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Childhood Stunting in South Asian Slums

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ABSTRACT

Accommodating the half of the global caseload, stunting in children is ubiquitous in South Asian countries, and the prevalence is as high as 50% among city slum dwellers. A review of the literature identified the existing gaps in determinants of childhood stunting and interventions to improve this condition in this population. This review identified three different research gaps and also developed three aims to address these issues.

The first aim was to explore whether chronic aflatoxin exposure is a predictor of childhood stunting in the slum children of South Asia. The second aim was to identify the burden of anemia and common micronutrient deficiencies among the slum children at different time points from birth to 5 years of age, and to investigate if the Hb and plasma levels of zinc, ferritin, and retinol in early childhood can predict their status at 5 years of age. The third aim was to test if a community-based dietary intervention with daily supplementation with egg, milk, and multiple micronutrients can improve the linear growth of stunted children living in slum settings.

The first study used birth cohort data (MAL-ED aflatoxin study) of slum children, where aflatoxin concentrations were measured from plasma samples collected at 7, 15, 24, and 36 months of age using mass spectrometry. This study observed that 62% of the children were exposed to aflatoxin at 36 months of age. However, no association between aflatoxin exposure and childhood stunting was detected in this study after adjusting the potential confounders.

The second study was a longitudinal cohort study (MAL-ED Bangladesh), where children were followed from birth to 60 months of age. This study observed that the proportion of children with anemia, and zinc, iron, and vitamin A deficiencies were high in the first couple of years and then the burdens were markedly reduced at 5 years of age. This study also revealed that children who had consistent upper levels of Hb and plasma zinc, ferritin, and retinol between 7 months to 24 months of age compared to children who had consistently lower levels of the Hb and other

micronutrients for the same period as detected by latent class growth modeling, had better concentrations of plasma micronutrient status at 60 months of age.

The third study was a community-based intervention study where stunted slum children aged from 12 months to 18 months were supplemented daily with one egg, 150 ml of milk, and 1 sachet of multiple micronutrient powder for three months. At the end of the diet, children in the intervention group had a significant improvement in length-for-age z-score, compared with the age, anthropometry, and area of residence matched historical comparison group, who did not receive any dietary intervention.

In conclusion, aflatoxin is not a predictor of childhood stunting in slum children of South Asia. However, as a substantial number of children are exposed to aflatoxin the source of exposure needs to be identified. In addition, anemia and multiple micronutrient deficiencies are common in young slum children. For children who already developed stunting, a nutrition package consisting of multiple micronutrient powder, egg, and milk may be effective to ameliorate the stunting burden in the slums of South Asia.

TIIVISTELMÄ

Puolet maailman lapsista, jotka kärsivät lyhytkasvuisuudesta, asuu Etelä-Aasiassa. Näiden maiden slummeissa jopa puolet lapsista on lyhytkasvuisia eli he eivät saavuta kasvupotentiaaliaan. Lyhytkasvuisuutta käsittelevä kirjallisuuskatsaus osoitti kolme puutetta aikaisemmassa tutkimustiedossa, joiden pohjalta tämän tutkimuksen kolme tavoitetta kehitettiin.

Ensimmäisenä tavoitteena oli selvittää, ennustaako krooninen altistuminen aflatoksiinille Etelä-Aasian slummeissa asuvien lapsille lyhytkasvuisuutta. Toisena tavoitteena oli tunnistaa anemian ja yleisten hivenravinteiden puutosten yleisyys slummissa asuvilla lapsilla syntymästä 5-vuotiaaksi sekä selvittää, voivatko hemoglobiinitaso sekä plasmasta mitattu sinkin, ferritiinin ja retinolin pitoisuus varhaislapsuudessa ennustaa lasten tilan 5-vuotiaana. Kolmas tavoite oli testata, voiko slummeissa tehtävä ruokainterventio, jossa päivittäin lisätään ruokavalioon kananmuna, maitoa ja hivenaineita, parantaa slummissa elävien lasten pituuskasvua.

Ensimmäisessä tutkimuksessa käytettiin MAL-ED syntymäkohortti-tutkimuksessa kerättyä slummissa asuvien lasten aineistoa. Aflatoksiini-pitoisuus mitattiin massaspektrometri-menetelmää käyttäen plasma-näytteistä, jotka oli kerätty lasten ollessa 7, 15, 24 ja 36 kuukauden ikäisiä. Lapsista 62 prosenttia oli altistunut aflatoksiinille 36 kuukauden ikäisinä. Kun analyysissa otettiin huomioon mahdolliset sekoittavat tekijät, yhteyttä aflatoksiini-altistuksen ja lyhytkasvuisuuden välillä ei havaittu.

Toinen tutkimus oli pitkittäiskohortti, jossa lapsia seurattiin syntymästä 60 kuukauden ikään (MAL-ED Bangladesh). Tässä tutkimuksessa havaittiin, että kahden ensimmäisen ikävuoden aikana suuri osa lapsista on aneemisia ja kärsii sinkin ja vitamiini A:n puutteista. Viiden vuoden iässä näistä puutoksista kärsivien lasten määrä vähenee huomattavasti. Tämä tutkimus osoitti myös, että lapsilla, joilla veren rauta-arvot ja plasman sinkki-, ferritiini- ja retinoli-tasot olivat korkealla 7 ja 24

kuukauden välillä, verrattuna lapsiin, joilla nämä arvot olivat matalat, plasmasta mitattujen hivenaineiden pitoisuudet 60 kuukauden iässä olivat korkeammat.

Kolmannessa tutkimuksessa slummeissa asuville lapsille annettiin päivittäin lisäruokaa 12 kuukauden iästä 18 kuukauden ikään. Lisäruokaan kuului yksi kananmuna, 150 millilitraa maitoa ja yksi pussillisen pulveria, joka sisälsi useita hivenaineita kolmen kuukauden ajan. Tutkimuksen loputtua, lisäruokintaa saaneet lapset olivat suhteellisesti pitempiä (length-for-age z-score) kuin historiallisen vertailuryhmän lapset, jotka eivät olleet saaneet lisäruokintaa.

Yhteenvetona voidaan sanoa, että aflatoksiini ei ennusta lasten lyhytkasvuisuutta Etelä-Aasiassa. Kuitenkin suuri osa lapsista on altistunut aflatoksiinille, ja altistuksen lähde pitäisi selvittää. Lisäksi anemia ja monien hivenaineiden puutteet ovat yleisiä slummeissa asuvilla lapsilla. Lapset, jotka jo ovat lyhytkasvuisia, lisäruokapaketti, joka sisältää useita hivenaineita, kananmunaa ja maitoa, voi tehokkaasti vähentää lasten lyhytkasvuisuutta Etelä-Aasian slummeissa.

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ABBREVIATIONS

AFB1-lys: Aflatoxin B1-lysine albumin adduct

AGP: Alpha-1-acid glycoprotein

aOR: Adjusted odds ratio

BDHS: Bangladesh Demographic and Health Survey

CI: Confidence interval

COVID-19: Coronavirus disease 2019

DID: Difference-in-differences

EED: Environmental enteric dysfunction

ERC: Ethical Review Committee

GEE: Generalized estimating equation

HAZ: Height-for-age z-score

Hb: Hemoglobin

HPLC: High-performance liquid chromatography

IGF: Insulin-like growth factor

LAZ: Length-for-age z-score

LBW: Low birth weight

LCGM: Latent class growth modeling

LiST: Lives Saved Tool

LMICs: Low-and middle-income countries

IYCF: Infant and young child feeding

MAL-ED: Malnutrition and Enteric disease

MNP: Multiple micronutrient powder

MPO: Myeloperoxidase

MUAC: Mid-upper-arm circumference

NEO: Neopterin

QIC: Quasi-likelihood under independence model criterion

RCT: Randomized controlled trial

RDA: Recommended daily allowance

RRC: Research Review Committee

RTI: Respiratory tract infection

SD: Standard deviation

VIF: Variance inflation factor

WAMI: Water-sanitation-hygiene, asset, maternal education, and monthly income

WLZ: Weight-for-length z-score

WASH: Water, sanitation, and hygiene

WHO: World Health Organization

ORIGINAL PUBLICATIONS

1. Mahfuz M, Hasan SMT, Alam MA, Das S, Fahim SM, Islam MM, Gazi MA, Hossain M, Egner PA, Groopman JD, Ahmed T. Aflatoxin exposure was not associated with childhood stunting: results from a birth cohort study in a resource-poor setting of Dhaka, Bangladesh. Public Health Nutr. 2020 Jul 3:1-10. doi: 10.1017/S1368980020001421.

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3. Mahfuz M, Alam MA, Das S, Fahim SM, Hossain MS, Petri WA Jr, et al. Daily Supplementation With Egg, Cow Milk, and Multiple Micronutrients Increases Linear Growth of Young Children with Short Stature. J Nutr. 2019 Oct 26. pii: nxz253. doi: 10.1093/jn/nxz253

Author's contribution

Publication-1	Analysis, writing, development of the research protocol and data collection tools, and supervision of study implementation. The author was the Principal Investigator of this study.
Publication-2	Analysis, writing, supervision of study activities, and development of data collection tools. The author was a co-Investigator of this study.
Publication-3	Analysis, writing, development of study protocol, training of research staff, supervision and monitoring of study activities, and development of data collection tools. The author was the co-Principal Investigator of this study.

1   INTRODUCTION

Stunting in children is pervasive in South Asian countries [1]. Linear growth faltering leads to stunting, which is defined as having a length or height-for-age more than two standard deviations below the median of a reference standard for given sex [2]. Stunting is a status of chronic undernutrition associated with increased rates of mortality, infectious diseases morbidity, poor mental development, poor school performance, risk of chronic diseases, and lower productivity in adulthood [1]. Also, it has been linked to an estimated 17% of all under 5-year mortality burdens, and an additional 138% and 118% of higher risks of deaths from diarrhea and pneumonia, respectively [3]. A pooled analysis of ten prospective studies showed that the mortality hazard ratio (95% CI) was 5.5 (4.6, 6.5) for severe stunting [3]. Despite a massive reduction in poor childhood growth, stunting during the past several decades has affected globally about 144 million children, and 97% of the burden was shared by the low-and middle-income countries (LMICs) [4], [5]. Moreover, the highest numbers of stunted children resided in South and Southeast Asia (56 million) [6]. Although Bangladesh, a South Asian nation and member of LMICs, has experienced an almost 20 percentage point reduction in the prevalence of stunting among children under the age of five years over the last two decades, it is still facing sprawling stunting in the country. According to Bangladesh Demographic and Health Survey (BDHS) 2017-18 report, about 31% of Bangladeshi children were short for their age or stunted [7]. Recently, the necessity for the reduction of stunting has emerged as a pressing global health challenge as the World Health Assembly has ambitiously aimed to reduce the prevalence of stunting by 40% between 2010 and 2025 [8]. Despite this massive burden of stunting, this can also be noted that growth faltering is present in many other million children who are not categorized as stunted as the whole z-score distribution for linear growth has been shifted to the left [9].

Multiple factors, both causal and contextual, are responsible for the pathogenesis of stunting through an intricate pathway [10]. Linear growth faltering in children, although the process may already start in utero, most commonly occurs between six months to 24 months of age, and sometimes may extend up to three years of age

[11], [12]. Some causes of stunting are direct, which encompass inadequate diet due to the absence of exclusive breastfeeding, poor complementary feeding, consumption of poor quality or fewer varieties of food groups, and micronutrient deficiencies [12]] [9]. Additionally, frequent exposure to disease pathogens, particularly those causing diarrhea and pneumonia, has been associated with the development of childhood stunting [13]. Furthermore, limited access to pure drinking water, absence of proper sanitation and hygiene practices, poor environmental sanitation, crowding, and lack of caregivers' knowledge have been detected as factors underlying these childhood morbidities [14], [15]. In addition to the above-mentioned predictors of stunting poor pregnancy weight gain, low birth weight (LBW) (birth weight of less than 2.5 kg), maternal short stature, and genetic causes are factors associated with childhood linear growth deficit [9], [16], [17]. Limited maternal education, maternal under-nutrition, maternal depression, household food insecurity, poverty, and limited access to health care leading to inadequate child care practice during early childhood have been reported to be associated with growth deficit in children [13], [18].

Resource-poor settings of urban slums in South Asian cities are the areas where poverty, stunting, and micronutrient deficiency are pervasive. Fifty percent of slum-dwelling children under the age of 5 years are stunted in Bangladesh [19] and in other South Asian countries such as India and Pakistan [20]. There is a paucity of data on some predictors, such as mycotoxin exposure, as a risk factor of stunting that is observed in Africa. Similarly, there is limited data on micronutrient status among the young children residing in these slums, particularly from longitudinal birth cohort data, where repeated measurements were obtained from the same group of children from birth to five years of age.

Analysis of data from 110 countries indicates that the current rate of reduction of stunting is 1.8% per year [21]. At this rate, stunting will be reduced by 18% over the next 10 years, which is far away from the goal set by World Health Assembly [21]. Despite its contribution to mortality and morbidity, no single intervention has been identified that can effectively ameliorate linear growth faltering and reverse the consequences of stunting. It has been estimated that 20% of childhood stunting can be averted after ensuring the highest coverage of all existing well-proven nutrition-specific interventions [22]. Since childhood stunting is a repercussion that arises from chronic exposure to several factors, any success is unlikely if inappropriate

feeding, repeated exposure to pathogens, and environmental toxins are not taken care of effectively [17].

This work addresses the above-mentioned issues in South Asian slums. First, a comprehensive review of literature on the risk factors of stunting and nutrition interventions was performed. Second, the knowledge gaps were identified and specific research questions were developed. Finally, longitudinal community-based studies were conducted in an urban informal settlement in Dhaka, Bangladesh. This work will help to understand and manage childhood stunting.

2 LITERATURE REVIEW

This chapter describes a review of the literature to explore existing evidence and to identify research gaps to address two major questions: the risk factors of childhood stunting in low- and middle-income settings, and the interventions to reduce childhood stunting.

2.1 The approach of the review

2.1.1 Search strategy

This review utilized a comprehensive search strategy to search and retrieve reports of studies using the electronic database PubMed. A systematic search was carried out on risk factors of childhood stunting (Table 1) and on interventions to reduce childhood stunting (Table 2) using appropriate keywords.

Table 1. Search strategy on risk factors of childhood stunting using PubMed

Search	Search query (Filters: from 1970/1/1 - 2018/5/31)	Result
#1	(((stunting) OR (LAZ)) OR (linear growth)) OR (linear growth faltering)	149,944
#2	((child*) OR (children)) OR (under five year)	2,331,511
#3	(malnutrition) OR (micronutrient deficiency)	158,940
#4	((developing country) OR (low-and middle-income countries)) OR (LMIC)	134,119
#5	(risk factors) OR (predictors) OR (determinants)	9,686,334
#6	(randomized controlled trials) OR (RCTs) OR (non-randomized controlled trials) OR (interrupted time series) OR (cohort) OR (case-control studies) OR (surveys)	3,677,091
#7	(old) OR (adult) OR (mature)	7,768,564
#8	#1 AND #2	38,112
#9	#8 AND #3	4,383
#10	#9 AND #4	864
#11	#10 AND #5	491
#12	#11 AND #6	276
#13	#12 NOT #7	169

Table 2. Search strategy on interventions to reduce childhood stunting using PubMed

Search	Search query (Filters: from 1970/1/1 - 2018/5/31)	Result
#1	(((stunting) OR (LAZ)) OR (linear growth)) OR (linear growth faltering)	128,006
#2	((child*) OR (children)) OR (under five year)	2,331,511
#3	(malnutrition) OR (undernutrition) OR (micronutrient deficiency)	162,433
#4	((developing country) OR (low-and middle-income countries)) OR (LMICs)	134,164
#5	(((((dietary intervention) OR (dietary supplementation)) OR (Food supplementation)) OR (intervention)) OR (feeding)	7,620,981
#6	((((((protein supplementation) OR (egg)) OR (animal protein)) OR (milk)) OR (poultry)) OR (dairy)	3,346,331
#7	(((((micronutrient) OR (iron)) OR (zinc)) OR (micronutrient powder)) OR (sprinkle)) OR (mnp)	716,322
#8	(randomized controlled trials) OR (RCTs) OR (non-randomized controlled trials) OR (interrupted time series)	638,175
#9	#1 AND #2	38,112
#10	#9 AND #3	4,815
#11	#10 AND #4	933
#12	#5 OR #6 OR #7	10,651,067
#13	#11AND #12	702
#14	#13 AND #8	63

The literature review for this thesis was carried out in 2018 and 2019. All the titles and abstracts from the final search were examined and the relevant papers were reviewed. Moreover, systematic reviews, meta-analyses, and other review articles were examined. Special emphasis was given on the global reviews, such as the 2018 Global Nutrition Report, the Lancet Series on Undernutrition 2014, and the results

from large multi-country studies. All the articles published before June 2018 were considered. In addition, a manual search was carried out to identify and retrieve articles using snowball techniques from the references obtained from research and the review articles, which were not obtained from PubMed search.

2.2 Epidemiology of stunting

2.2.1 The concept and epidemiology of stunting

Stunting is an indicator of chronic undernutrition. Children aged 0-59 months whose length/height-for-age is less than -2 standard deviation from the median of World Health Organization growth standard for particular age and sex are considered stunted [2].

Despite a gradual reduction in the prevalence of childhood stunting for the last two decades, as of the 2018 global nutrition report, 150 million children under the age of 5 years are stunted [5]. Although stunting has been defined by a cut-off and stunted children were identified by measuring their length/height and contrasting these with standard reference groups, however, that does not necessarily mean that short stature is the main problem. Therefore, some researchers described 'stunting syndrome' as linear growth retardation in children with different pathological alterations, resulting in increased mortality and morbidity, and reduced neuro-cognitive, physical, and economic capability [15].

The prevalence of childhood stunting has been reduced steadily at a global scale from 33% in 2000 to 22% in 2017. Regionally, the reduction was from 38% to 23% in Asia. In total 39% of all stunted children in the world live in South Asia [5]. The prevalence of stunting was 31% in Bangladesh, but within Bangladesh, the prevalence varies according to socioeconomic strata, as half of the children living in slums are stunted [7], [19]. In India, the prevalence of stunting among under 5-year-old children in urban informal settlements is 45% and in Pakistan, it is 56% [20]. Stunting is the most common manifestation of childhood undernutrition, which is related to higher mortality, increased susceptibility to infection, reduced corporal growth potential, and reduced intellectual development of children [18], [23]. Moreover, stunting in childhood is linked to long-term poor health and income

including reduced adult stature, poor school attainments, and an increased risk of chronic diseases in adulthood [18], [23]. Therefore, linear growth faltering has been considered the best marker of the well-being of a child and also the best indicator of social and child health inequality [9].

2.2.2   Mechanism of stunting

The development of stunting follows an intricate pathway and its pathogenesis is poorly understood [9], [11]. In 1990, UNICEF developed a conceptual framework on the etiology of undernutrition, which is still considered as the foundation of all the latest conceptual frameworks for undernutrition including stunting. This framework has divided the causes into three categories: 1) individual or immediate, 2) underlying at family or household level, and 3) basic or general level [24], [25]. Immediate causes include diseases and inadequate dietary intake. Underlying factors are insufficient access to nutritious food, inadequate care, sub-optimal water sanitation, and health care services. The basic causes include scarce and/or incongruous knowledge and inequitable attitudes to household actual resource allocation, and the overall socio-economic, cultural, religious, and political system that limit utilization of potential resources [25]. More recently developed World Health Organization's (WHO) Conceptual Framework on Childhood Stunting described childhood stunting as a product of multifarious interactions between different causal and background factors [10]. Causal factors are maternal features, household atmosphere, poor complementary feeding, inadequate breastfeeding, food safety, and infection. On the other hand, the contextual factors are social, economic, cultural, and environmental influences [10]. Although the WHO conceptual framework was based on the UNICEF framework, it also focused on children stunting rather than overall malnutrition, which was the main focus of the UNICEF framework. Besides, outcomes of linear growth and developmental faltering and the importance of complementary feeding were depicted in the WHO framework [10].

WHO conceptual factor has provided important insight into the determinants of stunting. If we consider the proximal factors, according to epidemiological data, this will include poor breastfeeding practices, particularly non-exclusive breastfeeding, sub-optimal complementary feeding along with lower dietary diversity [9]. The frequent infections include diarrhea, pneumonia, acute lower respiratory tract

infections, and micronutrient deficiencies [13]. Intermediate factors are maternal factors such as maternal nutritional status, particularly maternal stature, and indicators of poor caring practices: maternal schooling, maternal depression, and home environment [13], [18]. Family income and assets are important to ensure proper nutrition and access to health care. Food insecurity is another important determinant of stunting. Poor drinking water supply, sub-optimal sanitation facilities, and the absence of good hygiene practices are other important risk factors [14], [15].

2.2.3 Intergenerational effects of stunting

Development of stunting is a vicious cycle as stunted women give birth to stunted children, and in absence of proper care and nourishment, these children remained stunted as they grow older [9], [25], [26]. The process of stunting starts in the mother's womb and can be continued beyond the second birthday of life [10]. The growth of the fetus is controlled by compound interactions with the nutritional status of the mother, her metabolic and endocrine functions, and the development of the placenta [9]. Therefore, the size of newborns represents the overall intrauterine environment during the pregnancy period [9]. Across the studies, size at birth or birth weight was found to be one of the most important predictors of childhood stunting [13]. Similarly, epidemiological studies consistently observed the association between maternal short stature and size at birth or LBW and subsequent postnatal linear growth deficit. Although maternal short stature is known to be the result of earlier childhood and adolescent nutritional status. Mechanisms of intergenerational effects on childhood stunting can be explained by metabolic programming, epigenetics, and genetic traits. Moreover, poverty and deprivation can also be transmitted from one generation to the next generation [25]. Despite these above-mentioned intergenerational effects, profound improvement in height can be attained in offspring and short undernourished mothers by improvement in nutrition, health, and environment [27].

2.2.4 Determinants of stunting from multi-country cohort studies

In the recently completed MAL-ED birth cohort studies conducted in seven countries, a longitudinal analysis was performed to determine factors contributing to stunting [13]. Using the UNICEF conceptual framework, this analysis examined pre-

selected biological and socio-economic factors among cohorts of children in seven resource-poor areas in Bangladesh, Nepal, India, Tanzania, South Africa, Peru, and Brazil. A total of 1,868 participants were followed longitudinally from birth to 24 months of age. Variables included in the analysis were socio-demography, maternal factors, reported morbidities, child feeding data, anthropometry, enteropathogens detected in non-diarrhoeal stool samples, and the bio-markers of Environmental Enteric Dysfunction (EED) and systemic inflammation. This analysis showed that factors that contributed to a lower length-for-age z-score (LAZ) at 24 months were undernutrition at birth, short maternal stature, and lower energy consumption from protein (OR 1.4, 95% CI 1.1, 1.7) [13].

2.3   Interventions to reduce stunting

2.3.1   Nutrition interventions to reduce childhood stunting

Nutrition interventions can be nutrition-specific or nutrition-sensitive. Interventions designed to target the immediate determinants of malnutrition are known as nutrition-specific interventions. Interventions to improve the practice of exclusive breastfeeding, dietary diversity, and micronutrient powder supplementation are some examples of nutrition-specific interventions [22].

According to the Lancet review 2013, successful nutrition-specific interventions to reduce undernutrition include the promotion of complementary feeding, multiple micronutrient powder supplementation, preventive zinc supplementation, and facility-based management of severe acute malnutrition using World Health Organization guidelines. Meta-analysis of several RCTs and non-RCTs in children from food-insecure households showed that nutrition counseling on complementary feeding had a significant effect on reducing stunting (RR 0.7, 95% CI 0.6-0.8) and improving height-for-age z-score (HAZ) (mean difference 0.3, 95% CI 0.1-0.4) when compared to children who did not receive any intervention [28], [29]. A meta-analysis from data on 17 RCTs in LMICs showed that multiple micronutrient powder supplementation in children was associated with a reduction in iron deficiency anemia and improved hemoglobin status, however, no effects were observed on stunting or change of HAZ score [12]. Another meta-analysis of RCTs showed daily

10 mg zinc supplementation for 6 months was associated with a 0.4 cm of net height gain in children compared to placebo [30].

Interventions addressing the underlying factors of undernutrition are known as nutrition-sensitive interventions. Examples include income-generating activities, improvement of social safety, education, sexual and reproductive health, cognitive and mental health, child protection, childhood development, food safety, food security, and family planning services [12]. Interventions to improve water, sanitation, and hygiene have been considered one of the most important interventions to improve nutritional status. Recent trials like WASH-benefits and SHINE have looked into this important issue [31], [32]. Usually, nutrition-sensitive interventions act as a distribution platform for nutrition-specific interventions for better coverage, delivery, and effectiveness [33]. A recent review of nutrition-sensitive interventions in urban slums of LMICs observed that interventions targeting nutrition system strengthening compared to no intervention had improved HAZ (MD 0.3, 95% CI 0.1-0.4) of children at 18 months of age [34]. Recent studies recommended that improvement of nutritional outcomes was better when both nutrition-specific and sensitive interventions were combined [35], [36].

Interventions that can improve the nutritional status of young children include the promotion of breastfeeding, improved dietary diversity, appropriate complementary feeding, iron-folate, vitamin A, zinc, and multiple micronutrient powder supplementations [22]. The effects of scaling up ten nutrition-specific interventions in 34 countries with the highest burden of stunting using the Lives Saved Tool (LiST) have been modelled [22], [37]. The nutrition-specific interventions include maternal folic acid, calcium, multiple micronutrients, balanced energy protein supplementation, interventions to improve breastfeeding and complementary feeding practices, and zinc and vitamin A supplementation [22]. The analysis showed that if these interventions were implemented with 90% coverage, there would be a mean 20% reduction in childhood stunting [22]. There have been no plausible solutions for the rest 80% of childhood stunting even after incorporating nutrition-sensitive interventions.

2.4 Identification of knowledge gaps and research questions

2.4.1 Evidence and knowledge gaps identified from the literature review

The systematic literature review identified the knowledge gaps in evidence on determinants of childhood stunting (Table 3), and the interventions to reduce this condition in South Asia (Table 4).

Table 3. Summary of literature review on determinants of stunting in South Asia

Determinants of stunting	Evidence in South Asia
Immediate causes	
Absence of exclusive breastfeeding, poor complementary feeding, poor dietary diversity	Available evidence [13], [18], [22]
Illness, diarrhea, ARI, pneumonia	Available evidence [11], [13], [17]
Micronutrient deficiencies (vitamin A, zinc, hemoglobin)	Limited evidence Data present on individual and multiple micronutrient status and stunting [11], [22], [38], but - A paucity of longitudinal data on the plasma micronutrient status of the same child in early and later periods of childhood - Lack of data to examine the association between plasma micronutrient status between early and later years of childhood
Poor dietary intakes	Available evidence Animal-sourced protein consumption is scanty [39], [40]
Dietary mycotoxin exposure (aflatoxin)	Unavailable evidence Some data on exposure is present, however, no data on the association between aflatoxin exposure and childhood growth faltering [41], [42]
Underlying causes	

Maternal and neonatal factors Low maternal height Low-birth weight	Available evidence [13], [22]
Poor child care Poor maternal education Maternal depression	Available evidence [13], [22]
Household factors Poor socioeconomic status Poor water, sanitation, and hygiene practice	Available evidence [13], [22]
Basic causes	
Economic, political, and social contexts Bangladesh South Asia	Available evidence Limited in slum settlements in South Asia [22], [34]

Table 4. Summary on availability of data on interventions to reduce childhood stunting

Interventions in slums	Participants	Evidence
Multiple micronutrient supplementation	Children Pregnant women	Available evidence [11], [22], [38]–[40], [43] Increased length of children significantly [22] Reduced low birthweight [29] No effect by zinc supplementation[43]
Complementary feeding education and supplementation	Children Mother	Available evidence [28], [44], [45] Improved LAZ and stunting [28]
Breastfeeding promotion	Pregnant women Household Community	Available evidence [44], [45] Reduce LBW but no reporting on LAZ
Energy balanced protein and micronutrient supplementation	Pregnant women	Available evidence [30], [44], [46], [47]

		Reduced SGA, Improved birth weight [30]
Animal-sourced protein supplementation	Children	Unavailable evidence [48], [49] No evidence in South Asian slums
Health system	Private sector, local government	Available evidence [11], [33]–[36], [39] Inconclusive result in LAZ
Interventions, overall (global)		
Complementary feeding promotion Nutrition education (16 RCTs)	Mother, food-insecure family	Available evidence [22] Improvement of LAZ
Vitamin A supplementation (43 RCTs)	Children	Unavailable evidence [22] No data on LAZ
Iron supplementation in children (33 RCTs)	Children	Available evidence [22] No effect on LAZ
Multiple micronutrient supplementation (16 RCTs)	Children 6 months to 16 years	Available evidence [22] Improvement in length
Zinc supplementation (16 RCTs)	Children	Available evidence [22] Improvement in length

2.4.2 Knowledge gaps and development of a theoretical framework for stunting

Based on the literature review summarized in Table 3 and Table 4, a conceptual framework was developed (Figure 1). This framework identified variables related to immediate, underlying, and basic causes of stunting. However, there have been many gaps in current research that remained unexplored in this review. These include but are not limited to exposure to specific pathogens, the role of growth hormone, exposure to heavy metals, microbiota, genetic and epigenetic factors, etc. [15]. Considering the finding of the current literature review, the following three evidence gaps were considered for this dissertation. These were the role of aflatoxin exposure and childhood stunting (Publication 1); the prevalence of anemia, micronutrient deficiency, and its association between early and later years of childhood (Publication 2); and finally, the dietary intervention with animal protein and multiple micronutrients to improve linear growth of children with stunting (Publication 3).

Immediate Causes

Child exposures:
- Exclusive breastfeeding (EBF)
- Illness: diarrhea, acute lower respiratory tract infection (ALRI), fever
- Micronutrient status (vitamin A, zinc, hemoglobin)
- Gut inflammation/Environmental enteric dysfunction (EED): elevated stool myeloperoxidase (MPO), neopterin (NEO), alfa one anti-trypsin (AAT)
- Systemic inflammation: C-reactive protein (CRP)
- Dietary intake and energy from protein
- Aflatoxin exposure: Aflatoxin-lysin adduct (AF-lys) detected in plasma
- Sex

Intergenerational cycle of malnutrition

Baby low birth weight (LBW)

Low-pregnancy weight gain

Inadequate food, health, and care

Child Stunted

Short Mother

Short Adolescent

Determinants of stunting in under 2 years children
- No or insufficient EBF
- Poor complementary feeding
- Micronutrient deficiency
- Inadequate dietary protein
- Poor WASH-Diarrhea, EED
- Recurrent infections
- Exposure to mycotoxin, particularly aflatoxin
- Poor care
- Maternal depression

Underlying Causes

Maternal and neonatal factors:
- Maternal height
- Birthweight
Child care:
- Maternal education
- Maternal depression
Household factors
- Socio-economic factors
- Water sanitation and hygiene (WASH)
- Food insecurity

Basic Causes

Economic, social and political context
- Bangladesh
- South Asia
- LMIC

No or insufficient evidence in South Asian slums
- *Aflatoxin exposure and childhood stunting*
- *Micronutrient status longitudinal data*
- *Dietary intervention with protein*

Figure 1. A theoretical framework to understand factors associated with stunting as the starting point for the present work.

