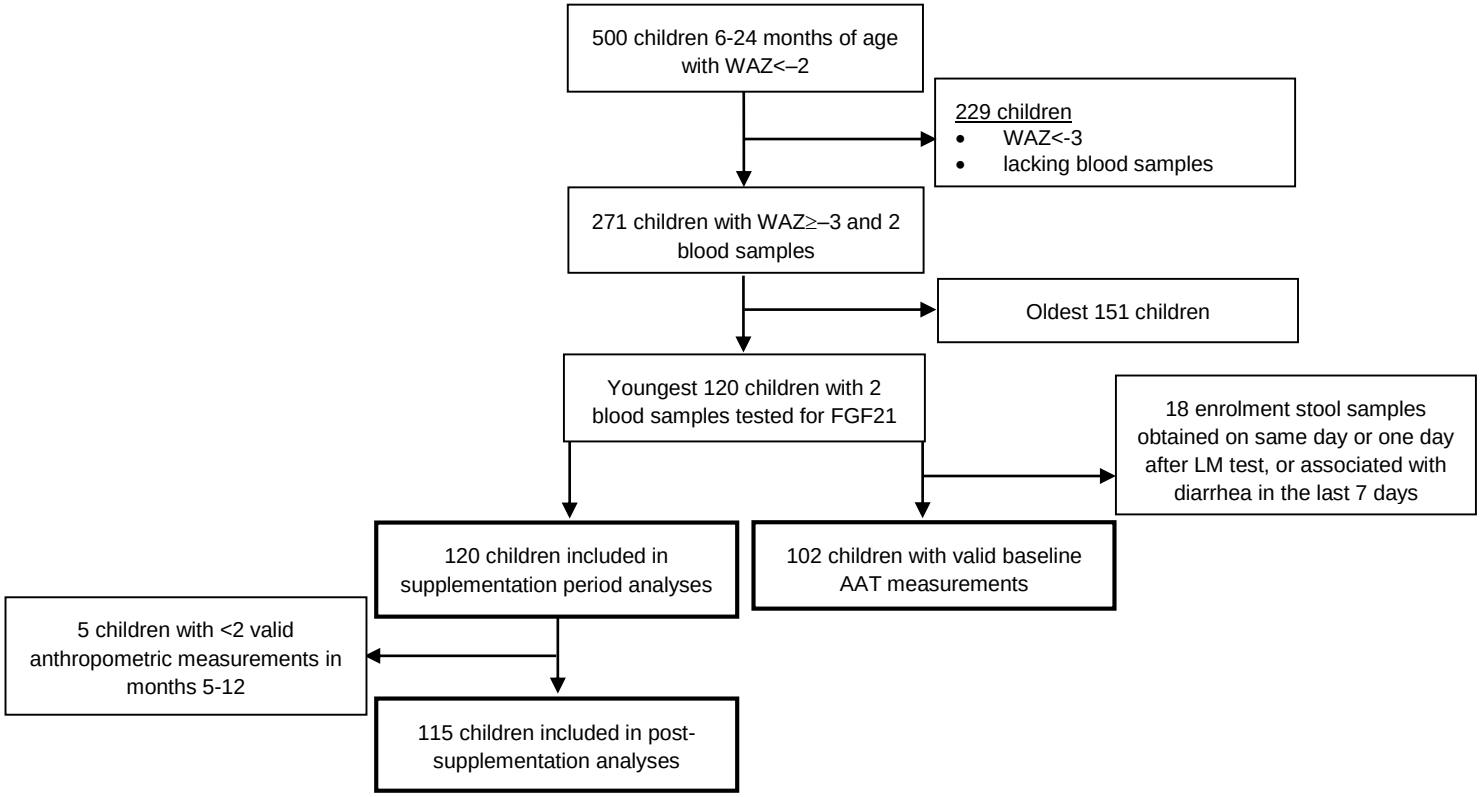
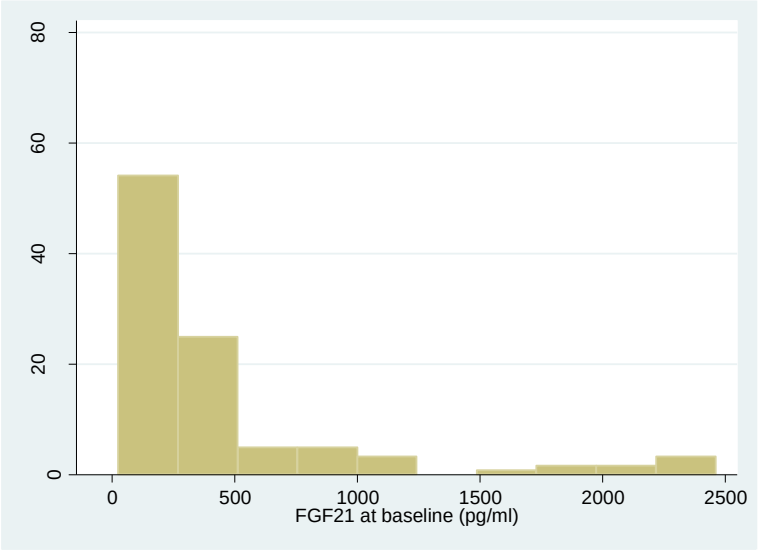


