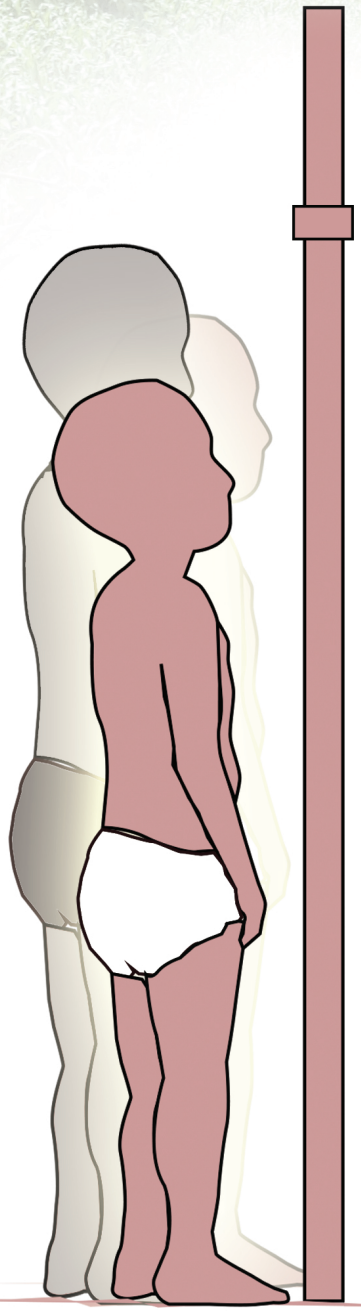
LINEAR GROWTH FAILURE OF ETHIOPIAN CHILDREN

the role of protein, zinc and mycotoxin intake



Masresha Tessema

Propositions

1. The lack of improvement in linear growth does not mean that introducing a biofortified crop is not important, as behavioral change should be sufficiently long to show impact.
   (this thesis)
2. Low quality protein and inadequate energy intake contribute to high linear growth failure in Ethiopia.
   (this thesis)
3. The present stunted economic progress in Ethiopia is the result of stunted children in the past.
4. Nutrition behavior change interventions alone without having food at the plate are a waste of time and resources.
5. The benefit of nutrition and medical research output in developing countries is much lower than its investment.
6. It is not what we publish, but what we translate into practice that benefits the planet.

Propositions belonging to the thesis, entitled

Linear Growth Failure of Ethiopian Children: the role of protein, zinc and mycotoxin intake

Masresha Tessema
Wageningen, 6 October 2020

Linear Growth Failure of Ethiopian Children

the role of protein, zinc and mycotoxin intake

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Linear Growth Failure of Ethiopian Children

the role of protein, zinc and mycotoxin intake

Masresha Tessema Anegago

Thesis

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Contents

Summary

Linear growth failure manifested as stunting is a major public health problem in developing countries. Stunting is often considered as an important marker of an adverse quality of population's life and child development. Over 90% of stunted children live in 10 developing countries, in Asia and Africa. Ethiopia is the country with the high burden of linear growth failure. In 2016, it was estimated that about 5 million children suffered from poor linear growth or stunting in Ethiopia. The prevalence of stunting in Ethiopia has been reduced from 52% in 2000 to 37% in 2019, however, the number of stunted children has increased by about 1 million in the same period. There is a high level of commitment to reducing stunting globally and nationally. Although the government of Ethiopia formulated ambitious goals to reduce stunting, the progress to reduce stunting in Ethiopia remains too slow partly due to the fact that the aetiology of linear growth failure is still poorly understood. Stunting or poor linear growth is caused by a diverse and complex interaction of household, environmental, socioeconomic and cultural influences, related to, amongst others, poor nutrition, infectious diseases, unfavorable prenatal conditions and genetic disorders. The aim of this thesis was to contribute to the understanding of the aetiology of poor linear growth and stunting in rural Ethiopia by studying the effect of household-level quality protein maize (QPM) promotion and consumption, and the role of protein, zinc, and mycotoxins intake on linear growth of Ethiopian children. The first chapter provides background information on the role of QPM, protein, and zinc (in soil and serum) in linear growth of children. Furthermore, the research questions were described in detail.

Chapter 2 describes a randomized controlled trial conducted in real practice in which households make their own decisions whether to adopt QPM, how much to adopt and cultivate, and whether and how to incorporate QPM into children's diets. The intervention had two components: a) nutrition-focused adoption encouragement and provision of free QPM seed (AE), and b) a consumption encouragement (CE) primarily targeting female caregivers and encouraging earmarking and integration of QPM into diets for infants and young children. Eligible children (n=873) aged 6-35 months at baseline were randomly assigned to 3 groups: a first intervention group receiving AE only; a second

intervention group receiving both AE and CE; and a control group. We hypothesized that promotion and consumption of QPM could improve the protein and amino acids status, which could, in turn, improve linear growth of children. Children consumed QPM based foods on average 4 days per week, while non-QPM based foods were consumed mostly. In addition, the quantitative intake of QPM was low (27 gram per day) contributing to only 5% of their total protein, 12% of lysine and 15% of tryptophan intakes, compared with conventional maize (80 gram per day) contributing to 16%, 9%, 13% of protein, lysine and tryptophan intakes respectively. Encouragement to adopt and feed QPM to infants and young children in a real-life setting had no effect on children's protein biomarkers (p> 0.05) or linear growth (p> 0.05). Further evaluation of multi-year interventions is needed to understand how biofortified crops promoted at scale could change behavior and increase intakes at the household level which in turn improve biomarkers and outcomes in target populations.

In chapter 3, we performed a cross-sectional analysis using baseline data of the QPM intervention study conducted in chapter 2. We investigated the association between protein intake, and protein and amino acids status with linear growth of children. The results indicated that protein intake (b=0.01, p=0.01) and protein status (b=2.58, p=0.04) as well as tryptophan intake/status (p<0.05) were positively associated with linear growth of children. Furthermore, most children had low energy intake (76%) coupled with high intestinal parasites (48%) and inflammation (35%). Also, protein and amino acids status were negatively correlated with inflammation, which suggests that the current requirement of protein and amino acids may not be adequate for children with low food intake or low energy intake and infection in Ethiopia. Linear growth failure in Ethiopian children is likely associated with low-quality protein intake and inadequate energy intake. Nutrition programs that emphasize improved protein quantity and quality and energy intake may enhance linear growth of young children.

In chapter 4, we assessed exposure to aflatoxins and fumonisins measured in serum in two seasons, post-harvest and pre-harvest, and we also assessed mechanisms through which linear growth of children was affected. Children (n=873) 6-35 months old were enrolled in an intervention trial on quality protein maize consumption in rural Ethiopia as described in chapter 2. These children were stratified by baseline stunting status, and 102 children (50 stunted and 52 non-stunted) were

randomly selected for this sub-study. Blood samples were collected during pre-harvest (August-September 2015) and post-harvest (February 2016) season. In the pre-harvest season, the proportions of children exposed to AFG1 (8%), AFG2 (33%) and AFM1 (7%) were higher than in the post-harvest season (4%, 28% and 4%, respectively). Likewise, the proportion of children exposed to any aflatoxin was higher in the pre-harvest than in the post-harvest season (51% vs. 41%). Exposure to fumonisins ranged from 0-11%, depending on the type of fumonisins. Exposure to any aflatoxin was not associated with inflammation (p>0.05), serum transthyretin (p >0.05) or serum IGF-1 (p >0.05), nor with linear growth (p >0.05) after adjusting for potential confounders. Our study revealed that exposure to most aflatoxins was high in pre-harvest season. Good practices in both post-harvest (to reduce accumulation of aflatoxins) and pre-harvest (to reduce aflatoxin levels) are needed for preventing contamination of aflatoxin. The mechanism in which aflatoxin affects linear growth of children is not clear. Aflatoxins are carcinogenic properties and the current exposure is a major public health problem that warrants intervention. Future studies on mechanisms between aflatoxin exposure and linear growth and sources of exposure with large sample size needed. In addition, future research is also needed on the complex and interacting pathophysiology of multiple mycotoxins and exposure management.

In chapter 5, we use data from the cross-sectional, nationally representative Ethiopian National Micronutrient Survey (n=1776), which provided anthropometric and serum zinc (n=1171) data on children aged 6–59 months. Data on soil zinc levels were extracted for each child from the Africa Soil Information Service. With these data, we assessed the geographic distribution of poor soil zinc, poor zinc status and growth faltering at the national level. Zinc deficiency in soil was prevalent (20%) at the national level, with a higher prevalence in low land of Ethiopia (87%). Nationally, one in four children was zinc deficient, as measured by serum zinc level. High zinc in agricultural soils was positively associated with zinc status (b=0.9, p=0.02), however, linear growth of children was not associated with soil zinc or serum zinc. The findings from our study suggest that agricultural biofortification of zinc could be an alternative strategy for reducing zinc deficiency in developing countries. In Ethiopia most households consume food that comes from own production, however, crop production on zinc-deficient soils and its effect

on human health has not yet been studied. Therefore, a future longitudinal experimental study on the effects of soil zinc application on crop zinc content and human serum zinc levels will help to elucidate this relationship. The phytate content of foods may affect zinc bioavailability. Future research is also needed on the effect of phytate on zinc bioavailability of crops grown on zinc-deficient soils.

Finally, chapter 6 discusses the main findings, and the internal and external validity of the studies addressed in this thesis. Furthermore, the public health perspective including recommendations for possible future research is presented. Overall, we can conclude that low protein (of low quality) intake, high prevalence of zinc deficiency and high exposure to multiple aflatoxins are public health problems in Ethiopia. Linear growth of children is positively associated with protein intake, energy intake, as well as protein status, but not with zinc soil levels, zinc status or multiple aflatoxin exposure. Our study has demonstrated that the implementation of QPM in real life had no effect on the protein and amino acids status nor on linear growth of children. Therefore, in our study and also in other nutrition intervention programs, measuring intermediate indicators as outcomes of improved linear growth may be a more feasible approach than measuring linear growth or stunting.

Chapter 1

General Introduction

Background

Poor linear growth of children under five years of age manifested as stunting is one of the most serious developmental obstacles [1-3]. Evidence has shown that linear growth is effected by a process named endochondral ossification in which the cartilage in the epiphyseal growth plate proliferates and grows, and is replaced by bone [4]. Stunting is often considered as the marker of an adverse quality of population's life and child development [5-7]. Globally, it is estimated that about 1491 million children under five years old were stunted in 2019 [8]. Over 90% of stunted children live in ten developing countries in, Asia and Africa [1, 8]. Linear growth faltering is a failure to reach one's linear growth potential, which may (but does not have to) lead to stunting it is defined as two standard deviations or more below the mean height-for-age when compared with the World Health Organization (WHO) growth chart [9]. Evidence has shown that the number of stunted children is much lower than the number of children who have linear growth failure [10]. Ethiopia has a high burden of linear growth failure [11]. In 2016, it was estimated that about 5 million children were stunted in Ethiopia [1, 11]. Stunting prevalence in Ethiopia has been reduced from 52% in 2000 to 37% in 2019, however, the number of stunted children has increased by about 1 million in the same period [11].

Stunting is associated with several important negative individual and societal consequences [5, 12]. Stunted children have a higher risk of morbidity, mortality, and delayed motor and cognitive development [3]. These effects may continue into adulthood and may also contribute to loss of intellectual performance, work capacity, life expectancy, and reproductive outcomes of adults [12]. There is a high level of commitment to reducing stunting globally, as expressed in the UN Decade of Action on Nutrition 2016–2025 and the Sustainable Development Goals (SDGs) [1]. The World Health Assembly and the UN target is to reduce globally by 40% the number of stunted children under five years of age by the year 2025 [2]. The government of Ethiopia has also formulated an ambitious goal to reduce stunting. The Seqota Declaration aims to reach zero stunting by 2030; the Growth and Transformation Plan (GTP) and

the National Nutrition Program (NNP) has set a goal to reduce stunting by 26% in 2020 [13]. However, progress in reducing stunting in Ethiopia remains too slow to achieve these goals due to the fact that the aetiology of linear growth failure is still poorly understood [14].

Stunting or poor linear growth is caused by a diverse and complex interaction of household, environmental, socioeconomic and cultural influences [9, 15, 16], related to, amongst others, poor or inappropriate nutrition, infectious diseases, unfavorable prenatal conditions and genetic disorders [3, 15]. A myriad of interventions has been implemented with varying success in reducing stunting. Nutrition specific programmes such as micronutrient and diet-based interventions have had little effect on linear growth of children [17, 18], while lipid-based supplementation seems to be more successful [19]. Nutrition-sensitive interventions proved to have shown a positive effect on dietary intake and nutrition outcomes, especially when combined with communication aimed at changing behavior, empowerment of women, improvement of water, sanitation and hygiene, and micronutrient fortified products, but their impact on stunting appeared hard to achieve [20]. Although the earlier emphasis on protein malnutrition waned in the seventies due to poor understanding of the role of the 'protein gap' in linear growth in the presence of energy deficiency, it has recently become apparent that quality protein and essential amino acids are missing in the diet [21]. It is still poorly understood what consequences protein malnutrition has for child growth and stunting [22]. Exposure to mycotoxins, especially aflatoxin, poses a major health problem in many developing countries, but its assumed role in linear growth faltering is still unclear [23]. Zinc is known to play an important role in growth and development [18, 24] and the association between zinc in the soil where foods grow, and the zinc status of children consuming these foods, has not yet been established.

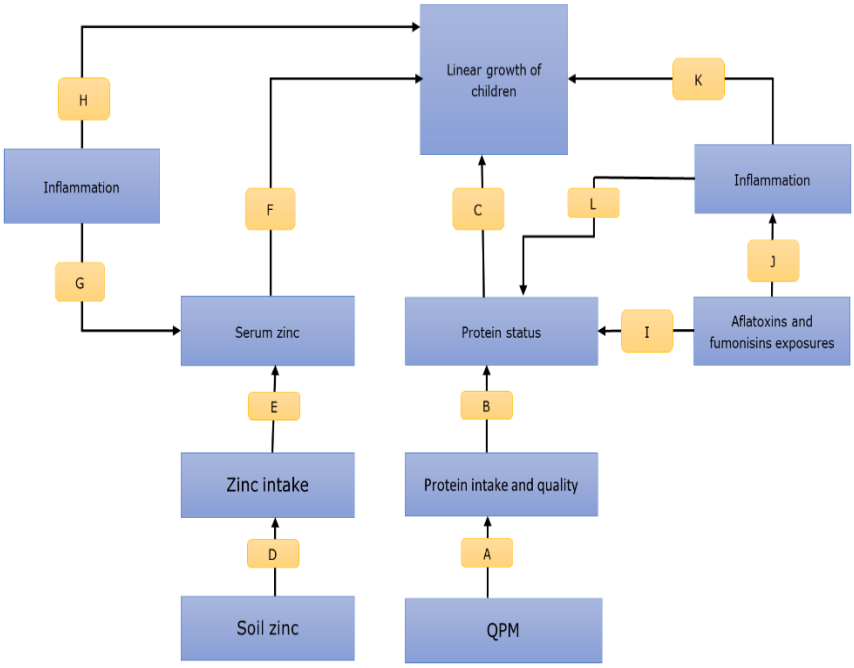

Fig.1. Conceptual framework showing the aetiology of poor linear growth: the roles of quality protein maize, protein, soil zinc, serum zinc and mycotoxin exposure on linear growth of children in Ethiopia.

Legend: A, B and C: Protein and amino acids play a biological role in linear growth of children [4, 25]. Consumption of QPM could improve quality protein intake, thereby improving protein and amino acids status, which may, in turn, improve linear growth of children [26]. D, E and F: Low soil zinc may lower grain zinc levels, thereby reducing dietary zinc intake, resulting in a higher prevalence of linear growth failure [27-29]. I and C: Exposure to aflatoxins and fumonisins may inhibit protein synthesis which in turn reduces the growth [30]. J, K and H: Exposure to aflatoxins and fumonisins may increase infection and inflammation which in turn reduces growth of children [30]. Inflammation may affect linear growth of children [14]. G and L: Inflammation may also affect zinc status [31, 32] and protein status [33-35].

In this thesis, we have addressed the above knowledge gaps through studying the role of quality protein maize (QPM), protein, soil zinc, serum zinc and mycotoxin exposure on linear growth of children in Ethiopia. Fig.1 shows the conceptual framework used and the pathways studied as explained in the following sections.

Protein, quality protein maize and linear growth of children

Protein is the major functional and structural component of all the cells which are a chain of amino acids, the building blocks of proteins, connected together [36]. Protein and amino acids are signaling factors in several regulatory pathways and in growth regulation [4]. The main growth regulation pathway is through gene regulation and is related to cell fate and differentiation; it is sensitive to the availability of protein and amino acids to regulate the growth of bone, skeletal muscle, nervous system, immune effector cells, organ size, and whole-body energy balance [37]. Lysine and tryptophan are essential amino acids that are not synthesized by humans, and therefore need to be ingested through the diet. Unfortunately, they are also the most limiting essential amino acids in human diets, especially in areas where cereals products are the major staple foods [38, 39]. An earlier study showed that reduction of protein deficiency could contribute to growth through insulin-like growth factor-I (IGF-1) [40]. A recent systematic review in developing countries showed that protein-based interventions were more effective than other interventions in improving linear growth in children above two years of age in developing countries [41]. Other studies from China and Pakistan have shown that the consumption of lysine fortified complementary food had a positive effect on linear growth of children aged 5 to 14 years [42, 43]. Similarly, an observational prospective study in a developed country (Dutch children) has shown that high protein intake from animal-based foods was positively associated with linear growth of children [44]. Conversely, protein intake in developed countries exceeds recommendations, and high protein intake in early childhood might lead to a higher risk of obesity [44, 45]. On the other hand, protein intake in developing countries is often low in quantity and quality [46, 47]. Furthermore, most children in developing countries with protein deficiency were also energy deficient [46]. Inadequate intake of energy in animals leads to reduced protein status and may also be related to diversion of some protein intake to meet energy requirements [48]. The daily protein requirement for children aged six months to three years for increasing

body weight and growth ranges from 0.9 to 1.3 gram of protein per kilogram body weight depending on the child's age [36, 49]. Evidence shows that infection may increase the requirement of protein and lysine [50]. The protein and amino acid requirements for children who have frequent infections and low energy intake are poorly studied in developing countries [51-53]. In addition, the role of protein and amino acids in linear growth of children in the presence of infection and low energy intake is still poorly understood [22, 37, 38, 54].

Maize is produced and consumed by a large number of smallholder farmers in Ethiopia and contributes to household food security [55]. However, conventional maize has a low concentration of the essential amino acids lysine and tryptophan and is referred to as having low-quality protein. To address this problem, QPM has been developed through conventional breeding [56] into a biofortified crop with a high lysine and tryptophan contents [57]. An earlier study on the effect of QPM consumption on child nutrition status has shown that it had a significant positive effect on the weight and weight-for-age of children under five [58]. In addition, a meta-analysis of community-based studies on QPM found that consumption of QPM instead of conventional maize led to a 12% (95% CI: 7–18%) weight increase and a 9% (95% CI: 6–15%) height increase in children under five who were mildly or moderately malnourished [59]. Despite this effect, the mechanism of how QPM improves linear growth of children is still not understood. Furthermore, most of the studies were carried out in a fully controlled setting and little is known about QPM’s impact on children’s linear growth in a natural setting in which households make their own decisions as to whether or not to adopt QPM, how much to adopt and cultivate, and whether and to what extent to incorporate QPM into children’s diets. In this thesis, we investigated the effect of household level QPM promotion and consumption on protein status and linear growth of children in the natural setting (Fig.1. pathway A, B, C).

Aflatoxin and fumonisin exposure, and linear growth of children

Aflatoxins are dietary mycotoxins produced by the fungi Aspergillus flavus and Aspergillus parasiticus, which commonly infect food crops in warm climates [60]. Aflatoxins are naturally occurring human carcinogens [61]. The main sources of aflatoxins in humans are staple foods, such as maize, sorghum, and groundnuts, where poor harvesting and storage are common in developing countries [60, 62]. Drought stress, insect damage, high temperatures and high humidity, and poor storage can all contribute to a higher occurrence of the moulds [63]. It is estimated that over 4.5 billion people are at risk of exposure to aflatoxin in developing countries [64]. Exposure to aflatoxins poses adverse health effects [65], and there is concern that maize-based complementary foods for children among maize-consuming populations of Ethiopia may lead to high concurrent exposure to aflatoxins. A study on Ethiopia on child complementary foods for children showed that most complementary foods were contaminated but the level of exposure was low and only a few food samples exceeded the maximum allowed level [66]. Similarly, other studies also reported aflatoxin exposure in foods or feeds [66-68]. Most exposures were measured through assessing the aflatoxins present in the food consumed, but exposure as assessed in human serum has been poorly studied so far. Two techniques are commonly used in biological sampling: aflatoxin products in urine that indicate 24 hours of exposure, and aflatoxin compounds in serum which indicates exposure over weeks or months [63]. In Ethiopia, there is no information on exposure to multiple aflatoxins among young children.

Fumonisins, another mycotoxin, are produced by the fungi Fusarium verticillioides and Fusarium proliferatum species, which also commonly infect maize and maize-based products in warm climates worldwide [60]. Fumonisin B1 (FB1), Fumonisin B2 (FB2) and Fumonisin B3 (FB3) are the major naturally occurring human carcinogens as identified by the International Agency of Research on Cancer [69]. Among these, the most poisonous is FB1 [69]. FB2 is a deoxy analogue of FB1 and, is less abundant than FB1 but has an important toxicological effect.

FB3 is present in lower concentrations in the diet of humans and has lower toxicological significance [70]. The exposure to FB1 in food samples has been reported in many countries [60, 71-75]. However, exposure to fumonisins measured in human serum has been poorly studied [76]. In this thesis, we study the level of exposure to multiple aflatoxins and fumonisins measured in serum in rural Ethiopian children where maize is the staple food.

Linear growth faltering in developing countries has not been reduced by dietary interventions on their own, as anticipated [1]. Although the aetiology of linear growth faltering is poorly understood [9], exposure to aflatoxins is believed to contribute to growth retardation among children in developing countries. Evidence from animal models has shown that exposure to aflatoxins leads to immune suppression, which may increase susceptibility to infections and incidence of diarrhea [30, 60, 77]. Furthermore, studies using cell cultures and animal models have shown that exposure to aflatoxins damages the production of IGF-1 and inhibits the synthesis of proteins, which may result in growth faltering [30, 78]. However, this relationship between mycotoxin exposure and linear growth is not well understood in humans. In this thesis, we study the biological mechanism of how exposure to aflatoxins affects protein status and linear growth of children (Fig.1. pathway J, I, C and J, L, K).

Soil zinc, zinc status and linear growth of children

Zinc participates in human cell division and growth, enzymatic catalysis, and functional modification of membrane protein, and is part of gene-regulatory proteins and hormonal receptors [31, 79]. Zinc contributes to DNA and RNA synthesis, protein metabolism, and overall growth and development [79, 80]. Globally, it is estimated that one in five children are at risk of low zinc intake [81]. Zinc deficiency is a major public health problem in developing countries and it is estimated that there is a high prevalence of zinc deficiency in sub-Saharan Africa and South Asia, where over 40% of children were found to have a low zinc intake [81]. In those regions, zinc deficiency in children causes morbidity [18], and increases

the risk of infections by reducing the immune response to pathogens [82]. Nationally representative data on zinc status and zinc deficiency among children is lacking in Ethiopia; only some small location-specific cross-sectional studies have been conducted, which showed that zinc deficiency among children was highly prevalent [83-85].

Globally, zinc deficiency in soils and crop systems is also widespread [86]. Many plant species are affected by zinc deficiency in a wide range of soil types in most agricultural regions of the world [87]. Soil type has been shown to influence crop mineral composition in Malawi [88]. Maize is the cereal crop most susceptible to zinc deficiency, although wheat grown on calcareous soils and lowland rice on flooded soils are also highly prone to zinc deficiency [89]. The main reason for high zinc deficiency in humans in developing countries is low dietary zinc intake [31, 90]. The human body has no long-term storage system for zinc, so consistent dietary intake is needed [31]. Living on zinc deficient soils producing zinc deficient foods may limit the dietary zinc intake. To reduce zinc deficiency in soils, adding Zn to fertilizers is a common strategy that enhances plant growth and development, and also increases yield [91]. Zinc soil fertilizers, may represent an effective approach to biofortifying cereal grains with zinc, and if zinc fertilizers increase the concentration of zinc in cereal grain, consumption of this biofortified crop may improve zinc status [92]. Several studies have been conducted on the effect of zinc application on foliage or soil [29, 93]. Experiments with wheat showed that foliar application alone or in combination with soil application significantly increased grain zinc concentrations [93, 94]. For maize, a study in Zimbabwe showed increases in yield and grain zinc through the application of zinc fertilizer [95]. However, the association between soil zinc and human zinc status is not yet well understood. A study in India showed that low zinc intake was observed in people living in areas with zinc-deficient soils; however, there was no clear correlation with the serum zinc levels of adults and growth of children [96]. In this thesis, we analyze the geographic distribution of zinc deficiency in soil and children in Ethiopia. Furthermore, we study the relationships among soil zinc, serum zinc and linear growth of Ethiopian children at the national level (Fig.1. pathway D, E, F).