2.4.3   Aflatoxin exposure and stunting

Aflatoxins are the most common mycotoxins related to childhood stunting [50]. Aflatoxin contamination has been observed in common staples like groundnut, maize, wheat, sorghum, pulses, dried fruit, and spices [51]. Hot-humid climate, rainfall, flooding, and poor storage conditions increase the likelihood of fungal growth and aflatoxin production [52], [53]. Dietary aflatoxin exposure is common in children through complementary food as well as breastmilk [51]. During metabolism, Aflatoxins break down in the liver into a highly reactive aflatoxin exo-epoxide. It interferes with protein, carbohydrate metabolism, and fatty acid synthesis. Aflatoxins also damage enterocytes causing nutrient malabsorption, hamper nutrient utilization, zinc deficiency, and systemic immune activation [50], [52]. Several studies have reported an association between chronic aflatoxin exposure and growth retardation and LBW in children [50], [54]. Moreover, few studies had shown a dose-response relationship between aflatoxin exposure and the degree of stunting in children [54], [55]. An observational study conducted in Benin and Togo measured plasma

aflatoxin-albumin adduct (AF-alb) in 475 children and detected AF-alb in 99% of samples with a mean concentration of 32 pg/mg albumin. The study revealed that children with stunting had a 30% higher mean concentration of aflatoxin and categorical analysis showed a clear and significant dose-response relationship with the concentration of aflatoxin metabolites[54]. Another longitudinal study in Benin examined post-weaning exposure to aflatoxin with child growth. Based on the mean concentration of AF-alb at three-time points over eight months period, children were categorized into quartiles. The study observed that children who belonged to the highest quartile of AF-alb had an average 1.7 cm length gain compared to the children from the lowest quartile over the study period [55]. In a longitudinal study in the Gambia, maternal aflatoxin levels during pregnancy were measured by assaying plasma AF-alb, and then after childbirth anthropometry of infants was performed at regular intervals for one year. The adjusted analysis demonstrated that a higher concentration of maternal AF-alb was associated with a shorter LAZ of infants and every one-unit increase in maternal AF-alb was associated with a twenty percent reduction of the standard deviation for LAZ of infants. These above-mentioned study series in West Africa provided astute data on longitudinal and dose-response aspects of the relationship between aflatoxin exposure and linear growth impairment in young children [56]. The most common biomarker for chronic aflatoxin exposure is the detection of the aflatoxin-lysine adduct in blood samples [42], [57].

2.4.4   Environmental enteric dysfunction (EED)

EED or environmental enteropathy is a sub-acute inflammation of the small intestinal mucosa pervasive among people living in countries with low-socio-economic status and sub-optimum sanitary conditions [58], [59]. EED is characterized by structural and functional changes in the absorptive surface of intestinal mucosa causing reduced absorption of nutrients, increased permeability, and bacterial translocation [58]. Despite its unclear etiology, EED is related to childhood growth faltering [58]. Data from Africa showed that as high as 43% of linear growth deficit in young children has been associated with small intestinal enteropathy [60]. Currently, many high-quality studies are underway to reveal the pathology and biomarkers of EED and its association with the undernutrition of children [61].

2.4.5 Multiple micronutrient deficiencies and stunting

Deficiencies of multiple micronutrients are one of the most common immediate causal factors of childhood stunting [38], [62]. Common micronutrients essential for growth are zinc, vitamin A, and iron [38]. Globally 50% of children under the age of 5 years are suffering from anemia, the majority of which is due to iron deficiency [38]. Anemic children are prone to suffer from frequent infections and undernutrition [63]. Additionally, Vitamin A is crucial for tissue accretion, infection prevention, and metabolism [64]. One hundred and forty million young children have sub-clinical vitamin A deficiency [38]. Zinc is also important for cellular growth and is essential for the production of enzymes necessary for RNA and DNA synthesis, however, 50% of the world population is at risk of zinc deficiency [65]. Poor complementary feeding practices like cereal-based diet, anti-nutrients in food, and lack of dietary diversity are the major reasons behind reduced bioavailability of micronutrients and subsequent multiple micronutrient deficiencies in young children [66]. In addition, an amalgamation of underlying risk factors like poor socio-economic status, poor maternal schooling, household food insecurity, inadequate access to pure drinking water, poor sanitation and hygiene practice, morbidity, and poor child caring practice increase the burden of micronutrient deficiency [67].

2.4.6 Multiple micronutrient powder supplementation

In LMICs, the diet of young children is mostly cereal-based, and therefore, deficiency of essential micronutrients like zinc, iron, and vitamin A is common [66]. Supplementation of a single-sachet micronutrients-mix is one of the most effective ways to control anemia and other micronutrient deficiencies [68], [69]. Therefore, incorporating MNP supplementation in other dietary interventions is essential for vulnerable groups like stunted children. The current recommendation in Bangladesh is to supplement children with a five-component MNP that includes iron, zinc, folic acid, vitamin C, and vitamin A for 2 months [68].

2.4.7 Animal source protein: Egg and milk supplementation

Compared to plant protein, animal protein is considered a better source of protein for children. Egg and milk are the most convenient and good sources of animal protein and other nutrients like essential fatty acids, minerals, and vitamins. Recent

studies have reported that a daily intake of one chicken egg can reduce stunting in young children [70]. In this RCT in Ecuador, infants 6 months to 9 months were provided daily with one chicken egg for 1 year, and compared to the control group, infants belonging to the intervention group had an effect size of 0.63 increase in LAZ [70]. Studies showed that circulating insulin-like growth factor (IGF-1) that improves linear growth, has improved substantially after consumption of cow milk [71], [72]. Bangladesh, India, and other South Asian countries are the countries where both egg and milk are easily available and traditionally acceptable.

2.4.8 Interrelationship between aflatoxin exposure, diet, and micronutrient deficiency in children with stunting

Stunting, multiple micronutrient deficiencies, and anemia, are global health problems. Many epidemiological studies have demonstrated the relationship between two separate closely related entities [38], [73]. The prevalence of anemia, and other micronutrient deficiencies like zinc, iron, and vitamin A deficiency are more common in areas where childhood stunting is also prevalent [38]. Both conditions can be the effect and outcome of each other and can be the end product of the same exposure and deficiency. Based on the present literature review, the relationship between chronic aflatoxin exposures, stunting, and micronutrient deficiencies might be explained by the mechanism of aflatoxin metabolism, EED, and poor dietary intakes [57]. After dietary consumption, aflatoxin is metabolized by the cytochrome P450 (CYP) enzyme system [74]; and the small intestinal enterocytes are the site for the CYP-catalyzed metabolism of orally ingested aflatoxins [74]. After metabolism, aflatoxin is converted into highly reactive exo-epoxide that damages enterocytes, possibly through inhibiting protein synthesis leading to altered intestinal architecture and nutrient malabsorption [75]. Moreover, aflatoxin hinders the metabolism of carbohydrates, protein, fatty acid, and interferes with the IGF1 pathways causing growth faltering [57].
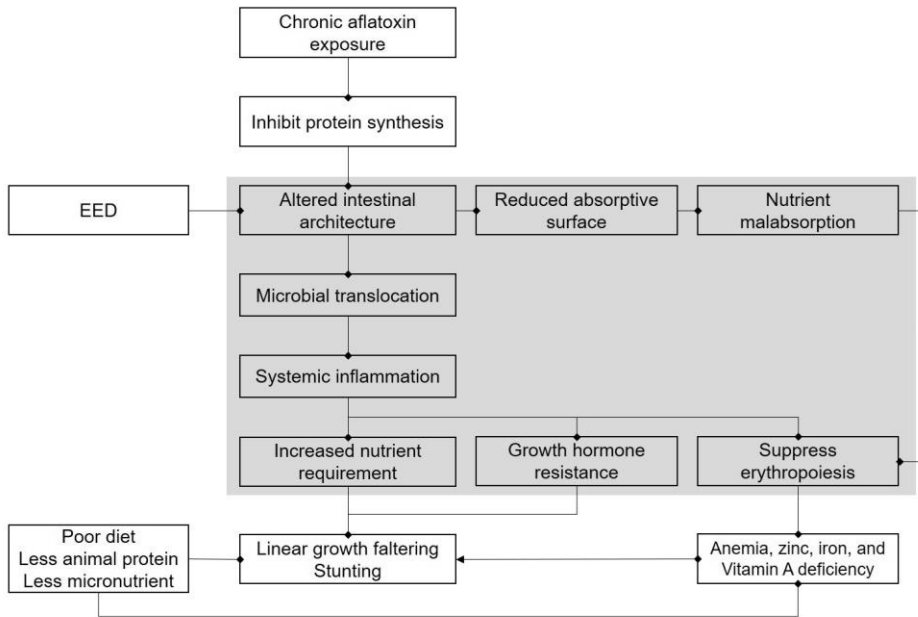
Figure 2. Pathophysiology of aflatoxin exposure, environmental enteric dysfunction (EED), and poor-quality diet in stunting and micronutrient deficiency.

EED that is present in most of the children living in the slums disrupts the small intestinal mucosa, reduces the absorptive surface area, and reduces efficient nutrient harvesting from dietary consumption [15], [57]. Due to increased gut permeability, bacteria traverse from gut to non-gut site causing local and systemic inflammation. Inflammation increases nutrient demands of the body [57], [60], [76]. Moreover, it causes growth hormone resistance and suppresses erythropoiesis [15]. All these factors in combination with the consumption of a poor-quality and less diverse diet with less animal protein and micronutrients cause growth faltering, anemia, and micronutrient deficiencies [57] (Figure 2).

3   AIMS

The overall aim of this PhD dissertation was to examine predictors of childhood stunting, dynamics of micronutrient status, and to test interventions to improve linear growth deficit in young children of South Asian slums.

Based on the literature review, the specific aims of this PhD dissertation were as follows:

1. To explore if chronic aflatoxin exposure is a risk factor for childhood stunting in slums of South Asia.
2. To determine the plasma micronutrient status of children living in the slums of South Asia from birth to sixty months of age, and to explore whether patterns of plasma micronutrient concentrations in early childhood can predict micronutrient status at a later age.
3. To examine if a dietary supplementation of socially acceptable and available animal-source protein and multiple micronutrient powder can improve the linear growth of short children living in the slums of South Asia.

The relationship between the publications and aims is presented in Figure 3.
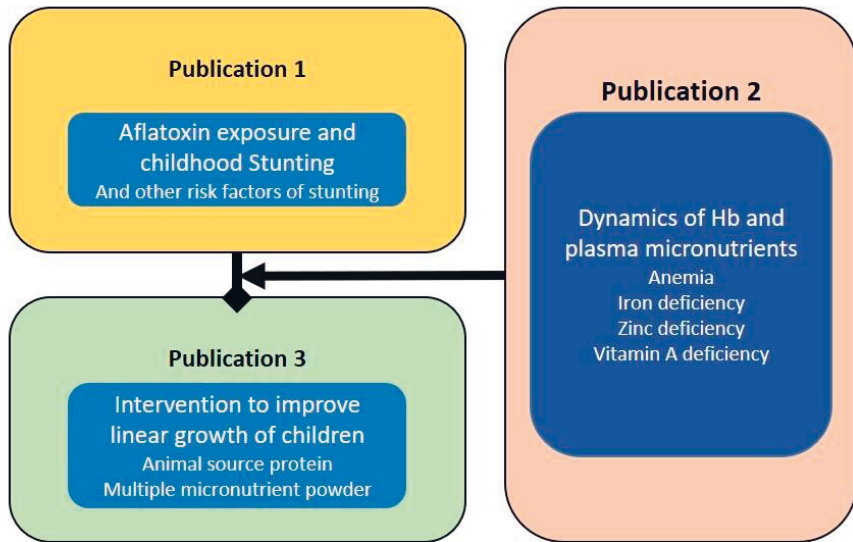
Figure 3. A schematic diagram of research questions

4 METHODS

4.1 Study design and oversight

In order to address the aims of the present thesis, data from three different studies were utilized. Study 1 was a longitudinal observational study, where assays for Aflatoxin B1-lysine albumin adduct (AFB1-lys) were done from plasma samples collected at the age of 7 months, 15 months, 24 months, and 36 months of age as the biomarker of chronic aflatoxin exposure.

Study 2 was planned to identify deficiencies of zinc, iron, and vitamin A; and anemia among children who were prospectively followed from seven months to five years of age. In this study, plasma zinc, ferritin, retinol, and Hb concentrations were measured at 7 months, 15 months, 24 months, and 60 months of age. This study also aimed to identify whether the patterns of plasma micronutrient status at less than 2 years could predict micronutrient status at five years of age. This was a longitudinal observational study.

Study 3 set out to examine whether daily supplementation of an egg, 150 ml cow's milk for three months, and multiple micronutrient powder for two months could improve the linear growth of undernourished Bangladeshi children aged 12 months to 18 months. This was a community based non-randomized comparative intervention study where children who underwent nutrition intervention were compared with age and anthropometry-matched children from a recently completed longitudinal study, where no intervention was provided.

4.2 Study premises

To address all three aims, the current study took the opportunity of three longitudinal studies conducted in a slum settlement in Dhaka city, Bangladesh.

MAL-ED-aflatoxin study: This longitudinal study was nested in the MAL-ED study, a birth cohort study where participants were tested for chronic aflatoxin exposure by laboratory assay of 'Aflatoxin B1-lysine albumin adduct (AFB1-lys)', a biomarker of chronic aflatoxin exposure, from blood samples, which was collected at 7 months, 15 months, 24 months and 36 months of age of the participants [42].

MAL-ED birth-cohort study, Bangladesh: The Etiology, Risk Factors, Interactions of Enteric Infections and Malnutrition, and the Consequences for Child Health and Development or MAL-ED study was a birth cohort study carried out to understand the risk factors of undernutrition, enteric diseases, and related health consequences in children of developing countries. Bangladesh was one of the research sites of the MAL-ED study, where participants were followed longitudinally from birth to 60 months of age [77], [78].

BEED study: Bangladesh Environmental Enteric Dysfunction Study or BEED study was a quasi-experimental community-based intervention study designed to evaluate less-invasive biomarker candidates of EED [61]. This study had an intervention phase designed to test whether a nutrition intervention consisting of egg, milk, and multiple micronutrient powder administered daily for 3 months could ameliorate linear growth deficit in children aged 12 months to 18 months in a slum of Dhaka city [61].

4.3 Study sites

All three studies were carried out in children living in Bauniabadh and adjacent slum areas of Mirpur, Dhaka. Mirpur is one of the administrative units of Dhaka city, Bangladesh. This selected area is inhabited by working-class people from lower socio-economic strata. It is a very congested area with underdeveloped sanitation and garbage disposal system. Population density is more than 50,000 per square kilometer compared to a mean population density of 1026/km2 in Bangladesh [77]. The average family size in this area was 4.5, with an average monthly family income of US$ 100. Maternal education status is poor as more than 30% of mothers were never admitted to a school and only 3% had 10 years of schooling [77]. The majority of the inhabitants worked as garment workers, day laborers, small business owners, and transport workers [77].
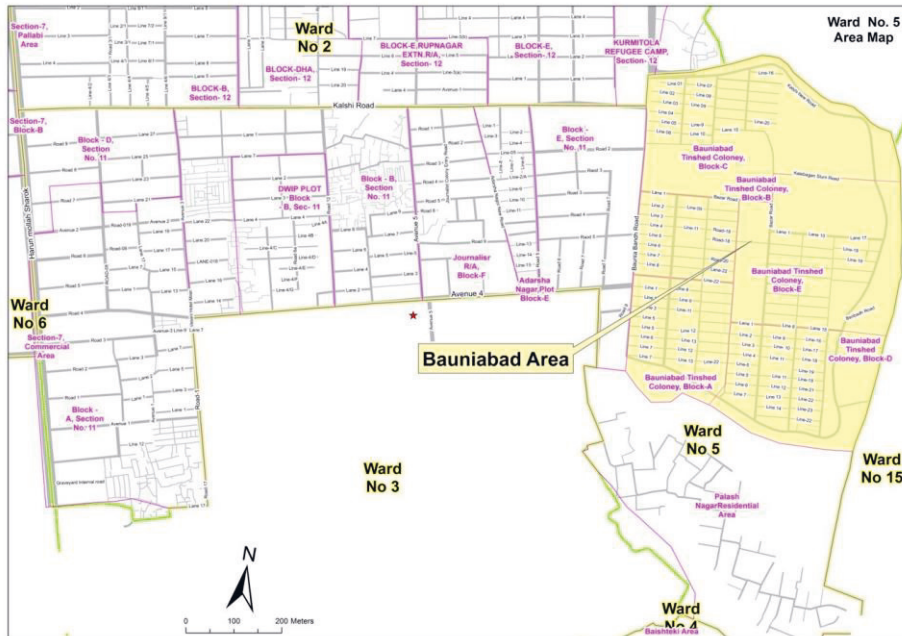
*Ward is an administrative unit of the city region; Dhaka North city corporation consists of 54 wards

Figure 4. Map of study location in the Bauniabadh slum of Dhaka, Bangladesh

4.4   Study participants

4.4.1   Study 1: Aflatoxin exposure as a determinant of childhood stunting

In this study, infants were enrolled after birth and then prospectively followed until 36 months of age [42]. Inclusion criteria in the study included: apparent healthy newborns within 17 days after birth, birth weight >1.5 kg, and the families had no plan to migrate out from the area of residence in the coming six months [77], [78]. Newborns were excluded if the age of the mother was less than 16 years, non-singleton infant, and presence of severe disease requiring hospitalization, chronic disease, or congenital and or developmental disorder. Those who had available plasma samples collected at 7, 15, 24, and 36 months of age were included in this study.

4.4.2 Study 2: Identify risk factors of anemia and the deficiencies of zinc, iron, and vitamin A among children from seven months to five years of age.

In the MAL-ED birth cohort study, using pre-set criteria for inclusion and exclusion, newborns were enrolled and prospectively followed from birth to 5 years of age. Inclusion and exclusion criteria were similar to the aflatoxin study described above: newborn singleton babies of either sex, with a birth weight more than 1.5 kg were enrolled within 17 days after birth, who did not have any severe or chronic disease or developmental disorders, and whose family had no plan to migrate out from the community in the next six months from the time of enrollment [77], [78]. Different plasma micronutrient levels were measured from samples collected at 7 months, 15 months, 2 years, and 5 years of age.

4.4.3 Study 3: Daily supplementation of egg, milk, and micronutrient powder to improve linear growth of children.

For the intervention group, children from both sexes, aged 12 months to 18 months, with LAZ <-1 were recruited from the Bauniabadh and nearby slums of Mirpur, Dhaka. Other inclusion criteria included: parents agreeing to sign a consent form to take children to community-based nutrition centers daily, six days a week for 90 days. Children were excluded if s/he had an acute infection, chronic disease, severe anemia, congenital anomaly, or known allergy to milk or egg [61].

Age and anthropometry-matched children enrolled in the MAL-ED birth cohort study were utilized as historical control for the intervention group. Here data were abstracted when the children were 14 months to 17 months old. Monthly anthropometry, socio-demography, food security, dietary intake, morbidity, and selected EED biomarker data were used. Comprehensive methods of data collection were reported elsewhere [77], [78].

4.5 Details of data collection

4.5.1 Study 1

Trained research staff collected all the data. Baseline data were collected at enrollment and subsequent follow-up data were collected through monthly household visits. At baseline, data on the child's birth date, sex, weight at birth, and breastfeeding status were recorded. Data on socio-economic status including monthly income, assets, food security status, household crowding, and water-sanitation and hygiene practices were collected [77], [78]. Moreover, maternal height and education status were recorded. Monthly anthropometry was done by trained research staff using study-specific standard operating procedures (SOPs). A portable stadiometer (Seca 213) was used for height measurement, a commercial measuring board from Seca was used for measuring length (Seca Infantometer; model 417, Hamburg, Germany), and a dual-purpose baby scale (Seca 354) was used for weight measurement. Calibration of all instruments was done daily using a standard weight and measuring rod. Detail methodology for anthropometry and QC has already been published [77], [78]. Biological samples used in this analysis were collected at 7 months, 15 months, 24 months, and 36 months of age. From the plasma samples collected at the above-mentioned time-points, concentrations for aflatoxin B1-lysine albumin adduct (AFB1-lys), a biomarker of aflatoxin exposure, were measured. AFB1-lys assays were done at Dr. Groopman's laboratory at Johns Hopkins University, USA using isotope dilution mass spectrometry. The limit of detection used in the study was 0·5 pg AFB1-lys per mg of albumin [42].

4.5.2 Study 2

After enrollment, baseline data on socio-demography, morbidity episodes, and child feeding data were collected. Anthropometry was done every month and data on water-sanitation-hygiene (WASH) and food security were collected every six-monthly until 5 years of age. We used a quantitative 24-hour dietary recall method to calculate energy and nutrient consumption from non-breast milk foods at 60 months of age. We also used data of plasma micronutrient concentrations measured from samples collected at 7, 15, 24, and 36 months of age. We performed assays to quantify plasma concentrations of zinc, retinol, ferritin, alpha-1-acid glycoprotein (AGP), and C-reactive protein (CRP). Plasma zinc concentrations were measured by flame atomic absorption spectrophotometry, plasma retinol was measured by

reverse-phase High-performance liquid chromatography (HPLC), and plasma ferritin, CRP, and AGP levels were measured using commercial kits by immunoturbidimetric methods. CRP and AGP values were used to adjust micronutrient concentrations for inflammation. Hb was measured by HemoCue 201 instrument [79]. All laboratory assays were done in the Nutritional Biochemistry laboratory of icddr,b, a state-of-the-art accredited laboratory.

4.5.3   Study 3

In this community-based intervention study, enrolled children were provided with daily supplementation of 1 egg, 150 ml of cow's milk for 90 feeding days, and one small packet of multiple micronutrient powder (MNP), which was equivalent to 1 recommended daily allowance (RDA) of zinc, iron, folic acid, vitamin C and vitamin A for 60 days. At baseline, data on socio-demography, maternal education, maternal anthropometry, and food security were collected for both the intervention and comparison groups. To compare the socioeconomic status between the groups, we used Water-sanitation-hygiene, asset, maternal education, and monthly income (WAMI) index which is a composite index computed by using data on WASH, maternal education, asset, and monthly income. This index has been used in different studies [80]. For comparison purposes, we used monthly anthropometry data. Trained research personnel performed all monthly anthropometry using study-specific SOPs. Weight was measured by a digital weighing scale (model 727, Hamburg, Germany) and length was measured by Seca Infantometer; model 417 (Hamburg, Germany). All measuring equipment was calibrated daily and 5% of anthropometry data were re-collected to ensure the precision of measurements [61]. We also collected data on food intake and its compliance throughout the intervention period. A sub-set of children (n=45) underwent a quantitative 24-hour dietary recall during and before the intervention period to estimate the energy and nutrient consumption from non-breast milk foods. Morbidity data were collected during each feeding session for the intervention group and twice-weekly for the comparison group through home visits.

4.6 Data analyses

4.6.1 Study 1

We performed descriptive analysis and reported data according to the distribution of data. The explanatory variable of interest for this analysis was aflatoxin exposure, which was measured at four-time points. Other explanatory variables were selected by using the UNICEF's theoretical framework for undernutrition adapted to include variables available in the MAL-ED study [14]. The Independent relationship between LAZ and aflatoxin exposure was done by mixed-effect multiple linear regression. Similarly, a mixed effect multiple logistic regression was fitted to examine the association between childhood stunting and aflatoxin exposure. We checked multicollinearity by a variance inflation factor (VIF) with a cut-off for VIF of ≈ 2 in the final model. Strength of association was stated as odds ratios with 95% confidence interval and a p-value of less than 0.05 was considered as statistically significant. Data analyses were done by STATA version 15.1.

4.6.2 Study 2

The distribution of different plasma micronutrient concentrations was examined and baseline data were reported as appropriate. We performed latent class growth modeling (LCGM) [81] to find distinct trajectories for each plasma micronutrient and Hb concentration tested from 7 months to 24 months of age. These analyses were performed among the children who had micronutrient data available at all three-time points (7, 15, and 24 months). Then, we fitted multiple linear regressions to investigate the association between the trajectories identified through LCGM and concentrations of hemoglobin and each plasma micronutrient at five years of age. We used STATA (version 15.1) and R (version 3.5.1) for the statistical analyses.

4.6.3   Study 3

We checked the distribution of LAZ by histogram and Q-Q plot. Baseline data between intervention and comparison groups were compared by Student's t-tests or Pearson chi-square tests or Mann-Whitney U test, according to the type of data. We used the Difference-in-differences (DID) analysis to compare the changes in LAZ before and after the interventions and within the groups. We regressed the effect of nutritional intervention using GEE after adjusting for age, sex, baseline LAZ, breastfeeding status, maternal height, morbidity, use of antibiotics, myeloperoxidase (MPO), neopterin (NEO), alfa-one anti-trypsin (AAT), and WAMI index. We used STATA (Version 13.1) to perform data analyses.

4.7   Ethical considerations

All three studies received ethical approval from the Institutional Review Board (IRB) of icddr,b in Dhaka, Bangladesh. The IRB of icddr,b has two committees: Research Review Committee (RRC) and the Ethical Review Committee (ERC). The RRC deals with the scientific soundness of submitted protocols and the ERC deals with the ethical perspective of the research, particularly the consenting process. Before submission to RRC, all three protocols were peer-reviewed externally and the reviewers' comments were addressed. Then these were presented before the RRC committee. After addressing RRC comments we received RRC approvals and then we submitted the revised protocols to ERC. Before enrolment, signed informed consents were obtained from the participants or their legal guardians. Since all participants of the studies were under-five-year-old children, we collected free informed written consent from the parents. The consent forms were written and formatted in Bangla language and were made easily understandable by the study subjects with even little or no educational background. The consent forms were read out to parents if he/she was unable to read them. Signed consent or the left thumb impression was obtained from the parents of the study subjects for participation in the study. The intervention study was registered in ClinicalTrials.gov with the following id: NCT02812615.

5   RESULTS

5.1   Enrollment and baseline characteristics

Both study-1 and study-2 were conducted among the same birth cohort from two different research studies. Baseline data was essentially the same, although few differences were observed due to differences in sample sizes between the studies.

In Study 1, baseline data were available for 229 children and we were able to follow 196 of them until the age of 36 months. Apart from baseline information required for the analysis, this study used data from 208, 196, 173, and 167 children with available assay results for aflatoxin metabolite (AFB-lys) from samples obtained at their ages of 7, 15, 24, and 36 months, respectively. In study 2, we used data of the same children followed from their enrollment (average 3.5 days after birth) to 5 years of age. We had 212 children at baseline who also had micronutrient data at any point of the above-mentioned time. In LCGM analyses, sample sizes for hemoglobin, ferritin, retinol, and zinc were 155, 153, 154, and 155, respectively. However, due to missing data, sample sizes in these models were further decreased to 142, 140, 138, and 138 for hemoglobin, zinc retinol, and zinc, respectively.

Baseline data collected at an average of 3.5 days after birth showed that 52% of participants were female. Fifty percent of the newborns had LBW (<2.5 kg) with a mean birth weight of 2.8 kg, and 22% were stunted. Children were exclusively breastfed for an average of 107 days. The average height of the mothers' (mean (SD)) was 149 (5) cm and the average (mean (SD)) maternal age was 25 (5) years. Twenty percent of mothers had 5 years of formal education, whereas 20% of them never went to school. The monthly family income was USD 100 and more than 25% of children came from families with food insecurity. Forty percent of families did not treat water for drinking and 25% had no access to an improved toilet.

In study-3, among the intervention group, data were available for 472 children, where 220 were stunted (LAZ <-2) and 252 were at risk for stunting (LAZ between -1 to -2). A total of 52 children discontinued, of them, 45 children left the

community, and 7 were withdrawn by their parents due to various reasons. The comparison group had age and LAZ matched data of 174 children. The enrollment scheme is illustrated in Figure 5.
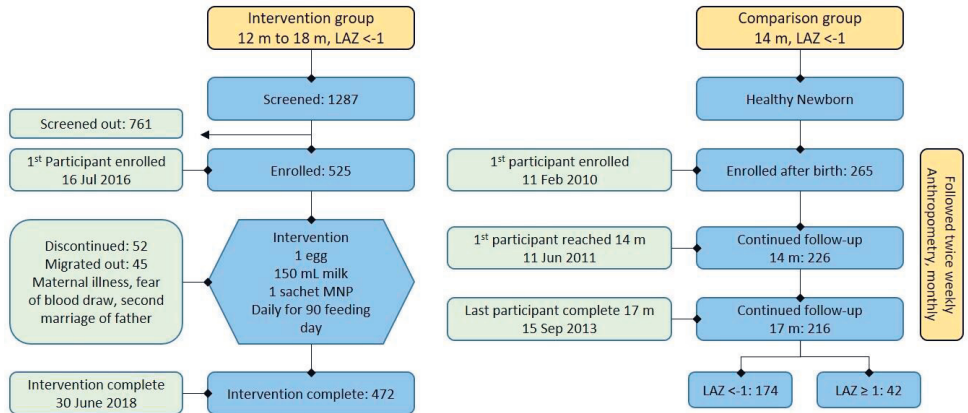
Figure 5. Enrollment scheme in intervention and a historical comparison group in study-3

In both of the groups, the median age of the children was 14 months and half of the children were female. Anthropometry indices including LAZ, maternal height, and maternal education were also comparable between the groups at baseline. The composite index, created from the data of the WAMI index was higher in the intervention group than in the comparison group. A higher WAMI index represents better socio-economy and WASH status. On the other hand, greater concentrations of stool α1-antitrypsin and stool myeloperoxidase were observed in the comparison group compared to the children who belonged to the intervention group. These baseline statistics of the participants are presented in Table 5.

Table 5. Features of children at baseline between intervention and comparison groups in Study-3

Characteristics[1]	Intervention (n=472)	Comparison (n=174)	p-value[2]
Age in month	14 [13, 16]	14 [14, 14]	0.37
Sex (male)	236 (50)	84 (48.3)	0.69
Breastfeeding status	431 (91.3)	168 (96.6)	0.02
WAMI index[3]	0.58 ± 0.1	0.52 ± 0.1	<0.05
Access to improved sanitation	297 (62.9)	174 (100)	<0.05
Access to pure drinking water	472 (100)	174 (100)	NA
Length-for-age z (LAZ) score	-2.15 ± 0.8	-2.10 ± 0.8	0.48
Weight-for-length z (WAZ) score	-0.90 ± 0.9	-0.76 ± 0.9	0.07
Weight-for-age z (WAZ) score	-1.71 ± 0.9	-1.65 ± 0.8	0.06
Monthly income, USD	157 [124, 241]	97 [72, 133]	<0.05
Maternal schooling, year	5 [2, 7]	5 [2, 7]	0.44
Height of mothers', cm	149.3 ± 5.2	148.5 ± 5.2	0.11
Fecal AAT[3], mg/g	0.3 [0.1, 0.6]	0.4 [0.2, 0.7]	<0.05
Fecal MPO[3], ng/ml	2300 [1340, 4380]	5250 [3030, 10800]	<0.05
Fecal NEO[3], nmol/L	2480 [1270, 3790]	1380 [714, 2190]	<0.05

[1]Values are mean ± SD, median [IQR], frequency (percentage).
[2]Student's t test, Pearson chi-square test and Mann-Whitney U test, as appropriate
[3]WAMI: Water-sanitation-hygiene, asset, maternal education, and monthly income; AAT: α1-antitrypsin; MPO: myeloperoxidase; NEO: neopterin

5.2   Study-1: Association of aflatoxin exposure and childhood stunting

5.2.1   Prevalence of stunting and chronic aflatoxin exposure

In this cohort, the prevalence of stunting at 7, 15, 24, and 36 months were 21%, 41%, 49%, and 49%, respectively. Based on the pre-defined level of detection, aflatoxin B1-lysine adduct (AFB1-lys) was measured in plasma samples collected at the above-mentioned four-time points. Chronic aflatoxin exposure was detected in 10%, 20%, 17%, and 62% of children at 7, 15, 24, and 36 months of age, respectively.

5.2.2   Factors associated with LAZ

Aflatoxin exposure was found not to be associated with LAZ after adjusting to other explanatory variables in the multivariable model (coefficient: 0.03, 95% CI -0.06 to 0.11; p= 0.54). It was also observed that female sex, maternal height, access to better-quality toilet, and fecal MPO level were positively associated with LAZ (p<0.05), and age, LBW, and the average number of people who slept in one room were negatively associated with LAZ (p <0.05). Detailed data are presented in Table 6.

5.2.3   Factors associated with childhood stunting

In adjusted analysis, no relationship was observed between the detection of aflatoxin in plasma samples and childhood stunting (aOR: 0.9, 95% CI 0.4, 1.9; p=0.82). However, age, LBW, MPO levels in fecal samples, and the average number of people who slept in a room were associated with higher odds of stunting. On contrary, a lower odds of stunting was found related to being a female child and the height of the mother. These associations were statistically significant (p <0.05).