Figure 2: FGF21 distribution at baseline, after 5 months of follow up



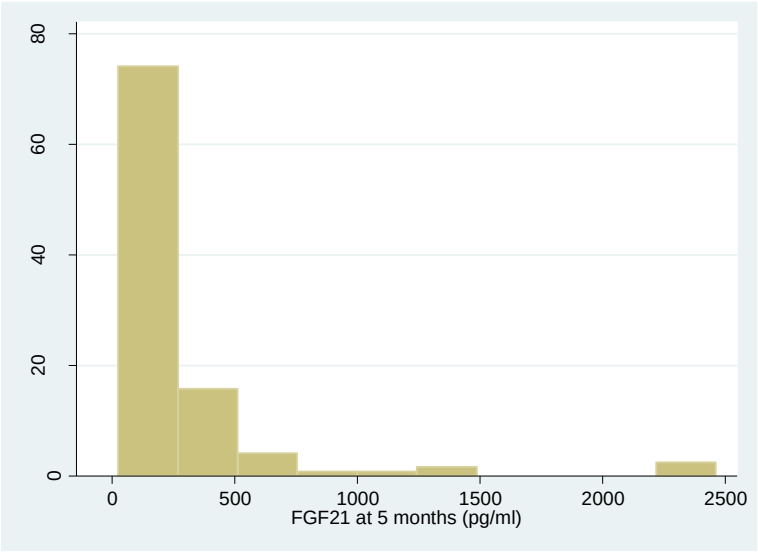


Figure 3: 12-Month anthropometry trajectories and Lowess curves by baseline FGF21 status (N= 120)