Rationale and objectives

Linear growth failure, a failure to reach one's linear growth potential that may lead to chronic undernutrition manifested as stunting is a major global health problem [9]. Linear growth failure and stunting among children under five years of age are most prevalent in sub-Saharan Africa. In Ethiopia, the prevalence of stunting among children under five years of age has declined in the last two decades (2000-2019); however, the prevalence is still unacceptably high and represents a major public health problem with negative short- and long-term consequences [3]. Currently, there is a high level of national commitment to address stunting in Ethiopia [2] and the government aims to end stunting among children under two years of age by 2030 as reflected in a commitment to the 'Seqota' Declaration [97]. This thesis aims to contribute to a better understanding of the aetiology of poor linear growth, by providing an insight into the effect of household-level QPM promotion and consumption on linear growth, and into the roles of protein, zinc, and mycotoxins (aflatoxins and fumonisins) in linear growth of children under five in rural Ethiopia. To achieve this overall aim, four specific questions were formulated:

- What is the effect of household-level QPM promotion and consumption on protein and amino acid status, and linear growth of children in a natural setting in which households make their own decisions?
- What are the relationships among protein intake, protein and amino acid status, and linear growth of children?
- What is the level of exposure to aflatoxins and fumonisins, and how is it associated with linear growth of children?
- What is the geographic distribution of poor soil zinc and serum zinc and what is the relationship with linear growth of children?

Outline of the thesis

To be able to reach our objective and answer our research questions, this thesis describes four sub-studies organized into six chapters. Chapter 1 is

a general introduction and explanation of key terms and conceptual framework used in the thesis. This chapter also provides background information for the sub-studies by describing the literature review, studies rationale and objectives. Chapter 2 explores the effect of household-level QPM promotion and consumption on protein and amino acids status, and linear growth of children. This randomized controlled trial (RCT) evaluates the effect on the protein status and linear growth of children in rural Ethiopia in a natural setting, of increasing household-level QPM production through encouraging adoption (AE) and increasing consumption through encouraging consumption (CE). Chapter 3 describes a study on the associations among the intake of protein, and especially of the essential amino acids tryptophan and lysine, serum levels of transthyretin (TTR), and IGF-1, with linear growth of Ethiopian children. Chapter 4 describes a study into exposure to multiple aflatoxins and fumonisins measured in serum in children. It studies whether high aflatoxin exposure is associated with low serum protein and IGF-1, with higher infection or inflammation and with lower linear growth of children. In chapter 5, we assess the geographical distribution of poor zinc soil, poor serum zinc status, and poor linear growth and their association among Ethiopian children. We also investigated whether a low soil zinc level was associated with a low grain zinc level in the diet and whether this was associated with a higher prevalence of linear growth failure among preschool children, mediated by lower serum zinc levels. Finally, in chapter 6 we summarize the main findings of the thesis and critically discuss theoretical, practical and methodological issues related to the studies described. Furthermore, this general discussion puts findings into a broader perspective and highlights implications for practice and directions for future research, ending with an overall conclusion.

Study setting and site selection

The studies described in chapters 2, 3, and 4 were superimposed upon the Nutritious Maize for Ethiopia (NuME) Project, which develops, promotes, and disseminates QPM varieties in the country's major maize-growing areas. NuME is a collaboration of the International Maize and Wheat Improvement Center with the Ethiopian Institute of Agricultural

Research, Sasakawa Global 2000, the Ethiopian Public Health Institute, and other national and international partners. Households in this study had at least one member who had been exposed to QPM varieties by attending field demonstrations organized by NuME.

Administratively, Ethiopia is divided into nine regional states, which are further divided into zones, then districts or *woredas*, and finally peasant associations or *kebeles. Kebeles* are the smallest official administrative units and comprise about 500 to 1000 or more households each. The NuME project was implemented over a five-year period, starting in 2012, in three agro-ecological zones (drought-prone, moist mid-altitude, and highland zones) where the impact was expected to be greatest, as identified by GIS analysis combining agro-climatic, nutritional and poverty databases [98].

Within the NuME project areas, the study team conducted extensive focus group discussions with more than 100 men and women in the Oromia and Amhara regions. Interviews with the women focused on existing child feeding habits (e.g., age of initiation to solid food, foods fed to young children, etc.), while interviews with the men focused on details of their planting seasons, including when and how choices of seed variety were made, as well as the general acceptance of and desire for QPM varieties. Based on the results of these focus group discussions, the study is being conducted in two zones of the Oromia region, where there is higher likelihood of potential impact. The entire Oromia region is a third of the total area of Ethiopia and has a population of 35 million people [99]. The average household size in the region is about five members. Agriculture is the primary economic activity of the region, engaging about 90% of the population, with home production used to meet a significant portion of household food needs.

The study area comprises one to two *kebeles* each from the *woredas* of Boneya Bushe, Gobu Seyo, Gudeya Billa, Guto Gida, and Sibu Sire in the East Wollega zone and two *kebeles* each of the *woredas* of Omo Nada and Mena from the Jimma zone. The 12 *kebeles* in total are in rural, maize-growing areas (Fig.2). The study described in chapter 5 was a secondary data analysis from the Ethiopian National Micronutrient

Survey. The survey was both national and sub-nationally representative (chapter 5, Fig.2).

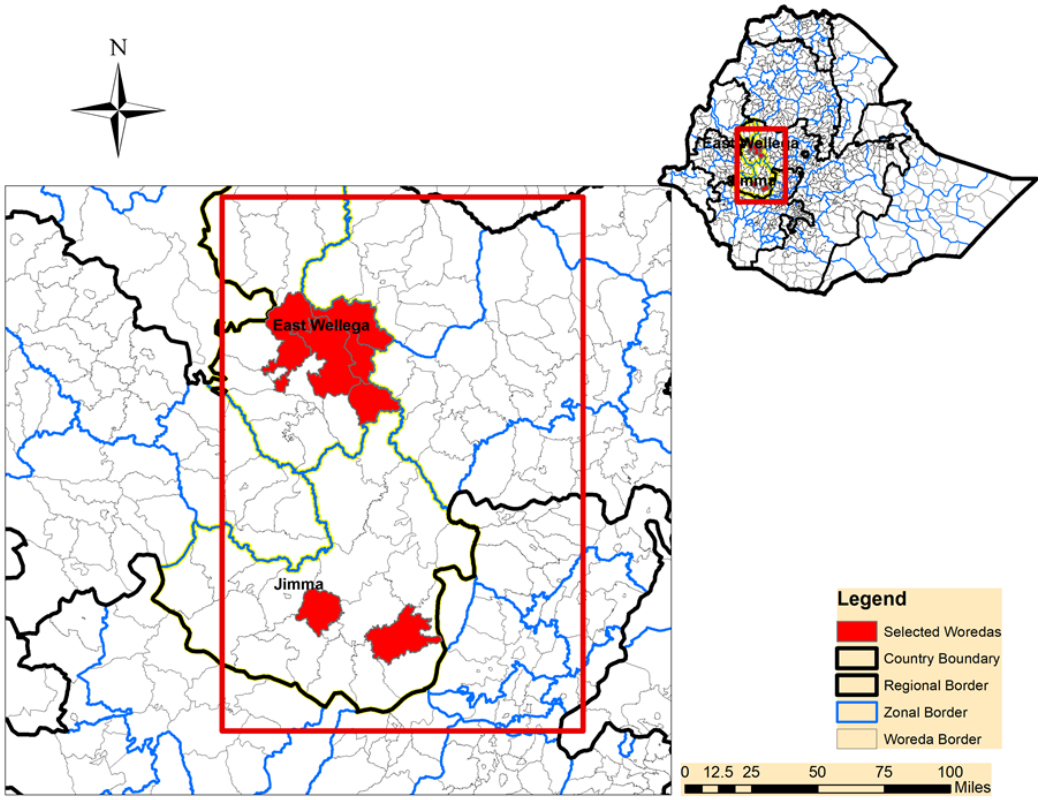

Fig. 2. Map of the study area for chapters 2, 3 and 4.

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Chapter 2

Effect of quality protein maize on protein status and linear growth of Ethiopian children: a randomized controlled trial

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Abstract

We aimed to assess the effect of promotion and consumption of quality protein maize (QPM, with increased lysine and tryptophan levels) on child protein status and linear growth in a real-life setting. A randomized controlled trial evaluated the effect of QPM promotion, production and consumption among Ethiopian children aged 6-36 months (n=873). The study had a control (no intervention) arm and two intervention arms: a) nutrition-focused adoption encouragement and provision of free QPM seed (AE), and b) AE plus a consumption encouragement (CE), primarily targeting female caregivers and encouraging earmarking and integration of QPM into the diets of infants and young children. Children's linear growth over the 12-month study was computed using length/height difference (HAD). Serum transthyretin (a protein biomarker) was determined using immunoturbidimetry. Serum amino acids and insulin-like growth factor-1 (IGF-1) were determined using Biochrom 30 amino acid analyzer and ELISA kits, respectively. Mixed linear regression models with unstructured covariance matrices based on intention-to-treat were used to assess the effect of the interventions on protein status and linear growth of children. The prevalence of stunting at baseline among AE, AE+CE, and control was 36%, 28%, and 22%, respectively. Inflammation (35%) and intestinal parasites (48%) were highly prevalent in the study population. There were no statistical differences between interventions and control in HAD (p=0.20) at baseline. The intake of QPM was low (27 gram/day) and most children were energy deficient (76%). No significant differences in change of intake of lysine or tryptophan, serum transthyretin, serum lysine, serum tryptophan, or IGF-1 were observed between participants who received interventions vs. control (p>0.05). The study found that both interventions, AE (p=0.10) and AE+CE (p=0.97), had no significant effect on linear growth of children. Encouragement to adopt and feed QPM to infants and young children in a real-life setting had no effect on children's protein status or linear growth. Inadequate QPM intake in the first year of adoption may have contributed to this finding. Multi-year interventions are needed to understand how biofortified crops promoted at scale in real-life settings could change intakes of target nutrients at the household level and in turn improve biomarkers and outcomes in target populations.

Background

Linear growth failure of children, manifested as stunting, is a major public health problem in developing countries [1]. Linear growth failure as a result of inadequate nutrition and infections is a risk factor for infant and child morbidity and mortality [2] Linear growth faltering is widespread among Ethiopian children [1]. The aetiology of linear growth failure is poorly understood, hampering the development of effective interventions [3,4].

The diet of Ethiopian children heavily depends on cereals such as teff and maize [5]; however, conventional maize has low levels of the essential amino acids lysine and tryptophan, resulting in poor protein quality and increased risk of inadequate intakes of utilizable protein and essential amino acids [6,7]. Quality protein maize (QPM), a biofortified crop with high levels of lysine and tryptophan, was therefore developed [8]. Despite previous research, knowledge gaps remain in assessing the potential of QPM to positively impact the nutritional status of children. Furthermore, little is known about QPM impact on children's nutritional status in a natural setting in which households make their own decisions whether to adopt QPM, how much to adopt and cultivate, and whether and how to incorporate QPM into children's diets.

QPM has shown positive effects on height- and weight-for-age in studies exerting control over children's diets [9-11]. While QPM is now being promoted in Ethiopia, no studies have analyzed the effect of QPM on protein status and linear growth of children in a real-life setting. Most prior interventions on QPM did not directly measure children's consumption of QPM or conventional maize, and all largely or exclusively relied on anthropometric outcomes, which are known to be affected by many factors beyond quality protein intake, hampering attribution despite using randomization [12]. Therefore, it is not possible to establish whether the provision of QPM led to children's consumption of a critical amount or whether consumption of QPM led to changes in protein or amino acid status, and, in turn, to improved growth. In this paper, we evaluate our hypothesis that household-level adoption encouragement (AE) and consumption encouragement (CE) of QPM in a real-life setting

will have an observable effect on biomarkers of protein (serum level of transthyretin) or amino acids (lysine and tryptophan), which in turn improve child linear growth.

Methods

Study design and site

We conducted a randomized controlled trial in rural Ethiopia, with two interventions related to household-level QPM production and consumption. The overall study had three intervention arms (Fig. 1): a control group that received no intervention, an intervention group that received household level AE ("Intervention 1"), and an intervention group that received both AE and CE ("Intervention 2"). Ethical approval was obtained from the Ethiopian Public Health Institute Scientific and Ethical Review Committee (SERO-006-02-2015) and the Harvard University Institutional Review Board (IRB14-3255). The trial was registered at ClinicalTrials.gov as NCT02710760 and AEA RCT Registry as AEARCTR # 0000786. Prior informed consent was obtained from children's parents.

The study areas and project descriptions were explained in detail elsewhere [13,14]. In brief, this study was superimposed upon the Nutritious Maize for Ethiopia (NuME) project, which develops, promotes, and disseminates QPM varieties in Ethiopia's major maize-growing areas. In the 12 selected *kebele/villages*, a list of households who participated in NuME field days and who were eligible for inclusion in the study was established with the help of the local administration, in particular, the *kebele*-level development agents, who are government extension officers for agriculture and rural development. A total of 1779 households were randomly assigned during screening to receive AE or no intervention, followed by confirmation of eligibility. Households assigned to AE and meeting eligibility criteria were further randomized to receive CE or no additional intervention.

Participants

Characteristics of the study population have been described in detail elsewhere [13,14]. In brief, the study population consisted of children who

were 6-35 months during recruitment in July-September 2015. The target age range excluded the first six months of life when exclusive breastfeeding is recommended but included the remaining critical first two years of life when children are particularly vulnerable to growth faltering and the third year when they are increasingly dependent on solid foods.

Households in the study area were eligible for inclusion if they met the following criteria: (1) the household had at least one child aged 6-35 months at recruitment in July-September 2015; (2) the household had at least one member who had attended a field demonstration of QPM conducted by the NuME project in November 2014-January 2015; and (3) the household provided informed consent to participate in the study. Households were excluded if (1) the primary caregiver or index child did not intend to remain in the study area for the study duration; (2) the household did not have access to land for crop cultivation in the main 2015 season; or (3) the household had previously produced QPM in an on-farm demonstration in the previous year. Additionally, households in the intervention groups were excluded if the primary caregiver for the target child was not in a 'one-to-five' group since this information was used for randomization between the two intervention groups. The one-to-five groups, formally called the Health Development Army (HDA), consist of about five women each and are formed to help Health Extension with an outreach of health and nutrition programming at the community level.

Procedures

Data and specimen collection were conducted at baseline (August-September 2015) prior to any green maize consumption, midline (February 2016) approximately four months after the grain harvest, and endline (June-August 2016) prior to the following harvest. Interviews with caregivers were conducted by trained enumerators using a pretested questionnaire, which was electronically administered with tablets using Open Data Kit (University of Washington, Seattle, WA, USA) software. Details about the data collection have been described elsewhere [13]. The length of younger children (6-23 months) was measured in a recumbent position to the nearest 0.1 cm using a measuring board designed by UNICEF (UNICEF Supply Division, Copenhagen, Denmark) with an upright wooden base and a movable headpiece. The height of children older than

23 months of age was measured in a standing position with the same measuring board, to the nearest 0.1 cm. The weight of children was measured with light clothing and without shoes to the nearest 100g using a standard UNICEF SECA 874 U digital scale (UNICEF Supply Division, Copenhagen, Denmark). The weight and height/length of the children were converted into Z-scores for height-for-age (HAZ) and weight-for-height (WHZ) according to 2006 WHO child growth standards using WHO Anthro software. Stunting was defined as LAZ or HAZ scores less than 2 standard deviations below median values. The height-for-age difference (HAD) was calculated as the difference between the actual height of a child and the median height of a child from the corresponding reference distribution; HAD is preferred to HAZ when assessing changes in height/length with age in a longitudinal study [15].

Venous blood samples were collected from children by trained phlebotomists. About 5 ml blood samples were collected from target children from the left arm as per blood collection protocol. Serum transthyretin, alpha-1-glycoprotein (AGP), and C-reactive protein (CRP) concentrations were determined by immunoturbidimetry using Cobas 6000 (Roche Diagnostics, GmbH, Mannheim, Germany) with fully automated clinical chemistry instruments. Inflammation was defined as having either elevated serum concentrations of CRP > 5.0 mg/L and/or AGP > 1.0 gram/L. Serum insulin-like growth factor-1 (IGF-1) concentrations were measured in duplicate using R&D Systems Quantikine Enzyme-linked Immunosorbent Assay (ELISA) kits (R&D Systems, Abingdon, UK) following the manufacturer's instructions. The analysis of serum amino acids (lysine and tryptophan) was conducted using a Biochrom 30 amino acid analyzer, and the method was based on ion exchange chromatography with post-column derivatization with ninhydrin, as described previously [16,17].

A quantitative 24 hours dietary recall and one-week food frequency questionnaires were used to estimate the amount of QPM-based foods consumed by children and the intake of total protein, lysine and tryptophan. The details of the data collection methods were described elsewhere [13].

The analyses of serum transthyretin, IGF-1, AGP, and CRP were conducted at the Ethiopian Public Health Institute laboratory, certified by the Ethiopian National Accreditation Office in accordance with the requirements of ISO 17025: 2005 and ISO 15189:2012. Serum amino acids were analyzed by Ansynth Service B.V., The Netherlands, a laboratory specializing in amino acids (http://www.ansynth.com/, Roosendaal, The Netherlands). The (inter-assay) Coefficients of variation (CV) for the various indicators were: serum transthyretin, 3.1%; IGF-1, 17%; AGP, 3.6%; CRP, 2.8%; and serum amino acids, 1.5%.

Randomization and masking

Randomization was conducted in two stages by a co-investigator at the Harvard T.H. Chan School of Public Health, Boston. In the first stage, households identified through the screening procedure were stratified by kebele (village), then individually randomized to the control (no intervention, one-third of households) or to receive AE (two-thirds of households). Households receiving AE were clustered based on community health group (HDA) membership; these clusters were randomized with equal probability to receive either CE or no further intervention. After confirmation of eligibility and informed consent, 873 households were enrolled across the three study arms (Fig. 1). Given the nature of the interventions, it was not possible to blind study participants, fieldworkers, or researchers. The sample size determination has been described elsewhere [14].

Intervention

Adoption encouragement

The study team, assisted by local development agents, visited households selected for the AE intervention in March–April 2015 and held a discussion with the head of the household and the caregiver for the household's young children, if she was available. This discussion focused on: (1) the nutritional benefits of QPM, compared to conventional maize varieties; (2) the special vulnerability children face regarding nutritional deficiency and malnutrition and that QPM could help mitigate these risks; (3) details

about the two varieties of QPM available – one, AMH760Q, has white grain and is late maturing and drought tolerant while the second, BHQPY545, has yellow grain, intermediate maturity, and drought tolerance; and (4) information about how QPM is similar to other maize varieties agronomically and for food preparation and consumption. After this discussion, the study team offered the option to order up to three 2-kg bags of QPM seed, emphasizing that the farmer had no obligation to order, but was also asked not to share the seed with anyone outside of his household if he did choose to place an order. If the farmer was interested, the study team took orders for QPM seed to be planted in the coming month. The seed was offered for free, but household heads were required to come to a central location to pick up the seed a few weeks later.

Prior to the intervention, we calculated that 2 kg of seed could yield enough grain to provide a target child with 150 gram of QPM per day for at least six months, with sufficient leftover grain for further household use; thus, this would be the target level of adoption for new adopters. This intervention was driven by the insight that while it is important for children to have nutritionally dense foods, they do not eat much food, particularly while they are also breastfeeding. Farmers are likely only willing to experiment with a small portion of their land when growing a new variety for the first time, but even growing a relatively small amount of QPM could impact their children's nutritional status.

Consumption encouragement

In the CE intervention, household heads and particularly caregivers for young children were offered (1) further guidance on the nutritional benefits of QPM for young children; (2) guidance on the importance of keeping QPM separate from conventional maize to prevent dilution of the nutritional benefits; and (3) tools to help them separate and 'earmark' QPM grain and flour for consumption by the child (Appendix 1, Fig.1a, Fig.1b and Fig.1c). The second component, guidance on QPM management, was based on recommendations by breeders and agronomists on production and utilization of QPM. The third component, tools for earmarking, was motivated by evidence from interventions in financial decision-making, which suggests that earmarking can have surprisingly large effects [18]. In this study, the CE intervention explored

the hypothesis that providing a way to separate nutritional resources (QPM) with a label with reference to children increases the quantity that reaches them.

The CE messages were presented during three different sessions over the study period: two one-on-one sessions during the baseline and midline surveys and one group session in between these surveys, prior to the harvest. The first CE message was offered at the household during the baseline survey in July-September 2015, immediately following data collection. The message was given prior to the availability of green maize in farmers' fields, at which point children may begin consuming QPM. During this visit, enumerators (1) discussed with the caregivers and heads of household the benefits of QPM relative to conventional maize and the special benefit young children receive from QPM consumption; (2) discouraged participants from selling QPM or feeding it to livestock; (3) discussed the importance of keeping QPM separate from other grains and flours; and (4) informed caregivers that they would be offered tools to help keep QPM grain and flour separate later in the year. Heads of household were encouraged to build separate cob storage cribs or to partition existing storage cribs, in order to keep their QPM separate from conventional maize while it was drying. The messages overall took less than 10 minutes. The second session was conducted in November 2015, prior to the grain harvest. Caregivers in the CE intervention group were invited to participate in a group meeting at a nearby location, e.g., a health extension post or a farmer training center. During this visit, enumerators used an educational poster to re-emphasize messages that had been presented earlier and engaged participants in a group dialogue to help identify ways to better target QPM to their young children. When participants identified aspects that might be difficult (e.g., cooking separate meals for their young children), the enumerator facilitated a group discussion to help them think of ways to overcome these challenges. At the end of the visit, caregivers were offered several tools to help them separate QPM grain and flour from other grains and flours and to remember to do so. Each caregiver was given four standard bags for storing grain (each capable of holding 100 kg), 1 bag for storing flour (capable of holding 50 kg), and a bowl and spoon for feeding the selected child (Appendix 1, Fig.1). All of these items were marked with a colorful

label that had a picture of an infant eating and images of white and yellow QPM named in the local language [14]. Additionally, each caregiver was given a poster (60 cm x 41 cm) displaying complementary foods that could be made with QPM. Overall, the group events took 30-35 minutes. Caregivers who were unable to attend a group session received a one-on-one session and all materials in their homes. In the third session, the CE educational messages were re-emphasized for caregivers for a final time during the midline survey, immediately after data collection. Enumerators reviewed a short set of key messages, focusing on the benefits of QPM consumption and targeting QPM-based foods for young children.

Outcomes

The primary outcome was HAD. Secondary outcomes included serum transthyretin, serum IGF-1, serum lysine, serum tryptophan, daily protein intake per body weight, daily lysine intake per body weight, daily tryptophan intake per body weight, and frequency of QPM intake in the past 7 days. All outcomes were measured at each time point, except nutrients intakes and status (protein, lysine, and tryptophan), which were measured only at baseline and midline.

Statistical methods

Statistical analyses followed the intention-to-treat principle and were conducted in accordance with the predefined statistical analysis plan using SAS versions 9.3 and 9.4 (SAS Institute, Cary, North Carolina, USA). We assessed the intervention effects by fitting linear mixed models to continuous dependent variables using the procedure "mixed" and to categorical dependent variables using the procedure "glimmix". All models controlled for stratification by kebele (village), clustering within HDA groups (assuming an exchangeable covariance structure), and repeated measures on children over time (assuming a first-order autoregressive covariance structure). Following the intention-to-treat analyses, additional models including a limited set of covariates (e.g., child age and sex) were estimated. All statistical tests were two-sided and used a significance level of 0.05.

Results

Baseline characteristics, by intervention group

Of 1779 households screened, 873 were enrolled (Fig. 1). Among participating children, 6% did not complete follow-up, most commonly because the household moved out of the study area (4%) or withdrew from the study (2%), while one child died. The baseline characteristics of the study sample showed that the study arms were largely balanced for most or important variables (Table 1). However, children in the AE + CE group were slightly older and taller.

The prevalence of stunting was higher in the AE group (36%) compare with AE + CE (28%) and control (23%). At enrollment, children were on average about 20 months old. We noted no significant difference between interventions and control for all biomarkers. Acute or chronic inflammation was prevalent in more than one-third of children (37%). Demographic and socioeconomic characteristics were balanced between groups. Most caregivers (65%) had no formal education. Most children (78%) had a daily energy intake below the estimated average requirement.