Table 6. Factors related to length-for-age between 7 months-36 months of age in multivariable analysis

	Multivariable model		
	Coefficient	95% CI	
Aflatoxin exposure	0.03	-0.06	0.11
Age, months (ref: 7 months)			
15	-0.58*	-0.66	-0.5
24	-0.77*	-0.87	-0.67
36	-0.74*	-0.84	-0.63
Female (ref: male)	0.22*	0.02	0.42
LBW	-0.69*	-0.94	-0.44
Height of mother	0.04*	0.02	0.07
Sanitary toilet	0.26*	0.04	0.49
Treatment of water for drinking	0.21	-0.002	0.41
Fecal MPO, mcg/ml	0.004*	0.0004	0.008
Average family members slept in one room	-0.14*	-0.22	-0.05
Asset Index (ref: poorest)			
Poor	0.25	-0.13	0.63
Middle	0.29	-0.08	0.67
Wealthier	0.13	-0.20	0.47
Wealthiest	0.29	-0.10	0.69

*Statistical significance at $P < 0.05$.

5.3   Study-2: Anemia, iron, zinc, and vitamin A deficiency

5.3.1   Burden of anemia and micronutrient deficiencies over-time

The distribution of Hb and plasma concentrations of ferritin, retinol, and zinc are illustrated in Figure 4. We can see that the mean Hb concentration in children at 7 months was 11 g/dL and this gradually rose to 11.7 g/dL at 24 months of age and then more steeply increased to 13.2 g/dL at 60 months of age. Plasma retinol concentration was gradually increased from 22 μg/dL at 7 months of age to 29

µg/dL at 60 months of age. Plasma zinc concentrations were similar at 7 months and 15 months of age (11.5 mmol/L) and then it was gradually increased to 12.5 mmol/L at 60 months of age. On contrary, the log of plasma ferritin concentration was higher at 7 months and then gradually reduced until 24 months of age, and then steeply increased at 60 months of age (Figure 6).
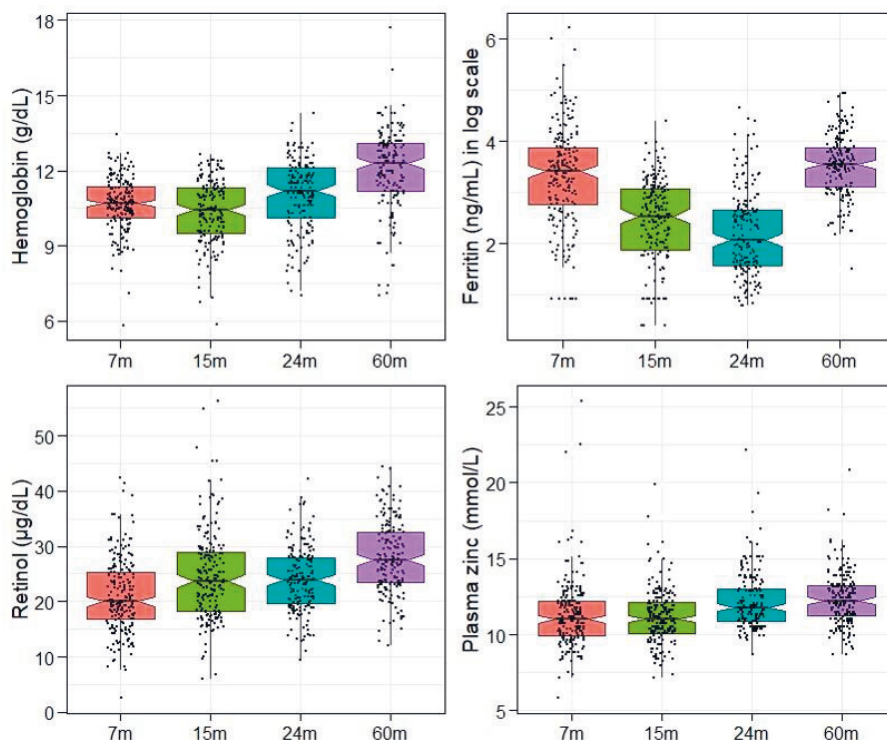
Figure 6. Distribution of Hb and concentrations of plasma micronutrients collected at different time-points

Using the pre-defined cut-offs, the prevalence of deficiencies of vitamin A, zinc, iron, and anemia were calculated. Among the study children, a higher prevalence of anemia and micronutrient deficiencies were detected in the first two years of life, and then the deficiencies were reduced markedly at 60 months of age. Anemia, retinol, and zinc deficiencies were uppermost at 7 months of age, and iron deficiency was highest at 24 months of age. With a prevalence of 15% of deficiency at 15 months,

zinc deficiency was markedly reduced to 1% at 24 months of age. The results are reported in Figure 7.
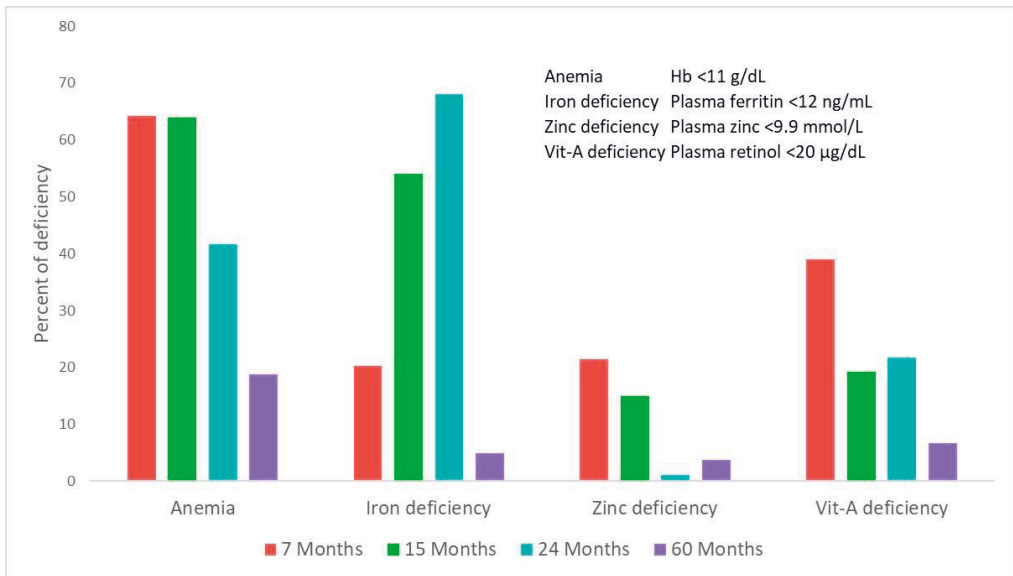
Figure 7. Anemia and deficiencies of iron, zinc, and vitamin A at 7, 15, 24, and 60 months

5.3.2 Group trajectories of plasma micronutrients in Latent Class Growth Model

Hemoglobin, ferritin, and retinol were best fitted by a two-class model. In these two-class models, Group 1 showed a general declining trend over the period and Group 2 specified an overall rising pattern. On the other hand, a one-trajectory model was identified for zinc. Estimated means for hemoglobin, retinol, ferritin, and zinc were based on their trajectories at different time points are described in Figure 8 and Table 7.

Table 7. Estimated trajectories for hemoglobin and plasma micronutrients in latent class growth modeling (LCGM)

Trajectory Group*	Mean (95% CI)		
	Age = 7 Months	Age = 15 Months	Age = 24 Months
Hb (g/dl)			
Group 1	10.3 (9.9, 10.8)	9.8 (9.3, 10.3)	10.2 (9.6, 10.8)
Group 2	11.4 (11.1, 11.6)	11.8 (11.6, 12.0)	12.4 (12.1, 12.7)
Ferritin (ng/mL)			
Group 1	24.2 (20.8, 27.7)	13.2 (10.3, 16.1)	13.9 (10.9, 16.9)
Group 2	82.9 (75.0, 90.9)	21.2 (12.9, 29.5)	18.2 (9.6, 26.8)
Retinol (µg/dl)			
Group 1	21.5 (20.3, 22.6)	25.3 (24.0, 26.6)	24.3 (23.3, 25.4)
Group 2	26.8 (23.6, 30.0)	44.0 (38.5, 49.5)	30.4 (26.9, 33.9)
Zinc (mmol/L)			
All children	11.7 (11.3, 12.1)	11.5 (11.1, 11.9)	12.3 (11.9, 12.7)

*Trajectory group: Group 1: rising pattern; Group 2: declining pattern
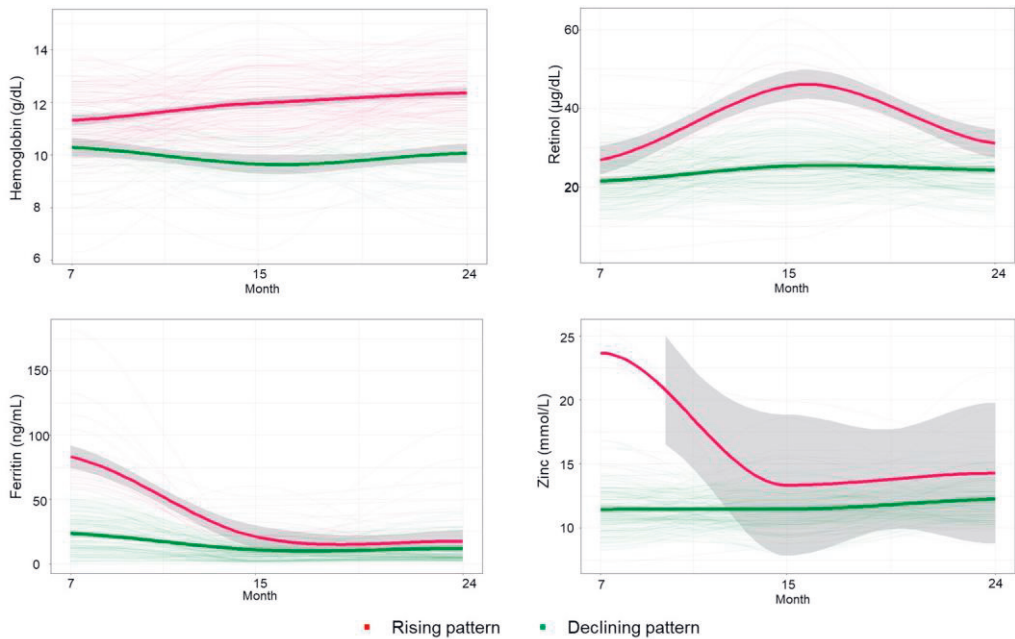
Figure 8. Group trajectories for Hb and plasma micronutrients derived from latent class growth modeling (LCGM) with 95% confidence interval during 7 to 24 months of age

5.3.3 Latent class trajectories in 7-24 months and its association with plasma micronutrient and Hb concentrations at 5 years of age

In multiple linear regression models, we did not observe any association between trajectories for Hb concentrations for 7–24 months of age with Hb concentration at 60 months. However, mean dietary intake of iron and percent of energy derived from dietary intake of protein at 60 months were related to Hb level at 5 years of age ($p < 0.05$).

For plasma ferritin, children who belonged to the upper trajectory compared to the lower trajectory, at 7–24 months, had a higher concentration of plasma ferritin at 5 years (coefficient 14, 95% CI 1–36, $p < 0.05$). Plasma zinc concentration at 24 months was positively related to the concentration of plasma ferritin at 5 years (coefficient 12, 95% CI 0.2–4, $p < 0.05$). Children who belonged to the upper trajectory for retinol at 7–24 months also had a higher concentration of plasma retinol at 5 years of age (coefficient 4, 95% CI 1–7, $p < 0.05$), and plasma zinc levels at 24 months was positively related to plasma zinc levels at 5 years of age.

5.4 Study-3: Effect of daily egg, milk, and micronutrient powder on length-for-age z-score of short children

Children consumed an average of 97% and 99% of the served egg and milk, respectively, daily for a median (IQR) intervention period of 108 (106, 112) days considering the weekends. This intervention package provided 6% of their daily RDA of carbohydrates, 92% RDA of protein, and 33% of the required kcal of energy.

The outcome indicator was a change in LAZ and it was compared with age and LAZ-matched cohort of children as a historical control. Children in the historical comparison group were taken from the same community and were followed for the same duration with a similar frequency of anthropometry and other data collection, however, did not receive any nutrition intervention.
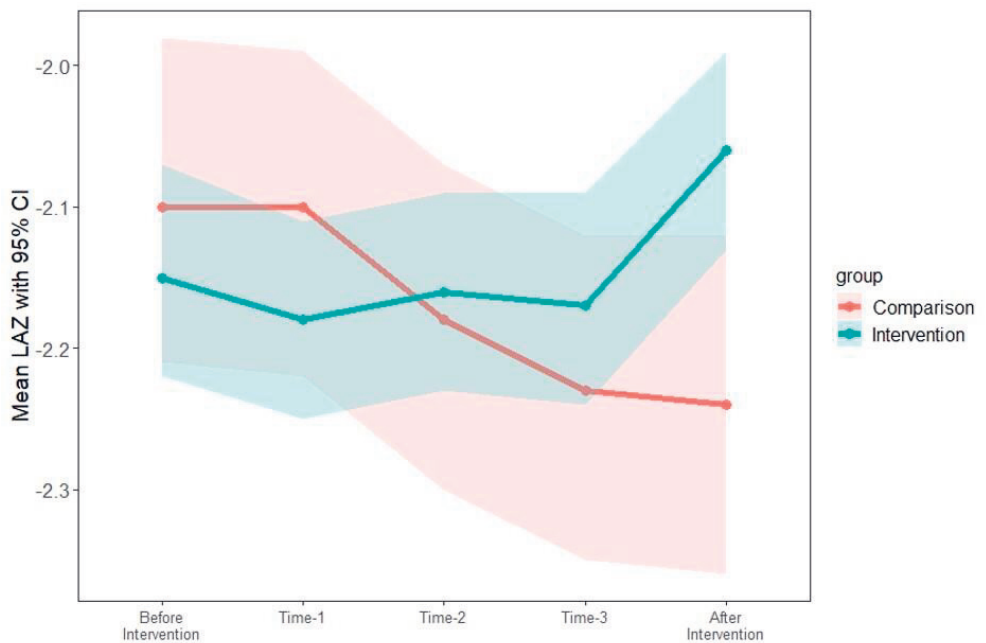
Figure 9. Mean LAZs of children between the study groups over time

The mean distribution of LAZ over the study period between the groups is illustrated in Figure 9. At the end of the intervention, changes of LAZ between the groups were contrasted using difference-in-difference methods, which were adjusted

for age, sex, mother's height, LAZ at baseline, the status of breastfeeding, reported morbidities, use of antibiotics, and WAMI index in the multivariable regression model. After the end of the intervention period, the group of children who received the nutrition intervention had a positive change of LAZ (coefficient 0.23, 95% CI 0.17, 0.28, p<0.05) compared to children who did not receive any intervention.

We repeated the same analysis after dividing the participants from both of the groups into stunted (LAZ <-2) and at risk of stunting (LAZ -1 to -2). In sub-group analysis, similar positive changes in LAZs were observed for both groups who received the intervention compared to children in the corresponding comparison groups. However, the positive change was enhanced in the stunted children (coefficient 0.27, 95% CI 0.18-0.35, p<0.001) but, attenuated in children who were at risk for stunting (coefficient 0.19, 95% CI 0.12-0.27, p<0.001).

6 DISCUSSION

South Asia, the home of 1.9 billion people, is also the residence of half of the stunted children in the world [82], [83]. Among the South Asian countries, India, Pakistan, and Bangladesh belong to the ten most populous countries in the world [83]. Most of the countries in this region have been observing extraordinary growth in human capital development including improvement of both economic and social indicators in recent years[84]. Surprisingly, this impressive economic growth, reduced poverty, and most importantly, the transition of several countries from this region to classified middle-income countries have not been translated into improved nutritional status justifying the phenomenon colloquially known as the "South Asian enigma" [84], [85]. Major underlying predictors considered for this were frail urban governance, the nonexistence of an actual system for social protection, improper resource allocation, limited access to public services, natural calamities, political instability, food insecurity, climate change, gender disparity, inequity, and mounting poverty among disadvantaged groups [83]. Hasty and unrestrained urbanization in South Asian countries, fueled by economic growth is downplaying the route to overall development including an increased prevalence of undernutrition in its most vulnerable population. Close to 37% (709 million) of people in South Asia live in urban areas and around 130 million of them live in slum areas [82], [85].

Like other South Asian countries, Bangladesh is undergoing significant socio-demographic changes, including rapid urbanization, which has resulted in a rise in the urban population from 5% to 40% between 1950 and 2017 [86]. The resident population has doubled in Dhaka between 1995 to 2015 and is forecasted to triple by 2025 [87]. With 160 million people, Bangladesh is one of the countries with the highest population density [88]. Fifty-three million of this population live in cities and of them, 40% are children [87]. Several issues such as unemployment, river erosion, and limited access to amenities like quality education and health are the reasons behind the rapid influx of poor migrants from rural areas to urban settlements. Inability to pay the formal housing rent due to inadequate income leads these migrants to choose informal settlements (slums) as their shelter. More than 30% of the estimated 21.7 million inhabitants in Dhaka presently reside in slum areas

and it has been anticipated that the proportion will be increased to 50% in the next decades [88]. This phenomenon is also true for other major South Asian countries like India and Pakistan.

Slums are predominantly inhabited by working-class people who are usually young to middle-aged. A recent survey in Bangladesh reported that more than 30% of slum people were less than 15 years old and 3% were more than 65 years old [89], [90]. More than 80% of the families live in one-room, with shared kitchen, water connection (95%) and toilets (90%) [89], [90]. The most recent slum household food consumption dataset identified that 52% of slum households were food insecure [91]. The under-nutrition rates are higher among the urban slum and fifty percent of children under the age of five in slums were stunted compared to one-third in non-slum areas [92]. A study conducted in Dhaka slums showed that dietary consumption of fat and animal protein is insufficient in young children despite an average calorie intake [40]. Moreover, the prevalence of anemia is high among these children with a marked micronutrient deficiency. This scenario reflects a sub-standard complementary feeding practice that is essential for child growth [40]. All of these attributes to a high burden of childhood stunting in South Asian slums. Mumbai is the largest city in India and 42% of the population there live in slums with a prevalence of stunting ranging from 34% to 47% [93]–[96].

The overarching aim of this PhD research was to understand childhood stunting in a typical slum-settlement of a major South Asian city. At the beginning of this dissertation work, an extensive literature review was carried out to discover the factors related to childhood stunting, and another review was done to identify successful and potential nutrition interventions to decrease childhood stunting in LMICs. The literature reviews led to the development of a theoretical framework. The review identified some of the key variables that were missing or not explored adequately in South Asian children and also came up with three research questions.

To address the first research question, we did not observe any relationship between aflatoxin exposure and stunting or LAZ in multivariable models after adjusting for other confounders. A similar lack of association was observed in a couple of recent studies carried out in Nepal and Tanzania, where they utilized the same methodology for the detection of aflatoxin concentration in plasma samples [41], [97]. In the current work, a child was considered to be exposed to aflatoxin, when a concentration of 0.5 pg of AFB1-lys was detected per mg of plasma sample.

Using this cut-off, the prevalence of aflatoxin exposure in our population was 62% at the age of 36 months. In the studies done in Nepal and Tanzania, the proportions for children who were exposed to aflatoxin were 91% and 72%, respectively [41], [97].

Although the detection rates were higher, the actual concentrations of AFB1-lys detected in all three studies were much lower with an average concentration of ≤5 pg per mg of plasma. On the contrary, previous studies conducted in Benin and Togo observed a much higher concentration of AFB1-lys with a median detectable concentration of >30 pg/mg, and they observed a significant association between aflatoxin exposure and linear growth of children [54], [55]. Few other epidemiological studies conducted in Asia and Africa also observed a similar association with a higher detectable concentration of aflatoxin [56], [98]. One study done in rural settings in the north of Bangladesh observed high concentrations of AFB1-lys in plasma samples of newborns, infants, and mothers, but they did not assess any relationship with growth outcomes [53]. In our assumption, the low detectable concentration of AFB1-lys was the reason for a non-association with linear growth deficit in study children. This also suggests the presence of a threshold for detection of aflatoxin metabolites in plasma to cause stunting, and if the exposure to aflatoxin goes past this threshold, it may contribute to linear growth faltering in children [41], [42].

Aflatoxin exposure usually occurs through dietary consumption of food contaminated with aflatoxin. Maize and peanut are the most common crops associated with aflatoxin contamination, however, the consumption of these is low in Bangladesh. This may be another reason for low aflatoxin exposure among the participants of the current study [42]. Previously, we utilized 24-hour dietary recalls to examine the dietary intakes of the study and did not observe any relationship between the consumption of lentils, bread, porridges, and rice with the presence of detectable aflatoxin metabolites in the plasma. However, consumption of flour-based sweet foods including biscuits, cakes, and pastries was associated with aflatoxin exposure among the participants (aOR: 2.2, 95% CI 1.3- 3.7, p <0.05) [42].

We have looked into the published papers about aflatoxin exposure and childhood stunting in South Asia and found several papers from India and Pakistan reporting aflatoxin detection in food samples [99], [100]. Unfortunately, except for Nepal and Bangladesh, no other study used a robust method to measure aflatoxin

from plasma samples and compared it with childhood stunting [100], [101]. One small Indian study (n=46) measured aflatoxin B1 in serum samples of young children using thin layer chromatography and observed a negative correlation with LAZ ($r = -0.46$, $p = 0.001$) [101]. Another recent study in Pakistan measured aflatoxin in the urine sample of children using the ELISA technique but observed no association with nutritional status [100]. Nonetheless, a recently published systematic review looked into the evidence generated on the relationship between aflatoxin exposure and child growth faltering. After reviewing 50 published papers, they concluded that the evidence was inconclusive and many studies did not follow proper design and methods [102]. Finally, it can be said that aflatoxin exposure is present in South Asia and no association was observed with childhood stunting. However, more evidence is required to completely refute its association with linear growth deficit in children.

Although aflatoxin exposure was not associated with childhood stunting, we have identified several factors that were significantly related to stunting. These were LBW, age, sex, height of the mother, number of family members who slept in one room, access to an improved toilet, and stool MPO concentration reflecting the role of EED. Low maternal height is a known determinant of stunting, which may represent poor nutrition during the adolescent period. Similarly, LBW is the result of poor maternal nutrition, poor pregnancy weight gains, and short maternal stature [103]. Children living in a crowded environment in a slum setting with sub-optimal water-sanitation and hygiene conditions are exposed to the transmission of pathogens and often develop enteropathy. EED is pervasive in this community as documented in other studies done in similar settings [104]. A meta-analysis explored factors attributable to childhood stunting between urban slum and non-slum areas of 28 countries including four South Asian countries, which showed that maternal schooling, asset, and available health services were the most explainable variables associated with stunting [20]. Similar findings were observed in several studies conducted in South Asian slums and other LMICs [13], [20], [96], [103]–[106].

Childhood stunting and multiple micronutrient deficiencies are interrelated and both conditions can be the cause, exposure, and manifestations of each other and often share the same causal pathways [107]. Anemia and deficiencies of vitamin A, zinc and iron are pervasive among children living in South Asian countries [43]. Children in our study cohort exhibited a high burden of anemia and other micronutrient deficiencies in their first two years of life, which was then reduced substantially at the age of 5-years. Compared to data generated from the Bangladesh

National micronutrient survey 2011, current study children had a higher prevalence of anemia (64% vs. 33%) and iron deficiency (68% vs. 3.9%), lower prevalence of zinc deficiency (21.5% vs. 44.6%), and similar prevalence of Vitamin A deficiency (21.7% vs. 20.5%) [108]. A recent study examining the dietary intake data of children in a slum of Dhaka observed that the mean adequacy ratio (MAR), developed by averaging the intakes of 13 vitamins and minerals, was poor in stunted children living there [40]. In India, according to different national surveys, the prevalence of anemia among under-5years children was 59%, iron deficiency was 72%, subclinical vitamin A deficiency was 62%, and zinc deficiency was 44% [109], [110]. In Pakistan, using data from half a million children from 6 to 23 months old, the calculated prevalence of iron deficiency anemia, and subclinical vitamin A deficiency was 43% and 51% respectively in children belonging to the poorest socio-economic strata [109].

Our analysis identified group trajectories for hemoglobin, plasma ferritin, and plasma retinol using latent class growth modeling from plasma samples collected at 7 months, 15 months, and 24 months of age. The trajectories for hemoglobin did not show any relationship with hemoglobin concentration at 60 months. However, consumption of food rich in iron and protein was linked to an increased Hb level at 60 months. Some other studies also showed similar results [111]. Higher trajectories of retinol, ferritin, and plasma zinc concentration at 24 months were related to higher levels of these plasma micronutrients at 60 months of age. These findings indicated that a better micronutrient status during the initial couple of years after birth may sustain the micronutrient status of children at 5 years of age. This is very important for South Asian countries because they can utilize their limited resources to provide interventions to improve micronutrient status only to a targeted age group.

Most of the published studies on interventions to reduce childhood stunting were done in rural settings, while limited data were available from studies done in urban slum dwellers [34]. On the other hand, the prevalence of stunting is more frequent in urban slum settings than the other areas of the city [112], [113]. A recent Cochrane review reviewed 15 RCTs mostly done in South Asian countries including Bangladesh and India [34]. Although they found a positive change in birth weight following interventions to improve maternal education, the effect of macro or micronutrient supplementation in stunting was found indecisive (mean difference -0.02; 95% CI -0.06, 0.02) [34]. Different epidemiological studies not only observed an association between childhood linear growth faltering and consumption of diets containing less animal protein and more starches but also demonstrated a positive

association between ingesting animal-source protein and increased rate of child growth velocity [72], [114]–[116]. A recent systematic review and meta-analysis of 19 RCTs observed that after food-based animal protein supplementation including either milk, fish, yogurt, or red meat, the HAZ score of young children had increased meaningfully compared to the formula-based supplementation (HAZ +0.06 vs. −0.11) [117].

We did not find any published study where a combination of these interventions was provided to children in this age group to examine the improvement of linear growth. The effect size observed in this study was modest compared to other community-based studies conducted to reduce childhood stunting [22], [28]. One study examined the reduction of stunting by providing one egg daily for six months and observed much higher effect size for the change of LAZ [46]. In that RCT, children were less than one year old and intervention was provided for a longer duration [46]. Since, the linear growth velocity is higher in children less than one year compared to older children, after a longer duration of egg intervention this finding was not unexpected [118]. Surprisingly the follow-up study did not show any differences in stunting and no change in LAZ was observed in a similar RCT conducted in Malawi [119].

Traditionally both egg and milk are regarded as good food by South Asian people and we observed very good compliance with egg and milk consumption by our study children. Most South Asian countries produce a lot of eggs and India today is one of the world's largest producers of eggs [120]. Egg and milk are better sources of animal-source protein and consumption of these proteins are lower in children living in LMICs [121]. An egg is also rich in choline, zinc, vitamin B12, vitamin B6, and folate [122]. Choline is highly abundant in eggs and is important for the neurocognitive development of children [123]. Milk improves the concentration of plasma IGF-1 and is a very good source of calcium and phosphorus, which are essential for bone remodeling and linear growth [124]. With the current burden of multiple micronutrient deficiencies, the addition of multiple micronutrient power in the study was also expected to ameliorate the linear growth deficit of study children.

We also observed an association between the concentration of EED biomarkers and stunting in the children living in slums. The results of a recent study supported a causal association between stunting and the presence of specific members of the small intestinal microbes and enteropathy in slum-dwelling children of Dhaka [104].

Another study observed an extremely high rate of duodenal inflammation in histopathology of stunted children living in the slums of Dhaka city [125]. This also reflected the fact that children were exposed to different pathogens. Therefore, in addition to nutrition supplementation, an intervention targeting reducing the pathogen burden also needs to be considered. Underdeveloped water sanitation and the sewerage systems in the slums of Dhaka or similar settings in cities of India and Pakistan might be contributing to this high pathogen exposure and enteropathy. Studies showed that piped water supply in slum areas of Dhaka city is heavily contaminated, presumably due to leakages, and unsafe handling [126]. Moreover, the absence of appropriate drinking water treatment, household waste disposal system, and food safety issues related to proper heating of complimentary food, improper storage of cooked food for children, and the longer time lapse between cooking and feeding that allows microbial contaminations as observed in other studies need to be addressed [127]. Three large trials, WASH-benefits in Bangladesh and Kenya, and SHINE in Zimbabwe failed to show any effect on linear growth by combining WASH interventions with nutrition interventions [31], [32]. To make the WASH intervention successful as we learned from the above-mentioned studies, we need to improve infrastructure related to WASH in urban slums of South Asian cities to expect a tangible effect on linear growth.

This PhD research was carried out in one of the 5000 slums in Dhaka city. Despite the fact, based on the similarity of socio-economic conditions across the slums, findings might be true for children of similar settlements in Dhaka or even for other cities in Bangladesh. On the other hand, South Asia is a huge sub-continent, harboring fifty percent of all stunted children under five years of age in the world and they live in communities with diverse social, religious, and culinary structures; and the results of this small research may not be inferred to all slum-dwelling children of South Asia. However, when we tabulated basic country-level indicators from major South Asian countries, it showed that the features like gross national incomes (GNI), levels of income inequalities as reflected by GINI co-efficient, stunting prevalence in urban slums, and the strength of association of being an urban poor and likelihoods of being stunted, we can see the indicators were not that different across the countries (Table 8).

The conceptual framework developed in this research was based on UNICEF's 1990 theoretical outline on causes of undernutrition, WHO's framework of childhood stunting, and the framework reported in the Lancet Series published in

2013 [24], [128]. However, substantial work has been done since 2013. Recently, articles published in The Lancet journal again examined the new evidence generated in the interim period. New evidence recommended that preventive small-quantity lipid-based nutritional supplement (SQ-LNS) is effective to reduce childhood undernutrition including stunting [43]. Strategies to improve and maintain optimal infant and young child feeding (IYCF) and vitamin A supplementation are still recommended, but, zinc supplementation has failed to show any real improvement in child growth outcomes [43]. Previously, depending on domains of determinants, interventions were categorized as nutrition-sensitive or nutrition-specific. New recommendations considered nutrition actions as direct and indirect, and interventions delivered in both health care and non-healthcare sectors were given equal emphasis [43].

Table 8. Country-level indicators and stunting in major South Asian countries

	GNI[1] per capita (USD) [129]	GINI index[2]* [130]	Stunting prevalence (%) [131]	Stunting among Under 5 urban non-poor (%, 95% CI) [20]	Stunting among Under 5 urban poor (%, 95% CI) [20]	aOR[3] of stunting among urban poor [20]
India	1900	35.7	35	29 (28, 30)	43 (41, 45)	1.4 (1.3, 1.6)
Bangladesh	2010	32.4	31	29 (25, 32)	48 (40, 55)	1.7 (1.3, 2.3)
Pakistan	1280	31.6	38	28 (25, 32)	56 (45, 66)	1.9 (1.2, 3.3)
Nepal	1190	32.8	32	28 (25, 32)	44 (38, 51)	1.8 (1.3, 2.4)

[1]GNI: gross national income; [2]Gini index or Gini ratio, is the most commonly used measure of income inequality. It ranges from 0 to 1, with every person earning the same amount, its Gini score would be 0 (0%). On the other hand, if one person earned all the income in a nation and the rest earned zero, the Gini coefficient would be 1 (100%) [132]. [3]aOR: adjusted odds ratio

The emergence of COVID-19 poses a substantial public health risk to the world. Modeling based on macroeconomic indicators, results of demographic and health surveys, and reduced coverage of health and nutrition interventions in LMICs has

led to the following projections in coming years: gross national income reduced by 8%, childhood wasting or acute malnutrition increased by 14%, and 128,000 deaths among under-five children due to COVID-19 associated food insecurity [133]. Another very recent modeling exercise in 118 LMICs estimated that COVID-19-associated disturbances may cause an additional 2.6 million stunted children by the end of 2022 [134]. One research done at the peak of the lockdown in Dhaka city showed that 90% of more than 200 households surveyed in a slum in Dhaka city suffered from food insecurity [135]. In India, as of 2020, 40% of all COVID-19 cases were from four megacities: Mumbai, Kolkata, Delhi, and Chennai [136]. Due to lockdowns, unemployment in Indian urban areas has increased to 15% in 2021 compared to the average national unemployment rate of 7%, however, there was a gap in data originating from slum areas [137], [138]. A similar finding was recorded in Pakistan as UNDP reported that people from the poorest strata were at risk of food insecurity and malnutrition due to the ongoing COVID-19 pandemic [139].