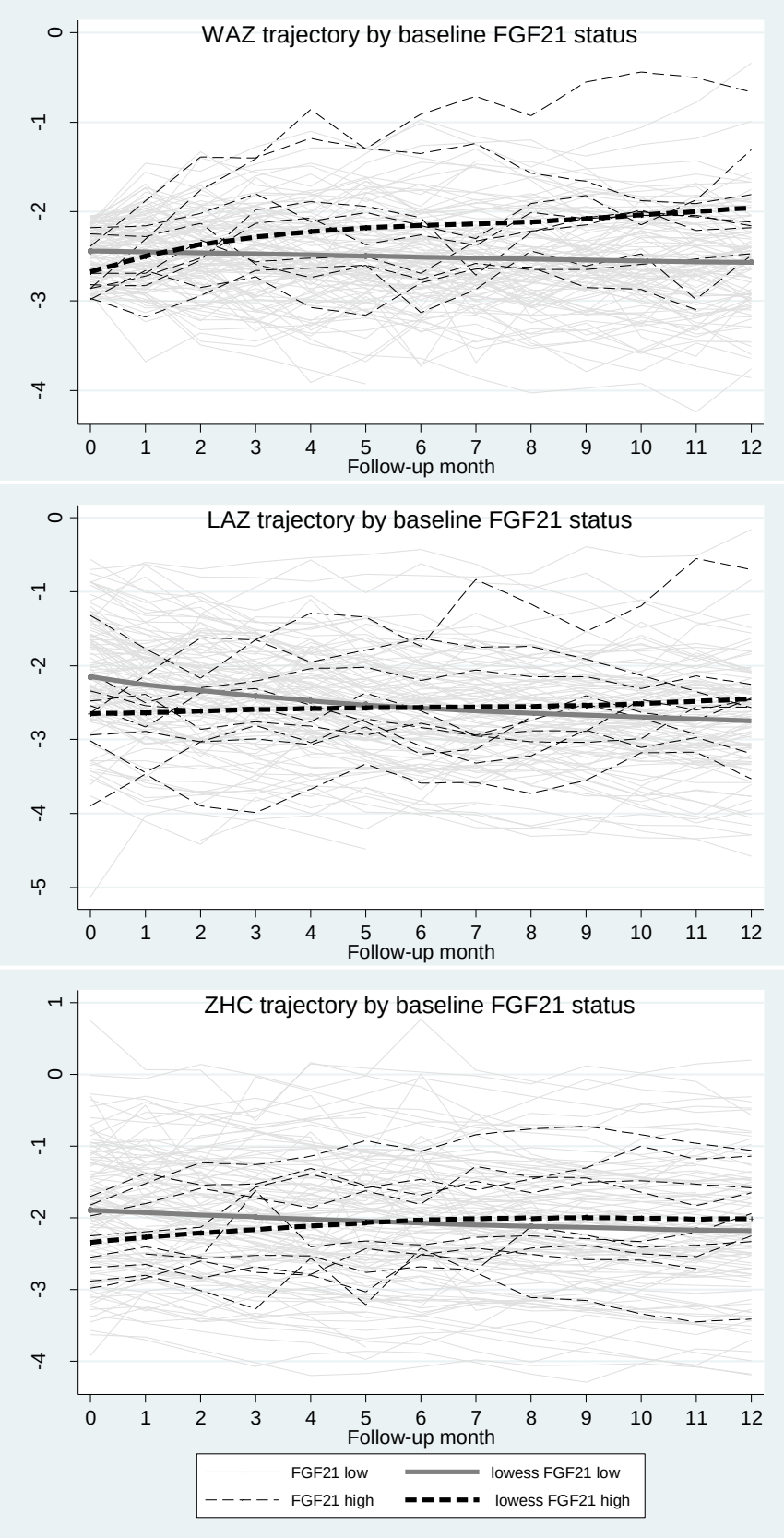
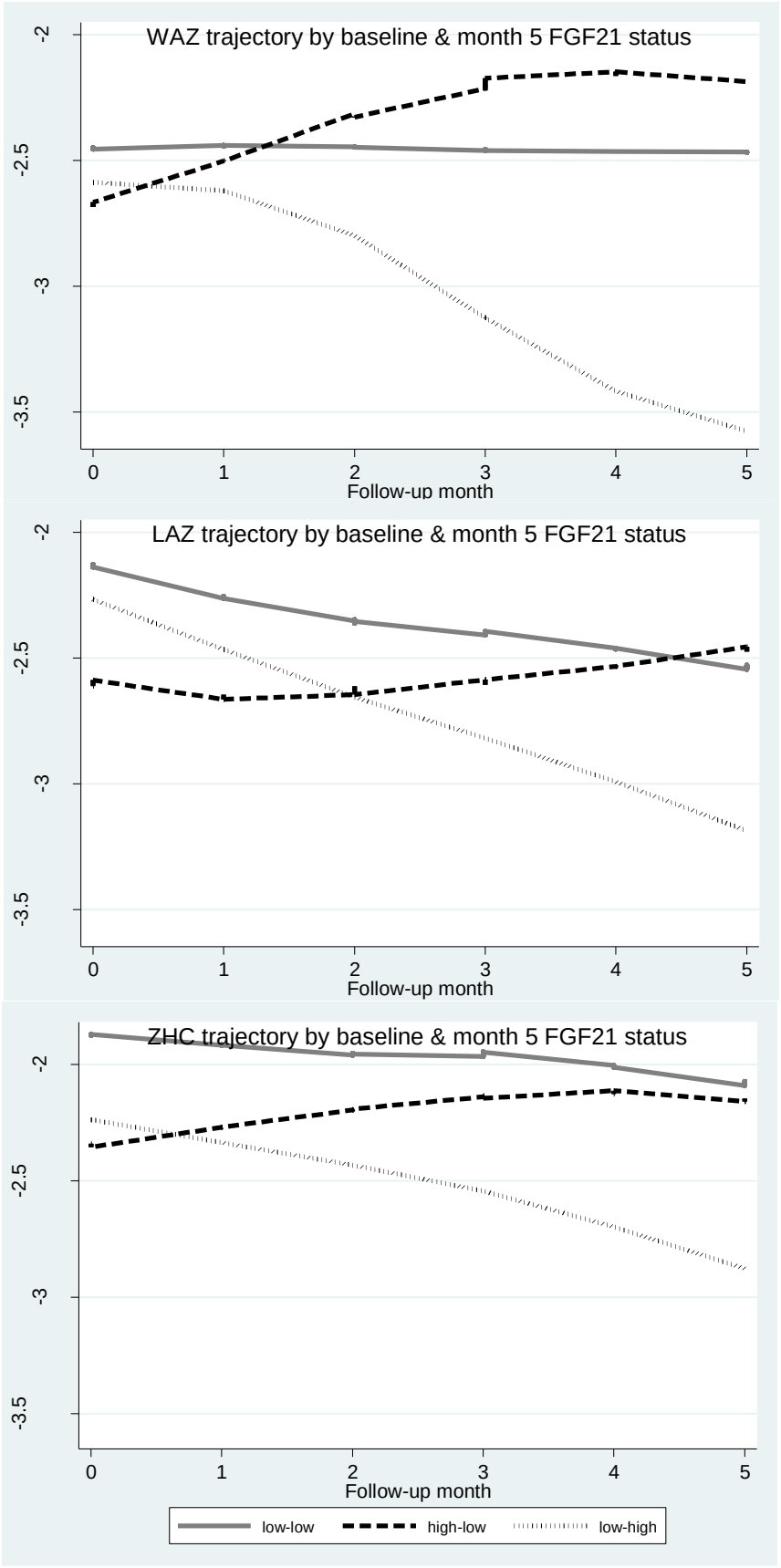