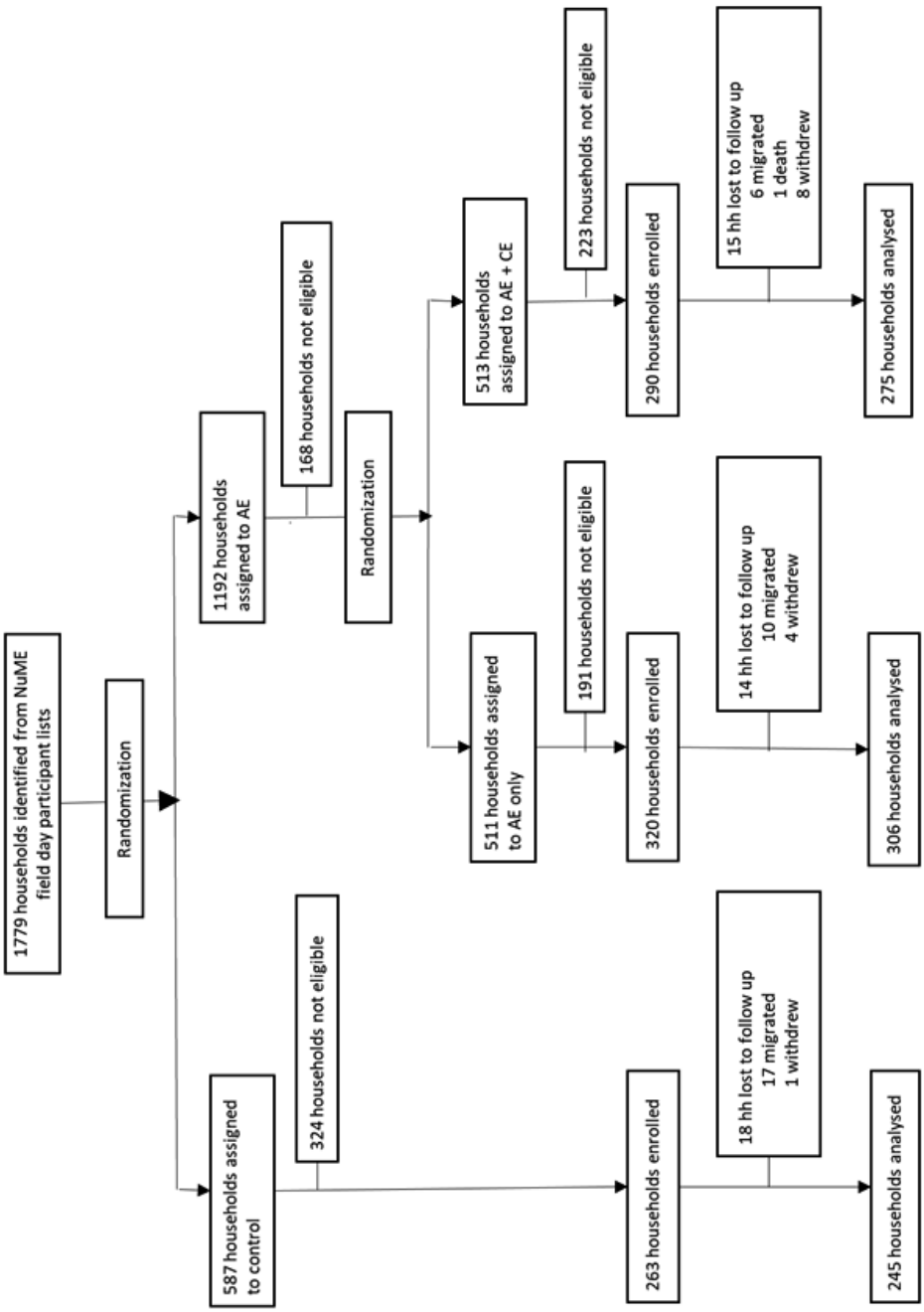

Fig. **1.** Trial profile

Effect of intervention on linear growth

We found the interventions had no significant effect on linear growth (HAD, HAZ, or height/length), and this remained so after adjustment for child age and sex (Table 2). Further, the interaction of interventions with sex and age had no effect on the linear growth of children. We conducted separate sub-group analyses for children with and without energy deficiency or inflammation (Appendix 2) and similarly found no significant intervention effects on linear growth.

Effect of intervention on secondary outcomes

Results show that the interventions had no significant effect on the protein biomarker, serum transthyretin (Table 3, Model 1). Inflammation (AGP and CRP) was significantly and negatively associated with serum transthyretin. Furthermore, we examined the effect of an intervention on other secondary outcomes: serum lysine, serum tryptophan, and serum IGF-1 (Table 4). We found that the interventions also had no significant effect on any of these outcomes. Inflammation biomarkers (AGP and CRP) showed significant negative associations with secondary outcomes.

Intermediate food consumption outcomes

QPM intake, based on 24-hour recalls, was low in the intervention groups, and consumption of conventional maize was higher than that of QPM. Child QPM intake on average was 27 gram/day for both intervention arms, i.e., QPM contributed only 5% of their daily food intake. The intake of conventional maize was about 80 gram/day in both arms, contributing about 16% of daily food intake. Furthermore, the contribution of legumes to the daily intake of protein, lysine, and tryptophan was high with no difference between intervention and control groups, while the intake meat and it's product was minimal (Appendix 3).

The results of the one-week food frequency questionnaire showed that during the season of high consumption (post-harvest), assessed at midline, consumption frequency of any QPM based foods among the intervention groups was on average four days per week, compared with five days per week for non-QPM based staple foods (Appendix 3). During

the less food secure or low consumption season, assessed at endline, children consumed QPM on average three days a week, compared with five days per week of non-QPM based staple foods (Appendix 3).

Table 1. Baseline characteristics, by intervention group.

Variables	Intervention group		Control, n=263	p-values
	AE, n=320	AE + CE, n=290		
Female, %	44	52	47	0.18
Age at enrolment (month)	19 (13, 25)	22 (14, 29)	20 (12, 27)	0.01
Length (cm)	78 (73, 82)	80 (74, 85)	78 (73, 84)	0.005
Length-for-age z score (HAZ)	-1.5 (-2.3, -0.6)	-1.4 (-2.1, -0.5)	-1.3 (-1.9, -0.4)	0.07
Height-for-age-difference (HAD)	-4.1 (-7.1, -1.7)	-4.2 (-6.4, -1.2)	-3.7 (-5.9, -1.1)	0.20
Stunted, length-for-age z score < -2 SD, %	36	28	23	0.002
Weight-for-length z score (WHZ)	-0.4 (-1.0, 0.4)	-0.3 (-1.0, 0.3)	-0.3 (-0.9, 0.4)	0.22
Protein intake (gram/kg/day)	1.6 (1.2, 2.3)	1.8 (1.2, 2.4)	1.8 (1.1, 2.4)	0.12
Proportion low protein intake[1], %	13	14	11	0.48
Lysine intake (mg/kg/day)	60 (36, 91)	63 (39, 94)	63 (42, 96)	0.29
Proportion low lysine intake[1], %	35	33	28	0.26
Tryptophan intake (mg/kg/day)	23 (16, 33)	23 (15, 36)	25 (15, 35)	0.12
Proportion low tryptophan intake[1],%	3	7	4	0.07
Proportion low energy intake[1], %	79	77	77	0.68
Serum IGF-1 (ng/mL)	29 (22.6, 41.2)	31 (22, 47)	30 (21, 44)	0.97
Serum Transthyretin (gram/l)	0.2 (0.1, 0.2)	0.2 (0.1, 0.2)	0.2 (0.1, 0.2)	0.09
Serum lysine (µmol/l)	142 (118, 161)	139 (111, 167)	141 (117, 167)	0.95
Serum tryptophan (µmol/l)	41 (32, 49)	42 (27, 51)	42 (34, 52)	0.44
Serum α-1-glycoprotein (gram/dL)	0.9 (0.7, 1.1)	0.8 (0.6, 1.1)	0.8 (0.6, 1.1)	0.25
Serum C-reactive protein (mg/L)	0.8 (0.3, 2.2)	0.8 (0.3, 1.8)	0.6 (0.3, 1.9)	0.42
Prevalence of inflammation (acute or chronic) among children, %	37	36	38	0.99
Caregiver having no formal education, %	70	61	65	0.19
Wealth tertiles, %				
1st tertile (poor), %	33	33	34	
2nd tertile (middle), %	34	34	32	
3rd tertile (less poor), %	34	33	30	

Data indicate median (25th, 75th percentiles) unless stated otherwise. Mixed models accounting for clustering and stratification by kebele (village) were used to assess baseline differences among study arms. [1]The recommended EARs [19] are: protein (0.87 gram/(kg·d)); lysine (45 mg/(kg·d)); tryptophan (6 mg/(kg·d)); energy (678 kcal, 764 kcal and 935 kcal for children aged 6–8 months, 9–11 months and 12–23 months, respectively).

Table 2. The effect of quality protein maize on linear growth.

Fixed effects	Height-for-age difference						Height-for-age z-score						Height/Length					
	model 1			model 2			model 1			model 2			model 1			model 2		
	Est	SE	P	Est	SE	P	Est	SE	P	Est	SE	P	Est	SE	P	Est	SE	P
Intervention																		
AE	-0.53	0.37	0.15	-0.57	0.35	0.10	-0.20	0.12	0.10	-0.19	0.11	0.09	-0.99	0.64	0.12	-0.49	0.40	0.22
AE+CE	-0.26	0.38	0.50	-0.01	0.36	0.97	-0.03	0.12	0.81	0.02	0.12	0.84	1.10	0.66	0.09	0.33	0.41	0.42
Time																		
Midline	0.28	0.22	0.20	0.34	0.21	0.11	0.00	0.07	0.99	0.00	0.07	0.98	-0.02	0.21	0.94	0.00	0.20	> 0.99
Endline	-1.46	0.17	<.0001	-1.45	0.17	<.0001	-0.28	0.05	<.0001	-0.27	0.05	<.0001	7.57	0.15	<.0001	7.59	0.15	<.0001
Intervention x Time																		
AE x Midline	-0.03	0.29	0.93	-0.07	0.29	0.80	0.00	0.09	0.997	0.00	0.09	0.995	0.01	0.28	0.96	0.00	0.27	0.99
AE x Endline	-0.18	0.23	0.44	-0.20	0.23	0.39	0.00	0.07	0.96	-0.01	0.07	0.93	-0.09	0.21	0.68	-0.10	0.21	0.64
AE + CE x Midline	-0.06	0.30	0.84	-0.13	0.30	0.66	0.00	0.09	0.96	0.00	0.09	0.999	0.02	0.29	0.93	0.00	0.28	> 0.99
AE + CE x Endline	-0.10	0.24	0.68	-0.11	0.23	0.63	-0.02	0.07	0.80	-0.03	0.07	0.68	-0.25	0.21	0.23	-0.26	0.21	0.23
Sex of child (female)				0.04	0.23	0.85				0.19	0.07	0.01				-1.25	0.28	<.0001
Age at enrolment																		
6-11 months				3.65	0.32	<.0001				0.93	0.10	<.0001				-15.86	0.39	<.0001
12-23 months				1.37	0.26	<.0001				0.25	0.08	0.002				-7.82	0.31	<.0001

All models controlled for stratification by kebele (village), clustering within HDA groups, and repeated measures on children over time. AE: adoption encouragement and CE: Consumption encouragement. Est: Estimate; SE: Standard error; and P: P-value.

Table 3. The effect of quality protein maize on serum transthyretin.

Fixed effects	Model 1			Model 2		
	Est	SE	P	Est	SE	P
Intervention						
AE	-0.004	0.01	0.46	-0.001	0.01	0.87
AE+CE	0.01	0.01	0.29	0.01	0.01	0.20
Midline	0.01	0.01	0.04	0.01	0.005	0.01
Intervention x Time						
AE x Midline	-0.001	0.01	0.90	-0.003	0.01	0.60
AE+CE x Midline	-0.005	0.01	0.50	-0.01	0.01	0.27
AGP				-0.04	0.004	<.0001
CRP				-0.001	0.0001	<.0001

All models controlled for stratification by kebele (village), clustering within HDA groups, and repeated measures on children over time. AE: Adoption encouragement and CE: Consumption encouragement. Est: Estimate; SE: Standard error and P: P-value. AGP: α-1-glycoprotein concentration; and CRP: C-reactive protein concentration.

Discussion

We found that the promotion of QPM adoption by farming households and QPM consumption by infants and young children in a natural setting did not have a significant effect on protein biomarkers or on linear growth of Ethiopian children. The lack of significant differences in serum transthyretin, lysine, tryptophan, or IGF-1 or in linear growth may be due to low intake of QPM and low energy intake coupled with inflammation and morbidity. We studied the effects of QPM on child protein status and growth in a natural setting, allowing target households and individuals to follow their typical behaviors while providing a small, scalable behavior change intervention to nudge families towards behaviors that could increase child QPM consumption. To our knowledge, there has been no study that investigated the effect of biofortified crops on nutrient intakes or biomarkers of nutrient status in natural life settings [20].

Previous studies on QPM involved young children or households with young children who were provided QPM or conventional maize in the

form of seed, grain, dough, or prepared food, with specific instructions to use the maize for child feeding. These studies found a positive effect on weight and height [9-11]. None of those studies looked at intervention at scale. Two previous studies in Ethiopia found that consumption of QPM instead of conventional maize led to positive effects on weight-for-height and height-for-age, reducing or preventing growth faltering that was prevalent among young children in developing countries [10]. A meta-analysis of nine studies from Africa, Asia, and Latin America found that QPM consumption was associated with a 12% greater rate of weight gain and a 9% greater rate of height gain; however, individual studies had several methodological differences and limitations [9]. All previous studies did not measure actual QPM intake, protein or energy intake, or biomarkers of protein status or inflammation. Similarly, other studies that have investigated utilization of biofortified crops in natural settings have generated limited evidence of changes in target nutrient intakes or biomarkers. However, a recent longitudinal cohort study among Kenyan women showed that the promotion of orange-fleshed sweet potato integrated with antenatal care could improve vitamin A intake [20].

Children's average daily intake of QPM in both intervention groups was low. Children were fed QPM, but in limited quantities, following the first season of adoption. Some did not consume QPM in the last 24 hours, while others consumed both QPM and conventional maize. In the first year of adoption, farmers continued to grow conventional maize alongside QPM, and caregivers continued to feed conventional maize along with QPM to their children. The biofortified crop and food may be treated as a new product rather than used to substitute for the conventional product. Research has found that QPM varieties are well-accepted by Ethiopian consumers, including mothers feeding young children; however, while acceptability may not be a barrier, familiarity and behavior change may take time [21,22]. If the interventions were continued longer, into subsequent seasons, consumption may increase as participants get more familiar with the biofortified crop, and interventions likely need to be longer to have sufficient change in child feeding behavior and practices. This suggests that sustained multi-year interventions may be required to see an effect on biomarkers and linear growth.

We have several potential explanations for the lack of effect of QPM promotion and consumption on linear growth and other outcomes. First, our hypothesis could have been incorrect – perhaps QPM does not improve linear growth. The pathogenesis of linear growth failure is poorly understood, and effective interventions to reduce stunting or promote healthy growth are limited [23, 24]. Dietary interventions have had minimal effect on linear growth of children [24]. Linear growth failure results from a complex interaction of household, environmental, socioeconomic, and cultural influences [25], and a dietary intervention alone may not be sufficient to improve the healthy growth of children in Ethiopia. Further, linear growth faltering begins in utero [26] and continues in particular through the first two years of life [27]. In Ethiopia, many mothers breastfeed for two years or longer [5]. This study included children under two years as well as slightly older children, who would be less dependent on breastmilk and may benefit more from a dietary intervention [28]. Agricultural strategies such as biofortification, which aim to function as dietary interventions, may be less effective at younger, albeit nutritionally vulnerable, ages.

Table 4. The effect of quality protein maize on secondary outcome biomarkers.

Fixed effects	Serum lysine						Serum tryptophan						Serum IGF-1*					
	model 1			model 2			model 1			model 2			model 1			model 2		
	Est	SE	P	Est	SE	P	Est	SE	P	Est	SE	P	Est	SE	P	Est	SE	P
Intervention																		
AE	-2.60	5.06	0.61	-0.56	5.22	0.91	-2.20	1.85	0.23	-1.37	1.84	0.46	0.02	0.07	0.76	0.06	0.07	0.39
AE+CE	-1.35	5.19	0.79	-0.47	5.33	0.93	-2.22	1.89	0.24	-1.89	1.88	0.31	-0.04	0.07	0.60	-0.03	0.07	0.72
Midline	-9.77	4.74	0.04	-9.43	4.82	0.05	-0.87	1.62	0.59	-0.56	1.63	0.73	-0.27	0.07	<.0001	-0.26	0.07	0.0001
Intervention x Time																		
AE x Midline	3.12	6.38	0.63	1.03	6.48	0.87	-0.88	2.17	0.69	-1.37	2.19	0.53	-0.14	0.09	0.12	-0.16	0.09	0.08
AE+CE x Midline	-0.66	6.52	0.92	-1.29	6.62	0.85	2.07	2.22	0.35	2.20	2.24	0.33	0.03	0.09	0.71	0.04	0.09	0.70
AGP				-6.10	4.30	0.16				-9.84	1.51	<.0001				-0.20	0.06	0.001
CRP				-0.29	0.14	0.04				0.04	0.05	0.38				0.001	0.002	0.65

All models controlled for stratification by kebele (village), clustering within HDA groups, and repeated measures on children over time. *IGF-1 was natural log-transformed prior to analysis. AE: Adoption encouragement and CE: Consumption encouragement. Est: Estimate; SE: Standard error; and P: P-value. AGP: α-1-glycoprotein concentration; and CRP: C-reactive protein concentration.

Second, our hypothesis could be correct, but the QPM promotion and consumption were not adequate to improve serum biomarkers and linear growth. Child QPM intake on average was low for both interventions, and overall energy intake was low in most children as well. Earlier studies on QPM consumption have shown a positive effect on the weight and height of infants and young children from populations in which maize is the major staple food [9]. However, individual studies were highly variable. A recent systematic review showed that among several nutrition-based interventions, high-protein based interventions had a greater positive effect on linear growth of children [29]. However, in this study, with insufficient intake of QPM and complementary food in general, positive changes to outcomes may not have been realizable.

Third, as most of our study children were energy deficient, their protein intake may have been diverted to meet energy requirements. Children in the study area were not getting adequate food, and the resulting energy deficit may also lead to growth retardation and increased morbidity and mortality [30]. Previous evidence has shown that both energy and protein restriction in children reduce IGF-1, which has a regulatory role in growth [31] An interventional study among Indian children found that an energy-rich low-protein supplement improved linear growth [32]. Thus, energy deficiency may be a major factor limiting child growth, masking any potential effect of QPM.

Another possible explanation is that poor appetite, a common response to infection or inflammation [3], contributed to low food intake by children. Subclinical infections caused by poor hygiene or parasitic infestations are common in developing countries and increase the loss of nitrogen from the human body [33-35]. Several pathways can be proposed to explain the potentially adverse effects of infection or inflammation on child malnutrition. These include decreased intake, consumption of nutrients by intestinal parasites, impaired absorption, and altered metabolism [36,37]. Both acute and chronic inflammation were prevalent in our study population and were associated with outcome variables. Inflammation negatively affects protein biomarkers [13], which could also affect the growth of children [3]. Earlier evidence suggests that bacterial infection increases protein requirements by about 30% [38] and lysine requirements by 50% [39] in malnourished children in India. A

recent study conducted among Indian school children showed that intestinal parasite infestation increased their lysine requirement by 20% [40]. In addition to low intake of QPM and low energy intake, inflammation may also contribute to the lack of positive effect.

Finally, the quality protein trait may be partially lost during cultivation when QPM is cross-pollinated by non-QPM maize [41]. As most families have relatively small farms, QPM plots were small and most QPM adopters also grew conventional maize simultaneously, such pollen contamination was a risk. QPM is visually indistinguishable from conventional maize, so mixing of QPM and conventional maize was also possible during harvest, storage, processing, or food preparation, leading to further dilution of the quality protein trait. These factors may also explain the lack of differences in the outcomes.

Our study had important strengths. This is the first RCT with a biofortified staple crop in a developing country that assessed nutritional impact under typical household behaviors. Previous studies did not target multiple biomarkers and nutritional outcomes as in this study. The randomized controlled trial design, the collection of data along hypothesized impact pathways, and assessment of nutritional status using both biomarkers and anthropometry allow greater understanding of the mechanisms of impact. This study also addressed important behavioral barriers between the development of a biofortified crop, QPM, and its impact on children's nutrition and health in a natural setting. Our study also had limitations. Due to the nature of the study interventions, blinding was not possible. Further, the study was conducted in only one agricultural cycle. Intervention and follow-up through a second agricultural cycle would illustrate whether adoption, utilization, and consumption behaviors were sustained or increased, but this was not possible given the study duration. The dietary data could also be affected by social desirability bias, which might have led to under-reporting.

Encouragement to adopt and feed QPM to infants and young children in a real-life setting had no effect on children's serum transthyretin, serum lysine, serum tryptophan, serum IGF-1, or linear growth. The intake of quality protein maize in the intervention arms was low, which may have resulted in a lack of improvement in quality protein

intakes and key outcomes. The interventions were designed to be scalable but may have been inadequate to stimulate sufficient behavioral change to increase QPM consumption and observe subsequent improvements in outcomes. Implementation and evaluation of multi-year interventions are needed to understand how biofortified crops promoted at scale in real-life settings could change intakes at the household level and for target individuals, which in turn can improve biomarkers and outcomes in target populations. These findings are also relevant to other biofortified crops. More research is needed, perhaps with mixed methods, to understand behaviors around adoption and child feeding when a biofortified crop is introduced.

Authors' contributions

MT, NSG, IDB, KD, and HDG drafted the manuscript. NSG and HDG conceived the original idea, the effect of QPM on nutritional outcomes within an RCT; MM and JC added the consumption encouragement intervention component; all critically commented on protocol. All authors contributed to the refinement of the study protocol and approved the final manuscript.

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Ethical approval and consent to participate

This study has been approved by Ethiopian Public Health Institute Scientific and Ethical Review Committee and the Harvard University

Institutional Review Board (IRB). Prior to any data collection, research staff sought written informed consent from study participants in their homes. If participants were unable to sign their name, they affixed their thumbprint to the consent form and a witness to the consent process signed the consent form.

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Appendices

Appendix 1

Study protocol for a randomized controlled trial.

Translating the Impact of Quality Protein Maize into Improved Nutritional Status for Ethiopian Children: Study Protocol for a Randomized Controlled Trial

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Abstract

Linear growth failure is the most common form of undernutrition. Childhood stunting impairs human development and health and productivity in adulthood. Ethiopia has a high prevalence of stunting, with diets reliant on staple crops with low nutrient content. Maize is the most highly produced crop in Ethiopia. Unfortunately, conventional maize has poor protein quality due to a poor balance of essential amino acids. Quality protein maize (QPM) varieties are biofortified with these essential amino acids and, in controlled trials, improve child growth. However, evidence on the impact of QPM adoption and consumption on protein status and linear growth of children under natural circumstances is not yet available. A randomized controlled trial was carried out to evaluate the impact of a) nutrition-focused adoption encouragement and provision of QPM seed in small seed packs, and b) a consumption encouragement intervention primarily targeting female caregivers and encouraging earmarking and integration of QPM into diets for infants and young children. The trial (n=1611) had three randomly assigned arms: a control group; a first intervention group receiving adoption encouragement only; and a second intervention group receiving both adoption and consumption encouragement. The primary outcomes of this study are QPM consumption, protein status, and linear growth of children, assessed using questionnaires, biological specimen collection, and anthropometry over one cycle of agricultural production and post-harvest consumption. Secondary outcomes include child stunting, acute malnutrition, underweight, total intake of utilizable protein, and caregivers' cooking and child feeding practices. This study addresses important behavioral barriers between the development of a biofortified crop, QPM, and its impact on children's nutrition and health in a natural setting. The randomized controlled trial design, collection of data in multiple domains along hypothesized impact pathways, and assessment of nutritional status using both biomarkers and anthropometry allow greater understanding on mechanisms of impact. This trial is the first such study to be conducted with a biofortified staple crop in a natural setting and supports the Government of Ethiopia's current targets for nutrition and agriculture. Prospectively registered in the AEA registry (AEARCTR # 0000786) on 24 July, 2015, and retrospectively registered on ClinicalTrials.gov (NCT02710760) on 30 January, 2016.

Background

Poor linear growth of children, manifested as stunting, is the most prevalent form of under-nutrition globally [1] and is associated with higher child mortality and morbidity [2,3], poorer motor and cognitive development [4], and lower educational attainment and economic productivity [5] as well as higher risk of metabolic diseases during adulthood [1]. Despite the international commitment to reduce the number of stunted children under five years by 40% by 2025 [6], current nutritional interventions alone are unlikely to meet this target [7]. Understanding the effectiveness of multi-sectoral approaches such as nutrition-sensitive agriculture in addressing the underlying determinants of malnutrition can accelerate progress in improving nutritional status globally [8].