Studies presented in this dissertation were conducted in the pre-COVID era, nevertheless, the results were consistent with studies done among South Asian slum-dwelling children in the pre-COVID period. If we consider the novelty of our research, for the first time in Bangladesh, this study showed that children in slums are exposed to aflatoxin. Although we did not observe any association between aflatoxin exposure and stunting, the exposure itself is a grave concern. To mitigate the problem in South Asia, well-designed research is essential to detect the source of its exposure in the food chain, and identification of the possible threshold value causing adverse health effects is essential. In addition to exploring the role of aflatoxin in stunting, our research observed a high prevalence of anemia and some important micronutrient deficiencies in study children in their early childhood and identified the importance of micronutrient sufficiency in the first couple of years of life. Moreover, our research suggested that supplementation of animal source food and multiple micronutrient powder might be helpful to mitigate childhood stunting in South Asian slums. However, the execution of these suggested actions will require proper policy, planning, and inter-departmental coordination at a national level. Moreover, different direct and indirect nutrition actions need to be implemented through active participation of both health care and non-healthcare sectors.

7   LIMITATIONS AND STRENGTHS

7.1   Limitations

Study-1

This study has several limitations. The most important issue is the unavailability of assay results of aflatoxin exposure biomarkers for some children in the birth cohort at different time points. This was mostly due to the absence of stored plasma samples from the MAL-ED cohort at different time points. The period of gradual transition of an infant from breast milk to family food is the most critical time for dietary aflatoxin exposure. Typically weaning started in this community from 3 months onwards. Since we had not collected blood samples before three months of age, we were unable to measure aflatoxin exposure before the weaning period. Therefore, with the available 7-month aflatoxin exposure data when weaning had already been initiated, we could not confirm that the detection of aflatoxin was due to the introduction of family food. Unavailability of data on aflatoxin concentration in common food items and aflatoxin concentration in breast milk was also limiting factors. Additionally, known predictors of childhood stunting like pathogen burden and protein intake data were also absent in this analysis.

Study-2

The limitations of this study include the missing values of plasma micronutrient assay results at different time points. Moreover, data on morbidity, pathogen burden, and environmental enteropathy biomarkers were available only between birth to 24 months of age. These important confounders could not be used in longitudinal analysis. In addition, plasma micronutrient status was not measured at birth and quantitative dietary intake data was unavailable before six months of age. Therefore, we do not know about the actual baseline micronutrient status of the children. Considering the time points of micronutrient assays, there was a long gap between 24 months and 60 months. As there was a sharp drop in anemia and micronutrient

deficiencies during this period, an additional time-point of micronutrient assays was required to identify the more specific time of change.

Study-3

There had been several limitations regarding measurement of the true efficacy of egg, milk, and micronutrient powder intervention in the study. The major limitation of this study was inherent to its non-randomized trial design and the absence of a control group that should be concurrent. Randomization prevents major biases and the absence of randomization increase the likelihood of both underestimation and overestimation of the intervention effect. The time difference between the intervention and historical control was three to five years. This could be a source of type-1 error and increase the possibility of overestimation of the intervention effect. In addition to dietary supplementation, the intervention group also received nutritional counseling, which was absent in the comparison group. Therefore, it was not possible to distinguish whether the change in linear growth was due to nutrition intervention or due to behavioral change following counseling. Moreover, all interventions were put into one intervention group, so which of the three components or which combinations were responsible for the change was not identifiable. Stunting is a long-term process and 90 feeding days might be too brief to measure the full potential of the nutrition intervention to improve the linear growth of children. We did not address the role of pathogen burden, which was observed in other studies to be an important predictor of stunting in this population. Finally, the effect size appeared modest considering the cost and complexity of the intervention, and called for further investigation before considering this at a programmatic level.

7.2 Strengths

Study-1

Recent multi-country cohort studies showed that the prevalence of stunting was highest at the age of 24 and 36 months. Despite this, most of the published studies focused on exploring stunting determinants for the first couple of years of life. This study longitudinally evaluated the association between aflatoxin and stunting from birth to 36 months of age. There has been a paucity of data on aflatoxin exposure in South Asia, and data on the relationship between aflatoxin exposure and linear growth faltering in young children were also missing in Bangladesh, India, and Pakistan. The current study was unique in the sense that it has a larger sample size and it performed the assays for aflatoxin metabolites using mass-spectrometry from plasma samples collected from the same children repeatedly at four time points, which is even unique in the world.

Study-2

During the literature review, we did not find any published data in LMICs, where plasma micronutrient status was measured from the same child repeatedly for at least 4-time points from children under the age of five years, which was done in this study. Considering the high infection burden, concentrations of all micronutrients were adjusted for acute-phase proteins. The availability of longitudinal data on socio-demography, water sanitation, morbidity, and dietary intake data was another strength of this analysis.

Study-3

In absence of similar data in the South Asian slum children, this directly observed intervention prevented sharing of food with other siblings in the family, and total consumption of the food and leftovers were weighed and recorded. The anthropometry was performed by trained research staff using a standard operating procedure. For quality control purpose 5% of all anthropometry were reperformed by research supervisors within 24 hours of primary measurement. Refreshers' training was organized at regular intervals to maintain consistency of anthropometry among the raters and an intra-class correlation coefficient was calculated between the raters. All anthropometry instruments were calibrated on daily basis.

Finally, the protocols of all three studies were reviewed by international panels of experts. Studies were conducted under strict ethical guidelines. Standard operating procedures were used for all activities of data collection. Data were collected in real-time and the data collection processes were verified by several tiers of supervisors. The double data entry method was used in the entry process and the consistency of data was checked routinely. All laboratory assays were done in accredited laboratories where routine quality controls were done. Therefore, the results reported in this dissertation are trustworthy and valid.

8   SCIENTIFIC IMPACT

Based on the research aims, findings, and literature review described above and considering the similarities between slums of Bangladesh and other South Asian countries, the following inferences can be made for the children living in South Asian slums:

South Asian children living in the urban slums are exposed to aflatoxin, but there is no association between aflatoxin exposure and childhood stunting. The concentration of the biomarker that reflects long-time exposure to aflatoxin is lower in the plasma of this population compared to the studies that show an association between aflatoxin exposure and growth faltering in young children. This indicates the presence of a threshold point for aflatoxin exposure level in plasma above which causes growth faltering and it's still unknown.

A large number of South Asian children living in slums suffer from anemia, and vitamin A, zinc, and iron deficiencies during the first couple of years of their life. Consuming foods rich in iron and protein could improve the poor micronutrient status of these children. Slum children who maintained plasma micronutrient adequacy during the first 2 years of life are more likely to have a better micronutrient status at 5 years of age.

The burden of childhood stunting is also exceedingly high in the South Asian urban slums. For children who are already stunted and who develop linear growth faltering, daily supplementation of an egg, milk, and multiple micronutrient powder for three months will improve their linear growth and ameliorate their growth deficit.

9 PUBLIC HEALTH IMPLICATIONS

This PhD thesis is housed in slums of South Asian cities, which are the gamut of all public health problems encountered by the developing world. The higher aim of this work is to study childhood stunting among this disadvantaged group and examine three different elements, which are intrinsically linked to determine and solve the burden of this grave condition. The results of this research have several implications for public health and also allude scopes for further research.

Aflatoxin is a food-born environmental contaminant of fungal origin known for its role in cancer and childhood malnutrition, particularly stunting. In this study, no association between aflatoxin exposure and childhood stunting was detected in slum children. However, by analyzing the aflatoxin metabolite in blood samples, we observed that South Asian children living in the slums are chronically exposed to aflatoxin. This long-time exposure to aflatoxin can cause nutrient malabsorption, micronutrient deficiency particularly zinc and iron deficiency, and intestinal inflammation. Stunting is a result of multiple insults from inside and outside of the body and these also include micronutrient deficiency and intestinal inflammation. In South Asian slums, food insecurity and inadequate consumption of essential micronutrients and animal protein-rich diets are common. Therefore, in the presence of most of the immediate, underlying, and distal causes of stunting such as poor socio-economy, lack of proper education, poor feeding practice, and frequent infections, a child born in a South Asian slum is the most susceptible person to develop linear growth faltering. In this vulnerable situation, additional exposure to aflatoxin can create havoc even if there is no association between the exposure and stunting. There is a paucity of data on the source of aflatoxin in the food chain of slum settings, which needs to be explored. Moreover, the threshold point of aflatoxin metabolite in the human body that most likely causes growth faltering, needs to be identified.

Another well-known and important public health problem is the high level of micronutrient deficiency in children dwelling in the slums, particularly in the first 24 months of their lives. Health, nutrition, and development of children in their initial

years greatly depend on the adequacy of essential micronutrients, namely iron, zinc, and vitamin A. Deficiencies of micronutrients are closely related to childhood stunting, which is also one of the three major standalone sub-categories of malnutrition along with undernutrition and overnutrition. Consumption of iron, protein and micronutrient-rich diets is poor among the South Asian children living in urban slums. Most importantly, micronutrient adequacy in early childhood can foretell better micronutrient status in later years. This is an important public health message generated from the aforementioned research, especially for the policymakers in South Asia who are planning to provide essential micronutrient supplementation in slum settings to determine which age group needs to be targeted for the intervention.

In addition to micronutrient inadequacy, consumption of balanced macronutrients is also poor in this group of children. Their diet is missing necessary animal-source protein, which is an important predictor of childhood stunting. This study demonstrated that daily supplementation of 1 sachet of multiple micronutrient powder, one chicken egg, and 150 ml of milk can improve the linear growth of children who are already short for their age. With half of all the slum children being stunted, supplementing them with a package of essential micronutrients, and an affordable, acceptable, and available food that is rich in animal protein can be a public health means to boost child growth, as this model may be used in slum settings as a targeted intervention to improve the nutritional status of children who are stunted. Since pathogen burden and sub-clinical enteric infections are high in slum communities, incorporating additional interventions to reduce the pathogen burden will most likely improve the robustness of the effect of dietary supplementation.

10 CONCLUSIONS

Considering the aims described in Chapter 3, the three research articles provide the following three conclusions:

1. Chronic aflatoxin exposure is not a risk factor for stunting among children living in the slums of South Asian cities.
2. Anemia and deficiencies of zinc, iron, and vitamin A are common in the first two years of life among slum children, and the overall patterns of their plasma micronutrient status over this period are positively associated with their plasma micronutrient status at five years of age.
3. A supplementation package with milk, an egg, and multiple micronutrient powder daily for three months can improve the linear growth of shorth children (LAZ <-1).

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PUBLICATION
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Aflatoxin exposure was not associated with childhood stunting: results from a birth cohort study in a resource-poor setting of Dhaka, Bangladesh

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Aflatoxin exposure was not associated with childhood stunting: results from a birth cohort study in a resource-poor setting of Dhaka, Bangladesh

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Abstract

Objective: Chronic aflatoxin exposure has been associated with childhood stunting (length-for-age/height-for-age < −2 SD), while data lacks for Bangladesh, a country with substantial burden of childhood stunting. This paper examined the association between aflatoxin exposure and childhood stunting in a slum setting of Dhaka city.

Design: In this MAL-ED aflatoxin birth cohort study, plasma samples were assayed for aflatoxin B1-lysine adduct (AFB1-lys) by MS at 7, 15, 24 and 36 months of age for 208, 196, 173 and 167 children to assess chronic aflatoxin exposure. Relationship between aflatoxin exposure and anthropometric measures was examined by mixed-effects logistic regression models.

Setting and participants: The study was conducted in Mirpur, Dhaka, where children were followed from birth to 36 months.

Results: Prevalence of stunting increased from 21 % at 7 months to 49 % at 36 months of age. Mean AFB1-lys concentrations at 7, 15, 24 and 36 months were 1·30 (range 0·09–5·79), 1·52 (range 0·06–6·35), 3·43 (range 0·15–65·60) and 3·70 (range 0·09–126·54) pg/mg albumin, respectively, and the percentage of children with detectable AFB1-lys was 10, 21, 18 and 62 %, respectively. No association was observed between aflatoxin exposure and stunting in multivariable analyses. Factors associated with childhood stunting were age, low birth weight, maternal height, stool myeloperoxidase and number of people sleeping in one room.

Conclusions: A relatively lower exposure to aflatoxin may not influence the linear growth of children. This finding indicates a threshold level of exposure for linear growth deficit and further investigation in other areas where higher concentrations of aflatoxin exposure exist.

Stunting (length-for-age *z*-score (LAZ)/height-for-age *z*-score (HAZ) < −2 SD of WHO growth standards) or chronic undernutrition is considered the most pervasive form of childhood malnutrition affecting 150 million children under the age of 5 years globally[1,2]. Recently published papers from the multi-country MAL-ED birth cohort study demonstrated that low birth weight, low maternal height, higher burden of non-diarrhoeal enteropathogens in stool samples, lower socioeconomic status (SES) and inadequate protein content in the diet are the predictors of childhood stunting at 24 months of age[3,4]. Additionally, poor water, sanitation and hygiene (WASH) behaviour as well as exposure to environmental toxins are the other known risk factors for childhood stunting[5–9]. Data from Africa showed that environmental toxin, particularly dietary aflatoxin exposure, is associated with linear growth faltering in children[6–11]. Aflatoxins are secondary metabolites of *Aspergillus* species, which are known to contaminate most of the African staples[12]. With an estimated prevalence of 36 %, Bangladesh is considered to be a country with

the highest burden of childhood stunting among the children <5 years of age[13]. Moreover, the prevalence of linear growth retardation is as high as 50 % in slum areas of the country[14]. Despite the limited availability of exposure data, it can be assumed that the hot and humid climate of Bangladesh is conducive for fungal growth and subsequent toxin production[6,15]. Aflatoxin exposure has been well documented in food commodities and human studies conducted in Bangladesh[16–18]. Recent data showed that 62 % of children in Dhaka slums were exposed to aflatoxin at 36 months of age, and the exposure was found to be associated with the end of rainy season and introduction of family food[19].

Aflatoxin is the most known and extensively studied mycotoxin for its role in the pathogenesis of liver cancer[20]. A number of animal studies and few human studies provided evidence for its association with fetal growth retardation, low birth weight and childhood stunting[20]. The possible mechanisms include its interference in the metabolism of carbohydrates, protein and fatty acid synthesis, damage to enterocytes leading to poor nutrient absorption and utilisation, Zn deficiency and systemic immune activation[9,10,21,22]. Aflatoxin exposure may also interrupt the insulin-like growth factor (IGF) pathway, which has been demonstrated in a study conducted in Kenya. The study results revealed an inverse relationship between aflatoxin-albumin adduct (AF-alb) and IGF1 concentrations, and showed that 16 % of child height deficits can be explained by low IGF1 levels[23]. A longitudinal study conducted in Benin and Togo provided explicit evidence on a dose–response relationship between aflatoxin exposures measured by AF-alb and HAZ of children, and ignited the public health community to further explore this relationship through a cascade of researches[9,10]. On the other hand, childhood stunting is believed to be associated with environmental enteropathy, a poorly understood chronic inflammatory condition that mainly affects the small intestine of an individual[24,25]. Environmental enteropathy is characterised by alteration of small intestinal structure, intestinal inflammation and increased gut permeability, owing to leakiness of intestine. Persistent dietary exposure to aflatoxin during childhood may induce enterocyte damage and partially explain the gut 'leakiness', which impairs efficient absorption and harvesting of nutrients from the diet, and ultimately results in malabsorption of essential nutrients[20]. Therefore, biomarkers of environmental enteropathy need to be considered to examine the association between aflatoxin exposure and childhood linear growth faltering.

This current analysis uses data from the MAL-ED aflatoxin study, a companion study of MAL-ED birth cohort study conducted in Dhaka, Bangladesh. In MAL-ED birth cohort, children were followed longitudinally from birth to beyond 36 months of age, and data were collected systematically on most of the variables associated with childhood growth, including sociodemography; maternal information; child feeding practices, including exclusive breastfeeding days; hand washing practice; treatment of drinking water; and presence of hygienic toilets[26]. Moreover, data on different biomarkers of environmental enteropathy are also available in children at different time-points[4,26,27]. On the other hand, the MAL-ED aflatoxin study utilised the blood samples collected from the children of MAL-ED birth cohort study at different time-points, and performed assays using MS to detect aflatoxin B1-lysine (AFB1-lys), a marker of chronic aflatoxin exposure. Most of the available literatures, particularly from birth cohort studies, examined the risk factors of stunting at 24 months of age. However, there remains paucity of data on the determinants of stunting at 36 months of age. Given the high burden of childhood stunting in the slum areas of Dhaka, this birth cohort study examined the association of aflatoxin exposure and childhood stunting from 7 to 36 months of age.

Materials and methods

Study design and participants

The study site was located in the Bauniabadh slum area of Mirpur, Dhaka, which is a densely populated slum settlement inhabited by people with low SES with sub-optimal sanitary conditions. Detailed information about the study site, geography and sociodemography has been published elsewhere[4,26,27]. In this birth cohort study, newborns were enrolled within 17 d of birth with an average age at enrolment of 3·4 d, and followed longitudinally beyond 36 months of age. Well-defined inclusion and exclusion criteria were used to enrol the participants. The inclusion criteria include apparently healthy newborn within 17 d of birth, parents had no plan to migrate out in the next 6 months, and caregiver agreed to be visited at home by research staff twice weekly. The exclusion criteria include the family having a plan to move outside the study area, newborn baby with very low birth weight (<1·5 kg), maternal age <16 years, multiple pregnancy, another child from the same family enrolled in the study, severe disease requiring hospitalisation and chronic disease or congenital anomalies[27]. Participant enrolment started in February 2010. To cover seasonal variations, enrolment continued till February 2012 with an average enrolment of ten participants per month. Among the 229 enrolled newborns with complete data at baseline, 212 children completed 24-month follow-up, and 196 of them were followed through 36 months of age. Blood samples were collected at the age of 7, 15, 24 and 36 months. Among the children with available plasma samples, 208 of them who provided consent to use their samples for aflatoxin assays were enrolled in MAL-ED aflatoxin study[19].

Data collection

During enrolment, date of birth and birth weight of each child were recorded, and data relating to breastfeeding status, including initiation of breastfeeding, baseline socio-demographic information and anthropometric measurements of children and mothers were collected[26–28]. Through twice-weekly home visits by research staff, intensive dietary and morbidity data were collected[29]. Detailed information regarding the methodology was published previously[27,28]. Anthropometric data were collected each month, and data on WASH behaviour, assets, income and food security were collected every 6 months[19,27].

Biological sample collection

Through longitudinal visits, blood and stool samples were collected using a standard MAL-ED protocol. Blood samples were collected at 7, 15, 24 and 36 months of age, and plasma was obtained by centrifugation of blood samples. Monthly stool samples were collected without a fixative, aliquoted and stored at –70°C[19].

Aflatoxin plasma biomarker assay

AFB1-lys is a well-established and sensitive biomarker of long-term aflatoxin exposure. We performed this assay at Groopman's Laboratory at Johns Hopkins University, using previously published methods[30]. We used isotope dilution MS to detect the concentration of plasma samples. In short, 200 µl of plasma was combined and vortexed with an internal standard (10 µl × 0·1 ng AFB 1-D4-lys per millilitre) and pronase (Millipore Corp.; Catalogue no. 537088 – 100 µM) and incubated at 37°C for 18 h. Samples were then passed across a solid-phase extraction column (Waters Oasis® MAX Cartridge; 1 cc/30 mg; Catalogue no. 186000366). The eluent was analysed using UPLC with an MS detection system. The AFB1-lys molecular ion (m/z 457·2) fragmented to yield a daughter ion at m/z 394·1, and the parent ion of internal standard ($(M + H)^+$, m/z 461·3) fragmented to yield a daughter ion at m/z 398·2. The limit of detection for this method was 0·5 pg AFB1-lys/mg albumin, and three quality control (QC) samples ran daily[19].

Biomarkers of environmental enteropathy

The biomarkers of environmental enteropathy, including α1 antitrypsin (AAT), neopterin (NEO), myeloperoxidase (MPO) and regenerating protein family member 1β (Reg1B), were measured from non-diarrhoeal stool samples. All assays were performed at icddr,b, Dhaka. AAT (Biovendor), NEO (GenWay Biotech), MPO (Alpco) and Reg1B (TechLab) were measured in stool samples using commercially available ELISA kits and following the manufacturers' instructions. The overall methodology of biomarker assays has already been described[31].

Anthropometry

The outcome of this analysis was linear growth of children measured in LAZ or HAZ. LAZ (0–24 months)/HAZ (36 months) was calculated from the length/height and weight of children collected during each monthly visit to the study field office. Anthropometry was conducted by two trained research staff following standard operating procedures. Length was measured using commercial measuring boards (Seca Infantometer; model no. 417); height was measured with Seca 213 portable stadiometer; and weight was measured with minimum clothing using Seca 354 Dual-Purpose Baby Scale. Anthropometric indices were calculated following WHO growth standards[32]. All the instruments were calibrated daily with standard weights and a measuring rod. Details of anthropometry, equipment and data on QC were published elsewhere[4,27].

Variable selection for analysis

Aflatoxin exposure is the explanatory variable of interest, which was measured at the ages of 7, 15, 24 and 36 months. Therefore, in addition to baseline information, data on covariates were considered, which were collected only at these particular time-points. The outcome variables of this analysis were LAZ/HAZ and stunting status at the ages of 7, 15, 24 and 36 months. In order to select explanatory variables, we considered the variables included in the MAL-ED study's pooled analyses to explore the predictors of stunting, which followed a modified version of the UNICEF malnutrition conceptual hierarchical framework and also used the maternal and household factors and childhood environmental exposures[4]. In addition, we considered variables used in other reported contemporary studies to explore the risk factors of childhood stunting. Name and availability of different variables across different time-points in this longitudinal study are described in online supplementary material, Supplemental Table 1.

Definitions

Asset index: A household asset index was constructed using household asset data obtained from the SES questionnaire. From these asset-related dichotomous variables, a common factor score for each household was generated using polychoric principal components analysis in STATA software. After ranking by their score, we divided the first principal component score into quintiles to create five categories where the first category represents poorest household, and the fifth category represents wealthiest household.

Improved toilet was defined as per WHO guidelines: presence of flush latrine to piped sewer system, septic tank, pit latrine; ventilated improved pit latrine; pit latrine with slab; or composting toilet[33].

Household food security status was categorised using the Household Food Insecurity Access Scale (HFIAS)

developed by Food and Nutrition Technical Assistance project[34].

Statistical analysis

We examined the distribution of variables and characterised their distributions using histograms, means and standard deviations, or frequency tables as appropriate. Continuous variables that were not normally distributed were characterised by median and interquartile range. A descriptive statistics was performed to present the characteristics of study participants. To investigate the independent relationship between aflatoxin exposure and LAZ/HAZ, we fitted a mixed-effect multiple linear regression model specifying a random effect at the child level to account for within-child correlations. To account for multiple measurements per child, we calculated robust standard errors. Similarly, we investigated the independent relationship between aflatoxin exposure and stunting by fitting a mixed-effect multiple logistic regression model. Multicollinearity was checked by calculating the variance inflation factor (VIF) in a series of single-level linear regression models. The variables in the final models had a VIF ≈ 2. The strength of association was measured by OR with 95 % CI. For multivariable model building, all the time-independent covariates, and the variables collected at four time-points (7, 15, 24 and 36 months) among the time-varying covariates, were considered. Statistical significance was set at $P < 0.05$. We performed all the statistical analyses using Stata/PC (StataCorp, version 15·1).

Results

A total of 228 children were enrolled in this study, and 196 were followed till 36 months of age. Baseline data showed that 52 % of the children were female; mean (SD) birth weight was 2·8 (0·4) kg, and 22 % of the children were born with low birth weight (<2·5 kg). Prevalence of stunting (LAZ/HAZ < −2 SD) was 22 % at birth, and median duration of exclusive breastfeeding was 107 (interquartile range 54, 155) d. The mean maternal height was 149 (SD 5) cm, and the average duration of formal education of the mothers was 5 years. Monthly family income of the enrolled children was $US 101, and the asset index showed that 21 % of the children were from the poorest families, and 18·7 % of the children came from poor families. Seventy-four percentage of the children came from food-secured households, and 7·7 % of the children came from households with severe food insecurity. Seventy-three percentage of the mothers washed their hands after helping the child defecate; 20 % of them washed their hands before food preparation; and 76 % washed their hands after using the toilet. Seventy-five percentage of the children had access to improved toilet, and 61 % of

their families treated water by any means before drinking (Table 1).

To detect aflatoxin exposure among the children, AFB1-lys assays were performed using the blood samples collected at the ages of 7, 15, 24 and 36 months. Assay results were available for 208, 196, 173 and 167 children, and aflatoxin was detected in 10, 20, 17 and 62 % of samples, respectively, at those time-points. The mean LAZ values were −1·29, −1·80, −2·03 and −1·99 at the ages of 7, 15, 24, and 36 months, respectively. The prevalence of stunting was 21 % at 7 months, 41 % at 15 months, 49 % at 24 months and 49 % at 36 months of age. Details on aflatoxin concentrations, LAZ/HAZ values, Hb concentrations and concentrations of enteropathy biomarkers, including stool MPO, NEO, AAT and Reg1B measured at 7, 15, 24 and 60 months, are reported in Table 2.

Association between aflatoxin exposure and length-for-age z-score/height-for-age z-score of children

To examine the association between aflatoxin exposure and LAZ/HAZ in mixed-effect linear regression models, unadjusted analyses showed that the detection of aflatoxin was inversely associated with LAZ/HAZ (−0·19, 95 % CI −0·28, −0·11, $P < 0.05$). Similarly, compared with children at 7 months of age, LAZ/HAZ values were lower in 15 months (−0·55, 95 % CI −0·63, −0·48, $P < 0.05$), 24 months (−0·77, 95 % CI −0·86, −0·67, $P < 0.05$) and 36 months (−0·71, 95 % CI −0·81, −0·62, $P < 0.05$) of age (Table 3). Among other explanatory variables, low birth weight and the number of people sleeping in one room were also inversely associated with LAZ/HAZ of children over the period from 7 to 36 months in bivariate analyses. On the other hand, maternal height, treatment of drinking water, MPO concentration in stool and asset index (wealthier compared with children from poorest households) were positively associated with LAZ/HAZ. The multivariable model did not show any association between aflatoxin exposure and LAZ/HAZ (0·03, 95 % CI −0·06, 0·11, $P = 0.54$). The multivariable model showed that age was inversely associated with LAZ/HAZ. Compared with children aged 7 months, LAZ/HAZ values were reduced in 15 months (−0·58, 95 % CI −0·66, −0·5, $P < 0.05$), 24 months (−0·77, 95 % CI −0·87, −0·67, $P < 0.05$) and 36 months (−0·74, 95 % CI −0·84, −0·63, $P < 0.05$) of age. Other factors positively associated with LAZ/HAZ in adjusted analyses were female sex (0·22, 95 % CI 0·2, 0·42, $P < 0.05$), maternal height (0·04, 95 % CI 0·02, 0·07, $P < 0.05$), access to improved toilet (0·26, 95 % CI 0·4, 0·49, $P < 0.05$) and MPO concentration (0·004, 95 % CI 0·0004, 0·008, $P < 0.05$) in stool. Factors inversely associated with LAZ/HAZ were low birth weight (−0·69, 95 % CI −0·94, −0·44, $P < 0.05$) and number of people sleeping in one room (−0·14, 95 % CI −0·22, −0·05, $P < 0.05$) (Table 3).

Table 1 Baseline characteristics of children enrolled in this study

Characteristic	Mean or n	SD or % or IQR
Sample size	229	–
Sample size of children included in the final analysis	205	–
Female sex, n and %	119	51·9
Birth weight (kg), mean and SD	2·81	0·41
Low birth weight (<2·5 kg), n and %	50	21·8
Length-for-age z-score < −2 at birth (%)	37	16·2
Maternal height (cm), mean and SD	148·8	5·12
Duration of exclusive breastfeeding (d), median and IQR	107	54, 155
Maternal education (years), median and IQR	5	2,7
Monthly family income (USD), median and IQR	101·3	75·9, 126·6
HFIAS category		
Food secure	171	74·5
Mild insecure	14	6·3
Moderate insecure	26	11·5
Severe insecure	18	7·7
Asset index*, n and %		
Poorest	49	21·4
Poor	43	18·7
Middle	48	20·9
Wealthy	46	20·3
Wealthiest	43	18·7
Number of people sleeping in one room, mean and SD	3·65	1·11
Hand-washing after helping child defecate, n and %	166	72·6
Hand-washing before preparing food, n and %	45	19·7
Hand-washing after using the toilet, n and %	174	75·9
Improved toilet†, n and %	173	75·5
Drink treated water, n and %	139	60·6

IQR, interquartile range; HFIAS, Household Food Insecurity Access Scale.
*Asset index: The household asset index was constructed using household asset data obtained from the Socioeconomic Status questionnaire. From these asset-related dichotomous variables, a common factor score for each household was generated using polychoric principal components analysis in STATA software. After ranking by their score, we divided first principal component score into quintiles to create five categories where the first category represents the poorest household and the fifth category represents the wealthiest household.
†Improved toilet was defined as per WHO guidelines: presence of flush latrine to piped sewer system, septic tank, pit latrine; ventilated improved pit latrine; pit latrine with slab; or composting toilet.

Association of aflatoxin exposure with stunting

We also examined the association of aflatoxin exposure and stunting. Results of both unadjusted and adjusted models are presented in Table 4. In unadjusted analyses, the detection of aflatoxin, age, low birth weight, maternal height, treatment of drinking water, MPO concentration in stool, number of people sleeping in one room and asset index were associated with stunting. Although the unadjusted analysis showed that the detection of aflatoxin was associated with increased odds of stunting (OR 2·2, 95 % CI 1·34, 3·67, $P < 0.05$), we did not find any association between these two in the multivariable model (adjusted OR (AOR) 0·9, 95 % CI 0·4, 1·9, $P = 0.82$). In the adjusted analyses, age was also associated with stunting as there were higher odds of being stunted at 15 months (AOR 24·4, 95 % CI 8·2, 72·4, $P < 0.05$), 24 months (AOR 38·5, 95 % CI 9·8, 151·3, $P < 0.05$) and 36 months (AOR 39·8, 95 % CI 10·2, 155·7, $P < 0.05$) of age compared with 7 months. Being female had 84 % lower odds of being stunted compared with being male (AOR 0·16, 95 % CI 0·04, 0·07, $P < 0.05$); low-birth-weight children had thirty-six times higher odds of being stunted compared with normal-birth-weight children (AOR 36·3, 95 % CI 5·29, 249·11, $P < 0.05$); and every unit increase in maternal height was associated with 21 % lower odds of being

stunted (AOR 0·79, 95 % CI 0·67, 0·93, $P < 0.05$). Moreover, MPO concentration in stool was inversely associated with stunting, and every unit increase in the number of people sleeping in one room had 2·2 times higher odds of a child being stunted (AOR 2·21, 95 % CI 1·14, 4·27, $P < 0.05$) (Table 4). We did not find an association between asset status and stunting.