Figure 4: Lowess curves for 5-month anthropometry by 5-month change in FGF21 status (N= 120)



Tables (Chapter 2)

Table 1: Characteristics of participants and their mothers (N= 120 unless otherwise noted)

			n (%) or median (IQR)	
Child	Anthropometry at enrolment[#]	Female	67	(55.8)
		Age at enrolment (days)	278	(221, 337)
		LAZ	-2.17	(-2.65, -1.60)
		Stunted	71	(59.1)
		WAZ	-2.41	(-2.74, -2.22)
		ZHC	-1.86	(-2.56, -1.28)
		Microcephalic (ZHC<-2)	51	(42.7)
		WLZ	-1.70	(-2.16, -1.27)
		Wasted	37	(31.1)
	Anthropometry (month 5)*	LAZ	-2.58	(-3.00, -2.02)
		Stunted	88	(75.9)
		WAZ	-2.49	(-2.81, -2.17)
		ZHC	-2.16	(-2.71, -1.48)
		WLZ	-1.60	(-2.04, -1.11)
		Wasted	31	(27.0)
	Anthropometry (month 12)^	LAZ	-2.67	(-3.20, -2.31)
		Stunted	88	(88.0)
		WAZ	-2.52	(-2.92, -2.16)
		ZHC	-2.19	(-2.75, -1.55)
		WLZ	-1.63	(-1.94, -1.10)
		Wasted	22	(22.0)
	FGF21 (pg/ml)	Baseline	241.4	(111.7, 451.3)
		5 months	139.1	(78.2, 272.0)
	AAT[+] (mg/g)	Baseline	0.268	(0.163, 0.673)
Mother		Age (years)	23	(20, 27)
	Marital status	Married- only wife	102	(85.0)
		Married- polygamous	17	(14.2)
		Age at first marriage	15	(17, 18.5)
	Maternal education	Never attended school	22	(18.3)
		Schooling completed (years)	4	(2, 6)