Recent evidence indicates that protein and amino acids play biological roles in protein and lipid synthesis, bone elongation, and the regulation of these and other processes necessary for linear growth. Similarly, linear growth is stimulated by insulin-like growth factor 1 (IGF-1), which is also responsive to dietary protein intake [9,10]. However, dietary intakes of utilizable protein, i.e., protein adjusted for quality (determined by the content of essential amino acids) and digestibility [11], may be inadequate, particularly in sub-Saharan Africa [12]. Furthermore, current estimates of protein requirements do not address (1) children's protein needs for optimal linear growth; (2) increased requirements due to frequent infections, growth faltering, or energy deficit; and (3)the roles of protein and amino acids in growth regulation and immune function [13-15]. Adjusting protein requirements to account for increased needs due to recurring infections and energy deficits significantly increase estimates of the prevalence of inadequate protein intakes in developing countries [12].

At the country level, the per capita supply of utilizable protein is significantly and negatively associated with the prevalence of child stunting, even after controlling for the supply of dietary energy [12]. In an observational cohort of Danish children, protein intake at nine months of age was correlated with length at that age and height at 10 years [16].

A cross-sectional comparison of children aged 12-59 months in rural Malawi found that stunted children had lower serum concentrations of most amino acids including all the essential amino acids, which are not synthesized by the body and therefore must be obtained through dietary intake [17]. Randomized controlled trials in China [18] and Pakistan [19] found that fortification of wheat flour with lysine, which is globally the most limiting essential amino acid [20], increased linear growth in children.

Growth faltering is widespread among Ethiopian children, and the annual cost of undernutrition to the country has been estimated at US$4.7 billion, which amounts to 16.5% of the gross domestic product (GDP) [21]. Among children under five years, 40% are stunted, 9% have acute malnutrition, and 25% are underweight [22]. The Government of Ethiopia has committed to significantly reduce child stunting by 2020 in its Second Growth and Transformation Plan (GTP II) and to eradicate child malnutrition by 2030 in its Seqota Declaration [23,24]. To achieve these goals, it has called for a multi-sectoral approach for implementation of the National Nutrition Program II (NNP 2) through the integration of nutrition into the agricultural and health sectors and development of a strategic plan for nutrition-sensitive agriculture [23,25]. However, despite these commitments, the evidence is still limited, both globally and in Ethiopia, on how agriculture can be effectively leveraged to improve nutrition and health [26].

Dietary quantity and quality are poor among infants and young children in Ethiopia, with less than half (49%) of all children aged 6–23 months receiving the minimum recommended number of meals and only 5% consuming a sufficiently diversified diet [27]. Children of this age who receive the minimum recommended a number of meals and the number of food groups (i.e., consuming a minimum acceptable diet) have significantly higher height-for-age Z-scores (HAZ, standardized for child age and sex), indicating better linear growth[28]. Diets of both children and adults in Ethiopia are heavily dependent on cereals and, in the last 20 years, maize has become the dominant source [29]. However, conventional maize has low levels of the essential amino acids lysine and tryptophan, and the resulting poor protein quality increases the risk of inadequate intakes of utilizable protein and essential amino acids [30,31].

Efforts to improve the protein quality of maize date back to the 1950s [32,33]. In the early 1960s, the natural *o2* mutation was identified as responsible for changing the protein composition of the maize endosperm, nearly doubling its lysine and tryptophan content [34]. As a result, *o2* maize grain had improved protein quality, while its protein quantity remained the same. Subsequent conventional plant breeding efforts (i.e., methods not using genetic modification) resulted in agronomically competitive maize varieties adapted to target environments, particularly in sub-Saharan Africa [35]. To differentiate them from the earlier *o2* maize varieties and from 'conventional' maize varieties, these new varieties are collectively referred to as quality protein maize (QPM) and are an example of biofortification, or the genetic improvement of the nutritional quality of food crops [36]. Several randomized controlled trials (RCTs) have been conducted in which young children or households with young children were provided QPM or conventional maize in the form of seed, grain, dough, or prepared food, with specific instructions to use the maize for child feeding [37-39]. A meta-analysis of these studies found that provision of QPM instead of conventional maize led to a 12% increase in the rate of growth in weight and a 9% increase in the rate of growth in height in infants and young children with mild to moderate undernutrition from populations in which maize is the major staple food [37].

Despite these efforts, knowledge gaps remain in assessing the potential for QPM to positively impact the nutritional status of Ethiopian children in practice. Most prior RCTs on QPM did not directly measure children's consumption of QPM or conventional maize, and all RCTs largely or exclusively relied on anthropometric outcomes, which are affected by many factors beyond quality protein intake [1]. Therefore, despite the randomization in these studies, it is not possible to establish whether provision of QPM led to children's consumption of a critical amount or whether consumption of QPM led to changes in protein or amino acid status, which in turn led to improved growth.

Furthermore, little is known about QPM's impact on children's nutritional status in a natural setting in which households make their own decisions whether to adopt QPM, how much to adopt and cultivate, and whether and how to incorporate QPM into children's diets. In Ethiopia,

maize seed is sold in a package with a mandated size of 12.5 kg, compared with 2-kg bags that are typically sold in other East African countries where seed markets are liberalized [40]. The larger seed package size may be a barrier to adoption of QPM or any other improved maize variety, particularly at the initial stage when farmers may prefer to allocate only a small area to a new variety. If farmers are convinced of the agronomic performance of a QPM variety and can access seed in appropriate quantities, additional gains in adoption could be achieved by nutrition-focused extension efforts in which farmers are provided with knowledge of the benefits, particularly for their children's nutrition.

Following adoption, QPM must be separated from conventional maize at all stages of production, harvest, post-harvest handling, storage, milling, cooking, and consumption to prevent dilution of the quality protein trait [41]. This requires knowledge of QPM, its nutritional benefit, and good management practices to maintain the quality protein trait. However, many of the steps between household adoption and children's consumption of QPM are handled by women, and women often have less access to agricultural extension and other sources of agricultural information [42]. Their lack of knowledge about the technology could lead to a reduction or loss of the quality protein trait in maize consumed by target individuals in the household, ultimately limiting the nutritional impact of QPM. Consumption by children in particular also depends on feeding practices, typically controlled by women, and on how women choose to incorporate QPM into children's diets in a natural setting. Therefore, this study aimed (1) to estimate the causal effect of adoption encouragement focused on nutritional benefits on adoption decisions and protein status among infants and young children in a major maize-growing area of Ethiopia; and (2) among QPM adopters in the same area, to estimate the causal effect of QPM consumption encouragement on children's QPM consumption, protein status, and linear growth.

Methods

Study Overview

This study is superimposed upon the Nutritious Maize for Ethiopia (NuME) Project, which develops, promotes, and disseminates QPM varieties in the country's major maize-growing areas. NuME is a collaboration of the International Maize and Wheat Improvement Center (CIMMYT) with the Ethiopian Institute of Agricultural Research (EIAR), Sasakawa Global 2000, the Ethiopian Public Health Institute (EPHI), and other national and international partners. Households in this study had at least one member who had been exposed to QPM varieties by attending field demonstrations organized by NuME.

This study evaluates two randomized interventions related to household-level QPM production and consumption. Both focus on children who were 6-35 months old at enrollment. The first intervention, QPM adoption encouragement (AE), consists of a household visit by the research staff. The visit was targeted towards the household head, the primary decision-maker on the adoption of new maize varieties [42], although the primary caregiver for the household's young children was encouraged to join if present, and in 81% of households was present. The message (supplementary material 1) focused on the nutritional benefits of QPM varieties for young children, and the households were offered seed of QPM varieties they had observed in NuME field demonstrations to plant during the coming agricultural season.

While encouraging the adoption and production of QPM is an important first step in increasing QPM consumption, the ultimate purpose of the study is to understand whether greater production and consumption of QPM can improve childhood nutrition. To examine how to nudge families to feed QPM to their children, half of households assigned to the AE intervention were selected to receive an additional encouragement campaign. In this second intervention, the consumption encouragement (CE), targeting the caregiver of the household's young children, the study team explained why households should prioritize children's consumption of QPM. The caregiver was provided with

extension materials and specific storage containers to 'earmark' QPM grain and flour for young children.

Both parts of this second intervention could plausibly have large effects on children's consumption of QPM. Primary caregivers tend to have relatively little information about how to use QPM effectively, but generally make food preparation decisions for the household, suggesting that provision of even minimal education about QPM could result in significant changes in behavior and consumption patterns. Additionally, studies on financial decision-making suggest that earmarking (e.g., labeling a cash transfer as being for education, but not enforcing how the money is spent) can have surprisingly large effects [43,44].

Besides testing for the effect of these two interventions on household-level adoption of QPM and consumption of QPM by children in the target age range, we ultimately aim to evaluate the impact of these interventions on nutritional outcomes for infants and young children, including biochemical indicators of protein status and linear growth. Additionally, we will consider how other relevant outcomes, including allocation of food within the household and knowledge of QPM, are influenced by these interventions.

Overall, the selected households will be followed over one agricultural cycle, starting prior to planting, when adoption decisions are made, through production, harvest, and the period of storage and consumption that follows. Data collection includes a baseline survey (July-September, 2015), a midline survey (February-March 2016) and an endline survey (June-August, 2016). Given the nature of the interventions, it was not possible to blind either study participants or staff.

We hypothesize that the first intervention, adoption encouragement, will increase the adoption and use of QPM, and have an observable effect on the outcomes, compared to households who only observed the varieties in the demonstration. Further, we hypothesize that the second intervention, consumption encouragement, with its focus on targeting the QPM to the young child, will have a larger effect compared to the adoption encouragement alone. Finally, as many confounders affect the outcomes, particularly overall dietary intake and morbidity, these variables will be included in the analysis.

The chronology of the study reflects the seasonality of the outcomes and main factors (Table 1). The households were first contacted after they participated in the demonstrations during the main season of 2014. They were visited again before the next planting season in 2015 and offered QPM seed in the adoption encouragement. The baseline survey took place at the peak of food insecurity, after planting but before harvest, in the main season of 2015, and included biological specimen collection. Consumption encouragement was offered immediately before the harvest of 2015. The midline survey, including collection of biological specimens, took place at roughly four months after the harvest, when the peak effect on biomarkers was expected, while the endline, with anthropometrics, will take place in 2016 during the same season as the baseline, between planting and harvest, as households' maize stores are diminishing.

Site Description

Administratively, Ethiopia is divided into nine regional states, which are further divided into zones, then districts or *woredas*, and finally peasant associations or *kebeles. Kebeles* are the smallest official administrative unit and comprise about 500 to 1000 or more households each. The NuME project is being implemented over a five-year period, starting in 2012, in three agro-ecological zones (drought-prone, moist mid-altitude and highland zones) where the impact is expected to be greatest, as identified by GIS analysis combining agro-climatic, nutritional and poverty databases [45].

Within the NuME project areas, the study team conducted extensive focus groups with more than 100 men and women in the Oromia and Amhara regions. Interviews with the women focused on existing child feeding habits (e.g., age of solid food initiation, foods fed to young children, etc.), while interviews with the men focused on details of their planting seasons, including when and how seed variety choices are made, as well as general acceptance of and desire for QPM varieties. Based on the results of these focus group discussions, the study is being conducted in two zones of the Oromia region, given higher likelihood for potential impact.

The entire Oromia region is a third of the total area of Ethiopia and has a population of 27 million people [46]. The average household size in the region is 4.8 members. Agriculture is the primary economic activity of the region, engaging about 90% of the population, with home production used to meet a significant portion of household food needs.

The study area comprises one to two *kebeles* each from the *woredas* of Boneya Bushe, Gobu Seyo, Gudeya Billa, Guto Gida, and Sibu Sire in the East Wollega zone and two *kebeles* each of the *woredas* of Omo Nada and Mena from the Jimma zone. The 12 *kebeles* in total are in rural, maize-growing areas.

Study Population

This study focuses on households in the target areas where QPM was demonstrated prior to the main growing season in 2015. The primary focus is on children who were 6-35 months during the baseline survey in July-August 2015. The target age range excludes the first six months when exclusive breastfeeding is recommended and otherwise includes the critical first two years of life when children are particularly vulnerable to growth faltering and the third year when they are increasingly dependent on solid foods.

Households in the study area were eligible for inclusion if they met the following criteria: (1) the household had at least one child aged 6-35 months at recruitment in July-September 2015; (2) the household had at least one member who had attended a field demonstration conducted by the NuME Project in November 2014-January 2015; and (3) the household provided informed consent to participate in the study. Households were excluded if (1) the primary caregiver or index child were not intending to remain in the study area for the study duration; (2) the household did not have access to land for crop cultivation in the main 2015 season; or (3) the household had previously produced QPM in an on-farm demonstration in the previous year. Additionally, households in the treatment groups were excluded if the primary caregiver for the target child was not in a 'one to five' group, since this information was used for randomization between the two treatment groups. The one to five groups, formally called the Health Development Army (HDA), consist of about five women and

are formed to help local health extension personnel with the outreach of the health and nutrition program at the community level.

Study design

The study is a randomized controlled trial, with two interventions related to household-level QPM production and consumption. The overall study has three treatment arms (Fig.1). A third of households were allocated to the control arm, where the household's participation was limited to data collection. The remaining households were split between those receiving the AE intervention only and those receiving both the AE and CE interventions.

Randomization and recruitment

In the 12 selected *kebeles*, a list of households who participated in the field days and who were eligible for inclusion in the study was established with the help of the local administration, in particular the *kebele*-level development agents, which are government extension officers for agriculture and rural development. These households (1779 in total) were randomly assigned to the control (one third) and two treatment groups (each one third), sequentially in two stages, and stratified by *kebele* (so resulting in the same proportions in each *kebele*). In the first stage, control vs. AE, randomization was done at the household level, while at the second stage, which was AE only vs. AE plus CE, randomization was done at the group or cluster level.

In the first stage of randomization, a third of study households (587) were assigned to the control group and the remainder (1192) to the AE treatment using simple randomization, stratified by kebele. This stage took place prior to the planting season in April 2015. In the second stage of randomization, half of the households that had participated in the AE intervention were assigned to the CE intervention. This occurred prior to the baseline survey, during which the initial CE messages were presented. Among the 1192 households that had been assigned to the AE intervention, 1024 met the eligibility requirements and ordered QPM seed, forming the set of potential households to be included in this stage.

Given that a large component of the CE intervention was based on information for the caregiver, the second stage of randomization was conducted at the 'one to five' group level. Therefore, all cases where the caregiver did not belong to a 'one to five' group were excluded, reducing the sample to 1024 households, now organized in 562 women's groups or clusters. Half of the clusters were assigned to each treatment group, stratified by *kebele*, using the Stata (software) command "randomize". This command maximized balance on each cluster's average values for caregiver being present during AE messages, household having a telephone number, and number of study households in the cluster (which ranged from 1 to 13 households, and averaged 3.0 households). After randomization, balance was confirmed at the household level on the number of bags of QPM of each variety that were ordered during AE, the total number of bags of QPM that was ordered during AE, and the three factors used during randomization. As in the first stage, this randomization was stratified by *kebele*. Overall, this resulted in 280 clusters (with 511 households) assigned to AE only, and 282 clusters (with 513 households) assigned to AE plus CE. Initially, 587 households were assigned to the control group, but power calculations suggested fewer households were needed to identify plausible treatment effects. Therefore, 467 of these households were selected by simple randomized sampling, stratified by *kebele*, for data collection.

A subsample was additionally selected from each study arm for biomarker collection, hemoglobin tests, and malaria rapid diagnostic tests. In the control group, this subsample was selected using simple random selection, stratified by *kebele*. In the treatment groups, this subsample was selected in two stages: (1) stratifying by *kebele*, an equal proportion of clusters was selected using simple random selection; and (2) within each of these randomly-chosen clusters, one household was chosen using simple random selection. After performing these two stages of random selection, balance was confirmed on the relevant household characteristics described above.

Given the multiple stages of randomization and the need to distribute QPM seed prior to the growing season, all randomization of

households to treatment groups was conducted prior to informed consent and enrolment. Provision of QPM seed to households in the groups receiving AE was not contingent on study participation, and households which were later found to be ineligible or declined to provide informed consent were free to use the QPM seed even though they did not participate in the study or data collection. Households that were allocated to receive CE but were found to be ineligible or declined to provide informed consent did not receive the CE intervention or otherwise participate in the study.

Interventions

Adoption encouragement

In the Adoption Encouragement (AE) intervention, households were offered guidance about the benefits of QPM consumption for young children and the opportunity to order a small amount of QPM seed to plant on their own land. Qualitative evidence suggests that, especially for new products, higher rates of adoption occur when farmers can try seeds in smaller quantities [47,48]. Prior to the intervention, we calculated that a package size of 2 kg—yielding enough to provide 150 gram of QPM grain per target child per day with sufficient leftover grain for further household use—would be the minimum level of adoption required to see meaningful growth in young children over a period of six months.

This intervention is driven by the insight that while it is important for children to have nutritionally dense foods, they do not eat much food, particularly while they are also breastfeeding. Farmers are likely only willing to experiment with a small portion of their land before they have experience growing QPM, but even growing a relatively small amount of QPM could greatly impact their children's health.

The study team, assisted by local development agents, visited the households selected for the AE intervention in March–April 2015, and discussed with the head of household and the caregiver for the household's young children, if she was available. This discussion focused on (1) the nutritional benefits of QPM, especially compared to

conventional maize varieties; (2) the special vulnerability children faced regarding nutritional deficiency and malnutrition and QPM's potential to mitigate these risks; (3) details about the two varieties of QPM available – one, AMH760Q, has white grain and is late maturing and drought tolerant while the second, BHQPY545, has yellow grain, has intermediate maturity and is also drought tolerant; and (4) information about how QPM is similar to other maize varieties agronomically and for food preparation and consumption. After this discussion, the enumerators offered the option to order up to three 2-kg bags of QPM seed, emphasizing that the farmer had no obligation to order, but was also asked not to share the seed with anyone outside of his household if he did choose to place an order. If the farmer was interested, the enumerators took orders for QPM seed to plant in the coming month. The seed was offered free of charge, but household heads were required to come to a central location to pick up the seed a few weeks later.

Consumption encouragement

In the Consumption Encouragement (CE) intervention, household heads and particularly caregivers for young children were offered (1) further guidance on the nutritional benefits of QPM for young children; (2) guidance on the importance of keeping QPM separate from conventional maize to prevent dilution of the nutritional benefits; and (3) tools to help them separate and 'earmark' QPM grain and flour for child consumption. The first component of the intervention, guidance on nutritional benefits for children, was adopted and developed based on the health belief model [49,50]. The second component, guidance on QPM management, was based on recommendations by breeders and agronomists on production and utilization of QPM. The third component, tools for earmarking, was motivated by evidence from interventions in financial decision-making, which suggests that earmarking can have surprisingly large effects. In Morocco, cash transfers with a non-binding education label were shown to lead to significant increases in school participation, similar to conditioning the payments on participation [43]. Experiments have shown that allowing for multiple accounts increased savings rates, and this was enhanced by earmarking one account with a visual reminder of children

[51]. In this study, the consumption encouragement intervention explores the hypothesis that providing a way to separate nutritional resources (improved maize) with a label with reference to children increases the quantity that reaches them.

The CE messages were presented during three different sessions over the study period: two one-on-one sessions during the baseline and midline surveys, immediately following data collection, and one group session in between these surveys prior to the harvest. The timing, content, and participants in these sessions depended on the agricultural calendar and the roles of men and women in agricultural, child care, and feeding practices.

The first CE message was offered at the household during the baseline survey in July-September 2015, immediately following data collection. The message was given prior to the availability of green maize in farmers' fields, at which point children may begin consuming QPM. During this visit, enumerators (1) discussed with the caregivers and heads of household the benefits of QPM relative to conventional maize and the special benefit young children receive from QPM consumption; (2) discouraged participants from selling QPM or feeding it to livestock; (3) discussed the importance of keeping QPM separate from other grains and flours; and (4) informed caregivers that they would be offered tools to help keep QPM grain and flour separate later in the year. Heads of household were encouraged to build separate cob storage cribs or to partition existing storage cribs, in order to keep their QPM separate from conventional maize while it was drying. The messages overall took less than 10 minutes.

The second session was conducted in November 2015, prior to the grain harvest. Caregivers in the CE intervention group were invited to participate in a group meeting at a nearby location, usually a health extension post or farmer training center. During this visit, enumerators used an education poster (Fig.2a) to re-emphasize messages that had been presented earlier and engaged participants in a group dialogue to help identify ways to better target QPM to their young children. When participants identified aspects that might be difficult (e.g., cooking

separate meals for their young children), the enumerator facilitated a group discussion to help participants think of ways to make these challenges easier to overcome. At the end of the visit, caregivers were offered several tools to help them separate QPM grain and flour from other grains and flours, and to remember to do so. Each caregiver was given four standard bags for storing grain (each capable of holding 100 kg), one bag for storing flour (capable of holding 50 kg), and a bowl and spoon for feeding the index child. All of these items were marked with a colorful label that had a picture of an infant eating and images of white and yellow maize, and "quality protein maize" written in the local language (Fig.2b). Additionally, each caregiver was given a poster (60 cm x 41 cm) displaying complementary foods that could be made with QPM (Fig.2c). Overall, the group events took 30-35 minutes. Caregivers who were unable to attend a group session received a one-on-one session and all materials in their homes.

In the third session, the CE educational messages were re-emphasized for caregivers for a final time during the midline survey, immediately after data collection. Enumerators reviewed a short set of the most key messages, focusing on the benefits of QPM consumption for young children and targeting QPM-based foods.

Outcome measures

The primary outcomes in this study are linear growth of the index child, measured as height-for-age Z-score (HAZ) [52], protein status measured using prealbumin (transthyretin) [53], and measures of QPM consumption. The secondary outcomes include child stunting (HAZ<-2), acute malnutrition (weight-for-height Z-score <-2) underweight (weight-for-age Z-score<-2), and total intake of utilizable protein measured using a 24 hours dietary recall [53].

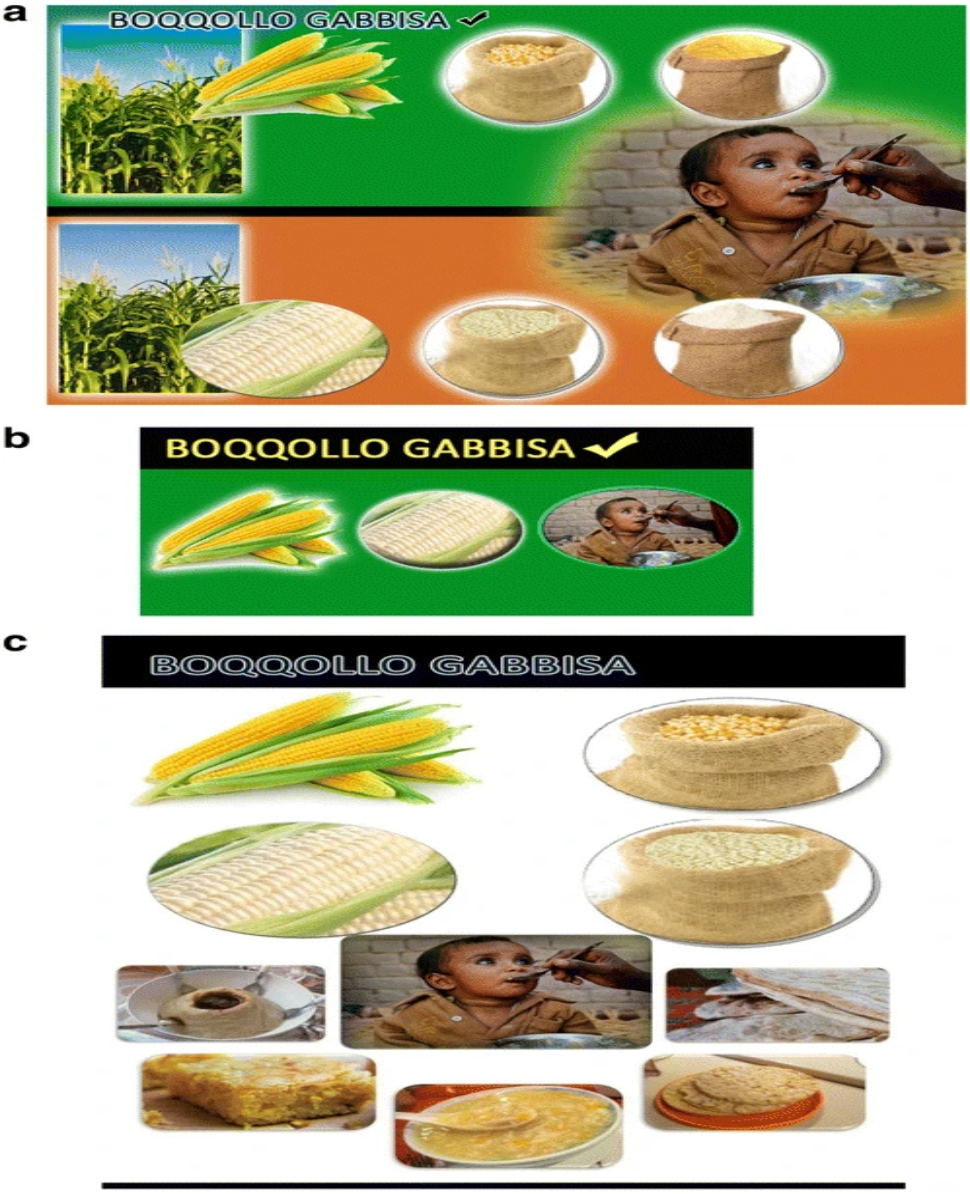

Fig. 1. Posters and label for consumption encouragement. "Boqqollo gabbisa" is the local term used for QPM in the Oromo language. **a** Poster for discussion during consumption encouragement group meeting, illustrating the yellow and white varieties offered in the study in the field, as grain and flour, and as complementary food; **b**) Label used for grain and flour storage bags; **c**) Poster for household use, illustrating complementary foods that could be made with QPM. Consent to use the image of the child in these posters and label was obtained from the child's mother by a staff member from the Ethiopian Public Health Institute (EPHI).