Discussion

The primary objective of this study was to examine the association of chronic aflatoxin exposure with childhood stunting from 7 to 36 months of age. We did not find any independent association between chronic aflatoxin exposure and childhood stunting after performing longitudinal data analysis in the multivariable model. Although aflatoxin exposure was found to be inversely associated with childhood stunting in the unadjusted analysis, the association became non-significant after adjusting for age. Since both aflatoxin and LAZ/HAZ data were collected at the ages of 7, 15, 24 and 36 months, and the rate of exposure to aflatoxin as well as the prevalence of stunting significantly increased with age (Table 2), the crude association between aflatoxin exposure and stunting more likely

Table 2 Growth and laboratory assay results of the children between 7 and 36 months

	Month 7		Month 15		Month 24		Month 36	
	Mean/*n*/median	SD/IQR/%	Mean/*n*/median	SD/IQR/%	Mean/*n*/median	SD/IQR/%	Mean/*n*/median	SD/IQR/%
Children with available data, *n*	208	–	196	–	173	–	167	–
LAZ/HAZ, mean and SD	−1·29	0·96	−1·80	0·93	−2·03	0·9	−1·99	0·8
Stunting prevalence, *n* and %	43	20·7	81	41·2	86	49·7	82	49·1
Aflatoxin detected, *n* and %	21	10·1	40	20·4	30	17·3	102	61·2
Concentration of AFB1-lys (pg/mg)								
Mean and SD	1·30	1·5	1·52	1·5	3·43	11·8	3·70	12·9
Median and IQR	0·83	0·3, 1·6	1·14	0·5, 2·0	0·95	0·6, 1·2	1·17	0·7, 2·9
MPO (mcg/ml), median and IQR	3·40	1·57, 6·83	4·34	2·21, 7·73	2·02	1·29, 2·88	2·75	1·08, 4·78
NEO (mcmol/l), median and IQR	1·36	0·51, 2·76	1·59	0·70, 2·78	0·67	0·27, 1·38	0·37	0·21, 0·73
AAT (mg/g), median and IQR	0·42	0·2, 0·8	0·38	0·19, 0·71	0·36	0·2, 0·7	0·28	0·15, 0·5
Reg1B (µg/ml), median and IQR	23·6	8·8, 52·5	76·9	42·9, 132·4	52·9	9·1, 95·7	21·1	6·4, 67·7
Hb (mmol/L), mean and SD	6·9	0·8	7·1	0·9	7·3	0·9	7·6	0·8

LAZ, length-for-age z-score; HAZ, height-for-age z-score; IQR, interquartile range; AFB1-lys, aflatoxin B1-lysine adduct; MPO, myeloperoxidase; NEO, neopterin; AAT, α1 antitrypsin.

represented an association between increase in age and stunting. The absence of a relationship between aflatoxin exposure and LAZ/HAZ or stunting became more evident when we examined this relationship by fitting multiple (linear and logistic) regression models separately for each time-point (see online supplementary material, Supplemental Tables 1 and 2).

Our finding was similar to recently published MAL-ED consortium studies conducted in Tanzania and Nepal, where they did not find any relationship between aflatoxin exposure and linear growth of children measured in LAZ/HAZ[35,36]. For the detection of aflatoxin exposure, we applied the same method (MS) used in Tanzania and Nepal, and assays were performed in the same laboratory[35,36]. In our study, 62 % of children had a detectable concentration of AFB1-lys in plasma at 36 months. The detection was much lower in samples collected at the age of 7 months (10 %), 15 months (20 %) and 24 months (17 %). In the Tanzanian study, Chen *et al.*[35] had performed AFB1-lys assays only at 24 months of age where 72 % of children had a detectable level of AFB1-lys. The Nepal study measured aflatoxin at three time-points, 15, 24 and 36 months, with an average 91 % of detectable AFB1-lys concentrations in plasma. Our sample size was much larger than both the studies. Compared with the total number of children with available aflatoxin assay results in our study (7 months: *n* 208; 15 months: *n* 196; 24 months: *n* 173; 36 months: *n* 167), the number of children with available aflatoxin exposure data in Tanzania (24 months: *n* 60) and Nepal (15 months: *n* 77; 24 months: *n* 85; 36 months: *n* 85) were much lower[35,36].

All three studies observed a lower concentration of AFB1-lys in plasma samples, which was 5 pg/mg or less compared with previous studies (median > 30 pg/mg)[9,10,35,36]. A lower concentration of AFB1-lys in detected plasma samples could be the main reason behind the non-association with the linear growth of children. The detectable concentrations were similar in Tanzania and Nepal studies. In Tanzania, the mean concentration of AFB1-lys at 24 months was 5·1 (range 0·28–25·1) pg/mg albumin, and this was 3·62 (range 0·58–22·7) pg/mg of albumin in Nepal[35,36]. In our study, the mean concentrations were 3·43 (range 0·15–65·6) pg/mg at 24 months and 3·70 (range 0·09–126·5) pg/mg at 36 months.

Recently, another cohort study conducted in Tanzania also found no association between LAZ and aflatoxin exposure[37]. They enrolled 166 children between the ages of 8 and 20 months and followed them for 12 months. ELISA was used to detect AFB1-alb, and the assays were done at three time-points: at baseline, at 6 months and at 12 months. The detected mean concentrations of AFB1-alb were 4·7, 12·9 and 23·5 pg/mg, respectively, at the three time-points[37]. Both Nepal and Tanzania studies used a scaling factor of 2·6 to adjust for methods between ELISA and MS based on previously published data, and found no difference with the concentration observed by

Table 3 Bivariate and multivariable analyses of factors associated with length-for-age/height-for-age over 7–36 months of age

	Unadjusted			Adjusted		
	Coefficient	95 % CI		Coefficient	95 % CI	
Aflatoxin detected	−0·19*	−0·28	−0·11	0·03	−0·06	0·11
Target months (reference: 7 months)						
15 months	−0·55*	−0·63	−0·48	−0·58*	−0·66	−0·5
24 months	−0·77*	−0·86	−0·67	−0·77*	−0·87	−0·67
36 months	−0·71*	−0·81	−0·62	−0·74*	−0·84	−0·63
Female sex	0·13	−0·09	0·37	0·22*	0·02	0·42
Low birth weight	−0·66*	−0·94	−0·39	−0·69*	−0·94	−0·44
Maternal height	0·06*	0·04	0·08	0·04*	0·02	0·07
Improved toilet	0·25	−0·02	0·52	0·26*	0·04	0·49
Treat drinking water	0·38*	0·16	0·61	0·21	−0·002	0·41
Myeloperoxidase (mcg/ml)	0·01*	0·004	0·02	0·004*	0·0004	0·008
Number of people sleeping in one room	−0·18*	−0·29	−0·08	−0·14*	−0·22	−0·05
Asset index (reference: poorest)						
Poor	0·54*	0·16	0·92	0·25	−0·13	0·63
Middle	0·66*	0·27	1·04	0·29	−0·08	0·67
Wealthier	0·49*	0·13	0·85	0·13	−0·20	0·47
Wealthiest	0·97*	0·59	1·34	0·29	−0·10	0·69

*Statistical significance at $P < 0.05$.

Table 4 Factors associated with stunting at 7–36 months of age: results from unadjusted and adjusted regression models

	Unadjusted			Adjusted		
	OR	95 % CI		OR	95 % CI	
Aflatoxin detected	2·22*	1·34	3·67	0·91	0·43	1·94
Target months (reference: 7 months)						
15 months	17·54*	6·73	45·69	24·35*	8·19	72·37
24 months	35·71*	10·81	117·97	38·51*	9·80	151·29
36 months	38·38*	11·76	125·28	39·78*	10·16	155·71
Female sex	0·40	0·14	1·12	0·16*	0·04	0·70
Low birth weight	8·94*	2·71	29·48	36·32*	5·29	249·11
Maternal height	0·79*	0·72	0·88	0·79*	0·67	0·93
Improved toilet	0·36	0·11	1·22	0·23	0·04	1·19
Treat drinking water	0·23*	0·08	0·63	0·29	0·07	1·26
Myeloperoxidase (mcg/ml)	0·93*	0·89	0·97	0·92*	0·87	0·97
Number of people sleeping in one room	1·83*	1·14	2·93	2·21*	1·14	4·27
Asset index (reference: poorest)						
Poor	0·20*	0·04	0·99	0·32	0·03	4·06
Middle	0·08*	0·01	0·42	0·11	0·01	1·43
Wealthier	0·18*	0·04	0·84	0·49	0·05	4·92
Wealthiest	0·04*	0·007	0·23	0·30	0·02	5·16

*Statistical significance at $P < 0.05$.

Shirima et al.[37]. It can be noted that ELISA and LC-MS/MS correlated strongly with each other, but ELISA usually quantified AFB-alb by a factor of 2·6 higher than LC-MS/MS as observed in previous studies[38]. Therefore, this 2·6 scaling factor was used previously to make the results comparable between ELISA and LC-MS/MS. Recently in the north of Bangladesh, a high level of AFB1-lys was detected by MS among 61 newborn infants and the same children at the age of 2 years[20]. The detected median concentration of AFB1-lys was 27·41 (range 3·88–81·44) pg/mg at birth and 13·79 (range 3·88–81·44) pg/mg at 2 years[18]. Unfortunately, no data on its association with child growth is available till date.

In the recent past, several epidemiological studies conducted in Africa and the Middle East showed an association between aflatoxin exposure and growth deficits in children[10,11,39–42]. The study conducted in Benin showed a significant inverse relationship between concentrations of AF-alb and different quartiles of LAZ of children. In contrast to our current research and other studies conducted in Nepal and Tanzania, the detected AFB1-alb concentration in Benin was as high as 100 pg/mg in children belonging to the lowest LAZ quartile[9,10]. Even after using the scaling factor of 2·6 in Benin and Togo study, the mean AFB1-alb concentrations became 32·8 pg/mg of albumin, a much higher concentration than the recent studies that found no association with growth. Therefore, we also concurred with the Nepal and Tanzania studies that exposure to a comparatively lower concentration of aflatoxin may not affect the linear growth of children[35,36]. It is possible that a threshold exists for

intake or exposure level, and a long-standing aflatoxin exposure above this threshold will cause growth deficits as seen in previous studies conducted in Benin and Togo.

Dietary habits may play an important role in the exposure of aflatoxin to children. Usually, children in Bangladesh do not consume maize and peanut as staples, like those in African countries[19]. Previously, using a 24-h recall method to calculate dietary intakes in the same population, we observed that the consumption of any sweet foods such as biscuits, pastries or cakes was associated with the detection of aflatoxin in plasma (AOR 2·17, 95 % CI 1·27, 3·70, $P < 0·05$)[19]. We did not find any association between the consumption of grains (rice, bread, porridges, noodles, etc.) and aflatoxin exposure. However, we observed that the introduction of family food as reflected by a cessation of breastfeeding was associated with the detection of AFB1-lys in plasma[19]. Similar to the current study, the rate of growth impairment increased with age in some African countries where they also found that cessation of breastfeeding and introduction of family food were associated with aflatoxin exposure. However, this exposure was mainly due to the consumption of weaning food prepared from maize and peanut[9,10,43].

In order to explore the true association of aflatoxin exposure with childhood stunting and LAZ/HAZ, we adjusted for other important confounding variables in the multivariable models. We observed that age, sex, low birth weight, maternal height, crowding as represented by the number of people sleeping in one room, presence of improved toilet and MPO concentration in stool were significantly associated with linear growth and stunting between the ages of 7 and 36 months. Low birth weight and short maternal stature are already considered as the most important predictors of childhood stunting[4]. The number of people sleeping in one room represents crowding, which also is related to poor hygiene and pathogen transmission. MPO is a biomarker of enteropathy, which was also found to be associated with linear growth[44]. It can be noted that a couple of papers used the MAL-ED data to explore the factors associated with childhood stunting[4,45]. Our findings are consistent with both the papers except that they did not examine the effect of aflatoxin exposure, and their study population was limited to 24 months of age.

So far, no data has been published from this current population to examine the factors associated with childhood stunting where children were followed until 36 months of age. Moreover, this is the first report from Bangladesh where the association of aflatoxin exposure and stunting is examined, and very few global studies with cohort data performed a robust MS assessment at four time-points with a reasonably large sample size. Among the other three recent studies, the Nepal study assayed AFB1-lys at three time-points (15, 24 and 36 months) with a small sample size (n 85); the Tanzania study performed aflatoxin

assays at a single time-point (24 months) with a small sample (n 60); and the second Tanzania study performed assaying using ELISA[35–37].

The findings of this study corroborate with recent studies that questioned the existing evidence linking aflatoxins with stunting. Earlier observational studies overlooked certain confounding factors, including SES[9,10]. Children in poorer households were often fed diets deficient in micro- or macronutrients, and suffer from frequent infections, both of which contribute to growth retardation[3]. Poverty is also associated with aflatoxin exposure, so, if not adequately controlled for, the association between aflatoxin/mycotoxin and stunting might be overestimated. A very recent RCT in Kenya also observed that providing aflatoxin-free maize can substantially reduce serum AFB1-lys, but it had no effect in improving linear growth faltering in children[46]. Therefore, it is possible that a true association between aflatoxin exposure and childhood growth faltering might not exist at all. Recently, one systematic review examined the association of mycotoxin exposure and child growth and other outcomes[47]. They have reviewed fifty articles and examined the evidence on aflatoxin and fumonisin exposure on child growth outcomes. They found that the results were inconsistent and inconclusive, and the evidence was considered very low due to study design and methodological issues[47].

This study has several limitations. First, aflatoxin exposure data were missing at several time-points. This was due to the unavailability of biospecimens due to some participants not turning out for blood collection. The weaning period is the most vulnerable time of aflatoxin exposure when a child is gradually exposed to family foods. Since we do not have aflatoxin exposure data at 2–3 months of age, we could not relate this to 7-month exposure data when weaning would have already initiated. The unavailability of aflatoxin concentration in common food is also a limiting factor. The absence of other important predictors, such as pathogen burden data after 24 months of age, is another limitation. Moreover, exposure to other mycotoxins, for example, fumonisin, which has a role in growth faltering[47], was not measured in this study.

Stunting at an early age is associated with an increased risk of childhood death, diseases and poor cognitive outcomes[48,49]. Despite substantial improvement, childhood stunting is pervasive in Bangladesh, particularly in slum settings. About half of children under the age of 5 years living in around 15 000 slums across Bangladesh are stunted[14,50]. Our analysis identified different modifiable factors associated with stunting among children aged ≤36 months, which are consistent with previously reported predictors of stunting under the age of 2 years. Improvement of birth weight and maternal height calls for improving maternal and adolescent nutrition on a large scale. Moreover, the improvement of SES of slum dwellers, provision of better living conditions without much

crowding, and proper sanitation and hygiene to prevent environmental enteropathy need to be ensured through proper policy and planning using a multi-departmental approach at the national level.

Similar to recent studies conducted in Tanzania and Nepal, we found that exposure to a low level of aflatoxin did not affect the linear growth of children. However, there can be a threshold level of the toxin to demonstrate any effect on linear growth during early years of life. We need to explore further in other rural settings where exposure to a high level of aflatoxin may be more likely among children and adults. Furthermore, well-designed studies are required to detect aflatoxin in food commodities and determine the threshold value of the toxin to possibly cause an adverse impact on health.

Supplementary material

For supplementary material accompanying this paper visit https://doi.org/10.1017/S1368980020001421.

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Supplementary table 1: Availability of variables at different time points in this longitudinal study

Factors	Variables	Baseline	7 month	15 months	24 months	36 months
Outcome	LAZ Stunting		+	+	+	+
Explanatory variables						
Explanatory variable of interest	Aflatoxin exposure		+	+	+	+
Inherent	Age	+	+	+	+	+
	Sex	+				
Immediate	Child exposures					
	Self reported illness, antibiotic use	+	+	+	+	–
	Gut inflammation					
	NEO, MPO, AAT		+	+	+	+
	Enteropathogen detected	+	+	+	+	–
	Micronutrient					
	Hb		+	+	+	+
	Zn, Retinol		+	+	+	–
	Total EBF days	+				
	Dietary intake, total protein		–	+	+	+
Underlying	Birth weight	+				
	Maternal height	+				
	Maternal age	+				
	WASH behavior		+	+	+	+
	Improved toilet		+	+	+	+
	Improved drinking water		+	+	+	+
	Food security access	+				
	Crowding	+				
Distant	Maternal education	+				
	Asset index	+				
	Monthly income	+				

Supplementary table 2: Association of aflatoxin exposure and length-for-age z-score at different time points by multiple linear regression

Months of measurement	Coefficient	95% Confidence Intervals	p- value
7 months	-0.05	-0.49, 0.39	0.83
15 months	0.03	-0.27, 0.34	0.83
24 months	-0.20	-0.52, 0.11	0.20
36 months	-0.06	-0.31, 0.20	0.67

All 4 models were adjusted for sex, concentrations of MPO in stool, low birth weight, maternal height, number of people sleep in one room, improved toilet, treatment of drinking water, asset categories

Supplementary Table 3: Association of aflatoxin exposure and stunting at different time points by multiple logistic regression

Months of measurement	Adjusted Odds ratio	95% Confidence Intervals	p- value
7 months	2.47	0.73, 8.36	0.15
15 months	0.67	0.27, 1.64	0.38
24 months	1.64	0.64, 4.21	0.30
36 months	1.13	0.55, 2.34	0.74

All 4 models were adjusted for sex, concentrations of MPO in stool, low birth weight, maternal height, number of people sleep in one room, improved toilet, treatment of drinking water, asset categories

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Why Do Children in Slums Suffer from Anemia, Iron, Zinc, and Vitamin A Deficiency? Results from a Birth Cohort Study in Dhaka

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Why Do Children in Slums Suffer from Anemia, Iron, Zinc, and Vitamin A Deficiency? Results from a Birth Cohort Study in Dhaka

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Abstract: Considering the high burden of micronutrient deficiencies in Bangladeshi children, this analysis aimed to identify the factors associated with micronutrient deficiencies and association of plasma micronutrient concentration trajectories from 7 to 24 months with the concentrations at 60 months of age. Plasma samples were collected at 7, 15, 24, and 60 months of age, and hemoglobin, ferritin, zinc, and retinol concentrations of 155, 153, 154, and 155 children were measured, respectively. A generalized estimating equation was used to identify the factors associated with micronutrient deficiencies, while latent class growth modeling identified the trajectories of plasma micronutrients from 7 to 24 months and its association with the concentrations of micronutrients at 60 months was examined using multiple linear regression modeling. Early (AOR = 2.21, $p < 0.05$) and late convalescence (AOR = 1.65, $p < 0.05$) stage of an infection, low ferritin (AOR = 3.04, $p < 0.05$), and low retinol (AOR = 2.07, $p < 0.05$) were associated with increased anemia prevalence. Wasting at enrollment was associated with zinc deficiency (AOR = 1.8, $p < 0.05$) and birth weight was associated with ferritin deficiency (AOR = 0.58, $p < 0.05$). Treatment of drinking water was found protective against vitamin A deficiency (AOR = 0.57, $p < 0.05$). Higher trajectories for ferritin and retinol during 7–24 months were positively associated with plasma ferritin (β = 13.72, $p < 0.05$) and plasma retinol (β = 3.99, $p < 0.05$) at 60 months.

Keywords: anemia; micronutrient deficiency; latent class growth modeling; children; Bangladesh

1. Introduction

Child undernutrition is the result of the interplay between multiple causal and contextual factors including poor complementary feeding which results in both macro and micronutrient deficiencies [1–3]. Diets of infants and young children in developing countries are usually cereal based, lacking adequately bioavailable micronutrients [4]. Micronutrients such as zinc, vitamin A, and iron are essential for growth, immunity, and cognitive development [5]. Globally, half of preschool children are anemic due mainly to iron deficiency, around 140 million preschool children have subclinical vitamin A deficiency [6], and approximately half the population is at risk of developing zinc deficiency [7].

Anemia is very common in preschool and school aged children and has implications for child nutrition, growth, and survival [8]. Vitamin A deficiency is also a public health problem, and vitamin

A is essential for various physiological functions, especially tissue development, metabolism, and resistance to infections [9]. Zinc is a trace mineral that plays a vital role in cellular growth, specifically in the production of enzymes necessary for the synthesis of RNA and DNA [10]. In addition to its protective role in diarrhea and acute respiratory tract infection, zinc is also essential for child growth and cognitive development [11]. There are ample data on the micronutrient status of children under the age of five years and on the risk factors for different micronutrient deficiencies at specific ages. However, there is a paucity of data on micronutrient status from cohort studies in which repeated samples were collected from infancy to five years of age.

Factors associated with different micronutrient deficiencies include low or inadequate dietary consumption, morbidity episodes, and socio-demographic status as well as child caring practices reflected by maternal education level. Some of these determinants are fixed and some are essentially time variant. Within a child, we can track variations in micronutrient status if we collect repeated measures longitudinally over a period of several months. At the population level, there is a possibility that these changes follow specific patterns or trajectories that may predict future micronutrient status. Therefore, it is important to explore the relationship between patterns of change in micronutrient status during early childhood and micronutrient status in later years.

The Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development (MAL-ED) study is a birth cohort study in which children were followed longitudinally from birth to 60 months of age [12]. At the Bangladesh site of the MAL-ED study, plasma micronutrient concentrations and hemoglobin were assessed at 7, 15, 24 and 60 months of age [13]. Most studies from low- and middle-income countries have not assessed child micronutrient status at four time points longitudinally while simultaneously collecting data on growth, nutrition, infection, and socio-demographic data.

The current analysis evaluated factors associated with the deficiencies of zinc, vitamin A, and iron, as well as anemia from seven months to five years of age and also aimed to identify whether distinct trajectories of micronutrient concentrations from 7 to 24 months predict micronutrient status at 60 months of age.

2. Materials and Methods

2.1. Study Design and Participants

This birth cohort study was conducted in a slum settlement located in Bauniabadh in the Mirpur area of Dhaka city. The area is inhabited by people with low socioeconomic status and limited sanitary conditions. The study site, location, demography, and socio-economic status have been reported previously [12,13]. In the MAL-ED birth cohort study in Bangladesh, children were enrolled, on average, 3.5 days after birth using predefined inclusion and exclusion criteria and were followed longitudinally until 60 months of age. The first child was enrolled on 11 February 2010 and the five-year follow-up was completed on 12 February 2017.

2.2. Inclusion and Exclusion Criteria

The MAL-ED study had well defined inclusion and exclusion criteria for enrollment. Inclusion criteria included apparently healthy infants, enrolled within 17 days after birth, parents agreed to be visited by the research staff in their household twice weekly during the study period and they did not have any plan to move outside of the study area for more than 30 days during the first 6 months of follow-up. The exclusion criteria included: family had a plan to migrate to another location; mother was less than 16 years old; the infant was not a singleton; birth weight was <1.5 Kg; child had acute or chronic clinical conditions, congenital anomalies, or developmental delays diagnosed by a physician; hospitalized after birth due to any clinical condition; and the parents failed to sign the consent form.

2.3. Ethical Issues

This study was approved by the Research Review Committee (RRC) and Ethical Review Committee (ERC) of icddr,b. Prior to enrollment, the parents were briefed about the study, study objectives and detailed methodology. Before any data were collected, written consent was obtained via signature from one of the parents.

2.4. Data Collection

After enrollment, baseline socio-demographic, feeding and morbidity data were collected and anthropometry was performed by trained research staff. The children were visited twice weekly to collect feeding and morbidity data and anthropometric measurements were taken every month until 24 months of age [14]. We used a qualitative 24 h food frequency questionnaire to collect feeding data from month 1 to month 8. From 9 to 24 months we switched to a monthly quantitative 24 h recall approach to estimate nutrient and energy intakes from non-breast milk foods. From 24 months onward, quantitative 24 h recall data were collected every six months until 60 months. This analysis used dietary intake data collected at 60 months of age using the 24 h recall method. Detailed information regarding the methodology and data collection has been published elsewhere [15]. Anthropometry data were collected on a monthly basis and data on water-sanitation and hygiene, assets, income, and food security were collected every six months [14,15].

2.5. Biological Sample Collection

Blood samples were collected at 7, 15, 24, and 60 months of age and plasma was obtained by centrifuging the blood. Plasma samples were stored at −80 °C until analysis [11,15].

2.6. Measurement of Plasma Micronutrient Status

Plasma zinc concentration was measured using flame atomic absorption spectrophotometry (Shimadzu AA-6501S, Kyoto, Japan) [16]. Plasma retinol concentration was measured by reverse phase HPLC using C18 column (Discovery C18, 25cmX4mm, 5μm, Cat# 504971) and detected at 325nm [17]. Plasma ferritin, C-reactive protein (CRP) and alpha-1-acid glycoprotein (AGP) concentrations were determined by immunoturbidimetric assay using commercial kits from Roche diagnostics on a Roche automated clinical chemistry analyzer (Hitachi −902, Boehringer Mannheim, Germany). A HemoCue 201 instrument was used to measure hemoglobin concentration [15]. Details are described in Supplementary Materials 2.1.

2.7. Infection Category and Adjustment of Ferritin, Zinc and Retinol for Inflammation

Using CRP and AGP concentrations, participants were divided into four groups: "incubation" (CRP > 5 mg/L and AGP < 1 g/L), "early convalescence" (CRP > 5 mg/L and AGP > 1 g/L), "late convalescence" (CRP < 5 mg/L and AGP > 1 g/L, and the "healthy/reference" (CRP < 5 mg/L and AGP < 1 g/L) group [18]. The geometric mean of plasma ferritin, zinc, and retinol were calculated for each of the infection groups described above. Then correction factors were calculated as ratios of geometric means of the "healthy/reference" group to that of the infection groups (incubation, early convalescence, late convalescence). Ferritin, zinc, and retinol concentrations in infection groups were then adjusted by multiplying by the group specific correction factors.

2.8. Definitions and Cut-offs

For the purpose of statistical analyses, cut-off values for each micronutrient deficiency were: anemia (hemoglobin concentration < 11.0 g/dL), iron deficiency (plasma ferritin < 12.0 ng/mL), zinc deficiency (plasma zinc < 9.9 mmol/L), and vitamin A deficiency (plasma retinol < 20 μg/dL) [19–22].

The Water-sanitation-hygiene, Asset status, Maternal education status, and monthly Income (WAMI) index is a composite score for assessing socioeconomic status. This index is widely used

in MAL-ED publications and the methodology is published elsewhere [23]. To examine the factors associated with the different micronutrient deficiencies we have not used the WAMI index, rather we used each of the components of WAMI as exposure variables. However, the WAMI index at 60 months was used as one of the explanatory variables to explore the association of trajectories of micronutrient concentrations from 7 to 24 months with micronutrient status at 60 months of age.

Improved toilet was defined per WHO guidelines: presence of flush latrine connected to sewer system, septic tank, pit latrine; ventilated improved pit latrine; pit latrine with slab or composting toilet [24].

Low birth weight: A birth weight of <2.5 kg was considered as low birth weight; those weighing <1.5 kg were excluded from the study. Although study children in the MAL-ED Bangladesh site were enrolled, on average, 3.5 days after birth, their original birth weight was recorded from the delivery/birth certificates.

Household food security access status was categorized by using the Household Food Insecurity Access Scale (HFIAS) developed by the Food and Nutrition Technical Assistance (FANTA) project [25]. Based on individual household food security access scores, the food security status was categorized to households with no food insecurity, mild food insecurity, moderate food insecurity, and severe food insecurity. We have used enrollment HFIAS for this analysis.

Asset index: The household asset index was constructed using household asset data obtained from the SES questionnaire. From asset related dichotomous variables, using principal components analysis in STATA software, a common factor score for each household was produced. After ranking by their score, we divided first principal component score into quintiles to create five categories where the first category represents the poorest and the fifth category represents the wealthiest ones.

2.9. Statistical Analysis

We examined the distribution of micronutrient concentrations by using histograms, box plots, Q-Q plots, or frequency tables as appropriate. Characteristics of the study participants at baseline were reported as mean and standard deviation for continuous variables and frequency distributions for categorical variables. Those that were not normally distributed were described by median and inter-quartile range (IQR). Continuous variables with normal distributions were compared between groups using Student's t-test after verifying the equality of variance (Levene's test). The difference in proportion was compared using a Chi-square test or the Fisher's exact test if the expected number in any cell was <5.

To investigate factors associated with anemia, iron deficiency, zinc deficiency and vitamin A deficiency from birth to five years of age, we used generalized estimating equation (GEE) regression. Our outcome variables were binary categorical variables measured at 7, 15, 24, and 60 months. GEE is a population specific method based on average changes in response over time and the impact of covariates on these changes. Therefore, the reported odds ratio is a pooled odds ratio of the effect of all the predictors over 7 to 60 months of age. We assumed an autoregressive (AR) covariance matrix with robust variance estimates. Initially, bivariate analysis was performed to identify the unadjusted effect of each predictor on each deficiency through individual GEE models. The variable selection was based on availability of data at different time points (Supplementary Table S1). Multi-collinearity between independent variables was examined using correlation matrix and variance inflation factor (VIF) values. In succeeding models, covariates whose p-values were less than 0.20 in bivariate analysis with the outcome variable were entered simultaneously to obtain the adjusted final model. The best models were selected based on the lowest quasi-likelihood under independence model criterion (QIC) value. A probability of less than 0.05 was considered statistically significant and the strength of association was determined by estimating the adjusted odds ratios (AOR) and their 95% confidence intervals (CIs).

We used latent class growth modeling (LCGM) to identify distinct clusters of children following similar trajectories with regard to the pattern of hemoglobin, ferritin, retinol, and zinc at 7, 15, and 24 months [26,27]. We built separate trajectory models for hemoglobin, ferritin, retinol, and zinc. The

analyses were restricted to the children for whom data on the outcomes were available at 7, 15, and 24 months (three time-points) [28]. In addition, for ferritin, three very high (unusual) values were dropped from the dataset. The sample sizes for LCGM were reduced to 155 for hemoglobin, 153 for ferritin, 154 for retinol, and 155 for zinc. We selected the final models with optimal number and shape of trajectories based on Bayesian information criteria (BIC), log Bayes factor, the statistical significance of quadratic terms, whether 95% confidence intervals of trajectories overlapped, and the percentage of the population in each trajectory group [29]. After selecting the final model, we calculated the posterior probabilities for each individual of belonging to each of the trajectory groups, and individuals were assigned to a trajectory group based on the maximum-probability assignment rule [30]. We reported the findings of the LCGM following the GRoLTS-Checklist: Guidelines for Reporting on Latent Trajectory Studies [31]. A detailed description of LCGM methodology and STROBE checklist for reporting cohort studies are included in Supplementary Materials 2.2.

In subsequent analyses, multiple linear regression models with robust standard errors were fitted to examine the association of concentrations of hemoglobin, plasma ferritin, retinol, and zinc at the age of 60 months with the trajectories identified through LCGM. As zinc was found to have a single trajectory (described in detail in the results section), zinc concentrations at 24 months were used instead of any trajectory to assess the predictive association with level of zinc at 60 months. We considered several covariates collected at the age of 60 months in building these models which include mean intakes of energy intake (kcal/d), protein (g/d), fat (g/d), carbohydrates (g/d), iron (mg/d), vitamin A (ug_RE/d), and zinc (mg/d). We also considered the phytate to iron ratio and the phytate to zinc ratio of the diet, as well as the percent of energy from carbohydrates (minus fiber), protein, and fat. Non-dietary covariates included socioeconomic and WASH variables, the WAMI score, and child sex. Details on the methodology are described in Supplementary Materials 2.2. The final regression models included only iron intake, the percentage of energy from protein, and WAMI score for hemoglobin, level of zinc at 24 months, total energy intake, protein intake, and ferritin, vitamin A, retinol, and zinc intake. Due to missing values for the outcome variables, the sample sizes for the linear models were reduced to 142 for hemoglobin, 138 for ferritin and retinol, and 140 for zinc.

The statistical analyses related to LCGM were performed using the "traj plugin" in Stata (StataCorp, College Station, Texas 77845 USA, version 14.1) [32], a Stata equivalent of the widely used "proc traj" in SAS [28]. Outputs of LCGM models were plotted using "traj" in Stata and the R packages "lcmm" and "ggplot2" in R (version 3.5.1). All other statistical analyses were performed with Stata/PC (StataCorp, College Station, Texas 77845 USA, version 14.1).