[#]119 children with valid ZHC & LAZ at enrolment
* 115 children with valid anthropometry from month 5 (116 with valid WAZ)
^100 children with valid anthropometry from month 12
[+]102 children with valid AAT measurement

Table 2: Age-standardized anthropometry during 5 months of supplementation and in the following 7 months grouped by FGF21 status

			Low baseline FGF21 Coefficient (95% CI)	**High baseline FGF21 Coefficient (95% CI)**
Supplementation (months 0-5; n=120)	**WAZ**	Month	-0.007 (-0.024, 0.009)	0.095† (0.027, 0.162)
		Constant	-2.454‡ (-2.534, -2.375)	-2.63‡ (-2.951, -2.309)
	LAZ	Month	-0.083‡ (-0.102, -0.065)	0.027 (-0.032, 0.087)
		Constant	-2.164‡ (-2.303, -2.025)	-2.628‡ (-3.034, -2.222)
	ZHC	Month	-0.042‡ (-0.059, -0.025)	0.047* (0.009, 0.085)
		Constant	-1.882‡ (-2.046, -1.718)	-2.316‡ (-2.705, -1.926)
Following supplementation (months 5-12; n=115)	**WAZ**	Month	-0.013* (-0.023, -0.002)	0.026 (-0.007, 0.06)
		Constant	-2.479‡ (-2.581, -2.376)	-2.207‡ (-2.617, -1.796)
	LAZ	Month	-0.025‡ (-0.035, -0.014)	0.000 (-0.039, 0.039)
		Constant	-2.574‡ (-2.709, -2.440)	-2.522‡ (-2.964, -2.08)
	ZHC	Month	-0.017† (-0.028, -0.005)	0.017 (-0.012, 0.046)
		Constant	-2.076‡ (-2.247, -1.906)	-2.102‡ (-2.56, -1.645)

* $p < 0.05$
† $p < 0.01$
‡ $p < 0.001$

Table 3: Age-standardized anthropometry during 5 months of supplementation stratified by change in FGF21 status (N=120)

		High-low FGF21 Coefficient (95% CI)	**Low-low FGF21 Coefficient (95% CI)**	**Low-high FGF21 Coefficient (95% CI)**
WAZ	Month	0.095† (0.027, 0.162)	-0.002 (-0.017, 0.013)	-0.229‡ (-0.294, -0.163)
	Constant	-2.630‡ (-2.951, -2.309)	-2.451‡ (-2.530, -2.372)	-2.449‡ (-2.651, -2.248)
LAZ	Month	0.027 (-0.032, 0.087)	-0.080‡ (-0.098, -0.062)	-0.184† (-0.308, -0.059)
	Constant	-2.628‡ (-3.034, -2.222)	-2.162‡ (-2.303, -2.021)	-2.269‡ (-2.908, -1.629)
ZHC	Month	0.047* (0.009, 0.085)	-0.039‡ (-0.056, -0.023)	-0.128‡ (-0.187, -0.069)
	Constant	-2.316‡ (-2.705, -1.926)	-1.873‡ (-2.038, -1.707)	-2.235‡ (-3.131, -1.340)

* $p < 0.05$
† $p < 0.01$
‡ $p < 0.001$

Supplemental Material (Chapter 2)

Supplemental Table 1: Baseline characteristics of participants by baseline FGF21 status

		Low baseline FGF21 (n=111)		**High baseline FGF21 (n=9)**	
		n (%) or median (IQR)		**n (%) or median (IQR)**	
Female		61	(55.0)	6	(66.7)
Age (months)		9.1	(7.2, 11.1)	8.9	(7.5, 10.6)
Anthropometry	LAZ	-2.13	(-2.61, -1.59)	-2.54	(-2.94, -2.34)
	Stunted	63	(57.2)	8	(88.9)
	WAZ	-2.40	(-2.71, -2.21)	-2.82	(-2.86, -2.39)
	ZHC	-1.81	(-2.55, -1.23)	-2.4	(-2.79, -1.9)
	WLZ	-1.7	(-2.17, -1.28)	-1.64	(-1.71, -1.10)
	Wasted	35	(31.8)	2	(22.2)
Stool	AAT^+ (mg/g)	0.27	(0.16, 0.68)	0.35	(0.21, 0.52)
Plasma micronutrient concentrations	Ferritin (μg/L)	23.6	(11.7, 44.0)	25.4	(14.8, 31.0)
	Retinol (μg/dl)	20.9	(17.4, 25.7)	14.2	(11.1, 17.7)
	Vitamin D (nmol/L)	66.6	(51.8, 81.4)	57.1	(51.3, 65.0)
	Zinc (mg/L)	0.74	(0.65, 0.83)	0.69	(0.65, 0.76)