Data collection

The main data were collected at three times: the baseline, midline and endline survey. Prior to beginning any data collection at baseline, written informed consent was obtained from all respondents. Much of the data collected focused on one target child (i.e., the "index child"), who was between 6 and 35 months old at the time of the baseline survey. In cases where there was more than one eligible child in the household, the youngest was selected to be the index child. All households received a small, non-monetary incentive such as soap and iodized salt at each major data collection event.

Questionnaires were administered to the caregiver and the household head at baseline and midline, and the caregiver alone at endline. Topics in the caregiver surveys included demographics; household roster (baseline only); 24 hours dietary recall for the index child; seven-day food frequency for key household members; cooking practices; growth perceptions; child health and illness; former pregnancies (baseline only); household food security; nutrition knowledge; QPM knowledge; water supply and sanitation access (baseline only); sources of information; gender responsibilities; and bargaining. Topics in household head surveys included demographics; household assets; details about crop production, area, and sale; specific information about maize production by variety; seasonality in crop storage, sale, purchase, and consumption; agricultural input use; livestock ownership; income sources; expenditures; growth perceptions; gender responsibilities; participation in rural institutions; and sources of information.

Anthropometrics

Anthropometrics (i.e., height or recumbent length, weight, and mid-upper arm circumference) were collected on all index children and their biological mothers (among the caregivers) during baseline, midline, and endline. Weight of index children was measured with light clothing and without shoes to the nearest 100 g. The scale was calibrated after moving

from one household to the next. The caregivers were weighed without ornaments, shoes and heavy clothes to the nearest 100 g using a standard SECA digital scale.

Length of younger children (6-23 months) was measured in a recumbent position to the nearest 0.1 cm using a locally made board with an upright wooden base and movable headpiece. Height of children older than 23 months of age was measured in a standing position to the nearest 0.1 cm using a locally made vertical board with a detachable sliding headpiece. Similarly, caregivers' height was measured by a portable measuring height board with moveable headboard.

Mid-upper arm circumference (MUAC) was measured for the index child and caregiver with a standard MUAC tape on the upper left arm. After locating the mid-point for measurement between the end of the shoulder (acromion) and the tip of the elbow (olecranon), this point was then marked. The arm was then made to hang freely and MUAC was measured at the marked mid-point. Referrals to the local kebele's health post were made whenever a participant was identified as severely malnourished (mid-upper arm circumference < 110 mm and/or bilateral oedema for children, < 210 mm for pregnant or lactating women, or BMI < 16 for non-pregnant, non-lactating women).

Dietary recall

Dietary recall interviews were used to collect the specific type and amount of food consumed by the index child during the full day (24 hours, sunrise to sunrise) prior to the survey. The questionnaire was developed based on the internationally-recognized multiple-pass method described by Gibson and Ferguson [54], adjusted to the Ethiopian context. Each interview involved a stepwise series of questions and typical household utensils and food substitutes (play dough, flour, lentils, water) to improve the memory of the respondents and assist in completing the questionnaires. A digital food scale was used to measure the gram amount of food consumed and of ingredients used in food preparation. The interviews were conducted on all seven days of the week to capture

changes in intakes across various days of the week. Collection days included market days and holidays that occurred while the team was in the study area. In addition to this, a seven-day food-frequency questionnaire asked the following details from the index child's primary caregiver: consumption of any QPM by the index child in the last 24 hours, amount of QPM consumed by the index child in the last 24 hours, index child's proportion of total maize consumption that was QPM in the last 24 hours, consumption of any QPM by the index child in the last week, and number of days in the last week that the index child ate any QPM. Other household behaviors related to QPM targeting include number of days in the last week that the caregiver cooked a QPM-based food that was primarily for target children, amount of QPM reserved for home consumption (both self-reported), and proportion of grain that is QPM in source most recently used to cook food for the index child.

This dietary recall module provides very rich data, but it only captures information about the previous day, which may not be representative of a child's overall eating habits. To identify the degree of within-person and between-person variation in food consumption, a subset of households was selected to revisit within one week of the midline survey, on a non-consecutive different day of the week, to conduct a repeated dietary recall [55]. Fifty households were randomly selected from each study group (control, AE and AE+CE) for the repeated dietary recall using simple random sampling. If the household was not available during the repeat dietary recall, it was randomly replaced with another household from the same *kebele*. This information will allow estimation of the usual intake of dietary protein and other nutrients.

Specimen collection and analysis

In the subset of households identified for specimen collection, caregivers and index children were assessed for anemia and malaria infection and venous blood and stool samples were collected from index children. These assessments and specimen collection were conducted at baseline and midline. Given the seasonal pattern of maize consumption, midline was chosen over endline as it represented a period of high maize consumption

approximately three to four months after harvest, which in turn followed a period of green maize consumption. At endline, it is expected that maize consumption will have tapered with declining maize stores. Venous blood samples were taken to assess serum prealbumin (transthyretin) and IGF-1 for protein status and alpha-1-glycoprotein (AGP) and C-reactive protein (CRP) for inflammation, which has been implicated in stunting [1,56].

Phlebotomists collected blood samples from participants' arm by venipuncture using a trace metal-free evacuated tube collection system, and collected whole blood into a vacutainer. The vacutainer contained a separator gel, free from trace metals, with a non-rubber stopper. If the caregiver refused collection of venous blood from the child, blood was taken by finger prick to assess hemoglobin concentration for diagnosis of anaemia and malaria infection using a rapid diagnostic test (RDT) only.

Anemia was assessed by measuring hemoglobin in red blood cells, using a HemoCue (Hb-201) instrument. Liquid controls (high, medium and low) were used at the beginning of each day for quality control of the HemoCue instrument. Hemoglobin concentrations were read immediately using the HemoCue. Participants were considered to have severe anemia if their hemoglobin level was less than 8 gram/dL and were referred to local health services [57]. Cut-off values for anemia will be adjusted per published recommendations [57,58] on the basis of age, sex, pregnancy status and the altitude where the person lived. The adjustment for altitude will be done (Hb adjustment = -0.032 x [altitude (m) x 0.0032808] + 0.022 x [(altitude (m) x 0.0032808)]^2) for children and caregivers living at an altitude of 1000 meters above sea level or higher [58], where the Hb adjustment will be the value subtracted from each individual's observed hemoglobin level. The malaria parasite burden was measured using RDT for *Plasmodium falciparum* and PLDH for other *Plasmodium* species [59].

Blood samples were transported from the household to the temporary field lab promptly after collection in cold boxes containing frozen gel packs (<8^{o}C) by local guides appointed specifically to assist each lab technician in rapidly carrying the samples to the centralized

temporary field laboratory site. The laboratory team vehicle maintained a self-contained field laboratory that included a portable centrifuge to allow for immediate centrifugation and aliquoting serum into cryovials. This vehicle included a -20^{o}C freezer, powered with electricity from the grid or a battery for fast freezing of serum samples in the field. This freezer was used to maintain frozen gel packs to be used with the cool boxes that went to the field during sample collection.

In each *kebele* a temporary field lab was set up in a central location such as a school, farmer training center, health center or other location for the technologist to immediately centrifuge samples transported from the field and aliquot the serum into appropriate cryovials. When electricity was not available, the field laboratory was set up in the vehicle. All samples were processed within two hours of collection. Cryovials were stored at −80°C. Specimen identifiers were labelled directly on the cryovial.

Stool samples were placed in a clean stool cap either during the visit with the lab team or by the caregiver later if no stool was available at the time of the visit. The examination of faeces for parasitological diagnosis was done to detect adult worms, cysts, ova and larvae using microscopes in the field using Kato Katz techniques [60,61]. The remaining stool samples were transported to the EPHI parasitology laboratory and stored at −80°C for later analysis of intestinal helminth infections [61].

AGP, CRP, IGF-1, and prealbumin (transthyretin)will be assessed using the immuno-turbidimetry method using Roche kits. The change in turbidity, proportional to the AGP and CRP concentration, will be measured on the modular Cobas Integra 600 clinical analyzer and the presence of inflammation will be determined by a standard method [62].

Grain sample

During the midline survey, the study team collected a sample of about 100 grains of maize from each household, from the source where the index child’s most recent maize-based meal was made, to analyse if the

grain came from a QPM variety. This is done by testing for QPM's endosperm modifiers along with the *o2* mutant allele using a rapid and low-cost method of selection, whereby light is projected through the vitreous grains or blocked by the opaque

grains respectively [35].

Sample size calculation

Sample size calculations were based on the nutritional outcomes for which effects of QPM were observed under controlled conditions and plausible biological mechanisms exist: height-for-age Z-score (HAZ), hemoglobin (Hb), and prealbumin (transthyretin). HAZ is standardized, so its standard deviation was assumed to be 1. Hb is typically symmetric and a standard deviation of 2.0 gram/dL is based on the expectation that physiologically plausible values will fall within a range of 12 gram/dL (six standard deviations) in the relevant age group. The mean and standard deviation for transthyretin were assumed to be 20 and 30, per published reference distributions [53]. All calculations are based on intent-to-treat analyses, which assumes a 78% overall QPM adoption rate in the treatment groups, and a 30% adoption rate in the control group (both are conservative estimates to account for potential spillovers). When comparing the treatment groups to each other, the effect of adoption was assumed to be 50% higher in the group receiving both adoption and consumption encouragement than in the group receiving adoption encouragement only.

Statistical analysis

Primary outcomes will be analyzed based on an 'intention-to-treat' principle. Baseline socio-demographic characteristics will be summarized with percentages for categorical variables and mean ± SD (or median and range) for continuous variables. To examine the impact of QPM adoption and consumption, generalized linear mixed effects models for repeated measures will be estimated for all outcomes. All hypothesis tests will be two-sided with a 0.05 significance level.

Discussion

Global commitment to reduce childhood stunting and improve nutritional outcomes is growing. There is an urgent need for evidence on the impact of agricultural interventions on nutrition and health, which requires rigorous assessments of effectiveness. Quality protein maize has the potential to improve the nutrition status of young children due to its higher lysine and tryptophan content. However, there are important challenges in ensuring appropriate adoption and use of QPM. This study seeks to address two important behavioral barriers between the development of QPM and its impact on children's nutrition and health in practice: the decision by households to adopt QPM, and the subsequent decision to allocate the improved maize to young children. It addresses the question of whether a biofortified crop can passively have an impact on children's nutritional status through adoption and typical household use, or whether additional intervention addressing behaviors affecting nutrition or targeting women as caregivers is needed for impact. Collection of data on production; decisions on storage, processing, intra-household food allocation and diets; and nutritional status using both biomarkers and anthropometry will provide greater understanding on the mechanisms through which QPM impacts child nutrition. This trial is the first such study to be conducted with a biofortified staple crop in a natural setting.

The Government of Ethiopia has set a target to have QPM varieties cultivated on 20% of the country's total maize area in the coming few years. The results of this randomized controlled trial will be used to inform the Ethiopian and other governments and other stakeholders and implementers addressing nutrition, agriculture, and rural development in maize-growing areas on how to integrate QPM and similar biofortified crops into their programming. This trial will further add to the global database on evidence for linkages among agriculture, nutrition, and health and for strategies to maximize the impact of nutrition-sensitive agricultural interventions.

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Appendix 2

Subgroup analysis for children with inflammation (A) and without inflammation (B).

Table A. Subgroup analysis for children with inflammation.

	Height-for-age difference			Height/Length			Height-for-age z-score		
	Inflammation			Inflammation			Inflammation		
Fixed effects	Est	SE	P	Est	SE	P	Est	SE	P
Intervention									
AE	-0.84	0.82	0.30	-0.49	1.30	0.71	-0.31	0.25	0.21
AE + CE	-1.70	0.83	0.04	0.44	1.32	0.74	-0.47	0.25	0.06
Time									
Endline	-2.00	0.56	0.0004	7.08	0.55	<.0001	-0.59	0.17	0.0004
Midline	0.0000006	0.55	1	0.01	0.54	0.99	0.002	0.16	0.99
AE X Endline	1.11	0.73	0.13	0.28	0.73	0.70	0.29	0.22	0.19
AE X midline	-0.02	0.72	0.97	-0.001	0.72	0.99	0.0001	0.21	0.99
AE + CE X Endline	1.40	0.75	0.06	0.55	0.74	0.46	0.43	0.22	0.05
AE + CE X midline	0.05	0.74	0.95	-0.0015	0.72	0.99	-0.0002	0.22	0.99

AE: Adoption encouragement and CE: Consumption encouragement. Est: Estimate; SE: Standard error; and P: P-value.

Table B. Subgroup analysis for children without inflammation.

	Height-for-age difference			Height/Length			Height-for-age z-score		
	No inflammation			No inflammation			No inflammation		
Fixed effects	Est	SE	P	Est	SE	P	Est	SE	P
Intervention									
AE	0.40	0.54	0.45	-1.10	1.02	0.27	0.07	0.17	0.63
AE + CE	0.26	0.54	0.62	0.18	1.02	0.85	0.06	0.17	0.71
Time									
Endline	0.27	0.37	0.46	8.02	0.32	<.0001	0.06	0.11	0.54
Midline	-0.01	0.37	0.96	0.004	0.32	0.99	-0.001	0.11	0.99
AE X Endline	-1.23	0.49	0.01	-0.57	0.43	0.18	-0.31	0.14	0.02
AE X midline	-0.23	0.49	0.63	0.0000002	0.42	1	-0.05	0.14	0.71
AE + CE X Endline	-0.77	0.50	0.12	-0.81	0.43	0.06	-0.16	0.14	0.24
AE + CE X midline	0.03	0.49	0.94	-0.0003	0.42	0.99	0.002	0.14	0.9

AE = Adoption encouragement and CE= Consumption encouragement

Appendix 3

Table.1. (A): Daily mean intake of QPM by interventions and percent of QPM contributions to total protein, lysine and tryptophan; (B): Daily mean intake of conventional maize (CVM) by interventions and percent of conventional maize contributions to total protein, lysine and tryptophan during the season of high consumption (post-harvest).

(A)

Intake	Interventions								
	AE			AE + CE			Control		
	Total in gram	From QPM in gram	% contributed by QPM	Total in gram	From QPM in gram	% contributed by QPM	Total in gram	From QPM in gram	% contributed by QPM
Total amount of food consumed (gram)	474.3	26.6	5.6	527.7	27.8	5.3	464.1	2.4	0.5
Protein intake in (gram)	18.9	1	5.4	22.3	1	4.6	17.3	0.1	0.6
Lysine intake (gram)	0.52	61.5	11.9	0.61	67.3	11.1	0.44	4.9	1.1
Tryptophan intake (gram)	0.12	18.2	15.6	0.14	20.8	14.9	0.10	1.8	1.7

(B)

Intake	Interventions								
	AE			AE + CE			Control		
	Total in gram	From CVM in gram	% contributed by CVM	Total in gram	From CVM in gram	% contributed by CVM	Total in gram	From CVM in gram	% contributed by CVM
Total amount of food consumed (gram)	474.3	76.6	16.1	527.7	81.5	15.4	464.1	96.5	20.8
Protein intake in (gram)	18.9	3.2	16.9	22.3	3.3	14.8	17.3	4.1	23.6
Lysine intake (gram)	0.52	47.9	9.2	0.61	50.9	8.4	0.44	68.6	15.4
Tryptophan intake (gram)	0.12	18	15.4	0.14	16.1	11.5	0.10	25.2	24.4

AE: Adoption encouragement and CE: Consumption encouragement

Table.1. (C): Daily mean intake of other cereals (OC) by interventions and percent of other cereals contributions to total protein, lysine and tryptophan, and (D): legumes by interventions and percent of legume contributions to total protein, lysine and tryptophan during the season of high consumption (post-harvest).

C	**Interventions**								
Intake	**AE**			**AE + CE**			**Control**		
	Total in gram	From OV in gram	% contributed by OC	Total in gram	From OC in gram	% contributed by OC	Total in gram	From OC in gram	% contributed by OC
Total amount of food consumed (gram)	474.3	108.4	22.8	527.7	134.3	25.5	464.1	112.5	24.2
Protein intake in (gram)	18.9	3.3	17.7	22.3	3.8	17.2	17.3	3.3	19.2
Lysine intake (gram)	0.52	66.9	12.9	0.61	92.3	15.2	0.44	71.3	16
Tryptophan intake (gram)	0.12	19.7	16.8	0.14	26.5	19.0	0.10	20.7	20

D	**Interventions**								
Intake	**AE**			**AE + CE**			**Control**		
	Total in gram	From legumes in gram	% contributed by legumes	Total in gram	From legume in grams	% contributed by legumes	Total in gram	From legumes in gram	% contributed by legumes
Total amount of food consumed (gram)	474.3	110.5	23.3	527.7	133.3	25.3	464.1	113.6	24.5
Protein intake in (gram)	18.9	3.7	19.8	22.3	4.7	21.0	464.1	113.6	24.5
Lysine intake (gram)	0.52	209.7	40.5	0.61	248.4	40.8	0.44	208.7	47
Tryptophan intake (gram)	0.11	35.6	30.5	0.14	47.2	33.9	0.10	36.3	35.1

AE: Adoption encouragement and CE: Consumption encouragement.

Table.1. (E): Daily mean intake of meat and meat products by interventions and percent of meat and meat products contributions to total protein, lysine and tryptophan during the season of high consumption (post-harvest).

Intake	Interventions								
	AE			AE + CE			Control		
	Total in gram	From meat in gram	% contributed by meat	Total in gram	From meat in gram	% contributed by meat	Total in gram	From meat in gram	% contributed by meat
Total amount of food consumed (gram)	474.3	2.2	0.5	527.7	2.2	0.4	464.1	0.3	0.1
Protein intake in (gram)	18.9	0.4	2.3	22.3	0.5	2.3	17.3	0.1	0.4
Lysine intake (gram)	0.52	39.1	7.6	0.61	45.4	7.5	0.44	5.9	1.3
Tryptophan intake (gram)	0.12	4.5	3.9	0.14	5.7	4.1	0.10	0.7	0.7

AE: Adoption encouragement and CE: Consumption encouragement.

Table.1 (F): QPM intake from one-week food frequency: during the season of high consumption (post-harvest) (i) and endline (ii).

	% consumed in a week during the season of high consumption (post-harvest)					
i Interventions	No of days	Any foods from QPM	Porridge from QPM	Injera from QPM	Quita/flat bread from QPM	Dabo/fermented bread from QPM
	0 (not consumed)	38	69	51	62	50
	1	1	12	2	9	11
	2	3	10	3	11	15
	3	9	7	6	10	12
	4	5	2	6	3	3
	5	4	1	4	1	2
	6	1	-	1	-	-
	7	39	-	26	3	7
AE	Mean no days	3.5	1	3	1	1
	0 (not consumed)	20	44	41	45	34
	1	2	17	2	13	14
	2	4	23	6	17	21
	3	12	13	9	12	15
	4	10	1	7	5	6
	5	8	2	4	2	3
	6	2	-	0	1	1
	7	43	-	31	4	6
AE + CE	Mean no days	4.4	1	3	1	2
	0 (not consumed)	89	95	94	94	91
	1	1	1	1	0	2
	2	1	2	1	1	3
	3	2	1	2	3	2
	4	3	0	0		2
	5	-	0		1	0
	6	1	-	1	-	-
	7	3	-	1	1	1
Control	Mean no days	0.5	0.1	0.2	0.2	0.3

AE: Adoption encouragement and CE: Consumption encouragement.

ii		% consumed in a week from endline				
Treatments	No of days	Any foods from QPM	Porridge from QPM	Injera from QPM	Quita/flat bread from QPM	Dabo/fermented bread from QPM
	0 (not consumed)	50	74	60	74	64
	1	0	12	1	7	8
	2	5	11	3	10	11
	3	5	3	4	6	7
	4	4	0	3	2	2
	5	3	0	1	1	1
	6	1	-	0	1	1
	7	32	-	28	-	6
AE	mean no of days	2.9	0	2	1	1
	0(not consumed)	42	65	55	66	54
	1	1	14	4	10	9
	2	6	15	4	11	14
	3	7	6	6	7	11
	4	7	0	4	2	3
	5	3	-	0	1	1
	6	1	0	1	-	1
	7	33	-	26	4	6
AE + CE	mean no of days	3.1	1	2	1	1
	0 (not consumed)	91	96	94	95	93
	1	1	3	0	2	1
	2	2	1	1	2	3
	3	2	0	0	0	1
	4	1		1	1	1
	5	-	-	-	-	-
	6	-	-	-	-	-
	7	4		3	0	1
Control	mean no of days	0.4	0.1	0.3	0.1	0.2

AE: Adoption encouragement and CE: Consumption encouragement.

Chapter 3

Associations among high-quality protein and energy intake, serum transthyretin, serum amino acids and linear growth of children in Ethiopia

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Abstract

Limited evidence is available on the associations of high-quality protein and energy intake, serum transthyretin (TTR), serum amino acids and serum insulin-like growth factor-1 (IGF-1) with linear growth of young children. Data collected during the baseline of a randomized controlled trial involving rural Ethiopian children aged 6–35 months (n = 873) were analyzed to evaluate the associations among height/length-for-age z-scores, dietary intakes, and these biomarkers (i.e., serum level of TTR, IGF-1, tryptophan and lysine, and inflammation). The prevalence of stunting was higher for children >23 months (38%) than ≤23 months (25%). The prevalence of inflammation was 35% and of intestinal parasites 48%. Three-quarters of the children were energy deficient, and stunted children had lower daily energy intake that non-stunted children ($p < 0.05$). Intakes of tryptophan, protein, and energy, and serum levels of tryptophan and IGF-1 were positively correlated with linear growth of children. Controlling for inflammation, intestinal parasites, and sociodemographic characteristics, daily tryptophan (b = 0.01, $p = 0.001$), protein (b = 0.01, $p = 0.01$) and energy (b = 0.0003, $p = 0.04$) intakes and serum TTR (b = 2.58, $p = 0.04$) and IGF-1 (b = 0.01, $p = 0.003$) were positively associated with linear growth of children. Linear growth failure in Ethiopian children is likely associated with low-quality protein intake and inadequate energy intake. Nutrition programs that emphasize improved protein quantity and quality and energy intake may enhance linear growth of young children and need to be further investigated in longitudinal and interventional studies.

Background

Globally, an estimated 151 million children were affected by linear growth failure in 2017 [1]. Linear growth failure (stunting) in early childhood as a manifestation of chronic undernutrition is a major public health problem in developing countries [2]. Over 75% of all stunted children under five years of age live in either the African or Southeast Asia regions [1,2]. Linear growth failure as a result of inadequate nutrition and infections is a major cause of morbidity and mortality in infants and children [1,3]. Growth failure in early life leads to permanent impairment and can affect future generations [4]. Several studies have been conducted on the role of micronutrients in linear growth of children [5]. However, the role of protein-energy and high-quality protein intake on linear growth of children has so far been poorly studied in developing countries [6,7]. Linear growth faltering is widespread among Ethiopian children [2,8].

Protein and essential amino acids are required for the growth of children [9–11]. The association between children's growth and high-quality protein intake (particularly intake of limiting essential amino acids lysine and tryptophan) is complex and influenced by several factors (See fig. 1). Childhood morbidity can cause inflammation as well as decreased appetite and can therefore reduce intake of nutrients including high-quality protein and energy. It can also lead to changes in caregivers' child feeding practices, which can also affect nutrient intakes [12]. Children are more sensitive to high-quality protein malnutrition than adults [9,10], probably due to the high requirement for various physiological functions and additional requirements during illness. Animal-based food products contain high amounts of protein, which are considered to be of excellent quality [13–15]. In developing countries such as Ethiopia, however, dietary protein is mainly limited to plant-based sources, which are deficient in certain essential amino acids such as lysine and tryptophan [16,17].