3. Results

3.1. Basic Socio-Demographic Characteristics

At enrollment, data were available for 212 newborns. Male children comprised 47% of the group and 22% had low birth weight (<2.5 kg). The mean age of the mothers was 24.8 years and 45% of them were either illiterate or did not complete the primary level of education. Three fourths of the participants were from food secure households and their average monthly family income was 120 USD. Sixty percent of the families used treated drinking water by any means and two-thirds of them had improved toilet facilities in their households. Prevalence of stunting was 17% at enrollment, and 30% had inflammation at 7 months, defined by high concentrations of acute phase proteins (Table 1).

**Table 1.** Baseline characteristics of the study population.

Characteristics	N = 212 (%/mean ± sd)
Male	47.6
Birth weight, kg	2.8 ± 0.4
Low birth weight (<2.5 kg)	22.17
Total days of exclusive breastfeeding	106.5 ± 57.4
Maternal age	24.8 ± 4.9
Maternal education	
None	19.3
Some primary	25.9
Primary complete	18.4
Some secondary	31.6
Secondary complete or higher	4.7
Treated drinking water	60.3
Improved toilet [a]	76.0
Hand wash after helping the child defecate	72.7
Hand wash before preparing food	22.3
Hand wash after using toilet	75.6
Food insecurity [b]	
Secure	74.5
Mild insecurity	6.13
Moderate insecurity	11.8
Severe insecurity	7.6
Monthly income (US$), median (IQR)	97.6 (73.1, 121.9)
Asset Index [c]	
Poorest	20.9
Poor	19.4
Middle	20.4
Wealthier	20.4
Wealthiest	18.9
Stunted (length-for-age z-score <−2)	16.9
Underweight (weight-for-age z-score <−2)	21.2
Wasted (weight-for-length z-score <−2)	16.9

[a] Improved toilet was defined as per WHO guidelines: presence of flush latrine to piped sewer system, septic tank; pit latrine; ventilated improved pit (VIP) latrine; pit latrine with slab; or composting toilet [23]. [b] Household food security access status was categorized by using the Household Food Insecurity Access Scale (HFIAS) developed by the Food and Nutrition Technical Assistance (FANTA) project [24]. [c] Asset index: The household asset index was constructed using household asset data obtained from the SES questionnaire. From these asset related dichotomous variables, we used polychoric principal components analysis in STATA software to produce a common factor score for each household. After ranking by their score, we divided the first principal component score into quintiles to create five categories of which the first category represents the poorest and fifth category represents the wealthiest.

3.2. Plasma Micronutrient Status

Results of the assays done for hemoglobin, plasma ferritin, plasma zinc, and plasma retinol are reported in Figure 1.
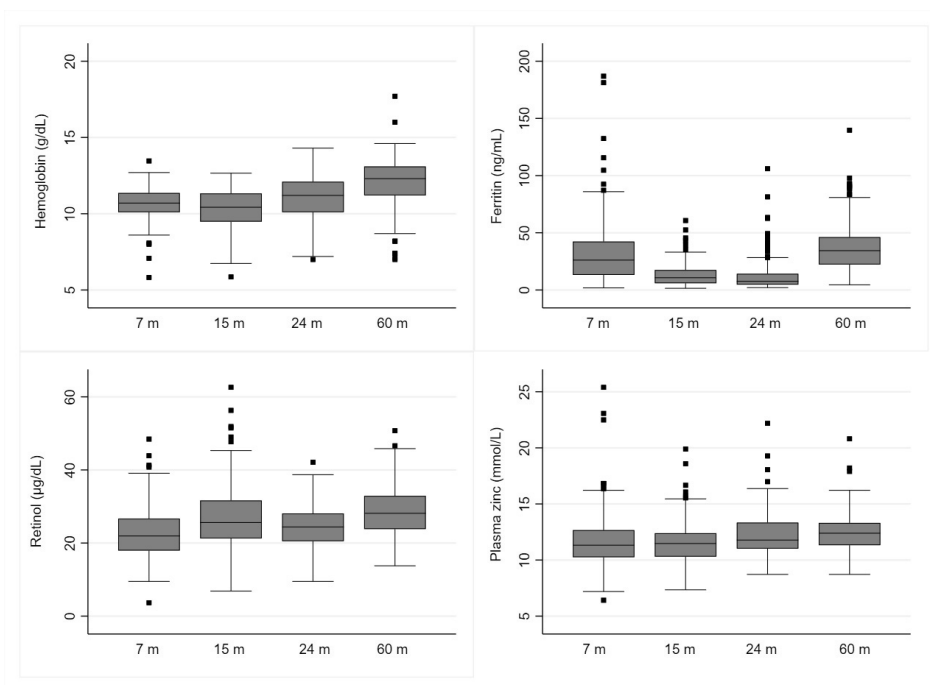
Figure 1. Hemoglobin and plasma micronutrient concentrations from 7 to 60 months.

Using the predefined cut-off values, it was observed that anemia, zinc deficiency, and vitamin A deficiency declined gradually from month 7 to month 60 whereas iron deficiency (low plasma ferritin) increased gradually from 7 months to 24 months and was then markedly reduced at 60 months of age. All of these changes were found to be statistically significant ($p < 0.05$) (Table 2).

Table 2. Anemia and adjusted * iron, zinc, and vitamin A deficiency at different time points.

	Cut-Off	**Percent (n)**			
		7 Months	**15 Months**	**24 Months**	**60 months**
Anemia	Hb < 11 g/dL	64.3 (117)	63.9 (106)	41.7 (68)	18.8 (31)
Low Ferritin	<12 ng/mL	20.3 (42)	54.1 (105)	68 (119)	4.9 (9)
Low Zinc	<9.9 mmol/L	21.5 (44)	14.9 (29)	1.1 (2)	3.8 (7)
Low Retinol	<20 µg/dL	39.0 (80)	19.2 (37)	21.7 (38)	6.7 (12)

* Adjusted for inflammation.

3.2.1. Factors Associated with Anemia from 7 to 60 months

Bivariate analyses showed that infection status, zinc deficiency, iron deficiency and vitamin A deficiency were positively associated with anemia. On the other hand, age, birth weight, monthly income and higher education of the mother were negatively associated with anemia ($p < 0.05$) (Supplementary Table S2). Multivariable analysis revealed that early (AOR: 2.21, 95% CI 1.14–4.27, $p < 0.05$) or late (AOR: 1.65, 95% CI 1.03–2.64, $p < 0.05$) convalescence stage of an infection, iron deficiency (AOR: 3.04, 95% CI 2.08–4.44, $p < 0.05$), and vitamin A deficiency (AOR: 2.07, 95% CI 1.31–3.28, $p < 0.05$) were associated with an increased prevalence of anemia. Whereas, age (AOR: 0.94, 95% CI 0.92–0.96, $p < 0.05$), female sex (AOR: 0.49, 95% CI 0.32–0.77, $p < 0.05$), higher maternal education (AOR: 0.38, 95% CI 0.15–0.92, $p < 0.05$) compared to no education were negatively associated with anemia (Table 3).

Table 3. Multivariable associations with anemia, low plasma ferritin, zinc, and retinol from 7 months to 60 months.

	Anemia		Zinc Deficiency		Vitamin A Deficiency		Iron Deficiency	
	AOR (95% CI)	*p*-Value	AOR (95% CI)	*p*-Value	AOR (95% CI)	*p*-Value	AOR (95% CI)	*p*-Value
Inflammation group								
Non-inflamed	Reference							
Incubation	2.43 (0.74, 7.94)	0.142						
Early convalescence	2.21 (1.14, 4.27)	0.019						
Late convalescence	1.65 (1.03, 2.64)	0.037						
Age in months	0.94 (0.92, 0.96)	<0.0001	0.95 (0.93, 0.98)	0.001	0.96 (0.95, 0.98)	<0.0001	0.98 (0.97, 0.98)	<0.0001
Female	0.49 (0.32, 0.77)	0.002					0.65 (0.47, 0.97)	0.013
Birth weight	0.67 (0.39, 1.14)	0.136			0.61 (0.36, 1.04)	0.072	0.58 (0.38, 0.88)	0.010
Wasting at birth			1.86 (1.11, 3.11)	0.018				
Food insecurity access								
Food secure					Reference			
Mild food insecurity					1.08 (0.42, 2.80)	0.87		
Moderate food insecurity					0.58 (0.29, 1.12)	0.105		
Severe food insecurity					1.49 (0.69, 3.19)	0.302		
Monthly income	0.99 (0.99, 1.00)	0.545	1.00 (0.99, 1.00)	0.079	0.99 (0.99, 1.00)	0.168		
Maternal education								
No	Reference		Reference				Reference	
Some primary	1.14 (0.63, 2.09)	0.661	0.69 (0.35, 1.37)	0.29			1.11 (0.71, 1.73)	0.65
Primary complete	1.17 (0.59, 2.30)	0.660	0.78 (0.36, 1.69)	0.53			1.29 (0.77, 2.16)	0.33
Some secondary	0.71 (0.39, 1.28)	0.250	0.53 (0.27, 1.0)	0.072			0.95 (0.62, 1.46)	0.814
Secondary complete or higher	0.38 (0.15, 0.92)	0.032	0.28 (0.06, 1.25)	0.096			2.40 (1.08, 5.34)	0.031
Treated drinking water			0.67 (0.39, 1.16)	0.15	0.57 (0.36, 0.89)	0.016		
Improved toilet			1.33 (0.65, 2.75)	0.44	1.63 (0.98, 2.70)	0.061		
Hand wash after helping the child defecate	1.63 (0.93, 2.86)	0.089						
Hand washing before food preparation	1.11 (0.66, 1.85)	0.700						
Hand washing after using toilet	1.44 (0.82, 2.52)	0.200	1.54 (0.79, 3.00)	0.21	0.79 (0.46, 1.38)	0.421	1.47 (0.99, 2.17)	0.052
Anemia			0.81 (0.49, 1.31)	0.39	1.69 (1.06, 2.69)	0.025	2.75 (1.87, 4.05)	<0.0001
Low zinc	0.79 (0.46, 1.38)	0.420			1.28 (0.71, 2.32)	0.406		
Low ferritin	3.04 (2.08, 4.44)	0.0001			1.16 (0.77, 1.75)	0.476		
Low retinol	2.07 (1.31, 3.28)	0.002	1.36 (0.78, 2.38)	0.27			0.81 (0.56, 1.19)	0.288

Explanatory variables with significance level of *p* < 0.2 in bivariate association with dependent variable were regressed in the multivariable model using generalized estimating equation (GEE). AOR: Adjusted odds ratio.

3.2.2. Factors Associated with Iron Deficiency (Low Ferritin) from 7 to 60 months

In bivariate analyses, age of the child, maternal age, birth weight, and female sex were associated with lower odds of iron deficiency, and being underweight at enrollment (weight-for-age z-score < −2), and anemia and vitamin A deficiency were associated with increased odds of iron deficiency (Supplementary Table S2). On the other hand, multivariable analysis showed that age in months (AOR: 0.98, 95% CI 0.97–0.98, $p < 0.05$), female sex (AOR 0.65, 95% CI 0.47–0.97, $p < 0.05$), and birth weight (AOR 0.67, 95% CI 0.38–0.88, $p < 0.05$) were associated with lower risk of iron deficiency and anemia (AOR 2.75, 95% CI 1.87–4.05, $p < 0.05$), and higher maternal education (AOR: 2.40, 95% CI 1.08–5.34, $p < 0.05$) was associated with greater risk iron deficiency (Table 3).

3.2.3. Factors Associated with Zinc Deficiency from 7 to 60 months

Bivariate analyses revealed that zinc deficiency was positively associated with anemia and the condition improved with an increase in age in months (Supplementary Table S2). After controlling for other explanatory variables, multivariable analysis showed that age in months (AOR: 0.95, 95% CI 0.93–0.98, $p < 0.05$) was negatively associated with zinc deficiency, and wasting (weight-for-length z-score <−2) at enrollment (AOR: 1.86, 95% CI 1.11–3.11, $p < 0.05$) was positively associated with risk of zinc deficiency (Table 3).

3.2.4. Factors Associated with Vitamin A Deficiency from 7 to 60 months

Anemia, low plasma zinc and severe household food insecurity (compared to children from food secure households) were associated with increased odds of vitamin A deficiency, and age in months, monthly income, and higher socio-economic status were protective of vitamin A deficiency in bivariate analyses (Supplementary Table S2). In the multivariable model, age (AOR: 0.96, 95% CI 0.95–0.98, $p < 0.05$) and treatment of drinking water (AOR: 0.57, 95% CI 0.36–0.89, $p < 0.05$) were significantly associated with lower odds of vitamin A deficiency, and anemia (AOR: 1.69, 95% CI 1.06–2.69, $p < 0.05$) was associated with increased odds of vitamin A deficiency (Table 3).

3.3. Latent Class Growth Model to Identify Group Trajectories of Plasma Micronutrients

Based on the criteria described in the Statistical Analysis Section (BIC, log Bayes factor approximation), the difference in the population distribution of the progression of hemoglobin concentration was best characterized by a two-class model with quadratic components for both trajectories. Group 1 indicated an overall decreasing pattern over the months with mean hemoglobin concentrations of 10.31 g/dL, 9.78 g/dL, and 10.16 g/dL at 7, 15, and 24 months, respectively, while Group 2 indicated an overall increasing pattern with mean hemoglobin concentrations of 11.36 g/dL, 11.83 g/dL, and 12.36 g/dL at 7, 15, and 24 months, respectively (Table 4 and Figure 2).

For ferritin, a two-class model with quadratic components for both trajectories was retained as the final and most parsimonious model. Mean ferritin concentration for the children belonging to Group 1 showed an overall lower value over the months, whereas Group 2 had higher mean values. Mean ferritin concentration in Group 1 was 24.2 ng/mL, 13.19 ng/mL, and 13.99 ng/mL, and in Group 2 was 82.97 ng/mL, 21.18 ng/mL, and 18.21 ng/mL at 7, 15, and 24 months, respectively (Table 4 and Figure 3).

Likewise, for retinol, a two-class model with quadratic components for both trajectories was retained as the final and most parsimonious model. Mean retinol concentration for the children belonging to Group 1 showed overall lower mean values over the months whereas Group 2 had higher mean values. Mean retinol concentration in Group 1 was 21.47 µg/dL, 25.27 µg/dL, and 24.33 µg/dL, and in Group 2 was 26.8 µg/dL, 44.01 µg/dL, and 30.45 µg/dL at 7, 15, and 24 months, respectively (Table 4 and Figure 4).

Table 4. Trajectory-wise estimated mean with 95% confidence interval for hemoglobin, ferritin, retinol and zinc at different ages derived using latent class growth modeling (LCGM).

Trajectory Group	Age = 7 Months	Age = 15 Months	Age = 24 Months
Hemoglobin (g/dL)			
Group 1 (Decreasing)	Mean = 10.31; 95% CI = 9.87, 10.75	Mean = 9.78; 95% CI = 9.29, 10.27	Mean = 10.16; 95% CI = 9.56, 10.77
Group 2 (Increasing)	Mean = 11.36; 95% CI = 11.12, 11.60	Mean = 11.83; 95% CI = 11.64, 12.02	Mean = 12.36; 95% CI = 12.07, 12.65
Ferritin (ng/mL)			
Group 1 (Lower)	Mean = 24.20; 95% CI = 20.76, 27.65	Mean = 13.19; 95% CI = 10.31, 16.07	Mean = 13.99; 95% CI = 10.99, 16.99
Group 2 (Higher)	Mean = 82.97; 95% CI = 75.01, 90.93	Mean = 21.18; 95% CI = 12.85, 29.51	Mean = 18.21; 95% CI = 9.64, 26.78
Retinol (μg/dL)			
Group 1 (Lower)	Mean = 21.47; 95% CI = 20.32, 22.62	Mean = 25.27; 95% CI = 24.0, 26.55	Mean = 24.33; 95% CI = 23.25, 25.41
Group 2 (Higher)	Mean = 26.80; 95% CI = 23.58, 30.02	Mean = 44.01; 95% CI = 38.54, 49.48	Mean = 30.45; 95% CI = 26.98, 33.93
Zinc (mmol/L)			
All children	Mean = 11.71; 95% CI = 11.33, 12.09	Mean = 11.53; 95% CI = 11.14, 11.91	Mean = 12.30; 95% CI = 11.95, 12.66
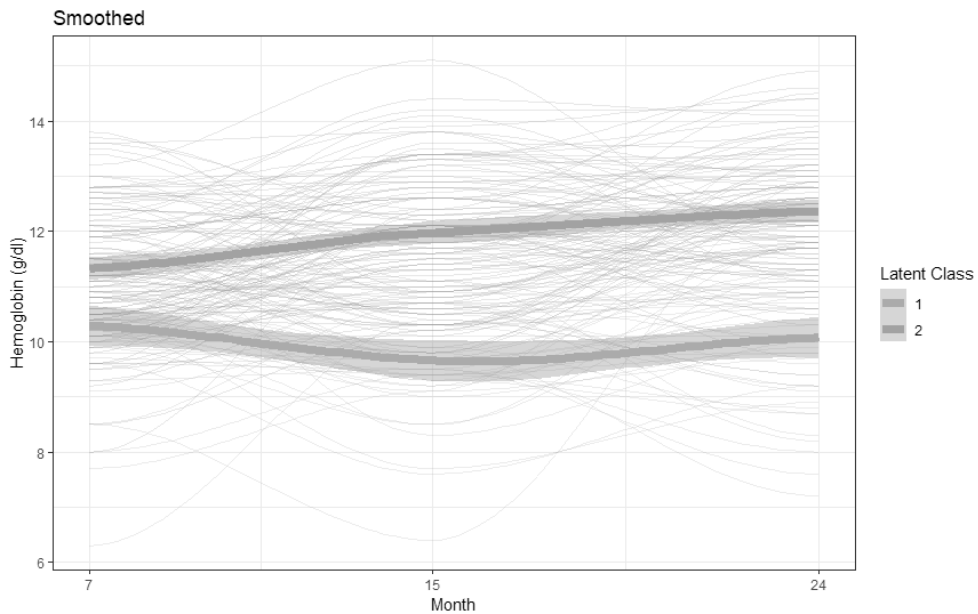
Figure 2. Latent growth class modeling (LCGM) derived latent trajectories with 95% confidence interval with individual trajectories of hemoglobin during age 7 to 24 months.
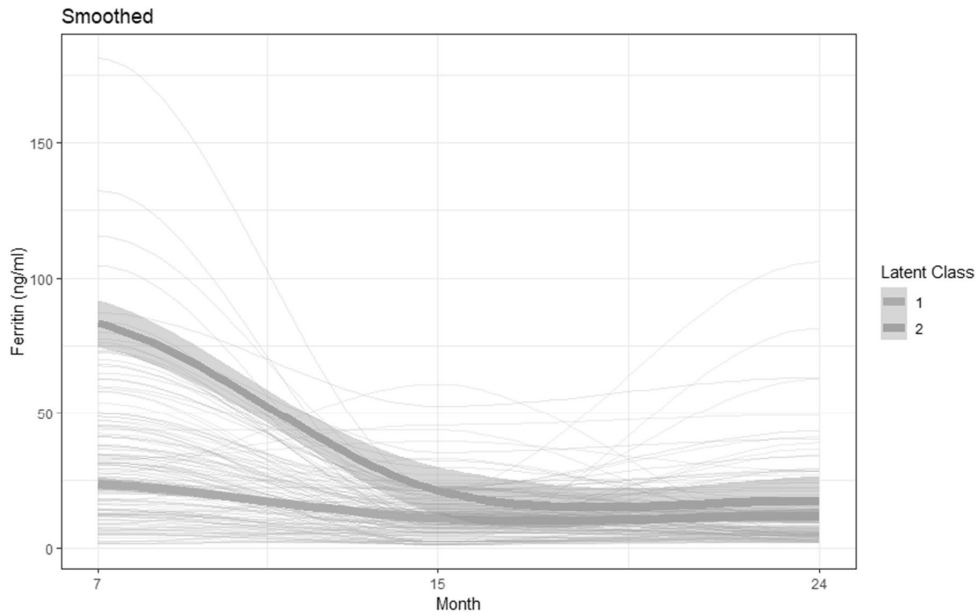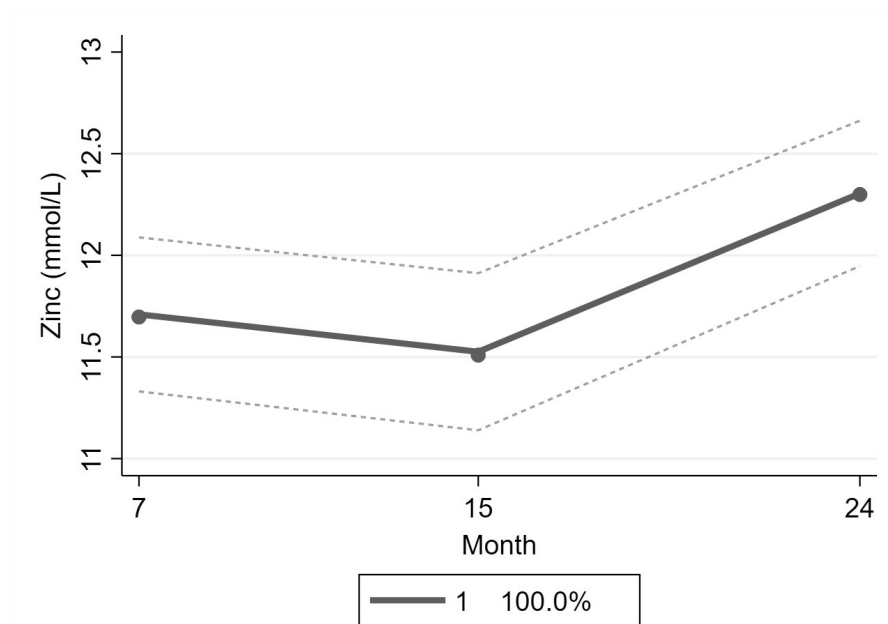
Figure 3. LCGM derived latent trajectories with 95% confidence interval with individual trajectories of ferritin during age 7 to 24 months.
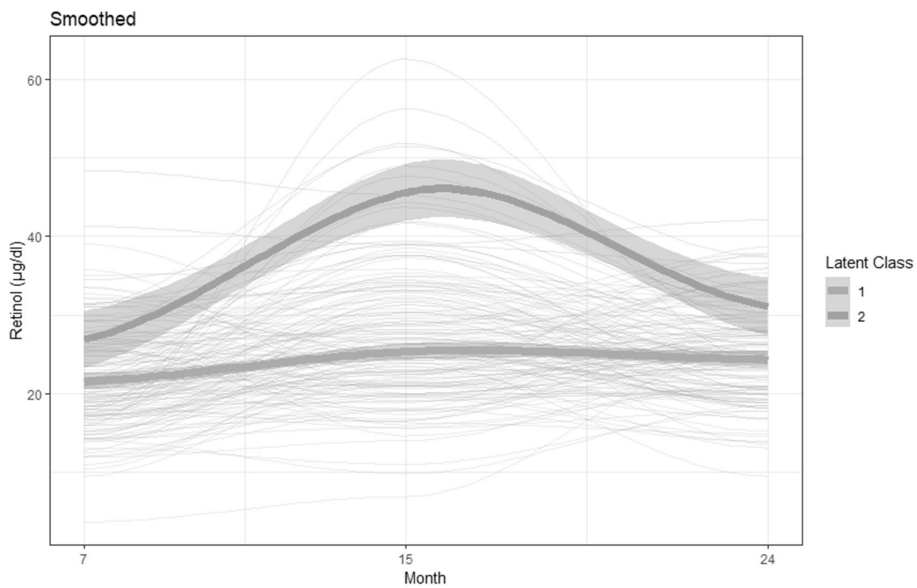
Figure 4. LCGM derived latent trajectories with 95% confidence interval with individual trajectories of retinol during age 7 to 24 months.

In contrast, a one-trajectory model with a quadratic component was retained as the final model for zinc. The more complex models with two, three, or four trajectories generated trajectories with insufficient cluster size (less than 5% of the study population). This result indicates that no significant distinct classes existed in the population for the progression of concentration of plasma zinc and all individuals followed a similar pattern over the months. Mean zinc concentration among the children

included in the analysis was 11.71 mmol/L, 11.53 mmol/L, and 12.3 mmol/L at 7, 15, and 24 months, respectively (Table 4 and Figure 5).

Figure 5. LCGM derived latent trajectory with 95% confidence interval of zinc during age 7 to 24 months.

For all the subgroups of hemoglobin, retinol, and zinc, the average posterior probability of group membership was above 0.8, odds of correct classification was more than 5, and modeled group probabilities were in good agreement with the proportions of group assignment following the maximum-probability assignment rule (Supplementary Table S3).

3.4. Multiple Linear Regression Models to Examine Association of Group Trajectories with Micronutrient Concentrations at 60 months

In multiple linear regression models, we observed that mean iron intake (coefficient: 0.29, 95% CI 0.04–0.55) and percent energy from protein (coefficient: 0.45, 95% CI 0.18–0.73) at 60 months were significantly associated with hemoglobin concentration at 60 months ($p < 0.05$). However, we did not find any significant association of hemoglobin at 60 months with the identified trajectories that the children's hemoglobin concentrations followed during 7–24 months. The children whose plasma ferritin concentrations followed a higher trajectory during 7–24 months were found to have higher plasma ferritin concentration at 60 months (coefficient 13.72, 95% CI 1.15–26.28, $p < 0.05$). Plasma zinc concentration at 24 months was associated with plasma ferritin concentration at 60 months (coefficient 1.98, 95% CI 0.24–3.71, $p < 0.05$). We also observed that the children whose plasma retinol concentrations followed a higher trajectory during 7–24 months had significantly higher plasma retinol level at 60 months (coefficient 3.99, 95% CI 1.04–6.94, $p < 0.05$). Mean vitamin A intake from food at 60 months was associated with plasma retinol concentration at 60 months ($p < 0.05$). One mmol/L increase in plasma zinc at 24 months was associated with 0.21 mmol/L greater plasma zinc concentration at 60 months (Table 5). Summary information generated from this study are recorded in Table 6.

Table 5. Association of sixty months plasma micronutrient concentration and group trajectories of the same micronutrients from 7 to 24 months.

Outcome	Explanatory Variables	Coefficient, 95% CI	p Value
Hemoglobin level at 60 months	Trajectories for Hb, 7 to 24 months		
	Group 1 (Decreasing)	Reference	
	Group 2 (Increasing)	0.21 (−0.56, 0.97)	0.54
	Mean iron intake at 60 mo in mg	0.29 (0.04, 0.55)	0.022
	Mean percent energy from protein at 60 mo	0.45 (0.18, 0.73)	0.002
	WAMI	−2.63 (−5.52, 0.25)	0.073
Adjusted plasma ferritin at 60 months	Trajectories for ferritin, 7 to 24 months		
	Group 1 (Lower)	Reference	
	Group 2 (Higher)	13.72 (1.15, 26.28)	0.033
	Plasma zinc (mmol) at 24 months	1.98 (0.24, 3.71)	0.026
	Mean energy intake at 60 mo (kcal)	0.002 (−0.02, 0.02)	0.84
	Mean protein intake at 60 mo	0.09 (−0.48, 0.65)	0.76
	WAMI	−4.65 (−27.12, 17.82)	0.68
Adjusted plasma retinol at 60 months	Trajectories for retinol, 7 to 24 months		
	Group 1 (Lower)	Reference	
	Group 2 (Higher)	3.99 (1.04, 6.94)	0.008
	Mean vitamin A intake at 60 mos in µgRE	0.003 (0.00007, 0.005)	0.004
	WAMI	4.48 (−3.75, 12.73)	0.28
Adjusted plasma zinc at 60 months	Zinc (mmol/L), 24 months		
	All children	0.21 (0.02, 0.39)	0.035
	Mean zinc intake at 60 mo in mg	0.02 (−0.18, 0.21)	0.87
	WAMI	−0.17 (−1.60, 1.27)	0.82

Table 6. Key information generated on plasma micronutrient status.

Factors Associated with Micronutrient Deficiencies from 7 Months to 60 Months	
Anemia	• Early and late convalescence of an acute infection, and low plasma ferritin and retinol concentrations were associated with higher odds of anemia. • Age, female sex, and higher maternal education were associated with reduced anemia.
Iron deficiency	• Anemia was associated with higher iron deficiency. • Age in months, female sex and birth weight were associated with reduced iron deficiency.
Zinc deficiency	• Wasting at birth was associated with higher odds of zinc deficiency. • Age in months was associated with reduced zinc deficiency.
Vitamin A deficiency	• Anemia was associated with an increased vitamin A deficiency. • Age in months and treatment of drinking water were associated with reduced odds of vitamin A deficiency.
Early life trajectories of micronutrient that can predict 60 months micronutrient concentrations	
Plasma ferritin concentration at 60 months	• The higher trajectory for plasma ferritin during 7 to 24 months was associated with higher plasma ferritin at 60 months.
Plasma zinc concentration at 60 months	• One mmol/L increase in plasma zinc concentration at 24 months was associated with 0.21 mmol/L greater plasma zinc concentration at 60 months. • Plasma zinc concentration at 24 months was associated with plasma ferritin at 60 months.
Plasma retinol concentration at 60 months	• Children belonged to higher trajectory of plasma retinol concentrations during 7 to 24 months had 3.99 µg/dL higher mean plasma retinol at 60 months than the children in lower trajectory.

4. Discussion

This analysis showed a high burden of anemia, and deficiencies of iron, zinc, and vitamin A during early childhood in the Mirpur area of Dhaka followed by a markedly lower prevalence of

all deficiencies at 60 months. Due to the unavailability of reliable, published longitudinal data on micronutrient status, we were unable to make any comparison with the changes we have observed in our study. According to the Bangladesh National micronutrient survey 2011, among children aged 6–60 months, 33% were anemic, 3.9% had iron deficiency, 20.5% had vitamin A deficiency, and 44.6% had zinc deficiency at the national level [33,34]. A recent review of the micronutrient status of under-five year South Asian children also reported similar burdens of anemia and iron, zinc, and vitamin A deficiencies [35].

Regarding the factors associated with anemia, and deficiencies of iron, zinc, and Vitamin A from the children followed longitudinally from 7 months to 60 months, we observed that infection, low ferritin, and low retinol were associated with a higher prevalence of anemia in multivariable analysis. In contrast, age, monthly income, and higher education of mothers compared to no education were associated with a lower risk of anemia. Iron is required to enhance immunity to prevent and protect the host from infections [36]. A previous MAL-ED multi-country pooled analysis also showed that the detection of pathogens and illness were inversely related to hemoglobin concentration [37]. Epidemiological studies also observed a positive association between vitamin A deficiency and anemia [38]. It can be explained through experimental studies that vitamin A deficiency is associated with increased hepcidin expression which directly acts on hepatic mobilization of iron stores essential for erythropoiesis [39]. As for iron deficiency, age in months, female sex, and birth weight were associated with higher plasma ferritin and anemia, and higher maternal education was associated with lower ferritin concentrations. We do not know the reason for a positive association between iron deficiency and higher maternal education. Perhaps this was due to the fact that a small number of mothers had secondary or higher level of education (4.7% of 212 mothers of children at 7 months). However, this research question should be followed up in a study consisting of both slum and non-slum children.

We also observed that age in months was negatively associated with zinc deficiency while wasting at birth was positively associated with zinc deficiency. Similarly, age and treatment of drinking water were significantly associated with lower risk of vitamin A deficiency, and anemia was associated with great risk of deficiency.

Considering the interrelation between different micronutrients, from 7 months to 60 months of age, low ferritin and low retinol concentrations were associated with greater odds of anemia. This was also evident in the Bangladesh national micronutrient survey of 2011, where higher serum ferritin was found to be associated with higher levels of hemoglobin in children less than five years of age [34]. They also reported a positive association between serum retinol and hemoglobin concentrations where the prevalence of anemia was 33% higher in children with low retinol concentrations [34]. Several other studies also found similar results [40,41]. Vitamin A most likely reduced anemia by lowering infection, augmenting erythropoiesis and releasing stored iron from the liver [34]. Our analysis also suggested a positive association between plasma zinc status at 24 months and plasma ferritin status at 60 months. If zinc is increasing erythropoiesis, then more iron will be taken out of ferritin to "keep up" with the erythropoietic needs and, therefore, the relation between plasma zinc and ferritin might be negative [42,43]. The positive relation that we see is likely due to intake where foods that are high in zinc are also high in iron. Despite the fact that zinc and iron compete for a shared absorptive pathway, iron generally does not hamper zinc absorption except at very high iron to zinc ratios [44].