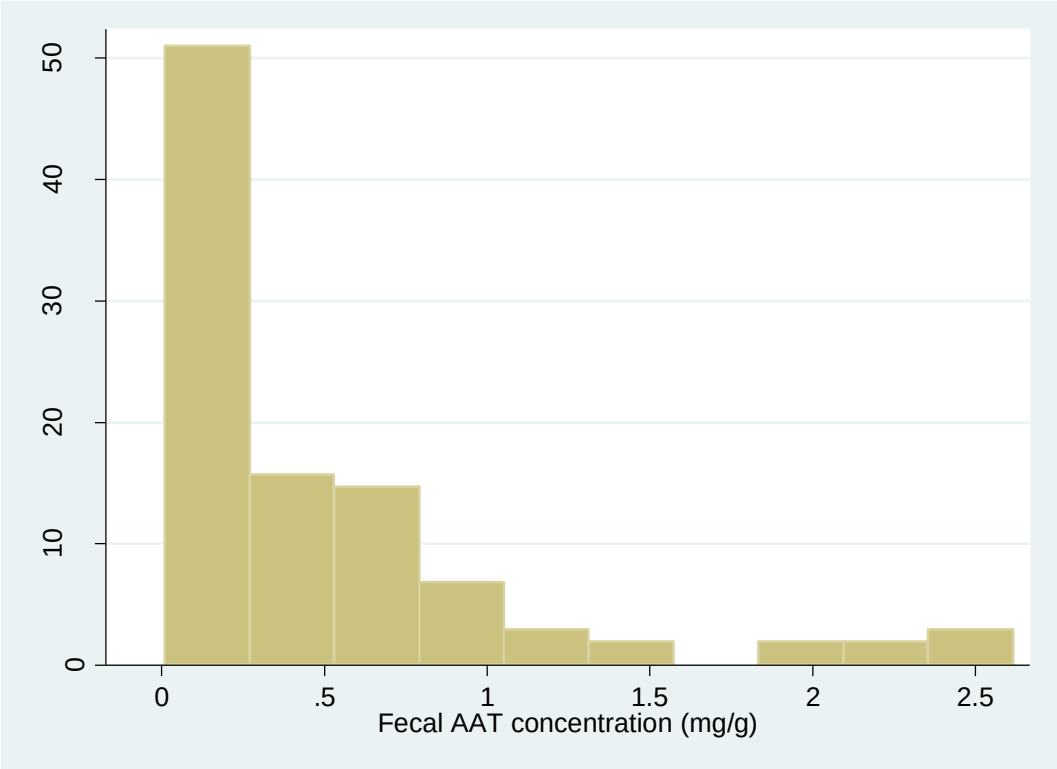
Observations missing for more than 5% of participants: AAT: 102 valid observations, hemoglobin: 100 observations, zinc: 114 observations

Supplemental Table 2: High FGF21 and anthropometry (metric scale) during 5 months of supplementation and in the 7 months following supplementation