The relationship between high-quality protein intake and children's growth in the context of energy deficit as well as illness is poorly understood in developing countries [6,18]. During illness, children need

additional protein and essential amino acids to recover [15,19]. The requirements for protein and essential amino acids are higher in the presence of chronic or acute infections [15,20]. Inflammation increases amino acids requirements three-fold [21]. However, the effect of inflammation on protein and amino acid requirements among children in developing countries is poorly understood [12,21]. Further, evidence suggests that energy deficit increases the need for protein and essential amino acids [22,23]. The current estimates of protein and essential amino acid requirements do not address the question of increased requirements due to frequent infections and energy deficit in children in developing countries [15].

Recent evidence suggests that stunted children might not be receiving adequate dietary intake of essential amino acids, and may have low circulating amino acids [24]. Insulin-like growth factor-I (IGF-I) is a protein hormone that mediates the effects of growth hormone and is reported to have numerous anabolic effects on skeletal muscles and other tissues [25–27]. When children have inadequate intake of protein and essential amino acids, their serum transthyretin (TTR), serum amino acids (AAs), as well as serum IGF-1 level may be low, which may, in turn, reduce the growth of children. However, this relationship has not been studied in developing countries with higher levels of inflammation. Furthermore, the role of inflammation on TTR, serum essential AAs and serum IGF-1 levels among children is poorly understood in developing countries [6].

A recent study on energy supply at the country level in developing countries has shown that energy supply was correlated with stunting among children [18]. Energy deficiency caused by inadequate food intake may lead to suboptimal nutritional status. Very little information is available on the relationship between energy intake and linear growth of children in Ethiopia. Evidence showed that the appropriate number of feedings depends on the energy density of local foods and that a higher meal frequency is needed with low energy density diets [28]. Findings on the effect of increased energy density of complementary foods on linear growth of children have been inconsistent [29].

To our knowledge, this study is the first to investigate the associations among the intakes of protein, energy, and the essential amino acids tryptophan and lysine; serum levels of TTR, lysine, tryptophan, and IGF-1; and linear growth of Ethiopian children. The association between the growth of children and high-quality protein intake is complex and influenced by several factors (See fig. 1). Linear growth may be sensitive to intake of high-quality protein through serum transthyretin, serum amino acids, and insulin-like growth factor-1 (IGF-1) [12], and the high-quality protein requirements of children may also be affected by inflammation and low energy intake [6]. We hypothesized that high-quality protein intake, energy intake, serum TTR, serum AAs, and serum IGF-1 are associated with linear growth of children in rural Ethiopia.

Materials and Methods

Study Design and Study Population

Data were collected from July–September 2015 as part of a baseline for a randomized controlled trial (RCT) of quality protein maize consumption of rural Ethiopian children aged 6–35 months (n = 873). The study protocol and population characteristics have been described elsewhere [30]. A total of 1491 households were screened, of which 873 households with children aged 6–35 months were eligible and selected for data collection. Five subjects were excluded from the analysis of primary outcome (HAZ) because their records were flagged as biologically implausible anthropometric values. Of eligible children, 611 were randomly selected for biomarker sampling, and 527 stool and 537 serum samples were collected for analysis. Ethical approval was obtained from the Ethiopian Public Health Institute Scientific and Ethical Review Committee (SERO-006-02-2015) and the Harvard University Institutional Review Board (IRB14-3255).

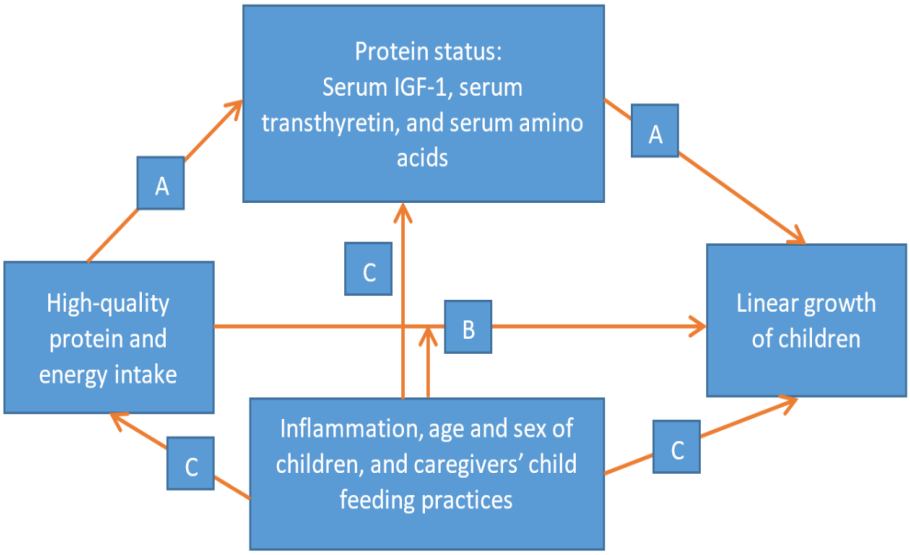

Fig. 1. Conceptual framework depicting pathways for associations between protein and energy intakes and linear growth of children: (**A**) Intake of high-quality protein improves protein status [12], which in turn improves linear growth of children; (**B**) The relationship between high-quality protein and energy intakes and linear growth of children is affected by inflammation [6]; and (**C**) Inflammation, together with characteristics of the child and caregivers' child feeding practices, reduces nutrient (protein and energy) intake, biomarkers of protein status, and linear growth of children [12].

Written informed consent was obtained from all adults who were interviewed, specifically the household head and caregiver. If participants were unable to sign their name, they affixed their thumbprint to the consent form and a witness to the consent process signed the consent form.

Data Collection

Interviews with caregivers were conducted by trained enumerators using a pretested questionnaire which was electronically administered with tablets using Open Data Kit (University of Washington, Seattle, WA, USA) software. Every day, collected data were sent to the central server and transferred from comma-separated values (CSV) files into the statistical software packages.

Venous blood samples were collected from children by trained phlebotomists. About 35 gram of fresh fecal samples were collected and placed in labeled clean plastic stool containers. A temporary field laboratory was set up in a central location i.e., school or health center for the laboratory technologist to immediately centrifuge and aliquot the serum into appropriate cryovials. All samples were transported for laboratory analysis promptly after collection in cold boxes containing frozen gel packs (−20 °C).

Dietary Assessment

Dietary recall interviews were used to estimate the amount of each food consumed by the children. High-quality protein intake was quantified as intake of lysine and tryptophan. High-quality protein intake, as well as the intakes of total protein and energy, were estimated based on the 24 hours dietary recall data.

Caregivers were interviewed about the food and beverage intake of their children during the preceding 24 hours defined as the time the child woke up the previous day until the time the child woke up the day of the interview. The multi-pass technique [31] was used after rigorous training and pre-tests conducted before dietary data collection. Each interview involved a stepwise series of questions, common household utensils, food substitutes (playdough, flour, lentils, and water, which were used as substitutes to estimate the quantities of the actual foods prepared and fed) and pictures of the most commonly consumed foods to improve the memory of the respondents and to assist in completing the recall. A digital food scale (Electronic Kitchen Scale EK 01) was used to measure the weight of the food consumed as well as the ingredients used in food preparation to the nearest 1 gram.

First, the caregivers were asked to report everything that their children had consumed the previous day, including during the night. The opening question was; "After you got up this morning/yesterday morning, when was the first time that you had given something to eat or drink to your child?", followed by the questions "What did your child eat or drink at that time?" and "Did the child eat or drink anything else at that time?"

The same three questions were repeatedly asked until the caregiver had recalled all the food and drink items consumed over the specified period. The first pass ended with the questions "Can you remember any other times you had given something to eat or drink to your child?". In the second pass, caregivers were asked to provide additional detailed information about each item of food and drink consumed by the children. This included the name of the food item (e.g., condiments, sugars), where they had eaten it, brand names, cooking methods, amounts served, and amount consumed. For homemade dishes, the caregivers were asked for the recipes and ingredients. The final pass reviewed all previously recalled information to confirm the accuracy of the record. During the final pass, the enumerators were also instructed to prompt for information about foods and drinks not mentioned that were considered to be easy to forget [32,33], such as snacks, fruits, water, and juices, which enumerators read from a list.

The interviews were conducted on all seven days of the week to capture variance in the intake across various days of the week. The content of protein and energy of foods consumed were obtained from the food composition databases compiled for Ethiopian National Food Consumption Survey (NFCS), which were primarily from the local food composition table (FCT) III and IV [34,35]. The values for lysine and tryptophan were borrowed from Tanzanian, UK, and the United States Department of Agriculture (USDA) food composition databases [36]. If the food was shared with other household members, FAO adult equivalent ratios were used to estimate the child's consumption [37]. Estimated average requirement (EAR) was defined as per the World Health Organization/Food and Agriculture Organization of the United Nations (WHO/FAO) [15]. The web-based software Compl-eat© (version 1.0, Wageningen University, Wageningen, The Netherlands, http://www.compleat.nl) was used to estimate protein, energy, lysine and tryptophan intakes.

Anthropometrics Assessment

Anthropometrics (i.e., height or recumbent length, and weight+) were collected on all selected children. Age of children in a month was taken

from their caregiver recall and further confirmed from immunization cards. The weight of children was measured with light clothing and without shoes to the nearest 100 gram using a standard UNICEF SECA 874 U digital scale (UNICEF Supply Division, Copenhagen, Denmark). The scale was calibrated using standard weights after moving from one household to the next.

The length of younger children (6–23 months) was measured in a recumbent position to the nearest 0.1 cm using a measuring board designed by UNICEF (UNICEF Supply Division, Copenhagen, Denmark) with an upright wooden base and movable headpiece. The height of children older than 23 months of age was measured in a standing position with the same measuring board, to the nearest 0.1 cm.

Biochemical Assessment

Serum TTR, alpha-1-glycoprotein (AGP), and C-reactive protein (CRP) concentrations were determined by immune-turbidimetry using Cobas 6000 (Roche Diagnostics, GmbH, Mannheim, Germany) with fully automated clinical chemistry instruments. Inflammation was measured using CRP and AGP and defined as having either elevated CRP > 5.0 mg/L and/or AGP > 1.0 gram/L. Serum IGF-1 concentrations were measured in duplicate using R&D Systems Quantikine Enzyme-linked Immunosorbent Assay (ELISA) kits (R&D Systems, Abingdon, UK) following the manufacturer's instructions. Serum samples were pre-treated prior to analysis to dissociate or release the IGF-1 from its binding proteins. The analysis of serum amino acids (lysine and tryptophan) was conducted using Biochrom 30 amino acid analyzer (the gold standard in amino acids analysis), and the method based on ion exchange chromatography with post column derivatization with Ninhydrin, as described previously [38–40].

During the data collection in the field site, a portion of each stool sample was processed by the Kato-katz techniques [41], and a direct mount was prepared to diagnose the presence of active motile trophozoites and larval stages of intestinal parasites. Lugol's iodine was added to observe cysts of the intestinal protozoan parasites. The leftover

samples were preserved using 10% of formalin to preserve the morphology of the parasite ova. A portion of the preserved stool sample was analyzed with the formol-ether concentration method as described by Ritchie [42], with some modification. In brief, the stool sample was sieved with cotton gauze and transferred to a 15 mL centrifuge tube. Then 12 mL of 10% formalin and 3 mL of diethyl ether was added and centrifuged for 5 min at 1500 rpm. The supernatant was discarded and the residue was transferred to microscopic slides and observed under a light microscope at 10× and 40× magnifications for the presence of cysts and ova of the parasites. The presence of parasites was confirmed when observed by any of the methods above.

The analyses of serum transthyretin, serum IGF-1, AGP, and CRP were conducted at the Ethiopian Public Health Institute (EPHI) laboratory, certified by the Ethiopian National Accreditation Office in accordance with the requirements of ISO 17025:2005 and ISO 15189:2012. The analysis of serum amino acids was done at Ansynth Service B.V., The Netherlands, an amino acid specialized laboratory (http://www.ansynth.com/, Roosendaal, The Netherlands). The CV (inter-assay) for the various indicators were: serum transthyretin, 3.1%; IGF-1, 17%; AGP, 3.6%; CRP, 2.8%; and serum amino acids, 1.5%.

Statistical Analysis

Statistical analyses were conducted with SAS version 9.3 (SAS Institute, Cary, North Carolina, USA). The weight and length of the children were converted into Z-scores for height/length-for-age (HAZ or LAZ), and weight-for-height (WHZ) according to 2006 WHO child growth standards using WHO Anthro software [43]. Stunting was defined as LAZ or HAZ scores less than 2 standard deviations below median values. The Mann–Whitney test was used to compare median high-quality protein and energy intake, serum TTR, serum IGF-1, serum lysine, and serum tryptophan between stunted and non-stunted children. Pearson correlation was used to investigate the correlation between high-quality protein intake, serum TTR, serum IGF-1, serum lysine, and serum tryptophan and linear growth of children. Multivariate linear regression was used to examine the associations between linear growth (HAZ or LAZ) as the dependent variable and serum

transthyretin, serum lysine, serum tryptophan, and IGF-1 as independent variables while controlling for inflammation, intestinal parasites, age and sex of children. A p value < 0.05 was considered statistically significant.

Results

Characteristics of the Study Population

The majority (96%) of caregivers were the spouse of the household head (Table 1). Caregivers' age ranged from 22 to 34 years, with a median of 28 years. Two out of three caregivers had no formal education, and caregiver education was similar between households with stunted and those with non-stunted children. Households with stunted children were more likely to be poor (Table 1).

Feeding Indicators and Child Characteristics

From children who participated in the study, 48% were female and the median age was 20 months (Table 2). Most children, stunted or non-stunted, had been supplemented with vitamin A in the last six months. There were no statistical differences in reported illness among stunted and non-stunted children. About 18% of children had complaints of diarrhea; 17% of cough; and 19% of fever in the two weeks prior to the study. About 22% of children had taken drugs for intestinal worms in the six months prior to the study. There were no statistically significant differences in infant and young child feeding practices indicators between households with and without stunted children (Appendix, Table S1). We found stunting was higher for older children and boys. The prevalence of wasting was about 5% (Fig. 2).

Table 1. Socioeconomic and demographic characteristics of participating households.

Indicators	All Households (*n* = 868)	Households with Stunted Child (*n* = 258)	Households with Non-Stunted Child (*n* = 610)
Caregiver age (years), Median (Q1, Q3)	28 (25, 32)	28 (25, 32)	28 (25, 32)
Caregiver relationship to household head, %			
Household head	3	2	4
Spouse	96	97	96
Other	1	1	-
Caregiver with no formal education, %	65	67	64
Religion, %			
Christian	62	61	63
Muslim	38	39	37
Family size, Median [Q1, Q3]	6 [5,8]	6 [5,7]	6 [5,8]
Wealth tertiles [1], %			
1st tertile (poorer)	33	37	31 *
2nd tertile	33	37	32 *
3rd tertile (wealthier)	33	26	37 *

* $p < 0.05$, households with stunted different from households without stunted children. [1]Wealth tertiles were constructed based on household assets using principal component analysis (PCA) techniques and the list variables used for wealth tertiles were sickle, hoe, shovel, axe, knap sack spray, ox plough, horse or mule cart, donkey or oxen cart, horse or mule saddle, bicycle, motor bike, car track, grinding stone, motorized, charcoal, kerosene, water carrier, refrigerator, watch clock, table, chair, bed, electric,, kerosene, radio, tape player, mobile phone, non-mobile phone, television, and owned land.

Table 2. Children's health characteristics.

Variables	Total (*n*=868)	Stunted (*n*=258)	Non-Stunted (*n*= 610)
Female, %,	48	44	49
Age in months, Median (Q1, Q3)	20 (13, 27)	23 (16, 28)	19 (12, 26)
Vitamin A supplementation in the last six months, %	83	84	83
Any multivitamin in the last six months, %	4	6	4
Iron tablets/syrups in the last six months, %	1	2	1
Any drugs for intestinal worms in the last six months, %	22	24	21
Diarrhea in the two weeks before survey, %	18	17	18
Cough or breathing problems in the two weeks before the survey, %	17	15	18
Fever in the two weeks before the survey, %	19	20	19
Height-for-age (HAZ) (overall), Mean ± SD	−1.3 ± 1.3	−2.8 ± 0.7	−0.7 ± 1.0

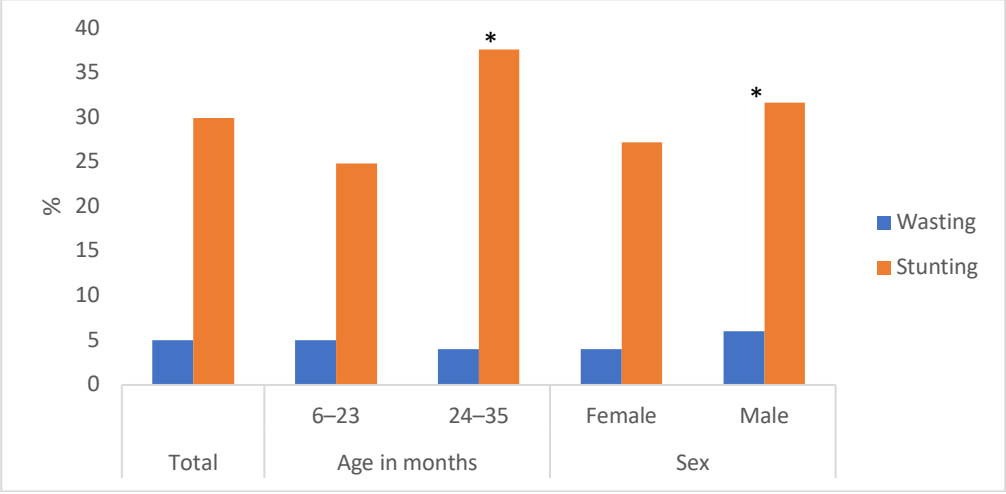

Fig. 2. Nutritional status of children by age and sex. * $p < 0.05$, nutritional status different by age and sex.

Dietary High-Quality Protein and Energy Intake of Children

No difference was found in the median protein intake between stunted and non-stunted children ($p > 0.05$), but intake of tryptophan of stunted children was significantly lower than that of non-stunted children (Table 3).

The energy intake of stunted children was significantly lower than that of non-stunted children. Furthermore, most children's energy intake in both stunted and non-stunted children was below the estimated average requirement. All children with protein deficiency were also energy deficient. The median energy density of the child's complementary foods was 1.4 kcal/g with no significant difference between stunted and non-stunted children (Table 4). We found that the contribution of cereals to the total protein and high-quality protein intake, as well as energy intake, was about 80% (Fig. 3). The consumption of animal foods such as meat, poultry, and fish was very limited.

Protein Biomarkers of Children

Serum tryptophan and serum IGF-1 were lower for stunted than for non-stunted children ($p < 0.005$) (Table 5). No difference was found in serum lysine and serum transthyretin between stunted and non-stunted children. Over one-third of children had acute and/or chronic inflammation and about half of children had one or more intestinal parasites (Table 5).

Correlations Among Intake of Essential Amino Acids, Serum Transthyretin, Serum Amino Acids, Serum IGF-1 and Children's Growth

HAZ was positively correlated with intakes of tryptophan ($r = 0.12$, $p < 0.0001$), protein intake ($r = 0.10$, $p = 0.011$), energy intake ($r = 0.20$, $p < 0.001$), serum tryptophan ($r = 0.18$, $p = 0.001$) and serum IGF-1 ($r = 0.12$, $p = 0.004$) (Table 6). Further, we found WHZ positively correlated with serum transthyretin ($r = 0.12$, $p = 0.006$), and serum IGF-1 (r =

0.16, p = 0.0003). We found that inflammation (AGP) was negatively correlated with serum TTR (r = −0.37, p < 0.0001), serum tryptophan (r = −0.23, p < 0.0001) and serum IGF-1 (r = −0.10, p = 0.019).

Table 3. Dietary protein and essential amino acids intake of children [1].

Variables	Total (*n* = 868)	Stunted (*n* = 258)	Non-Stunted (*n* = 610)
Protein intake (gram/day) [2]	16 (12, 22)	16 (11, 21)	16 (12, 22)
Lysine intake (mg/day) [2]	589 (349, 859)	541 (333, 813)	597 (356, 868)
Tryptophan intake (mg/day) [2]	233 (164, 343)	205 (142, 284)	246 (173, 369) *
Prop. children with low protein intake (below EAR), % [3]	10.5	10	11
Prop. children with low lysine intake (below EAR), % [3]	31	30	31
Prop. children with low tryptophan intake (below EAR), % [3]	4	4	4

* p < 0.001, stunted different from non-stunted children, tested with Mann–Whitney test. [1] Intake includes both diet and breast milk. [2] Median [25th, 75th]. [3] The recommended EARs [44] are: protein (0.87 gram/(kg·d)); Lysine (45 mg/(kg·d)); Tryptophan (6 mg/(kg·d)); energy (678 kcal, 764 kcal and 935 kcal for children aged 6–8 months, 9–11 months and 12–23 months, respectively).

Table 4. Energy intake of children [1].

Variables	Total (*n* = 868)	Stunted (*n* = 258)	Non-Stunted (*n* = 610)
Energy intake (kcal/day) [2]	695 (519, 870)	643 (463, 818)	703 (550, 891) *
Proportion of children with low energy intake (below EAR), % [3]	76	85	72 *
Energy density (kcal/g) [2]	1.4 (1.2, 1.6)	1.4 (1.2, 1.6)	1.3 (1.2, 1.6)

* p < 0.001, stunted different from non-stunted children, tested with Mann–Whitney test. [1] Intake includes both diet and breast milk. [2] Median [25th, 75th]. [3] The recommended EARs [44] are: (678 kcal, 764 kcal and 935 kcal for children aged 6–8 months, 9–11 months and 12–23 months, respectively).

Association among High-Quality Protein Intake, Energy Intake, Serum Transthyretin, Serum Amino Acids, and Serum IGF-1 with linear growth (Height-for-Age, HAZ) of Children

After adjustment for inflammation status, intestinal parasites, age, sex, and household wealth, protein intake (b = 0.01, p = 0.005), energy intake (b = 0.0003, p = 0.0002), serum TTR (b = 2.58, p = 0.04), and serum IGF-1 (b = 0.01, p = 0.003) were each significantly associated with HAZ (Table 7A).

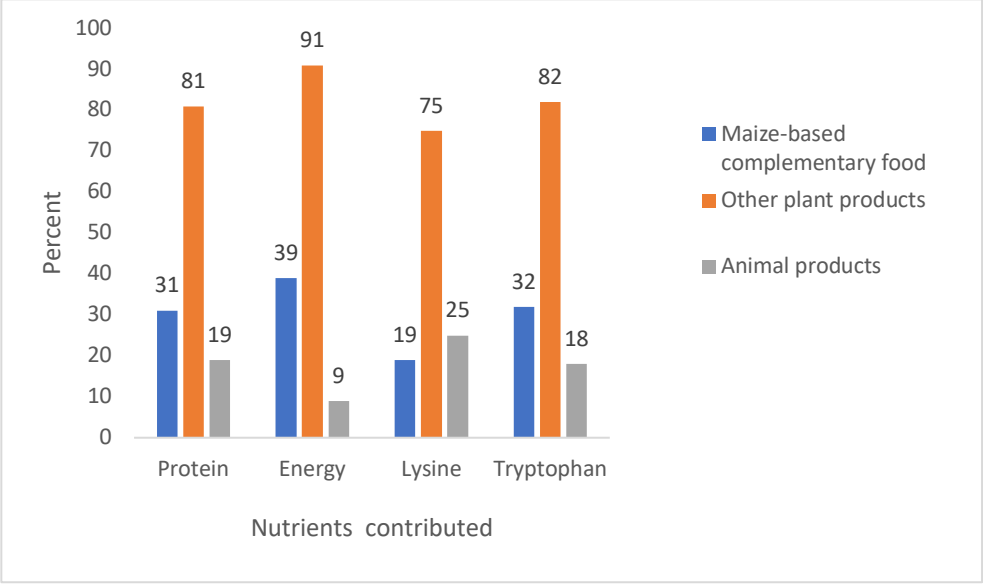

Fig. 3. The contribution of plant- and animal-based complementary foods to nutrient intakes of children.

Table 5. Protein and inflammation biomarkers and intestinal parasites of children [1].