We identified distinct group trajectories of hemoglobin and several plasma micronutrients between 7 months to 24 months of age and examined their predictive role in the micronutrient status at five years of age. Hemoglobin trajectory in early life did not predict the presence or absence of anemia in later childhood. This is perhaps because hemoglobin is a composite of several constituents that can be affected in different ways that can make it unpredictable. We observed that concurrent consumption of iron-rich food and protein are associated positively with hemoglobin at 60 months. Other studies also observed a positive association of consuming iron rich food and animal protein with hemoglobin status [45]. In contrast, higher concentrations of ferritin, retinol, and zinc at 24 months were each

associated with concentrations at 60 months. Our findings suggest that children whose plasma ferritin concentration was around 20 ng/mL at ages 15 and 24 months continued to have better plasma ferritin level at 60 months. In our study, children who had a plasma retinol concentration of around 30 µg/dL at ages 7 and 24 months went on to have higher plasma retinol level at 60 months. This may indicate that ensuring adequate micronutrient status in the first two years of life is important for micronutrient status at five years of age, or that dietary intakes track to a certain degree over time. The consumption of micronutrients was very poor in this population. Recently, we conducted a study in the same community to explore the overall quality of the diet and calculated the nutrient adequacy ratios (NARs) for different micronutrients including vitamin A, iron, and zinc from 9 months to 24 months of age. The NAR was calculated as the ratio of average daily intake and the recommended dietary allowance of that specific nutrient. The NAR values for vitamin A, iron, and zinc were 0.21, 0.23, and 0.42, respectively, well below a ratio of 1 for adequate intake [4].

Considering the water, sanitation and hygiene (WASH) practices, we observed that children from families who treat drinking water by any means had lower risk of vitamin A deficiency. Treatment of drinking water has a direct role in preventing diarrhea and subclinical infections which can lower serum retinol concentrations by as much as 25% [46]. We did not find an association of other WASH practices with any of the outcomes. No association was observed between the overall socio-economic status and micronutrient status among the children as WAMI-score was statistically insignificant in all the analyses.

Limitations of this analysis include a reduced number of plasma micronutrient data and the unavailability of data on morbidity, enteropathy biomarkers, and pathogen burden at 60 months.

5. Conclusions

The results point to the importance of the first 1000 days of life. A rich endowment of micronutrients in early life is important to sustain the requirements of pre-school age. It is, therefore, crucial to have a well-nourished infant at birth through improvement of maternal health, to start complementary feeds at the optimum time and to have a diet with sufficient nutrient density to ensure adequacy.

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Supplementary Materials-1

S1. Supplementary Tables

Table S1: Availability of different variables across different time points

Variables	7 months	15 months	24 months	60 months
SES, WAMI	+	+	+	+
Maternal age, education, age at marriage	+	+	+	+
Household income, asset	+	+	+	+
WASH behavior	+	+	+	+
Morbidity and antibiotic use	+	+	+	-
Stool microbiology	+	+	+	-
EED: MPO, NEO, AAT	+	+	+	-
Anthropometry	+	+	+	+
Zn, retinol, ferritin, Hb, sTFR, CRP, AGP	+	+	+	+
Child feeding: EBF, complementary feeding	+	+	+	+

Table S2 Bivariate analysis for anemia and other micronutrient deficiencies

	Anemia	Zinc deficiency	Vitamin A deficiency	Iron deficiency
	OR (95% CI), p-value	OR (95% CI), p-value	OR (95% CI), p-value	OR (95% CI), p-value
Inflammation group				
Non-inflammed	Reference	Reference	Reference	Reference
Incubation	2.77 (1.09, 7.04), 0.032	1	1.37 (0.46, 4.08), 0.577	1.15 (0.45, 2.91), 0.770
Early convalescence	2.53 (1.56, 4.10), <0.001	1.04 (0.48, 2.24), 0.923	0.99 (0.55, 1.75), 0.962	0.96 (0.59, 1.58), 0.877
Late convalescence	2.16 (1.46, 3.17), <0.001	1.36 (0.73, 2.55), 0.335	1.14 (0.74, 1.77), 0.557	1.07 (0.70, 1.62), 0.764
Age in months	0.94 (0.93, 0.96), <0.001	0.95 (0.93, 0.98), <0.001	0.96 (0.95, 0.97), <0.001	0.97 (0.49, 0.91), 0.011
Female	0.63 (0.44, 0.89), 0.010	0.79 (0.50, 1.25), 0.313	0.85 (0.58, 1.26), 0.422	0.67 (0.44, 0.89), 0.010
Birth weight	0.64 (0.40, 1.03), 0.066	0.93 (0.55, 1.59), 0.792	0.73 (0.47, 1.13), 0.160	0.62 (0.41, 0.94), 0.024
Underweight (WAZ <-2) at birth	1.21 (0.78, 1.87), 0.388	1.02 (0.61, 1.72), 0.936	1.13 (0.73, 1.75), 0.586	1.67 (1.16, 1.41), 0.006
Stunting (LAZ <-2) at birth	1.19 (0.71, 2.02), 0.503	0.89 (0.49, 1.61), 0.696	0.96 (0.59, 1.55), 0.866	1.09 (0.72, 1.67), 0.663
Wasting (WLZ <-2) at birth	1.39 (0.93, 2.09), 0.108	1.56 (0.95, 2.55), 0.080	0.92 (0.56, 1.52), 0.753	1.37 (0.91, 2.08), 0.131
Days of exclusive breast feeding	1.00 (0.99, 1.00), 0.442	0.99 (0.99, 1.00), 0.437	0.99 (0.99, 1.00), 0.315	0.99 (0.99, 1.00), 0.785
Food insecurity access				
Food secure	Reference	Reference	Reference	Reference
Mild food insecurity	1.09 (0.47, 2.57), 0.828	0.84 (0.32, 2.16), 0.712	1.22 (0.5, 2.69), 0.626	0.87 (0.40, 1.86), 0.713
Moderate food insecurity	0.75 (0.44, 1.28), 0.291	0.82 (0.40, 1.67), 0.591	0.91 (0.48, 1.73), 0.775	1.18 (0.77, 1.81), 0.446
Severe food insecurity	1.39 (0.77, 2.50), 0.274	0.59 (0.22, 1.57), 0.296	2.62 (1.35, 5.07), 0.004	1.33 (0.74, 2.38), 0.347
Monthly income	0.99 (0.99, 1.00), 0.138	1.00 (0.99, 1.00), 0.105	0.99 (0.99, 0.99), 0.016	0.99 (0.99, 1.00), 0.776
Asset index				

Poorest	Reference	Reference	Reference	Reference
Poor	0.68 (0.39, 1.17), 0.162	1.34 (0.66, 2.74), 0.420	0.75 (0.41, 1.37), 0.354	0.87 (0.52, 1.47), 0.609
Middle	0.81 (45, 1.48), 0.496	0.97 (0.41, 2.32), 0.950	1.11 (0.63, 1.94), 0.726	1.06 (0.64, 1.77), 0.818
Wealthier	0.71 (0.41, 1.23), 0.217	1.14 (0.53, 2.45), 0.743	0.58 (0.33, 1.02), 0.057	1.12 (0.69, 1.79), 0.642
Wealthiest	0.64 (0.35, 1.19), 0.158	1.71 (0.85, 2.45), 0.131	0.29 (0.15, 0.57), <0.001	1.01 (0.58, 1.75), 975
Maternal education				
No	Reference	Reference	Reference	Reference
Some primary	0.96 (0.58, 1.59), 0.865	0.73 (0.38, 1.39), 0.341	0.64 (0.34, 1.18), 0.152	1.11 (0.70, 1.76), 0.651
Primary complete	0.81 (0.45, 1.48), 0.496	0.77 (0.38, 1.55), 0.461	1.09 (0.59, 2.05), 0.766	1.40 (0.85, 2.32), 0.187
Some secondary	0.69 (0.42, 1.14), 0.151	0.64 (0.34, 1.21), 0.169	0.78 (0.44, 1.41), 0.420	0.82 (0.59, 1.42), 0.696
Secondary complete or higher	0.59 (0.24, 1.44), 0.247	0.34 (0.09, 1.32), 0.120	0.98 (0.39, 2.50), 0.973	2.05 (0.98, 4.29), 0.055
Treat drinking water	0.93 (0.64, 1.34), 0.698	1.31 (0.82, 2.12), 0.260	0.45 (0.31, 0.67), <0.001	1.13 (0.81, 1.58), 0.470
Improved toilet	0.95 (0.62, 1.45), 0.813	0.66 (0.41, 1.08), 0.100	1.56 (0.97, 2.49), 0.064	1.02 (0.72, 1.46), 0.905
Hand washing after help the child defecate	1.81 (1.18, 2.77), 0.007	1.78 (0.96, 3.17), 0.052	0.82 (0.53, 1.26), 0.364	1.26 (0.87, 1.82), 0.219
Hand washing before food preparation	1.31 (0.87, 1.99), 0.198	1.11 (0.64, 1.94), 0.703	0.99 (0.63, 1.58), 1.00	1.34 (0.91, 1.96), 0.137
Hand washing after using toilet	1.59 (1.02, 2.51), 0.043	1.75 (0.99, 3.06), 0.051	0.69 (0.43, 1.11), 0.124	1.26 (0.87, 1.85), 0.212
Age of mother in year	0.98 (0.93, 1.00), 0.064	0.99 (0.95, 1.04), 0.797	0.98 (0.94, 1.02), 0.317	0.96 (0.93, 0.99), 0.040
Anemia		1.56 (1.01, 2.42), 0.047	2.89 (2.04, 4.12), <0.001	3.79 (2.72, 5.29), <0.001
Zinc deficiency	1.59 (1.04, 2.45), 0.33		1.70 (1.06, 2.72), 0.027	0.94 (0.59, 1.48), 0.790
Iron deficiency	3.59 (2.59, 4.99), <0.001	0.96 (0.69, 1.52), 0.849	1.39 (0.99, 1.95), 0.050	
Vitamin A deficiency	2.86 (2.02, 4.03), <0.001	1.61 (0.99, 2.64), 0.057		1.40 (1.00, 1.97), 0.049

Table S3 Fit statistics of the final models derived using LCGM for Hemoglobin, Retinol and Zinc

Trajectory group	n	Average posterior probability	Odds of correct classification	Estimated group probability	Proportion of group assignment
Hemoglobin (g/dl)					
Group 1 (Decreasing)	39	0.88	7.5	0.28	0.25
Group 2 (Increasing)	116	0.93	12.8	0.72	0.75
Ferritin (ng/ml)					
Group 1 (Lower)	132	0.99	115.5	0.86	0.86
Group 2 (Higher)	21	0.93	13.9	0.14	0.14
Retinol (µg/dl)					
Group 1 (Lower)	138	0.98	39.0	0.88	0.90
Group 2 (Higher)	16	0.90	9.1	0.12	0.10
Zinc (mmol/L)					
All children	155	1.0	-	1.0	1.0

Supplementary Materials-2

2.1 Measurement of plasma micronutrient status

Plasma zinc was measured using flame atomic absorption spectrophotometry (Shimadzu AA-6501S, Kyoto, Japan). A four point calibration curve was prepared in every lot from the commercial zinc standard solution (Cica-Reagent, Kanto Chemical Co. INC), in concentrations of 0.1, 0.2, 0.3 and 0.4 mg/L. Diluted plasma was then aspirated into the AAS. Results were calculated from a standard curve [1].

For retinol assays, plasma was deproteinized with methanol containing retinyl acetate (Sigma Chemical Co., St Louis, MO, USA) as an internal standard and retinol was extracted twice with hexane. The hexane layer was pooled and evaporated under nitrogen gas. Then residue was re-dissolved in the mobile phase (95% methanol) and injected into HPLC (SPD-10A vp uv-visible detector, LC-10AT vp solvent delivery system and Chromatopac C-R8A integrator, Shimadzu, LC, Kyoto, Japan). Retinol was separated by reverse phase HPLC using C18 column (Discovery C18, 25cmX4mm, 5µm, Cat# 504971) and detected at 325nm. A pooled human plasma sample was calibrated against standard reference material (fat-soluble vitamins, carotenoids and cholesterol in human serum, 968c; National Institute of Standards and Technology, Gaithersburg, MD, USA). Three aliquots of the plasma pool were analyzed with each set of samples, and the retinol concentration was calculated based on the known concentration of retinol in the plasma pool [2].

Ferritin, CRP and AGP were determined by Immunoturbidimetric assay using commercial kits from Roche diagnostics on Roche automated clinical chemistry analyzer Hitachi -902 (Boehringer Mannheim, Germany). A HemoCue 201 machine was used to measure hemoglobin concentration [3].

2.2 Latent class growth modeling (LCGM) and multiple linear regression models

We used latent class growth modeling (LCGM), also called group-based trajectory modeling, to identify distinct clusters or classes of children following similar trajectories with regard to the pattern of hemoglobin, ferritin, retinol and zinc during the age of 7 to 24 months. LCGM is a semi-parametric, finite mixture modeling technique which analyzes longitudinal data using maximum likelihood to identify meaningful and distinct groups of individuals who follow similar progression over time for a given variable. LCGM relaxes the assumption that all individuals are drawn from a single population and allows for differences in growth parameters across unobserved subpopulations. However, it assumes that the intercept and slope are fixed for all individuals within each distinct group [4-5].

We built separate trajectory models for hemoglobin, ferritin, retinol and zinc. LCGM requires at least three measurement time-points for each case to generate reliable parameter estimates of trajectories [6]. Therefore, the analyses were restricted to the children for whom data on the outcomes were available at 7, 15 and 24 months (three time-points). In addition, for ferritin, three very large and unusual values were dropped from the dataset. The sample sizes for LCGM were reduced to 155 for hemoglobin, 153 for ferritin, 154 for retinol and 155 for zinc.

We built and compared several models for each outcome with 1 to 4 trajectories. A censored normal distribution approach was used for modeling all three outcomes. To identify the optimal number of trajectories, we fit models of increasing complexity starting from a single trajectory and finalized the model that best fit the data. In identifying the distinct trajectories of the outcomes, linear and quadratic functions of time (age in months) were examined. Non-significant quadratic terms were removed from a model for a given trajectory. Models generating

trajectories with insufficient cluster size (less than 5% of the study population) were not considered.

We selected the final models with optimal number and shape of trajectories based on Bayesian information criteria (BIC), log Bayes factor, the statistical significance of quadratic terms, whether 95% confidence intervals of trajectories overlapped, and the percentage of the population in each trajectory group. The smallest absolute value of the BIC indicated the best fit. A value greater than 6 for the estimated log Bayes factor, which is equal to two times the difference in the BIC values calculated by subtracting the BIC of the simpler model from that of the more complex model, was interpreted as strong evidence for the more complex model [7]. After selecting the final model, we calculated the posterior probabilities for each individual of belonging to each of the trajectory groups, and individuals were assigned to a trajectory group based on the maximum-probability assignment rule [8].

We used the following criteria to assess the goodness of fit of the final models: whether the average posterior probability of assignment was greater than 0.8 for each of the subgroups, whether the odds of correct classification was greater than 5, and whether the estimated/modeled group probabilities were in good agreement with the proportions of group assignments [27]. We reported the findings of the LCGM following *The GRoLTS-Checklist: Guidelines for Reporting on Latent Trajectory Studies* [9].

In subsequent analyses, multiple linear regression models with robust standard errors were fitted to examine the association of levels of hemoglobin, ferritin, retinol and zinc at the age of 60 months with the trajectories identified through LCGM. As zinc was found to have a single trajectory (described in detail in the results section), level of zinc at 24 months was used instead

of any trajectory to assess the predictive association with level of zinc at 60 months. We considered several covariates collected at the age of 60 months in building these models which include sum of energy in Kcal for the day of food recall, sum of protein in grams for the day of food recall, sum of fat in grams for the day of food recall, sum of carbohydrates in grams for the day of food recall, sum of iron in mg for the day of food recall, sum of Vitamin A in ug for the day of food recall, sum of zinc in mg for the day of food recall, phytate to iron ratio of the diet, phytate to zinc ratio of the diet, energy in Kcal from carbohydrates (minus fiber) as percent of total energy, energy in Kcal from protein as percent of total energy, energy in Kcal from fat as percent of total energy, socioeconomic and WASH variables including WAMI score, and child sex. However, the final regression models included only iron intake, the percentage of energy from protein, and WAMI score for hemoglobin, level of zinc at 24 months, total energy intake, protein intake and WAMI score for ferritin, vitamin A intake and WAMI score for retinol, and zinc intake and WAMI score for zinc. Due to missing values for the outcome variables, the sample sizes for the linear models were reduced to 142 for hemoglobin, 138 for ferritin and retinol and 140 for zinc.

The statistical analyses related to LCGM were performed using the "traj plugin" in Stata (StataCorp, College Station, Texas 77845 USA, version 14.1) [10], a Stata equivalent of the widely used "proc traj" in SAS [28]. Outputs of LCGM models were plotted using "traj" in Stata and the R packages "lcmm" and "ggplot2" in R (version 3.5.1). All other statistical analyses were performed with Stata/PC (StataCorp, College Station, Texas 77845 USA, version 14.1).

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Checklist

STROBE Statement—Checklist of items that should be included in reports of *cohort studies*

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	5,6
Objectives	3	State specific objectives, including any prespecified hypotheses	6
Methods			
Study design	4	Present key elements of study design early in the paper	6,7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5,6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	7,8,9
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7,8,9
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	7,8,9
Bias	9	Describe any efforts to address potential sources of bias	9,10,11,12
Study size	10	Explain how the study size was arrived at	12
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	10-12
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	9-11
		(*b*) Describe any methods used to examine subgroups and interactions	11
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how loss to follow-up was addressed	
		(*e*) Describe any sensitivity analyses	11, S1.2
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	13
		(b) Give reasons for non-participation at each stage	11

		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Table 1
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) Summarise follow-up time (eg, average and total amount)	12
Outcome data	15*	Report numbers of outcome events or summary measures over time	12-13
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	12-15
		(*b*) Report category boundaries when continuous variables were categorized	14-16
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	14-17
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	14-17, supple

Discussion

Key results	18	Summarise key results with reference to study objectives	17-20
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	20
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	17-20
Generalisability	21	Discuss the generalisability (external validity) of the study results	17-20

Other information

Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	21

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

PUBLICATION
III

Daily Supplementation With Egg, Cow Milk, and Multiple Micronutrients Increases Linear Growth of Young Children with Short Stature

Mahfuz M, Alam MA, Das S, Fahim SM, Hossain MS, Petri WA Jr, Ashorn P, Ashorn U, Ahmed T

Daily Supplementation With Egg, Cow Milk, and Multiple Micronutrients Increases Linear Growth of Young Children with Short Stature

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ABSTRACT

Background: Childhood stunting is the most prevalent public health nutrition problem in low- and middle-income countries.

Objective: This study aimed to determine whether daily supplementation in 12–18-mo-old undernourished Bangladeshi children with egg, cow milk, and multiple micronutrients improves linear growth.

Methods: In the Bangladesh Environmental Enteric Dysfunction (BEED) study, a community-based intervention study, 12–18-mo-old children with length-for-age z score (LAZ) <1 were supplemented daily with an egg and 150 mL of milk for 90 feeding days, and 1 sachet of multiple micronutrient powder was provided daily for 60 feeding days. The change in LAZ over this period was compared with that in children of the same age and same baseline LAZ who were enrolled in the recently completed Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health (MAL-ED) Dhaka birth cohort study conducted in the same community where no nutrition intervention was provided. Difference-in-difference (DID) analysis was done and the effect size was adjusted for other possible covariates using a generalized estimating equation in a regression model.

Results: A total of 472 children with LAZ <1 completed the intervention and data were available for 174 children in the comparison group. Compared with the comparison group, adjusted DID analysis revealed a change in LAZ in the intervention group of +0.23 (95% CI: 0.18, 0.29; $P < 0.05$). In a subgroup analysis, the changes were +0.27 (95% CI: 0.18, 0.35; $P < 0.05$) in stunted (LAZ <2) children and +0.19 (95% CI: 0.12, 0.27; $P < 0.05$) in children at risk of stunting (LAZ −1 to −2). No allergic reactions or other adverse events related to milk and egg consumption were observed.

Conclusions: Daily directly observed milk, egg, and multiple micronutrient supplementation may improve linear growth of stunted children. A randomized controlled trial with longer duration of supplementation coupled with an additional intervention aimed at reducing pathogen burden is warranted to confirm these results. This trial was registered at clinicaltrials.gov as NCT02812615. *J Nutr* 2020;150:394–403.

Introduction

Stunting [length-for-age z score (LAZ) <2 of the WHO growth standard] (1) is currently the most common form of childhood malnutrition worldwide (2). Stunting was found to be associated with negative health and economic outcomes in later life, including shorter adult height, impaired cognitive development, lower attained schooling, and reduced adult income (3). Together with low birth weight, stunting was responsible for 2.1 million deaths and 91.0 million disability-adjusted life years in 2008 (4). Globally, 151 million (22%)

children aged <5 y are stunted (5), while in Bangladesh, this proportion was 36% in 2014 (6). Despite being a relatively neglected area of research for many years, stunting has become a major public health priority as the World Health Assembly has called for reducing stunting by 40% between 2012 and 2025 (7).

Stunting is believed to be a result of interplay between multiple causal and contextual factors in resource-poor settings, and has been associated with increased susceptibility to infection and impaired neurocognitive development (8). In

affected children, such growth faltering usually manifests as a steady decline in LAZ between the ages of 6 and 24 mo (9, 10). During this period, the child transitions from predominant breastfeeding to complementary foods that are often deficient in key macro-/micronutrients and low in diversity, while diarrheal disease and exposure to enteropathogens is common (5, 11, 12).

A recent analysis including data from 110 countries showed that the average annual relative reduction in the rate of childhood stunting is 1.8% per year (13). This is far behind the rate of 3.9% that is required to meet the global target set by the World Health Assembly to cut the number of children with stunting to 100 million (7). Despite potential contributions to reductions in morbidity and mortality, no single intervention has demonstrated substantial efficacy in normalizing the linear growth velocity and reversing the consequences of stunting. Based on an assessment of multiple approaches to combat stunting, it has been suggested that scaling up of nutrition-specific interventions might correct about 20% of stunting (5). Given that stunting is a consequence of exposure to a complex mix of insults over time, successful treatment is likely to require addressing the problems of consumption of low-quality/diversity diets and recurrent pathogenic and adverse environmental exposures (14).

Micronutrient deficiencies are an important determinant of childhood undernutrition (8). Cereal-based diets of young infants in developing countries lack essential micronutrients such as zinc, vitamin A, and iron (12). In Bangladesh, micronutrient adequacy is poor and a national survey showed that 33% of children aged <5 y were anemic, 20.5% had vitamin A deficiency, and 44.6% had zinc deficiency (15, 16). Dietary supplementation with multiple micronutrient powder was effective in controlling anemia and other micronutrient deficiencies (17). In Bangladesh, the current recommendation as per the National Strategy for Prevention and Control of Anaemia is to provide a 5 component (zinc, iron, folic acid, vitamin A, and vitamin C) multiple micronutrient powder for 2 mo (18).

A recent study revealed that daily provision of a chicken egg can improve linear growth of young children and reduce stunting (19). Egg is a good source of protein, fatty acids, and a range of vitamins and minerals (20). Cow milk is a good source of essential nutrients and its consumption is associated with increased circulating insulin-like growth factor (IGF-1) that promotes linear growth of children (21, 22). Both egg and milk are culturally acceptable and locally available in Bangladesh. Here, we used data from the Bangladesh Environmental Enteric Dysfunction (BEED) study to explore whether daily supplementation with a chicken egg and 150 mL of cow milk and essential micronutrients could improve linear growth of 12–18-mo-old Bangladeshi children who were already stunted, or who were at risk for stunting (23).

Methods

Study design

This is a community-based nonrandomized comparative intervention study which is a part of the ongoing BEED study with a historical comparison group from the recently completed Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health (MAL-ED) study (23, 24).

Description of parent studies.
Intervention group. In the BEED study, children aged 12–18 mo with LAZ <1 were enrolled from the study area through community screening. The first child was enrolled in July 16, 2016 and children enrolled until June 30, 2018 were included in the current analysis. After enrollment, the children were provided with directly observed nutrition intervention for 90 feeding d (23).

The inclusion criteria for enrollment in the BEED study include children of either gender, aged between 12 and 18 mo, LAZ <1, and parent(s) or caregivers willing to sign the consent form and bring the child to the study site every day, 6 d/wk, for 90 d of nutritional therapy. Children with severe acute malnutrition, severe anemia (<8 g/dL), tuberculosis, other chronic diseases or any congenital disorder or deformity, an ongoing episode of diarrhea, a history of persistent diarrhea in the past month, or known allergy/intolerance to eggs or milk were excluded (23).

Comparison group. The MAL-ED Bangladesh study was an observational birth cohort study in which infants were enrolled within 3.5 d of birth and followed longitudinally until age 60 mo without any nutrition intervention (24). Data used in the current analysis were collected between June 11, 2011 and September 15, 2013, covering the period in which included children were aged between 14 mo and 17 mo. During the first 24 mo of the study, the children were followed by twice weekly home visits for dietary and morbidity surveillance. Anthropometry was collected monthly from all participants, while data on sociodemography and food security were collected during enrolment and at 6-mo intervals. The detailed methodology of data collection is described elsewhere (25–27).

The inclusion criteria for enrollment in the MAL-ED study were healthy newborn children of either gender, and parent(s) or caregivers willing to sign the consent form and have a plan to stay in the community for the next 2 y. Exclusion criteria were very low birth weight (<1500 g), extremely ill, severe acute malnutrition, severe anemia (<8 g/dL), tuberculosis, other chronic diseases or any congenital disorder or deformity, nonsingleton infant and mother aged <16 y (24–27).

Study settings.
Description of field site. Both BEED and MAL-ED studies were conducted among the residents of Bauniabadh and adjacent slum areas of Mirpur, Dhaka. The population density of the area is one of the highest in Dhaka city, with more than 38,000 people living in each square kilometer. The sociodemographic details of the field site have already been reported (23, 24).

Participants.
Intervention groups. Children aged 12–18 mo were enrolled from the study area with a LAZ of <1. This child cohort was further divided into 2 groups: stunted children (LAZ < −2) and children at risk of being stunted (LAZ <1 to −2).

TABLE 1 Nutrition and health intervention with delivery method provided to children of intervention group[1]

Intervention	Delivery method
Egg	— 1 boiled egg daily, 6 d/wk
Milk	— UHT processed 150 mL whole milk daily, 6 d/wk
Multiple micronutrient powder	— 1 sachet of multiple micronutrient powder a day administered to the child during each feeding session for 2 mo
	— Each sachet contained 12.5 mg iron, 5 mg zinc, 300 µg vitamin A, 150 µg folic acid, and 50 mg of vitamin C (Monimix)
Nutritional counseling	— Parents/caregivers were given nutritional counseling with particular emphasis on adding vegetable oil to the cooked diet as a source of energy, as well as sources of animal-based products (small fish, chicken, or meat) along with regular intake of vegetables

[1]UHT, ultra-high temperature.

Comparison group. The median age of children in the intervention group at baseline was 14 mo. The MAL-ED study was a longitudinal birth cohort with monthly follow-up data available for all children. We extracted data from MAL-ED children with LAZ <1 at 14 mo and follow-up growth data were collected for the subsequent 4 mo (14–18 mo). The comparison group was matched with the intervention group for age and LAZ. In addition, the following features were comparable between the groups: sex, community information, similar frequency of contact with research staff, anthropometry measurement using the same scales, and follow-up performed by the same research team.

Intervention group.

Enrollment, screening, and consenting. Census, screening, and enrollment of the subjects was conducted in the Mirpur area of Dhaka city through household visits. Based on predefined inclusion criteria, field research assistants enrolled the children whose parent(s) or caregivers were willing to consent and bring their child to the nutrition center every day for 90 feeding d to receive nutritional therapy. Parents of the children who met the eligibility criteria were invited to participate and the study was explained to them in detail by a trained research staff. A written informed consent was signed by the mother or the primary caregiver of the participants before enrollment in the study (23).

Detailed procedure of directly observed nutritional therapy. After enrollment, participants were asked to attend the designated nutrition center (established in the Bauniabadh area, Mirpur) daily for nutritional therapy between 10:00 and 11:30 for 90 feeding d to avoid the issue of food sharing at home. The consumption of milk and egg was directly observed by the field staff. Before serving, each shell-less boiled egg was weighed and 150 mL of milk was measured and the total amounts of food offered, consumed, and left over were measured and recorded at each feeding session. One sachet of multiple micronutrient powder (Monimix, Renata Pharmaceuticals) was mixed with milk by 1 of the research staff during daily feeding for 2 mo. This food was given in addition to the regular diet, and the mothers were instructed to continue usual feeding including breastfeeding. The intervention provided in the study was free of cost. In addition to this feeding intervention, caregivers were provided nutritional counseling during each of the feeding session. Nutritional counseling sessions were conducted using communication materials including posters, pictures, and pamphlets. They discussed how to provide a balanced diet, dietary diversity, minimum meal frequency, importance of animal source protein and iodized salt, water-sanitation-hygiene practice, etc. Details of the nutrition intervention are described in **Table 1** and the composition of the supplemental meal is described in **Table 2**. The nutrition intervention started on July 25, 2016 and this analysis includes all the children in the BEED study who completed nutrition intervention by June 30, 2018 ($n = 472$).

This directly observed nutrition intervention was provided for 90 feeding d, 6 d/wk, except for the weekends. Furthermore, some children were followed for >108 d who missed or were reluctant to take the intervention on scheduled feeding d. However, a child was discontinued from the study and referred for medical evaluation if he/she did not take at least 50% of the food offered for 7 consecutive d (23).

Primary outcome.

The primary outcome of this analysis was the change in LAZ of intervention group compared to children in comparison group, conducted in the same settings in Bangladesh.

TABLE 2 Characteristics of the children at baseline in intervention and comparison groups[1]

Indicators	Intervention ($n = 472$)	Comparison ($n = 174$)	P value[2]
Age, mo	14 [13, 16]	14 [14, 14]	0.37
Female	236 (50)	90 (51.72)	0.69
Currently breastfeeding	431 (91.31)	168 (96.55)	0.02
WAMI index	0.58 ± 0.14	0.52 ± 0.12	<0.001
Improved sanitation	297 (62.92)	174 (100)	<0.001
Improved source of drinking water	472 (100)	174 (100)	NA
Maternal education, y	5 [2, 7]	5 [2, 7]	0.44
Length-for-age z score at baseline	−2.15 ± 0.80	−2.10 ± 0.75	0.48
Weight-for-age z score at baseline	−1.71 ± 0.85	−1.65 ± 0.82	0.06
Weight-for-length z score at baseline	−0.90 ± 0.87	−0.76 ± 0.94	0.07
Income, US$/mo	157 [124, 241]	97 [72, 133]	<0.001
Maternal height, cm	149.26 ± 5.21	148.52 ± 5.23	0.11
Stool α1-antitrypsin, mg/g	0.30 [0.12, 0.57]	0.42 [0.24, 0.66]	<0.001
Stool myeloperoxidase, ng/mL	2300 [1340, 4380]	5250 [3030, 10,800]	<0.001
Stool neopterin, nmol/L	2480 [1270, 3790]	1380 [714, 2190]	<0.001

[1]Values are means ± SDs, median [IQR], or frequency (percentage). WAMI index, Water-sanitation-hygiene, Asset status, Maternal education status, and monthly Income index.
[2]Student's *t* test, Pearson chi-square test, and Mann-Whitney *U* test, as appropriate.

Data collection, management, and storage.