Outcome		**Months 0-5 (N=120) Coefficient (95% CI)**	**Months 5-12 (N=115) Coefficient (95% CI)**
Weight (kg)	Month of follow-up	0.18‡ (0.16, 0.19)	0.14‡ (0.13, 0.15)
	FGF21 high	-0.20 (-0.53, 0.14)	0.16 (-0.25, 0.58)
	FGF21 X month	0.08† (0.03, 0.13)	0.05† (0.01, 0.08)
	Baseline age^	0.01‡ (0.00, 0.01)	0.00‡ (0.00, 0.01)
	Constant	5.84‡ (5.67, 6.00)	6.89‡ (6.68, 7.10)
Height (cm)	Month of follow-up	0.9‡ (0.9, 0.9)	0.8‡ (0.8, 0.8)
	FGF21 high	-1.4* (-2.7, -0.0)	-0.0 (-1.5, 1.4)
	FGF21 X month	0.3† (0.1, 0.4)	0.1 (0.0, 0.2)
	Baseline age^	0.0‡ (0.0, 0.0)	0.0‡ (0.0, 0.0)
	Constant	63.0‡ (62.3, 63.6)	68.0‡ (67.2, 68.7)
Head circumference (cm)	Month of follow-up	0.3‡ (0.2, 0.3)	0.2‡ (0.1, 0.2)
	FGF21 high	-0.8 (-1.7, 0.2)	-0.2 (-1.2, 0.7)
	FGF21 X month	0.1† (0, 0.2)	0.1 (0, 0.1)
	Baseline age^	0‡ (0, 0)	0‡ (0, 0)
	Constant	40.8‡ (40.3, 41.2)	42.4‡ (41.9, 42.9)

* $p < 0.05$
† $p < 0.01$
‡ $p < 0.001$
^age in days beyond 182 (which was the youngest child)

Supplemental Figure 1: Fecal AAT concentrations at enrolment (N=102)



Conclusions

Due to the complexity of early child growth, it is unlikely that a single intervention will be sufficient to prevent or reverse chronic undernutrition globally. Rather, a suite of interventions and approaches will be necessary (9, 18). Successful approaches in public health must often account for the epidemiological context, and chronic undernutrition is no different. This dissertation highlights several areas warranting further investigation.

Chapter 1 emphasizes the potential importance of epidemiological context in the relationship between EE and growth faltering. In contrast to the multi-site MAL-ED study, we found that of the three fecal EE markers (MPO, AAT, and NEO), only MPO was associated with decreased linear growth, and only in the second year of life. None of the included EE markers provided a direct measure of the persistent intestinal dysfunction thought to underlie EE's relationship with chronic undernutrition, and in our study, the magnitude of the association between composite EE score and linear growth was marginally larger than MPO alone. Our findings suggest that EE, as currently measured, may not be the chief determinant of linear growth faltering during the first year in Bangladesh where many children are small at birth (20). Postnatal catchup growth is one of many competing metabolic demands in children with limited nutritional resources(69), and children in this cohort were vulnerable to repeated infectious diseases. EE was common in these infants and levels of their EE markers were higher than normal values from non-resource-limited settings. However, EE in these Bangladeshi children was less severe than in children from other sites within the MAL-ED cohort. Population or region specific EE markers or cut-offs may need to be evaluated for use in different settings.

Identifying accurate biomarkers for EE is important, as EE may be a surrogate marker for risk of chronic undernutrition. These surrogate markers could be useful in clinical practice and for the

development of effective interventions. A natural next step would be to repeat this analysis using data from the Karachi, Pakistan MAL-ED site to substantiate the generalizability of these findings to other urban settings in South Asia.

In chapter 2, high FGF21 levels identified a group of underweight children who demonstrated improved growth during 5-months of daily supplementation with a rice and lentil based food. If these results are replicated in other settings, the information provided by FGF21 could be useful for developing interventions which target the specific macronutrient deficits responsible for high FGF21 levels. FGF21 may also improve the targeting of undernourished children for non-nutritional interventions in addition to food supplements. Potential interventions are being investigated in many areas, including therapeutic treatments for underlying EE, restoration/implantation of a functional microbiome, and the use of prophylactic antibiotics to reduce the risk of intestinal and respiratory infections. It would also be useful to evaluate FGF21 across a range of settings to confirm its usefulness as a predictive biomarker of growth responsiveness to nutritional supplementation, and to determine if children with high FGF21 levels would demonstrate further improvements in growth when treated with a supplement higher in protein and essential amino acids.

In conclusion, the timing of growth insults related to EE and the performance of these fecal EE markers may be setting-specific, and circulating FGF21 may be a useful biomarker for predicting which children are likely to grow in response to nutritional supplementation. A strategy which incorporates information from both FGF21 and the EE markers may be especially useful for improving the treatment and prevention of poor growth in children, and warrants investigation.

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