Variables [1]	Total (*n* = 868)	Stunted (*n* = 258)	Non-Stunted (*n* = 610)
Serum transthyretin (gram/L)	0.17 (0.14, 0.20)	0.17 (0.14, 0.19)	0.17 (0.14, 0.21)
Serum IGF-1 (ng/mL)	30 (22, 44)	26 (19, 36)	32 (23, 46) *
Serum lysine (µmol/L)	141 (116, 164)	138 (116, 159)	142 (117, 167)
Serum tryptophan (µmol/L)	42 (32, 51)	39 (23, 49)	42 (34, 51) *
AGP (gram/L)	0.84 (0.65, 1.12)	0.83 (0.65, 1.11)	0.85 (0.65, 1.12)
CRP (mg/L)	0.67 (0.32, 2.03)	0.75 (0.32, 1.77)	0.65 (0.31, 2.11)
Prevalence of inflammation (acute and/or chronic), % [2]	35	35	35
Prevalence of one or more intestinal parasites, %	48	50	46

* $p < 0.05$, stunted different from non-stunted children checked by Mann–Whitney test. CRP: C-reactive protein. AGP: α-1-glycoprotein protein concentration. [1] Values are Median [25th, 75th] unless stated otherwise. [2] Inflammation: CRP > 5 mg/L and/ or AGP > 1 gram/L

While dietary intake of tryptophan per kg of body weight was positively associated with HAZ (b = 0.01, p = 0.001), dietary intake of lysine per kg of body weight was not (p = 0.69), and adjustment for inflammation status, intestinal parasites, age, sex, and household wealth resulted in no significant associations between HAZ and dietary intake or serum levels of lysine or tryptophan (p > 0.05, Table 7B). Dietary intake of tryptophan per kilogram body weight decreased among older children, resulting in collinearity with child age.

Discussion

We found that over one-third of children had growth failure in our study area. In Ethiopia, the prevalence of linear growth failure in children decreased from 57% in 2000 to 38% in 2015, about 1.3 percentage point reduction each year [2,8]. The existing prevalence rate still remains among the highest in the world indicating that growth failure is still a public health problem in Ethiopia, despite recent gains. In order to meet the goals for reduction in the prevalence of 40% set by the World Health Assembly [45], there is a need for country-specific evidence on the causes of child's linear growth failure and potential interventions to address the problem.

The present study showed that complementary foods consumed by study children were mainly prepared from cereals, suggesting low-quality protein intake. The highest digestibility of protein and biological values are found in food from animal origin [18,46]. Lysine and tryptophan are considered essential amino acids because they are not synthesized by humans, and they are the most limiting essential amino acids in human diets, particularly those reliant on cereals and other plant products [16,18,24].

These two amino acids are estimated to be particularly lacking among children in sub-Saharan Africa, including Ethiopia [8], because complementary foods here are primarily maize- or otherwise plant-based [13,16,18]. Thus, interventions to improve the intake of high-quality protein from child complementary food are warranted. Energy deficit in children may also lead to growth retardation, loss of fat and muscle, and increased morbidity and mortality [47]. Evidence has shown that when

children experience energy restriction, there is a significant decrease in nitrogen balance and decline in IGF-1 concentrations [48].

An interventional study among Indian children found that an energy-rich low-protein supplement improved linear growth [49]. Furthermore, inadequate intake of energy in animals leads to both reduced protein synthesis and degradation of muscle protein [50]. Our data suggest that energy deficiency is a major factor limiting child growth and may result in the diversion of some protein intake to meet energy requirements. We also found that the energy density of children's diets is reasonable as it fell in the ranges reported elsewhere for children receiving normal breastfeeding [28], indicating that the low energy intake was probably due to low food intake rather than low energy density of the food. Another possible explanation is that poor appetite is a common response to inflammation and therefore could be a major cause of low food intake by children. In view of the bulkiness of the diet, increasing the intake of complementary foods is not a feasible option in our population, and, hence, increasing energy density of consumed foods may be a better option [51]. However, there is limited recent evidence on the relationship between energy density and growth of children. A review of five studies on increased energy density of children's complementary foods in developing countries found that only two had positive impact on linear growth of children [29]. Further study is needed to better understand whether consumption of higher energy density food among children's complementary foods improves linear growth

Table 6. Pearson correlations between child's growth and other variables.

Indicators	HAZ	WHZ	Serum Transthyretin (gram/L)	Serum Lysine (µmol/L)	Serum Tryptophan (µmol/L)	Serum IGF-1 (ng/mL)	AGP (gram/L)	CRP (mg/L)	Lysine Intake (mg/kg/ Day)	Tryptophan Intake (mg/kg/ Day)	Protein Intake (g/Day)	Energy Intake (kcal/Day)	Intestinal Parasites
WHZ	0.11 **												
Serum transthyretin (gram/L)	0.08	0.12 **											
Serum lysine (µmol/L)	0.06	0.04	0.13 *										
Serum tryptophan (µmol/L)	0.18 **	0.01	0.25 ***	0.55 ***									
Serum IGF-1 (ng/mL)	0.12 **	0.16 **	0.22 ***	0.02	0.07								
AGP (gram/L)	−0.02	−0.02	−0.37 ***	−0.11 *	−0.23 ***	−0.1 ***							
CRP (mg/L)	0.005	−0.05	−0.32 ***	−0.13 *	−0.11 ***	−0.08 *	0.51 ***						
Lysine intake (mg/kg/day)	−0.01	−0.1 *	−0.03	0.01	−0.08	−0.09	0.01	0.09					
Tryptophan intake (mg/kg/day)	0.12 **	−0.069	−0.055	0.071	0.074	−0.042	−0.002	0.019	0.68 ***				
Protein intake (gram/day)	0.10 *	0.06	−0.01	0.04	−0.05	−0.07	0.03	0.04	0.74 **	0.47 **			
Energy intake (kcal/day)	0.13 **	0.07	−0.02	0.01	−0.03	−0.08	0.01	0	0.68 **	0.55 **	0.88 ***		
Intestinal parasites	−0.05	−0.1 ***	−0.05	0.05	−0.01	0.01	0.03	0.04	0.01	0.03	−0.03	−0.01	
Wealth index	0.1 **	0.11 **	0.01	0.04	0.01	0.04	0.004	0.05	0.09 *	0.03	0.01	0.01	0.01

***: $p < 0.001$, **: $p < 0.01$, * $p < 0.05$. HAZ: Height-for-age Z-score. WZ: Weight-for-height-Z-score. CRP: C-reactive protein. AGP: α-1-glycoprotein protein concentration. IGF-1: insulin-like growth factor-1.

Table 7. (**A**). The relationships of protein and energy intake, serum transthyretin, and serum IGF-1 with linear growth (height-for-age, HAZ) of children.

Models	Model 1*			Model 2*			Model 3*			Model 4*		
Fixed Effects	**B**	**SE**	**P**	**B**	**SE**	**P**	**B**	**SE**	**P**	**B**	**SE**	**P**
Intercept	−1.04	0.24	<0.0001	−1.10	0.26	<0.0001	−1.07	0.33	0.001	−0.89	0.24	0.0002
Protein intake (gram/day)	0.01	0.005	0.01									
Energy intake (kcal/day)				0.0003	0.0002	0.04						
Serum transthyretin (gram/L)							2.58	1.24	0.04			
Serum IGF-1 (ng/mL)										0.01	0.003	0.0004
Serum AGP(gram/L)	0.11	0.18	0.54	0.11	0.18	0.53	−0.06	0.16	0.70	−0.12	0.16	0.44
Serum CRP (mg/L)	−0.001	0.01	0.88	−0.0005	0.01	0.92	0.01	0.005	0.25	0.004	0.005	0.37
Intestinal parasites	−0.07	0.12	0.55	−0.08	0.12	0.51	−0.10	0.12	0.40	−0.08	0.11	0.47

* Model was adjusted for sex of child, age of child (in months), and household wealth tertile. AGP: α-1-glycoprotein protein concentration; and CRP: C-reactive protein concentration

Table 7.(B). The relationships of lysine and tryptophan intake and serum lysine and tryptophan with linear growth (height-for-age, HAZ) of children.

Models	Model 1			Model 2			Model 3 *			Model 4 *			Model 5 *			Model 6 *		
Fixed effects	**B**	**SE**	**P**	**b**	**SE**	**P**	**B**	**SE**	**P**	**B**	**SE**	**P**	**b**	**SE**	**P**	**b**	**SE**	**P**
Intercept	−1.26	0.09	<0.0001	−1.549	0.0948	<0.0001	−0.70	0.24	0.004	−0.63	0.29	0.03	−0.40	0.39	0.31	−0.92	0.39	0.02
Lysine intake per kg body weight (mg/kg/day)	−0.0004	0.001	0.69				−0.002	0.001	0.11									
Tryptophan intake per kg body weight (mg/kg/day)				0.01	0.003	0.001				−0.004	0.003	0.23						
Serum lysine (µmol/L)													−0.0003	0.002	0.89			
Serum tryptophan (µmol/L)																0.01	0.005	0.10
Serum AGP(gram/L)							0.10	0.18	0.58	0.10	0.18	0.58	−0.32	0.19	0.09	−0.25	0.19	0.20
Serum CRP (mg/L)							0.0002	0.01	0.96	−0.00002	0.01	1.00	0.01	0.01	0.13	0.01	0.01	0.14
Intestinal parasites							−0.08	0.12	0.52	−0.07	0.12	0.57	−0.01	0.14	0.96	−0.01	0.14	0.95

All models were adjusted for sex of child, age of child (in months), and household wealth tertile. AGP: α-1-glycoprotein protein concentration; and CRP: C-reactive protein concentration

In this study, we found that the proportion of children with total protein intake (both from diet and breast milk) below the estimated current average requirement was low (10%), while a higher proportion of children had deficient lysine intakes (30%). Protein and energy intakes were highly correlated, making it difficult to separate their relation to linear growth. Early evidence has shown that people with an energy deficit will need additional protein; even a modest energy deficit of 5% increases protein needs by about 10% [22]. The associations with protein biomarkers (serum TTR and IGF-1) suggest that there may be a biological mechanism between protein status and linear growth. Our analysis shows that over one-third of children had acute or chronic inflammation, and about half of children had one or more intestinal parasites. Although protein intake was found to be largely adequate, the current protein and essential amino acid requirements may not be adequate for energy-deficient children and those affected by high levels of inflammation and intestinal parasites. Earlier evidence suggests that bacterial infection increases protein requirements by about 30% [15] and lysine requirements by 50% [52] in malnourished children in India. A recent study conducted among Indian school children showed that intestinal parasite infestation increased the lysine requirement by 20% [53]. Inflammation resulting from morbidities and energy deficit [20] should be taken into account when calculating the requirements for protein and essential amino acids among children in Ethiopia. Our study population is energy deficient and will, therefore, have increased protein requirements. Moreover, the children mostly consume plant-based protein with a lower utilizability. Therefore, we may conclude that our population is also protein deficient.

To our knowledge, this is the first study to assess the pattern of linear growth failure in relation to protein, lysine, tryptophan, and energy intakes, while controlling for inflammation and intestinal parasites in Ethiopia. A simple comparison between stunted and non-stunted children did not reveal a difference in the intake of protein and lysine. The regression, however, did show a significant positive association between protein intake and linear growth of children. Evidence suggests that high-quality protein has a significant impact on gene expression, especially

IGF-1, which plays an important role in growth promotion [54], and in this study, serum TTR and IGF-1 were positively associated with linear growth. A recent review in developing countries showed a significant negative association between utilizable protein and stunting [18], emphasizing the need to address the low quality of dietary protein in developing countries. A longitudinal intervention study in Guatemalan children with high-protein food supplements showed an improvement in linear growth [55]. Evidence from animal trials showed that when lysine provision is inadequate, protein synthesis is unable to proceed efficiently and the rate of oxidation of all amino acids other than lysine increases disproportionately [10]. Studies in China [56] and Pakistan [57] found that fortification of wheat flour with lysine increased linear growth in children. While energy and protein intakes and biomarkers related to protein status (TTR and IGF-1) were associated with linear growth, this study did not find significant relationships between dietary or serum amino acids and linear growth. Most children in the study were breastfeeding, and breastmilk, therefore, provided a significant source of high-quality protein, particularly for younger children. Changes in dietary intake of complementary foods and breastmilk as children age may have confounded possible relationships between amino acids and linear growth. A further longitudinal study of the relationships between amino acid intakes and nutritional status is warranted, particularly as and after children cease breastfeeding.

The relationship between linear growth and serum TTR, serum lysine, and serum tryptophan were not previously studied in Ethiopia. A recent cross-sectional study among Malawian children suggests that stunted children have significantly lower circulating essential amino acids than non-stunted children [24]. In our study, we also found a positive association of linear growth of children with serum TTR, controlled for inflammation, intestinal parasites, age and sex of children, and household wealth. Evidence has shown that serum TTR is an indicator of the availability of essential amino acids in the body [25]. Previously it was used as a tool to screen patients with high risk of protein-energy malnutrition [25]. There are several possible explanations for these positive associations. First, protein and amino acids have biological roles

in protein and lipid synthesis, bone elongation, and the regulation of these and other processes necessary for linear growth [9,11,58]. Secondly, sufficient availability of amino acids potentially regulates cell and organismal growth [11]. Further, availability of amino acids is sensed via the master growth regulatory pathway of the cell, the mechanistic target of rapamycin complex 1 (mTORC1) [11,59], that will stimulate protein synthesis, cell, and organismal growth when amino acids are sufficient [60]. Inadequate dietary intakes of protein and essential amino acids may adversely affect serum amino acid status, which may, in turn, reduce the growth of children. Our data, however, did not show an association between serum lysine and tryptophan and linear growth of children. This needs further investigation.

The association between serum IGF-1 concentration and linear growth of children in developing countries is poorly understood. IGF-1 is a growth-promoting polypeptide that is essential for normal growth and development of children [27]. It is a major regulator of muscle protein and glucose homeostasis [26]. IGF-1 is also an important growth hormone, mediating protein anabolism and linear growth [27]. IGF-1 serum levels are responsive to improved nutritional status [61] and high-protein intake [48,62]. Protein restriction in children results in declined IGF-I concentrations [48]. Evidence from an animal model suggests that loss of IGF-1 signaling impairs muscle growth [26] and inactivation of IGF-1 causes linear and radial skeletal growth retardation [63]. We also found that serum IGF-1 concentration was positively associated with linear growth of children. Possible reasons are that low quantity and quality protein intake might affect stimulation of serum IGF-1, which mediates linear growth of children. However, further longitudinal interventions studies are needed to understand the stimulating effect of high-quality protein intake on serum IGF-1 and children's linear growth.

This study has several limitations. First, we could not establish causality between the observed associations, because of the cross-sectional character of the study. Second, the present study did not measure all factors that may be important for children's linear growth, e.g., environmental factors related to health and hygiene or child caregiving practices and resources [64]. Third, the misreporting of food

consumption is a potential issue for all dietary assessment methods, and it is not known to what extent parents underreport the dietary intakes of young children. The potential for underestimating leftovers, resulting in over-reporting of actual consumption, is a particular risk in this age group [65].

Conclusions

Inadequate protein and energy intake may be a predictor of childhood linear growth failure in rural Ethiopia. Nutrition programs that emphasize food security, recommended child feeding practices, and increased nutrient density of complementary foods, including density of high-quality protein and energy, may improve child's linear growth, especially in areas characterized by high inflammation and infections. Further, the calculated requirements for protein and essential amino acid intakes for children should account for inflammation, energy deficiency, and intestinal parasites in Ethiopia. The effect of consumption of high-quality protein food on linear growth in children will have to be further investigated in longitudinal intervention studies, including whether consumption of high-quality protein-enriched complementary foods, such as cereals with increased protein quality (e.g., quality protein maize), increase serum transthyretin and serum amino acid status, which in turn may lead to improved linear growth.

Author Contributions

M.T. designed the study protocol, performed the statistical analyses, composed the draft manuscript, and is responsible for the final content of the manuscript. H.D.G., N.S.G., and I.D.B. designed the study protocol, supervised the statistical analysis and draft manuscript preparation, and critically reviewed the manuscript. J.L.C., K.D., T.B., M.M., and D.B. participated in study protocol development. All authors read and approved the final manuscript.

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Ethical approval and consent to participate

Ethical approval was obtained from the Ethiopian Public Health Institute Scientific and Ethical Review Committee (SERO-006-02-2015) and the Harvard University Institutional Review Board (IRB14-3255). Written informed consent was obtained from all adults who were interviewed, specifically the household head and caregiver. If participants were unable to sign their name, they affixed their thumbprint to the consent form and a witness to the consent process signed the consent form.

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Appendix

Table S1. Infant and Young child Feeding Indicators

Variables	Total (n=868)	Stunted (n=258)	Non-stunted (n=610)
Prevalence of child ever being breastfed, %	99.7	99.6	99.7
Prevalence of initiation of breastfeeding (within 1 hour), %	71	67	73
Prevalence of colostrum/ first milk feeding, %	74	71	76
Prevalence of pre-lacteal feeding, %	3	4	3
Prevalence of exclusively breastfeeding for 6 months, %	53	53	53
Complementary feeding started At 6 month, %	50	50	49
Prevalence of bottle feeding, %	4	4	4

Chapter 4

Exposure to aflatoxins and fumonisins and linear growth of children in rural Ethiopia: a longitudinal study

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Abstract

Aflatoxins and fumonisins are common food contaminants in many developing countries that pose a major public health risk and may reduce linear growth in children. We hypothesize that exposure 1) inhibits protein synthesis, reducing serum protein and insulin-like growth factor 1 (IGF-1), and 2) increases inflammation and infection, leading to linear growth failure. Children (n=873) 6-35 months were enrolled in an intervention trial on quality protein maize consumption in rural Ethiopia (ClinicalTrials.gov Identifier: NCT02710760). These children were stratified by baseline stunting status, and 102 children (50 stunted and 52 non-stunted) were randomly selected for this study. Blood samples were collected in the pre-harvest (August-September 2015) and post-harvest (February 2016) seasons. Exposure was assessed using liquid LC-MS/MS by measuring serum aflatoxins, fumonisins, and metabolites: aflatoxin B1-lysine (AFB1-lys), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin M1 (AFM1), hydrolyzed fumonisin B1, fumonisin B1, fumonisin B2, and fumonisin B3. Linear growth was assessed using height-for-age difference. Proportions of children exposed to aflatoxin G1, aflatoxin G2, and aflatoxin M1 were higher in the pre-harvest season (8%, 33%, and 7%, respectively) compared to the post-harvest season (4%, 28%, and 4%, respectively). Similarly, the proportion of children exposed to any aflatoxin was higher in the pre-harvest than in the post-harvest season (51% vs. 41%). Exposure to fumonisins ranged only from 0-11%, depending on the type, and was not further analyzed. Exposure to any aflatoxin was not associated (p>0.05) with inflammation, serum transthyretin, IGF-1, or linear growth. Prevalence of aflatoxin exposure among rural Ethiopian children was high across seasons, with large variation between seasons and between individual aflatoxins. Exposure to fumonisins was relatively low. Aflatoxin exposure was not associated with protein status, inflammation, or linear growth of children. A larger study may be needed to examine potential biological interactions between aflatoxin exposure and linear growth.

Background

Poor linear growth of children, manifested as stunting, is the most prevalent form of undernutrition globally and has been associated with adverse health outcomes [1]. Despite the international commitment to reduce the number of stunted children under five years by 40% by 2030 [2,3], current nutritional interventions alone are unlikely to meet this target [4]. The aetiology of linear growth failure is poorly understood, which has hampered the development of effective interventions [5,6].

Many children in developing countries are not only stunted but also chronically exposed to mycotoxins — toxic fungal metabolites such as aflatoxins and fumonisins [7]. Both are common contaminants of maize and maize-based products in tropical countries [8]. Aflatoxins are produced by *Aspergillus flavus* and *Aspergillus parasiticus* on different crops, both in the field and in storage [9]. Aflatoxins are the most potent genotoxic and carcinogenic mycotoxins [10], and exposure to aflatoxins, mainly aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2), can cause hepatocellular cancer [11]. Exposure to multiple aflatoxins measured in serum is poorly studied. Fumonisins are produced mainly by *Fusarium verticillioides* (*Sacc.*) Nirenberg and *Fusarium proliferatum* (Matsush.) [9]. Contamination of children's complementary food with fumonisins has been reported in some countries [8,12-15]; however, presence of fumonisins in children's serum has not been studied.

Based on associations in observational studies in humans, there is a growing concern that exposure to aflatoxins is associated with impaired linear growth of children in developing countries [16-21]. However, research findings are inconsistent and the mechanisms are not clear. Observational studies in West Africa (Benin, Gambia, and Nigeria) found an inverse association between AFB1 exposure and linear growth of children as measured by height-for-age Z-score (HAZ) [16-21]. Likewise, a study from Mexico showed that a low level of aflatoxin exposure, measured using aflatoxin B1-lysine (AFB1-lys), was positively associated with linear growth [17]. In contrast, studies in East Africa [22-24] and

Nepal [25] did not find significant association between aflatoxin exposure (measured by aflatoxin-albumin or AFB1-lys/mg albumin) and linear growth of children. A recent intervention study among Kenyan children suggested that improving household access to aflatoxin-free maize reduced aflatoxin biomarker concentration in serum, but showed no effect on children's linear growth [22].

The biological mechanisms through which aflatoxin exposure affects linear growth of children are not clear. It has been proposed that exposure to aflatoxins may inhibit the synthesis of proteins including insulin-like growth factor 1 (IGF-1) or that it may increase inflammation and risk of infection. Each of these effects could in turn reduce child growth [26]. However, this hypothesis has not been formally investigated to date in humans. Therefore, we assessed children's exposure to aflatoxins and fumonisins, as measured in serum, in both pre-harvest and post-harvest seasons. Given the high prevalence of aflatoxin exposure, we then investigated associations between aflatoxin exposure and biomarkers of inflammation and protein status as well as linear growth.

Methods

Conceptual framework

We hypothesized that exposure to aflatoxins could lead to linear growth failure either by inhibiting protein synthesis or by increasing inflammation or incidence of infection (Fig. 1). Household wealth and child demographic characteristics such as sex and age could influence both exposure to aflatoxins and linear growth.

Study setting

The study used a subsample of children aged 6-35 months, stratified by baseline stunting, from an intervention trial on the consumption of quality protein maize (QPM) in rural Ethiopia (ClinicalTrials.gov Identifier: NCT02710760) [27], where maize is the predominant staple used in children's complementary foods. In total, the trial enrolled 873 children. These children were stratified by baseline stunting status, and a random

subsample of children were selected in each stratum (50 stunted and 52 non-stunted for a total of 102 children).

The objective of the trial was to assess the effect of promoting adoption and consumption of QPM on protein status and linear growth of children. The study protocol and sample characteristics are described elsewhere [27,28]. Children were eligible for inclusion in the overall trial if they met the following criteria: (1) the household had at least one child aged 6–35 months at recruitment in July-September 2015; (2) the household had at least one member who had attended a field demonstration conducted by the Nutritious Maize for Ethiopia (NuME) project in November 2014-January 2015; and (3) the household provided informed consent to participate in the study.

Data collection

Data were collected at three points: baseline (pre-harvest season, August-September 2015), midline (post-harvest season, February 2016), and endline (June-August 2016). Questionnaires were administered to the household head at baseline and midline, and to the caregiver at all three points of time. Topics in the caregiver surveys included demographics, household roster (baseline only), and child health and illness. Topics in the household head surveys included demographics and household assets. Anthropometrics (i.e., height/length combined with sex and age) were collected from all children at all three points of time following standard measurement procedures [29]. As mycotoxin exposure was only assessed at baseline and midline, this study only used questionnaires and anthropometric data from these two time points.

Venous blood (5 mL) was collected in the pre-harvest (baseline) and post-harvest (midline) seasons by trained phlebotomists using trace-element-free collection tubes (Vacutainer, Becton Dickenson, Franklin Lakes, NJ, USA). A temporary field laboratory was set up for the laboratory technologist to centrifuge and aliquot the serum immediately into appropriate cryovials. All samples for laboratory analysis were transported promptly after collection in cold boxes containing frozen gel packs (−20°C) and stored at −80°C prior to analysis.

Laboratory analyses

Aflatoxins (AFB1-lys, AFB1, AFB2, AFG1, AFG2, and AFM1) and fumonisins (hydrolyzed fumonisin B1, fumonisin B1, fumonisin B2, and fumonisin B3) were analyzed with liquid chromatography-tandem mass spectrometry (LC-MS/MS) [30]. Isotope-labelled $^{13}C_{15}$ aflatoxin was used as the internal standard. A protein precipitation method with few variations was used to prepare samples for analysis [30]. The details about materials and reagents used for the analyses of aflatoxins and fumonisins and about sample preparation are described in Appendix 1. The analyses of serum transthyretin, IGF-1, AGP, and CRP were described by Tessema et al. [28]. Serum transthyretin, IGF-1, AGP, and CRP were analyzed at the Ethiopian Public Health Institute laboratory, certified by the Ethiopian National Accreditation Office in accordance with the requirements of ISO 17025:2005 and ISO 15189:2012, while the analysis of serum amino acids was done at Ansynth Service B.V., a laboratory specializing in amino acids (http://www.ansynth.com/, Roosendaal, The Netherlands). Serum aflatoxins and fumonisins were analyzed at the Centre of Excellence in Mycotoxicology and Public Health, Faculty of Pharmaceutical Sciences, Ghent University, Belgium.