Data collection tools for this study included case report forms. All the forms were de-identified by coded numbers to maintain participants' confidentiality and to enable tracking throughout the study.

Anthropometry was performed bi-weekly in the intervention group and monthly in the comparison group. To make the groups comparable, we used anthropometry data of the intervention group collected every 28 d. Because children in the intervention group were followed for a median of 108 d to complete 90 d of feeding, anthropometric measurements at 5 time points were available for this analysis. Anthropometric data were collected by trained personnel, through use of scales with high precision. Weight was measured with a Seca digital weighing scale (model 727, Hamburg, Germany) and length was measured with a Seca infantometer (model 416, Seca, Hamburg, Germany). The same weight and length measurement scales were used for both the intervention and comparison groups (23, 24).

In both groups, socioeconomic and demographic data, maternal information, and maternal anthropometry data were collected at baseline. To measure and compare socioeconomic status between the intervention and control groups, we used the composite Water-sanitation-hygiene, Asset status, Maternal education status, and monthly Income index. This index was used in the MAL-ED study and details have been reported elsewhere (28).

The food intake and compliance data for the nutritional intervention (including data on breastfeeding) were collected daily throughout the intervention period (23). A quantitative 24-h recall approach to estimate nutrient and energy intakes from nonbreast milk complementary foods was carried out before and during intervention in a subset of children ($n = 45$). Dietary assessments by 24-h recall were performed twice: before onset of intervention and during intervention. Experienced research staff who were trained by experienced dieticians conducted the dietary assessments. To assist mothers to provide precise quantification of food intake by their children in the last 24 h, standardized measuring utensils and picture of foods with different portion sizes were used. These interviews were conducted without prior notification to mothers. The methods we used have been published elsewhere (15).

Reported daily morbidity data were available for both the intervention and comparison groups. Diarrhea was defined as passage of ≥ 3 loose stools in 24 h, and fever was defined as an axillary temperature of $>99°F$ ($37.2°C$) by a mercury thermometer. In the intervention group, morbidity data were collected during daily feeding sessions, and in the comparison group, the morbidity data were collected through twice-weekly home visits (24). As a measure of environmental enteric dysfunction (EED), stool neopterin (GenWay Biotech), myeloperoxidase (Alpco), and α-1 antitrypsin (Biovendor) were assayed with use of commercial ELISA kits. The laboratory methodologies for the assays have already been published (29, 30). In this paper, we used baseline biomarker results for both the intervention and comparison groups.

Quality control of anthropometric data and refresher training.

Anthropometric measurements were conducted by trained field staff following established standard operating procedures. To maintain the precision of the measurements, 5% of the participants were measured a second time within 24 h of each data collection. The measuring equipment was calibrated daily according to the manufacturers' instructions. To ensure the consistency of an anthropometric measurement from 1 rater to another, refresher training was provided on a regular basis to the field staff and the intra-class correlation coefficient was determined every 3 mo. Such training resulted in significant improvement of raters pertaining to anthropometric measurements with a coefficient >0.9 for each of the scales.

Data analysis

Statistical analyses were performed using STATA (Version 13.1, StataCorp). Statistical significance was defined as $P < 0.05$. The distribution of LAZ was checked for normality with a histogram, QQ plot, and tests for kurtosis and skewness. We compared baseline characteristics between intervention groups and comparison group using Student's t tests, Pearson chi-square tests, and Mann-Whitney U tests, as appropriate. Both parametric and nonparametric approaches were used for analyses and reported as medians and IQR, or mean and SD (31).

Standard biostatistical modeling techniques were used, including review and stabilization of the variance of the outcome distributions, identification of outliers, and inclusion of confounders in the models. Anthropometry data were collected at 5 time points: at baseline before the beginning of the intervention, at time-1, at time-2, at time-3, and after completion of the intervention. The comparison group data were collected every 30 d and the intervention group data were collected every 28 d. The change in LAZ before and after the interventions was compared within each group and between the groups. The difference-in-difference (DID) analysis technique, a quasi-experimental design that uses data from intervention and control groups to make a counterfactual to estimate the intervention effect, was used to measure the true effect of nutritional intervention on LAZ with the following formula:

$$DID : [(B - A) - (D - C)] \tag{1}$$

where, A = baseline mean LAZ of intervention group, B = endline mean LAZ of intervention group, C = baseline mean LAZ of comparison group, and D = endline mean LAZ of comparison group.

To assess the true effect of nutritional intervention, we used a regression model with a generalized estimating equation as follows:

$$Y_{it} = \beta_0 + \beta_1 Time + \beta_2 Group + \delta (Time \times Group) + \beta_3 X + \varepsilon \tag{2}$$

where, Y_{it} = outcome variable of interest for individual i at time t, Time = (1) if endline and (0) if baseline, Group = (1) if intervention group and (0) if comparison group, δ = the effect of nutritional intervention, X = other covariates, and ε = error term.

An additional analysis was done similarly where BEED stunted children were compared with MAL-ED stunted children, and BEED at risk of stunting children were compared with MAL-ED at risk of stunting children.

For dietary assessment, 24-h dietary recall data were converted to nutrients using available food composition tables. Details on these food composition tables have been published elsewhere (15). We have calculated nutrient adequacy ratio, which is the ratio of average daily intake and the RDA (15). We used the age-specific RDA from the recommendation developed by the Institute of Medicine, USA (32, 33). We have reported consumption of energy and nutrients as the percentage of RDA, which is also the percentage of nutrient adequacy ratio. Differences between energy and nutrient intakes before and during intervention were tested with use of a sign test, and differences between energy and nutrient intakes between breastfed and nonbreastfed children were tested with a Mann-Whitney U test.

Ethical approval

Ethical approvals was obtained from the Institutional Review Board of icddr,b (protocol no: PR-16,007; Version 1.03; March 1, 2016).

Results

A total of 1287 children with LAZ <1 were screened for BEED, of which 616 children were stunted and 670 children were at risk of stunting. A total of 761 children did not meet the inclusion/exclusion criteria and 525 children were enrolled in the study. Among 761 children who were screened out following exclusion criteria, 74% of parents did not sign the consent, 18% of children had severe anemia (Hb <8 g/dL), 5% of children were suffering from illness, and 3% had developmental delays or congenital anomalies. The parents did not sign the
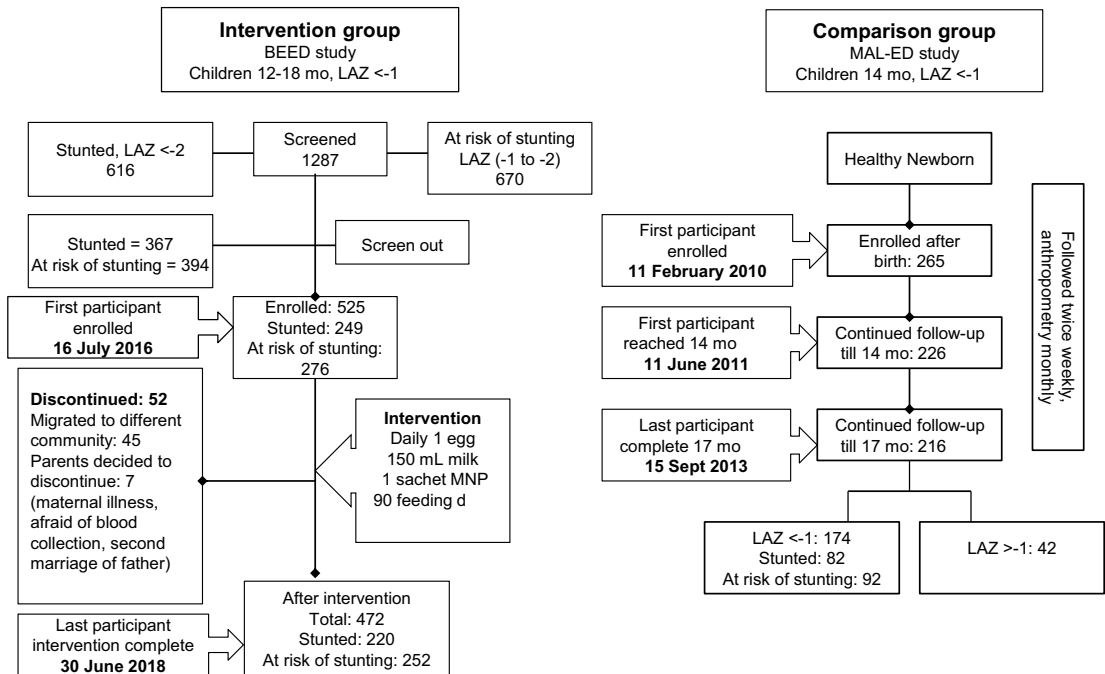
FIGURE 1 Flow diagram of study. BEED, Bangladesh Environmental Enteric Dysfunction study; LAZ, length-for-age *z* score; MAL-ED, Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health; MNP, micronutrient powder.

consent forms for the following reasons: 129 simply refused to enroll their children, 259 refused to provide blood samples, 152 had plans to migrate out from the community, and 3 were working parents. Complete data were available for 472 children (220 stunted children and 252 children at risk of stunting). Fifty-two participants failed to complete the intervention. Of these, 45 migrated out from the community with their family and 7 were withdrawn from the study by their parents for other reasons. The comparison group had complete data for 209 children, collected between the ages of 14 mo to 17 mo of which 174 children had LAZ <1. The enrollment scheme is shown in **Figure 1**.

At baseline, the median age of children from both groups was 14 mo, and baseline anthropometry including LAZ was comparable between intervention and comparison groups. Stool α-1 antitrypsin and myeloperoxidase concentrations were significantly higher in comparison children compared to intervention, and concentration of neopterin was higher in intervention children than the children from comparison group. Baseline characteristics are described in Table 2.

To achieve directly observed nutrition intervention in the nutrition centers for 90 feeding d, the children were followed for a median (IQR) of 108 (106, 112) d. The average (mean ± SD) number of visits to the nutrition centers made by the stunted children was 89.9 ± 0.18 d, and for the at risk children, it was 86.8 ± 1.2 d. Children were offered daily a boiled egg with a mean (SD) shell-less weight of 56.4 ± 0.9 g and the average egg consumptions of the stunted and at risk of stunting children were 54.7 ± 2.9 g and 54.9 ± 2.9 g, respectively. Compliance of egg consumption was 97% and compliance of milk consumption was 99% for both the groups (**Table 3**).

Table 4 describes the energy and nutrients provided by each component of the nutritional intervention and the percentage

of the RDA provided by the intervention for this age group (32, 33). One egg, 150 mL of milk, and 1 sachet of micronutrient powder provided 33% of the required kcal of energy, 92% RDA of protein, 6% RDA of carbohydrate, 40% RDA of calcium, 188% RDA of iron, and 206% RDA of zinc.

We also collected quantitative dietary intake data of the children before and during nutrition intervention using the 24-h dietary recall approach (**Table 5**). We observed that before intervention the dietary intakes for energy, protein, carbohydrate, vitamins, minerals, and trace elements were much lower than the age-specific RDAs. On the other hand, dietary intake data collected during nutrition intervention showed a marked improvement of intakes for energy, protein, iron, zinc, vitamins, and other trace elements (Table 5). At baseline, before the start of the intervention, 91% of children were breastfed, and at the end of intervention, still 88% children continued to breastfeed (**Supplemental Table 1**). We also compared nutrient intakes between the breastfed and nonbreastfed children.

TABLE 3 Compliance of daily milk and egg consumption in children of intervention group[1]

Indicators	Values ($n = 472$)
Egg offered, g	56.4 ± 0.96
Egg consumed, g	54.8 ± 2.95
Egg consumption compliance, %[2]	97.2 ± 4.78
Milk offered, mL	150 ± 0
Milk consumed, mL	148 ± 2.78
Milk consumption compliance, %[2]	98.8 ± 1.86

[1]All values are means ± SDs.
[2]Percentage of egg or milk consumption compliance calculated by [(consumed/offered) × 100].

TABLE 4 Nutritional content and estimated percentage of RDA of each component of nutrition intervention provided to the children of the intervention group[1]

	RDA	Egg (56 g)	Milk (150 g)	MNP (1 g)	Total intake	% of RDA
Energy, kcal/d	548	86.8	92.6	0.00	179	32.7
Protein, g/d	13	7.04	4.97	0.00	12.0	92.4
Fat, g/d	ND	5.94	5.02	0.00	10.9	—
Carbohydrate, g/d	130	0.63	6.98	0.00	7.60	5.85
Calcium, mg/d	500	28.0	175	0.00	203	40.6
Iron, mg/d	7	0.67	0.05	12.5	13.2	189
Zinc, mg/d	3	0.59	0.62	5.00	6.21	207
Copper, mg/d	0.34	0.01	0.02	0.00	0.03	7.50
Vitamin C, mg/d	15	0.00	0.00	30.0	30.0	200
Thiamin, mg/d	0.5	0.04	0.06	0.00	0.10	19.6
Riboflavin, mg/d	0.5	0.29	0.28	0.00	0.57	114
Niacin, mg/d	6	0.04	0.17	0.00	0.20	3.35
Vitamin B-6, mg/d	0.5	0.07	0.05	0.00	0.12	23.6
Folate, μg/d	150	24.6	6.56	160	191	128
Vitamin B-12, μg/d	0.9	0.62	0.54	0.00	1.16	129
Vitamin A, μg/d	300	94.6	43.2	300	438	146
Vitamin E, mg/d	6	0.58	0.09	0.00	0.67	11.2

[1] MNP, micronutrient powder; ND, no available data. RDA is the average daily dietary intake amount, sufficient to meet the nutrient requirements of nearly 97–98% healthy individuals in a group (32, 33).

Energy intake and carbohydrate consumption were significantly higher in nonbreastfed children than the breastfed children. All other estimated nutrients were comparable between the groups (**Supplemental Table 2**).

The mean LAZ of children in the intervention group before, during, and after nutritional intervention in comparison to children in the MAL-ED birth cohort for the same duration are illustrated in **Figure 2**. After adjusting for baseline LAZ, age, sex, mother's height, diarrhea, fever, cough, use of antibiotics, breastfeeding status, and the Water-sanitation-hygiene, Asset status, Maternal education status, and monthly Income index, in a multivariable regression model, DID analysis showed a positive change in LAZ (intervention compared with comparison: coefficient 0.23, 95% CI: 0.17, 0.28, $P < 0.001$) (**Table 6**). After categorizing the children in the intervention and comparison groups into those who were stunted and at risk of stunting, a subgroup DID analysis was performed between stunted children in the 2 groups and the children at risk of stunting in both groups. This analysis yielded similar results (stunted intervention compared with stunted comparison: coefficient 0.27, 95% CI: 0.18, 0.35, $P < 0.001$; at risk of stunting intervention compared with at risk of stunting comparison: coefficient 0.19, 95% CI: 0.12, 0.27, $P < 0.001$) (**Table 7**).

TABLE 5 Nutrient intake of children of intervention group before and during intervention estimated from quantitative 24-h recall data[1]

		Daily intake			% of RDA		
	RDA	Before intervention (n = 45)	During intervention (n = 45)	P value[2]	Before intervention (n = 45)	During intervention (n = 45)	P value[2]
Energy, kcal/d	548	396 (43.6, 852)	636 (255, 1120)	<0.001	72.2 (7.9, 155)	116 (46.5, 205)	<0.001
Protein, g/d	13	11.0 (0.6, 32.8)	24.3 (5.7, 46.9)	<0.001	84.8 (4.7, 252)	187 (44.0, 361)	<0.001
Fat, g/d	ND	9.7 (0.2, 36.7)	19.6 (5.7, 39.2)	<0.001	—	—	—
Carbohydrate, g/d	130	56.7 (9.7, 182)	88.1 (36.9, 157)	<0.001	43.6 (7.5, 140)	67.8 (28.4, 121)	<0.001
Calcium, mg/d	500	65.6 (3.7, 645)	258 (23.0, 995)	<0.001	13.1 (0.7, 129)	51.6 (4.61, 199)	<0.001
Iron, mg/d	7	2.2 (0.12, 8.00)	15.3 (13.8, 19.8)	<0.001	31.2 (1.8, 114)	219 (197, 283)	<0.001
Zinc, mg/d	3	1.3 (0.1, 3.89)	7.69 (5.7, 9.5)	<0.001	43.5 (2.7, 130)	256 (189, 316)	<0.001
Copper, mg/d	0.34	0.26 (0.03, 0.6)	0.38 (0.1, 0.8)	<0.001	77.4 (9.7, 201)	112 (32.5, 249)	<0.001
Vitamin C, mg/d	15	6.9 (0.1, 78.2)	40.0 (30.0, 318)	<0.001	46.3 (0.8, 521)	267 (200, 2120)	<0.001
Thiamin, mg/d	0.5	0.17 (0.01, 0.8)	0.34 (0.1, 0.8)	<0.001	34.9 (0.3, 154)	67.7 (27.2, 171)	<0.001
Riboflavin, mg/d	0.5	0.31 (0.002, 1.5)	0.76 (0.1, 1.9)	<0.001	61.5 (0.4, 298)	153 (28.8, 381)	<0.001
Niacin, mg/d	6	3.46 (0.02, 9.8)	4.02 (0.5, 10.7)	0.016	57.6 (0.4, 164)	67.1 (8.04, 178)	0.016
Vitamin B-6, mg/d	0.5	0.4 (0.003, 0.9)	0.53 (0.2, 1.1)	<0.001	74.4 (0.8, 176)	106 (33.9, 212)	<0.001
Folate, μg/d	150	26.2 (0.3, 3)	225 (178, 378)	<0.001	17.5 (0.2, 204)	150 (119, 252)	<0.001
Vitamin B-12, μg/d	0.9	0.5 (0.00, 8.1)	1.49 (0.15, 5.5)	<0.001	59.4 (0.0, 900)	166 (16.1, 608)	<0.001
Vitamin A, μg/d	300	57.8 (0.4, 1820)	486 (307, 1380)	<0.001	19.3 (0.1, 607)	162 (102, 461)	<0.001
Vitamin E, mg/d	6	0.79 (0.005, 3.3)	1.30 (0.4, 7.06)	<0.001	13.2 (0.07, 54.7)	21.7 (7.4, 118)	<0.001

[1] All values are median (min, max). ND, no available data. (An RDA is the average daily dietary intake amount, sufficient to meet the nutrient requirements of nearly 97–98% healthy individuals in a group) (32, 33).
[2] Sign test was performed to test statistical difference.
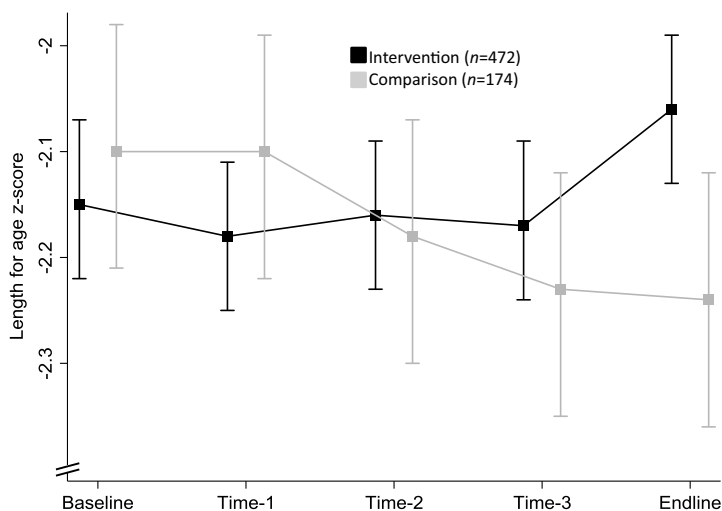
FIGURE 2 Length-for-age *z* score of children in intervention and comparison groups over study period with 95% CI. The comparison group data were collected every 30 d and the intervention group data were collected every 28 d.

We did not observe any allergic reactions or other adverse events related to milk and egg consumption.

Discussion

Our results indicate that stunted and at risk of stunting children who underwent daily dietary supplementation with 150 mL of milk and 1 egg for 90 d and 1 RDA of multiple micronutrient powder for 60 d had better length-for-age *z* scores. We are not aware of any prior study in which stunted children aged 12–18 mo were provided with egg, milk, and multiple micronutrient powder for 90 d to examine changes in LAZ. There are some studies involving intervention with egg, milk, or multiple micronutrient powder alone, although the age group and duration of intervention were not similar to our study (17, 19–22).

In the Lulun project, a randomized controlled trial conducted in Ecuador, 80 infants aged 6–9 mo were provided with 1 egg/d for 6 mo. Egg intervention increased LAZ by 0.63 compared to the control group (19). This is the only published study in which egg was given to young children to improve the linear growth. The effect size for LAZ in the Ecuador trial was substantially higher than any complementary feeding trial conducted earlier (19); the systematic reviews reported earlier showed that the experimental studies with supplementary foods for at least 6 mo among children aged <2 y in food insecure households was associated with a significant improvement in LAZ (standardized mean difference 0.39; 95% CI: 0.05, 0.73) (34, 35). In

our study, we observed a positive change in LAZ of 0.24 (95% CI: 0.19, 0.30) in children receiving intervention. It should also be noted that we provided nutrition intervention for only 90 d, every feeding session was directly observed and amounts of egg and milk offered and consumed by children were measured and recorded by research staff.

Egg is a nutritious food and an average 55 g egg contains 75 calories, 7 g protein, 5 g of fat, 1.6 g of saturated fat, vitamins, minerals, and carotenoids (20). Choline, an important precursor of phospholipid, abundant in eggs, is required for cell division and growth (20, 36, 37). In children aged 7–12 mo, an average 50 g egg provides 57% of RDA of protein, 98% Adequate Intake (AI) of choline, and 88% of AI of vitamin B-12, 25–50% of AI for vitamin B-6, folate, phosphorus, and around 20% of AI for zinc (20). In addition to its role in improving linear growth of young children, studies have shown that egg supplementation can improve choline and docosahexaenoic acid status, both are important for growth and neurocognitive development (20). Currently, there are no data on the acceptability of egg in Bangladeshi children. In this study, we observed excellent compliance with egg consumption among children and we did not encounter families that were hesitant to feed their children eggs.

Each 100 g of whole cow milk provides 64 kcal of energy and 3.5 g of protein (21). It also contains important micronutrients and bioactive components that likely contribute to its effects on growth. Studies conducted in developing countries have underscored the considerable benefits of cow milk in stimulating linear growth (21). Observational studies

TABLE 6 DID analysis of LAZ scores between children in intervention and comparison groups[1]

	n	Intervention	*n*	Comparison	DID	*P* value[2]
Baseline	472	−2.15 (−2.22, −2.07)	174	−2.10 (−2.21, −1.98)		
Endline	472	−2.06 (−2.13, −1.99)	174	−2.24 (−2.36, −2.12)	0.23 (0.18, 0.29)	<0.001

[1]All values are mean difference (95% CI) or DID (95% CI). DID, difference-in-difference; LAZ, length-for-age *z* score; WAMI, Water-sanitation-hygiene, Asset status, Maternal education status, and monthly Income index.
[2]Effect of intervention was adjusted for other possible covariates using generalized estimating equation. Adjusted covariates are: age, baseline LAZ, sex, breastfeeding status, mother height, diarrhea, fever, cough, antibiotic use, myeloperoxidase, neopterin, α-1 antitrypsin, and WAMI index. Baseline: before intervention, endline: after completion of intervention.

TABLE 7 Subgroup analyses of stunted and at risk of stunting children in intervention and comparison groups to examine changes in LAZ between the groups[1]

		n	Intervention	n	Comparison	DID	P value[2]
Stunted children	Baseline	220	−2.82 (−2.90, −2.73)	82	−2.71 (−2.85, −2.58)		
	Endline	220	−2.68 (−2.76, −2.59)	82	−2.84 (−2.98, −2.71)	0.27 (0.18, 0.35)	0.0001
Children at risk of stunting	Baseline	252	−1.56 (−1.59, −1.52)	92	−1.54 (−1.60, −1.49)		
	Endline	252	−1.52 (−1.57, −1.47)	92	−1.70 (−1.79, −1.61)	0.19 (0.12, 0.27)	0.0001

[1] All values are mean difference (95% CI) or DID (95% CI). DID, difference-in-difference; LAZ, length-for-age z score; WAMI, Water-sanitation-hygiene, Asset status, Maternal education status, and monthly Income index.
[2] Effect of intervention was adjusted for other possible covariates using generalized estimating equation. Adjusted covariates are: age, baseline LAZ, sex, breastfeeding status, mother's height, diarrhea, fever, cough, antibiotic use, myeloperoxidase, neopterin, α-1 antitrypsin, and WAMI index. Baseline: before intervention, endline: after completion of intervention.

have also demonstrated the growth-stimulating effect of milk in a nutrient-adequate population (21). Growth hormone is less important in early childhood, although its role in child growth starts as early as age 9 mo (38). Growth hormone and nutritional status regulate the synthesis of the insulin-like growth factor-1 (IGF-1) in the liver. In a cohort of stunted Zimbabwean children, amounts of IGF-1 were low and negatively correlated with markers of systemic inflammation (39). IGF-1 increases the uptake of amino acids which are incorporated into new protein including in bone tissue, thereby facilitating bone growth (21). IGF-1 is also involved in calcium and phosphate homeostasis (40), playing an essential role in bone remodeling (41). Hence, by increasing amounts of IGF-1, consumption of cow milk is expected to lead to an increase in linear growth velocity (22, 23).

Micronutrient deficiency, particularly iron, zinc, and vitamin A deficiency, is common in children in developing countries where growth faltering is also high. Zinc deficiency is associated with childhood stunting and supplementation of multiple micronutrient powder can reduce anemia and morbidity, and improve nutritional status (42). In addition to milk and egg, 1 sachet of multiple micronutrient powder containing the RDAs of vitamin A, vitamin C, folic acid, iron, and zinc was provided daily for 2 mo during nutrition intervention. The 24-h recall data indicate that intake of both macro- and micronutrients were improved through the intervention regimen.

Children in the comparison group from the MAL-ED study exhibited gradually worsening LAZ scores between the ages of 15 and 18 mo. The prevalence of stunting in the MAL-ED birth cohort was 18% at 6 mo and increased to 48% at 24 mo (27). In the absence of nutritional supplementation, this is a typical pattern of linear growth faltering in young children living in slum areas of Bangladesh. Nutritional supplementation with milk, eggs, and multiple micronutrient powder improved LAZ scores of children in the intervention group. Despite the comparatively short duration of intervention, this directly observed nutritional supplementation was effective.

Postnatal growth is characterized by an increased linear growth velocity that reduces gradually after birth. The velocity of linear growth of a healthy child is approximately 25 cm per year in the first year and 12 cm from 12 to 24 mo (43). This means that between 12 and 18 mo, a healthy child is expected to grow by 1 cm/mo. We observed that the mean ± SD length increment for stunted and at risk of stunting children were 3.63 ± 0.87 cm and 3.54 ± 0.94 cm, respectively after 90 d of intervention, a higher rate than that of healthy children. On the other hand, during the first year of life, the length velocity

is 2.1 cm/mo; this might be the reason why the Lulun trial observed a larger LAZ increase in children who were enrolled at age 6–9 mo.

EED biomarkers between the intervention and comparison groups showed mixed results. α-1 antitrypsin and myeloperoxidase were higher in the comparison group, which means MAL-ED children had more protein loss and intestinal inflammation (30, 44). On the other hand, children in the intervention group had higher concentrations of neopterin. Neopterin is a biomarker of intestinal inflammation and cellular immune activation (30, 44, 45). This also means that over the period of time children in the slums were similarly exposed to pathogens (46, 47).

The egg and milk supplementation provided here was accepted by the children and mothers as judged by the low dropout rates. There were no records of any adverse event, including allergic reactions associated with egg and milk consumption. There are limitations of this study. This was a nonrandomized trial with the absence of concurrent control group. Absence of randomization is prone to major biases and also known to be associated with both overestimation and underestimation of treatment effect (48, 49). The comparison data from the MAL-ED study, collected 3 y before the current study, can be a source of type 1 error and overestimation of treatment effect (50). No nutritional counselling was provided in the control group, thus preventing us from separating the effect of behavior change from the nutritional intervention itself. On the other hand, the duration of supplementation was 90 feeding d, which may be too short to assess the full potential of the intervention on linear growth. Moreover, we did not measure amounts of IGF-1, which may have provided insight regarding potential longer-term efficacy of treatment. Dietary assessment done from a subsample of 45 children might not be representative of all participants under intervention and absence of data on energy and nutrient intakes from breastfeeding is another limitation of this study. Considering cost and complexity of this intervention, and also compared to other research in the same area, the effect size seemed modest. Therefore, justification of this intervention at the programmatic level is subject to further investigation. The role of pathogen burden and its association with childhood stunting observed in the same population (27, 47) were also not addressed. Finally, the inability to identify which of the 3 components of the nutritional intervention was driving the change (or whether all three are required) adds to the cost of the treatment, which may in turn preclude its application in resource-limited programs.

In resource-poor settings with poor maternal education level and high burden of infectious morbidity, directly observed

nutrition therapy with locally available and culturally acceptable nutritious foods (cow milk and egg) with micronutrient supplementation improved linear growth of children aged <2 y. A randomized controlled trial that evaluates the same intervention provided for a longer duration with an additional intervention to reduce pathogen burden with post-intervention follow-up and appropriate control group(s) is warranted to test this hypothesis and further assess the durability of the response to treatment.

Acknowledgments

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Supplemental Table 1: Prevalence of breastfeeding among children of intervention and comparison group before and after intervention period[1]

	Intervention (n=472)			Comparison (n=174)		
	Before	After	p-value[2]	Before	After	p-value[2]
Breastfeeding status	431 (91.31)	417 (88.35)	0.132	168 (96.55)	162 (93.10)	0.146

[1] All values are n(%)

[2] Proportion test

Supplemental Table 2: Energy and nutrient intake of children between breastfed and non-breastfed children estimated from quantitative 24-hour recall data[1]

	RDA	Breastfed (n=39)	Non-breastfed (n=6)	P value[2]
Energy, Kcal/d	548	396 (43.6, 852)	761 (313, 852)	0.012
Protein, gm/d	13	10.9 (0.6, 32.8)	14 (10.8, 29.7)	0.071
Fat, gm/d	ND	9.77 (0.22, 23.3)	11.7 (1.73, 36.7)	0.504
Carbohydrate, gm/d	130	52.0 (9.74, 111)	89.3 (32.29, 182)	0.042
Calcium, mg/d	500	58.7 (3.73, 394)	240 (29.7, 645)	0.161
Iron, mg/d	7	2.19 (0.13, 8.00)	2.40 (0.52, 4.02)	0.713
Zinc, mg/d	3	1.29 (0.08, 3.89)	1.66 (1.25, 3.56)	0.083
Copper, mg/d	0.34	0.26 (0.03, 0.68)	0.33 (0.12, 0.59)	0.404
Vitamin-C, mg/d	15	7.21 (0.12, 78.2)	5.13 (2.14, 10.4)	0.593
Thiamin, mg/d	0.5	0.17 (0.01, 0.46)	0.18 (0.03, 0.77)	0.463
Riboflavin, mg/d	0.5	0.30 (0.002, 1.49)	0.42 (0.03, 1.35)	0.483
Niacin, mg/d	6	3.03 (0.023, 9.82)	4.02 (0.52, 5.42)	0.815
Vitamin-B6, mg/d	0.5	0.35 (0.01, 0.88)	0.40 (0.04, 0.57)	0.841
Folate, µg/d	150	29.3 (0.34, 306)	21.2 (6.77, 64.9)	0.815
Vitamin-B12, µg/d	0.9	0.54 (0.00, 8.10)	0.74 (0.00, 2.49)	0.537
Vitamin-A, µg/d	300	55.4 (0.39, 1820)	82.0 (8.62, 253)	0.463
Vitamin-E, mg/d	6	0.79 (0.004, 3.28)	0.659 (0.05, 2.50)	0.92

[1] All values are median (min, max); RDA, Recommended dietary allowance (An RDA is the average daily dietary intake level; sufficient to meet the nutrient requirements of nearly 97–98% healthy individuals in a group)( ); ND, Not determined.

[2] Mann-Whitney U test was performed to test statistical difference