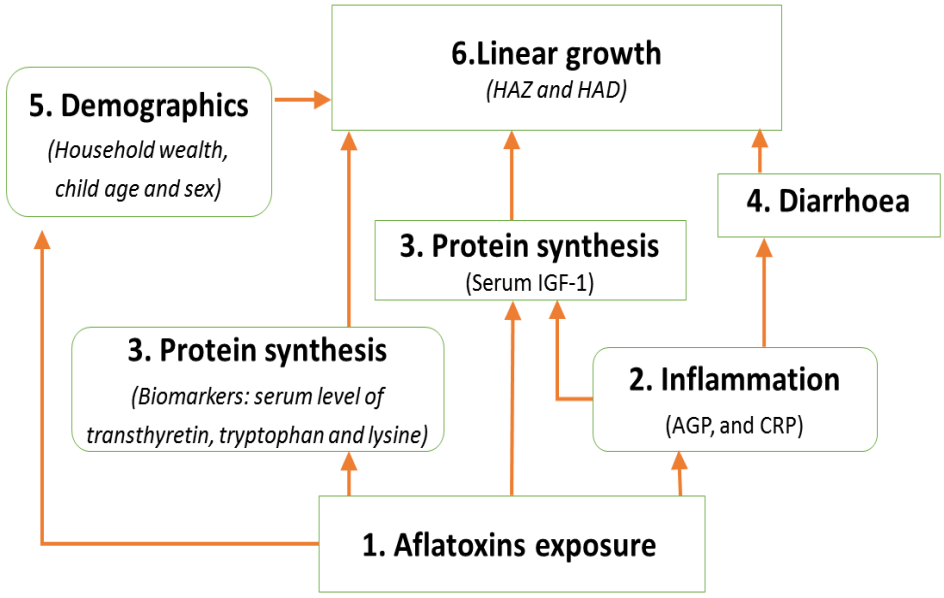

Fig. 1. A conceptual framework describing the relationship between aflatoxin exposure and linear growth in children.
1.Aflatoxin exposure was assessed by measurement of AFB1-lys, AFB1, AFB2, AFG1, AFG2, and AFM1 in serum samples.
2.Inflammation was assessed using C-reactive protein (CRP) and α-1-glycoprotein protein (AGP) concentrations in serum.
3.Protein synthesis was assessed by serum IGF-1 and transthyretin concentrations, as well as by serum tryptophan and lysine, the two most limiting amino acids in the diet.
4.Diarrhea was assessed by maternal recall for the two weeks prior to data collection.
5.Wealth tertiles were constructed based on household fixed assets using principal component analysis (PCA). Ownership of the following assets were used to represent wealth: sickle, hoe, shovel, ax, knapsack sprayer, ox plough, horse or mule cart, donkey cart, horse or mule saddle, bicycle, motorbike, car, stone grinder, charcoal stove, kerosene lamp, water carrier, refrigerator, watch or clock, table, chair, bed, electricity, radio, tape player, mobile phone, non-mobile phone, television and owned land.
6.Linear growth was measured using both height-for-age Z-score (HAZ) and height-for-age difference (HAD).

LC-MS/MS analysis

LC-MS/MS analyses were performed using an Acquity UPLC coupled to a Xevo TQ-S (Waters, Manchester, UK), equipped with a positive

electrospray ionization source (ESI). Two mobile phases were used: mobile phase A (95% water and 5% methanol) and mobile phase B (95% methanol and 5% water). Both phases were also adjusted with 5mM of ammonium acetate and 0.1% formic acid. The gradient elution program started at 100% mobile phase A. After an isocratic phase for 0.5 minutes at initial conditions, mobile phase B increased to 37% in 2.5 minutes. Then, during a further 13 min, phase B reached 75%. Later, it was enhanced for 2 minutes with 100% mobile phase B. An equilibration step for 1.5 minutes was introduced, resulting in a total run time of 19.5 minutes. The flow rate was set at 0.4 mL/min. The mass spectrometer was operated in positive electrospray ionization mode (ESI^+). The capillary voltage was 30 kV, and nitrogen was applied as spray gas. The source and desolvation temperatures were set at 150 °C and 200 °C, respectively. The argon collision gas pressure was 9×10^{-6} bar, the cone gas flow 50 L/h and the desolvation gas flow 500 L/h. Two selected reaction monitoring transitions with a specific dwell-time were optimized for each analyte, in order to increase the sensitivity and the selectivity of the mass spectrometric conditions (Appendix 2).

Method validation

The developed LC-MS/MS method was successfully validated, based on the European Commission Decision 2002/657/EC, which provides rules for the analytical methods to be used to test official samples [31]. Matrix-matched calibration plots were constructed for the determination of the analytes. MassLynx 4.1 and TargetLynx 4.1 software (Micromass, Manchester, UK) were used for data acquisition and processing. The real compounds were identified using peak ratio (relative ion intensity), the retention time and the signal-to-noise ratio [31]. After the completion of all criteria, the response, which is expressed as the ratio of the compound divided by the ratio of the area of the internal standard, was calculated.

Specificity was checked to ensure there was no interference or any peaks for the identification and quantification of the target compounds in the ±2.5 % margin of the relative time in 5 blank samples. Evaluating the linearity, the homogeneity of variance was checked before fitting the

linear model. The linearity was interpreted graphically using a scatter plot with the r^2 threshold set to ≥0.95. Recovery was calculated after measuring the concentration and the actual (spiked) concentration. The observed concentration was calculated in triplicate from a matrix-matched calibration curve. The precision was calculated in terms of the intraday (RSD_r) and interday (RSD_R) precision. Limit of detection (LOD) was calculated as three times the standard error of the intercept, divided by the slope of the standard curve; the limit of quantification (LOQ) was similar, differing by six times the standard error. The calculated LOD and LOQ, which should be more than 3 and 10, respectively, were verified by the signal-to-noise ratio (s/n), according to the IUPAC guidelines. The results of the performance characteristics of the LC-MS/MS method complied with the criteria outlined in European Commission Decision 2002/657/EC (Appendix 3) [31]. Briefly, four identification points should be fulfilled to allow confirmation of the identity of the detected compound; one precursor and at least two product ions should be monitored; the relative intensities of the detected ions should correspond within accepted deviations to those of the calibration; detected ions should have a s/n of at least 3; and the relative retention time of the detected ions must range within a margin of 2.5%.

Statistical Analyses

Exposure to any aflatoxins was defined as a binary variable indicating one or more aflatoxin biomarkers found at a detectable level in the child's serum. We used weighted prevalence [of exposure to aflatoxin], i.e., adjusted for baseline stunting prevalence to estimate aflatoxin and fumonisin exposure. To assess whether the exposure to aflatoxins was associated with linear growth, we measured height-for-age difference (HAD) and height-for-age Z-score (HAZ). HAD is preferred over HAZ when assessing changes in height/length with age in longitudinal data [32]. Stunting was defined as HAZ scores less than 2 standard deviations below median values [29].

Statistical analyses were conducted with SAS version 9.4 (SAS Institute, Cary, North Carolina, USA). Spearman's rank correlation was

used to investigate correlations among individual aflatoxins, inflammation (AGP and CRP), protein status (serum transthyretin, IGF-1, lysine, and tryptophan), and growth of children (HAZ and HAD). To examine these associations further, mixed linear models accounting for repeated measures on children were fitted using the SAS procedure 'proc mixed' with restricted maximum likelihood estimation and the Kenward-Roger method to compute degrees of freedom [33]. All regressions were repeated using sample weights; weighting did not materially change any of the study findings. Independent variables in the models were those known or suspected to be important predictors of the outcomes. A wealth index was created using principal component analysis [34] based on household assets, and households were assigned to wealth tertiles. Model diagnostics were checked to ensure that assumptions of normality of error terms and homogeneity of error variance were met. The level of significance was set at 0.05.

Results

At baseline (pre-harvest season), children were 21 months old on average and 65% were male. Fourteen percent of mothers reported that their child had diarrheal symptoms in the two weeks prior to data collection. Stunted and non-stunted children did not differ significantly in age, sex, diarrheal incidence, or household wealth (Table 1).

Half of the children (51%) were exposed to some aflatoxins during the pre-harvest season (Fig. 2a). Exposure to any aflatoxin type was lower in the post-harvest season ($p < 0.0001$), but still remained high (41%). Across seasons, exposure was most prevalent for AFB1 (19-22%), AFB2 (29-30%), and AFG2 (28-33%). Prevalence of AFB1-Lys, AFB1, and AFB2 in serum did not significantly change across seasons ($p>0.05$ for each); however, AFG1 ($p = 0.0048$), AFG2 ($p = 0.0192$), and AFM1 ($p = 0.0049$) were all less prevalent in the post-harvest season, although prevalence of exposure to AFG2 remained high (28%) (Fig. 2a). The proportion of children exposed to fumonisins was found to be low, ranging from 0-11% by type of fumonisin (Fig. 2b). Therefore, exposure to fumonisins was not further analyzed in this study.

Concentrations of individual aflatoxins varied in the serum of sampled children (Table 2). However, concentrations did not differ (p>0.05 using Wilcoxon tests) by baseline stunting status at either time point for any of the tested aflatoxins or AFB1-lys (Table 2). AFB1-lys was not correlated with any other aflatoxins in the pre-harvest season, but in the post-harvest season, AFB1-lys was positively correlated with AFB1 (r=0.20, p=0.04) and AFM1 (r=0.27, p=*0*.001) (Table 3). AFB1 and AFB2 were positively correlated in both seasons (p<0.001 for both). AFG1 was correlated with AFB2 (r=0.27, p=*0*.005) in the pre-harvest season and with AFB1 (r=0.25, p=*0*.01) in the post-harvest season, while AFG2 was positively correlated with AFB1 and AFB2 in both seasons (p<0.001 for both).

Table 1. Demographic and socioeconomic characteristics of study participants at baseline.

	Growth status			
Characteristics	**Stunted (*n*=50)**	**Non-Stunted (*n*=52)**	**All**	***P*-value[1]**
Child's age in months (mean ± SD)	21.6±7.6	19.8±8.7	20.7±8.2	0.27
Child's sex (male), %	72	58	65	0.13
Child had diarrheal illness in preceding two weeks, %	12	15	14	0.62
Household wealth status, %				
Less poor	16	34	25	
Medium	38	30	34	0.12
Poorest	46	36	41	

[1] No weighting was used for p-values.

Linear growth of children was not correlated with any aflatoxins in the pre-harvest season (all p>0.05) (Appendix 4). Furthermore, none of the measured aflatoxins were correlated with a biomarker of protein in

the pre-harvest season (Appendix 4). AFB1 was inversely correlated with IGF-1 (r=-0.26, p=0.009) and AFB2 was inversely correlated with serum tryptophan (r=-0.33, p=0.001) in the post-harvest season (Appendix 5).

Inflammation (AGP) was inversely correlated with the protein biomarkers serum transthyretin (r=-0.31, p=0.002), serum tryptophan (r=-0.23, p=0.02), and IGF-1 (r=-0.25, p=0.01) in the pre-harvest season (Appendix 4). Similarly, inflammation (AGP) was inversely correlated with the protein biomarkers serum transthyretin (r=-0.39, p<0.0001) and serum tryptophan (r=-0.25, p=0.012) in the post-harvest season (Appendix 5).

Height-for-age Z-scores (HAZ) in the pre-harvest season did not differ by exposure to aflatoxins in the same season (p=0.16, Fig. 3a). Similarly, HAZ in the post-harvest season did not differ by aflatoxin exposure in the pre-harvest (p=0.14) or post-harvest seasons (p=0.15).

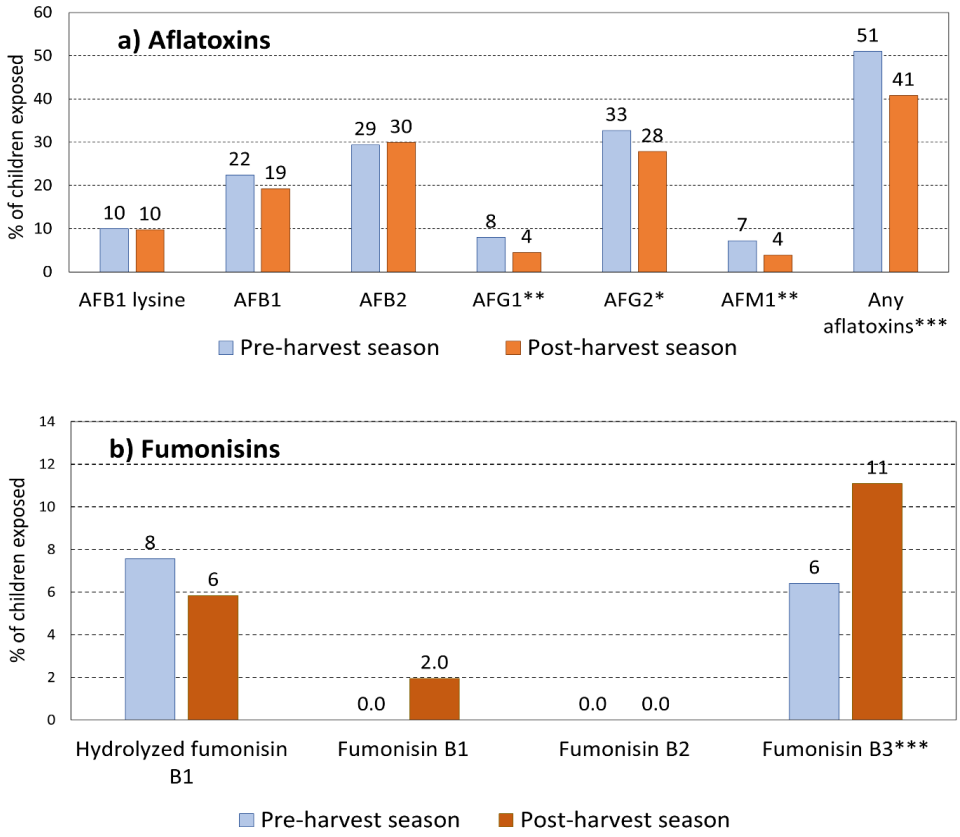

Fig. 2. Pre-harvest and post-harvest weighted prevalence of exposure to aflatoxins (a) and fumonisins (b) as measured in serum. P-values are from McNemar's tests using sampling weights. Difference between seasons $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

Table 2. Upper percentiles[1] of aflatoxin concentrations measured in serum.

Serum aflatoxin	Pre-harvest (pg/mL)				Post-harvest (pg/mL)			
	Stunted[2] (*n*=50)		Not stunted[2] (*n*=52)		Stunted[2] (*n*=50)		Not stunted[2] (*n*=52)	
	75%ile	Max	75%ile	Max	75%ile	Max	75%ile	Max
AFB1-lys	<LOD	167.0	<LOD	182.0	<LOD	290.0	<LOD	987.6
AFB1	<LOD	18.5	8.400	19.3	<LOD	18.9	<LOD	21.0
AFB2	5.700	11.3	6.000	11.3	6.500	14.3	2.700	10.9
AFG1	<LOD	5.6	<LOD	5.9	<LOD	5.8	<LOD	6.1
AFG2	0.500	5.6	1.200	5.9	0.400	5.1	0.600	5.6
AFM1	<LOD	11.1	<LOD	13.2	<LOD	10.9	<LOD	10.8

[1]As all aflatoxins were found in <50% of children, all median values were below the limit of detection (LOD). [2] As measured at baseline

Table 3. Correlations among aflatoxins and AFB1-lys in each season.

	Pre-harvest season					Post-harvest season				
	AFB1-lys	AFB1	AFB2	AFG1	AFG2	AFB1-lys	AFB1	AFB2	AFG1	AFG2
AFB1	0.07					0.20*				
AFB2	0.01	0.63***				0.13	0.43***			
AFG1	-0.08	-0.01	0.27**			0.10	0.25*	0.13		
AFG2	-0.004	0.41***	0.56***	0.38***		0.19	0.49***	0.65***	0.12	
AFM1	0.04	-0.13	-0.07	0.09	-0.08	0.27***	0.04	0.12	-0.05	0.05

Spearman's rank correlations, unweighted. Values <LOD were set to LOD/2. *p<0.05, p**<0.01, ***p<0.001.

However, when stunting (HAZ<-2) was specifically considered, children exposed to aflatoxins in the pre-harvest season were less likely to be stunted in the same season (p=0.01), contrary to expectation, though pre-harvest exposure was not associated with stunting in the post-harvest season (p=0.19) (Fig. 3b). In contrast, children exposed to aflatoxins in the post-harvest season were marginally more likely to be stunted in the same season (p=0.08) (Fig. 3b).

Exposure to any aflatoxin was not associated with acute or chronic inflammation (Table 4) or biomarkers of protein status (Table 5) in young children. However, AFB2 exposure measured in serum was inversely associated with serum tryptophan (p=0.0002). Children's exposure to any aflatoxins was not associated with linear growth , as measured using HAZ or HAD (Table 6).

Discussion

We found high prevalence of exposure to one or more aflatoxins in nearly half of our children during both the pre- and post-harvest seasons. In our study population, all the carcinogenic aflatoxins could be detected in variable amounts. Exposure to AFG1, AFG2, and AFM1 was higher in the pre-harvest season than in the post-harvest season (p<0.05). According to the International Agency for Research on Cancer (IARC), AFB1, AFB2, AFG1, AFG2, and AFM1 are highly carcinogenic for humans [35]. In general, the level of carcinogenicity is categorized in decreasing order as AFB1>AFG1>AFM1>AFB2>AFG2 [36]. Although exposure to aflatoxins in serum in our population was lower than in some other African studies [17,22], the current exposure data suggest that aflatoxin contamination is a public health problem in Ethiopia as zero tolerance for aflatoxin exposure is desirable.

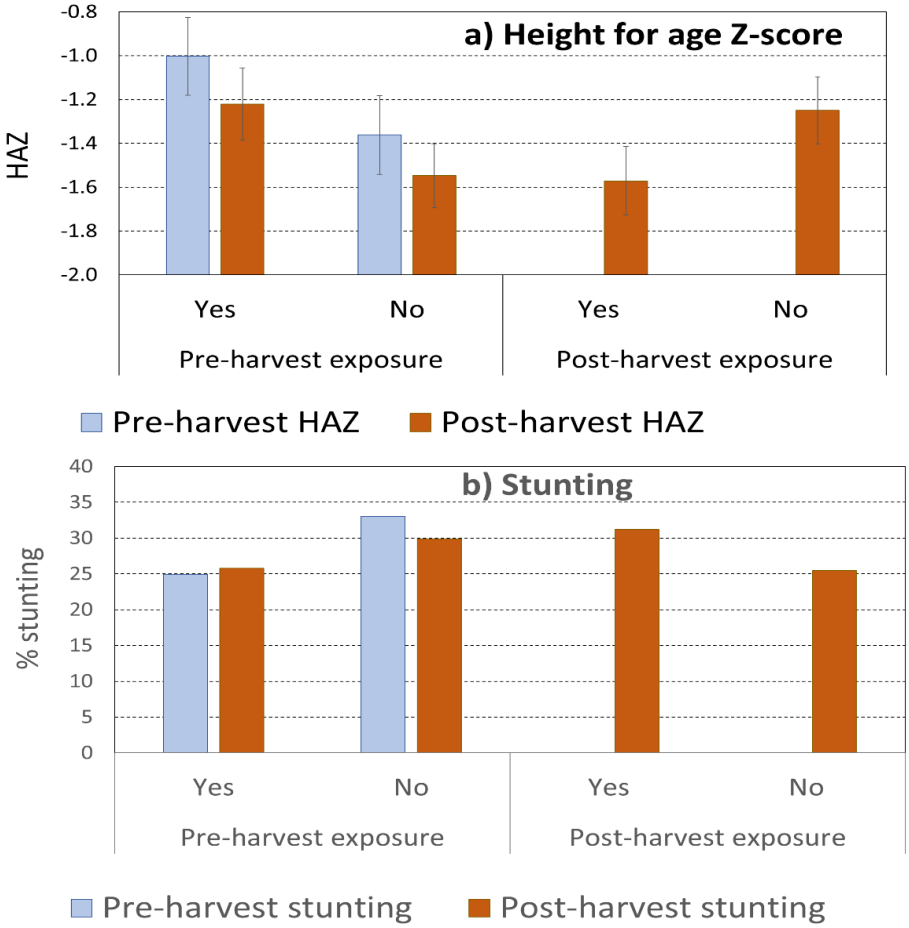

Fig. 3. Mean weighted height-for-age Z-scores (HAZ, panel a) and weighted prevalence of stunting. Error bars indicate standard error of the mean. (panel b) in pre- and post-harvest seasons, by exposure to any aflatoxins in either season.

To our knowledge, this is the first study that assesses exposure to aflatoxins using multiple serum biomarkers, allowing us to compare the relative contribution of each to exposure risk. AFB1 is the most frequently reported carcinogen in many cereal-consuming populations [37]. However, in our population, it was third-highest, with one out of five children exposed to AFB1. The serum concentration of AFB1-lys, an indicator of exposure over longer periods of time [37], was not high, contrary to our expectation but comparable to some other studies

[17,25]. AFB1-lys in our studied population was much lower than the exposure found in several West African studies [16-21]. In our study, exposure to AFB2 and AFG2 were the most common, found in nearly a third of our study children.

The prevalence of exposure to AFM1, a metabolite found in milk and milk products when animals are fed contaminated feeds, was low in our study. An experimental study of animals showed that both AFB1 and AFM1 were detected in the plasma of cows after the ingestion of AFB1 on corn-based feed [38]. As reported in our previous paper [28], milk consumption in our study area was minimal, which probably led to low levels of AFM1 contamination. AFG2 was quantitatively the most important type of aflatoxin, detected in about a third of our population. AFG1, on the other hand, was present in only a few samples, although both AFG1 and AFG2 are metabolites of AFB1 [38].

Table 4. Associations between aflatoxin exposures and inflammation among Ethiopian children. The quantiative effect of each aflatoxin or aflatoxin biomarker on AGP and CRP was individually assessed ("individual models") and assessed in a joint model ("joint model").

Aflatoxin	AGP						CRP					
	Individual models			Joint model			Individual models			Joint model		
	Estimate	SE	P-value	Estimate	SE	P-value	Estimate	SE	P-value	Estimate	SE	P-value
AFB1-lysine	-0.59	0.68	0.39				0.44	2.05	0.83			
AFB1	-1.49	7.40	0.84	2.72	8.14	0.74	-17.61	21.91	0.42	-12.20	24.28	0.62
AFB2	-17.54	10.53	0.10	-16.08	12.52	0.20	-34.55	30.60	0.26	-19.07	37.42	0.61
AFG1	-4.93	33.99	0.88	13.86	34.93	0.69	-55.37	102.21	0.59	-16.97	105.23	0.87
AFG2	-30.82	22.27	0.17	-21.77	24.46	0.37	-56.35	62.47	0.37	-36.95	70.41	0.60
AFM1	-19.38	12.14	0.11	-19.92	12.26	0.11	-61.44	36.51	0.09	-62.75	36.96	0.09

Individual models are controlled for child sex, child age, time of assessment (pre- or post-harvest), and intervention arm. Joint models are controlled for all aflatoxins except AFB1-lys, child sex, child age, time of assessment (pre- or post-harvest), and intervention arm. In all models, the natural logarithm was taken of the response variable (AGP or CRP) prior to analysis.

Table 5. Associations between aflatoxin exposures and serum biomarkers among Ethiopian children. The quantiative effect of each aflatoxin or aflatoxin biomarker on serum biomarkers was individually assessed ("individual models") and assessed in a joint model ("joint model").

Aflatoxin	Transthyretin						IGF-1[1]					
	Individual models			Joint model			Individual models			Joint model		
	Estimate	SE	P-value	Estimate	SE	P-value	Estimate	SE	P-value	Estimate	SE	P-value
AFB1-lysine	0.09	0.07	0.18				0.69	1.11	0.54			
AFB1	-0.20	0.73	0.78	0.03	0.82	0.97	-8.33	11.87	0.48	-9.37	13.42	0.49
AFB2	-0.73	1.08	0.50	-1.19	1.26	0.35	0.68	16.77	0.97	1.92	20.91	0.93
AFG1	-0.60	3.34	0.86	-0.99	3.48	0.78	0.15	58.01	1.00	-3.86	59.96	0.95
AFG2	2.29	2.38	0.34	3.22	2.59	0.21	19.88	32.78	0.55	21.09	37.79	0.58
AFM1	-0.56	1.22	0.65	-0.49	1.23	0.69	4.32	20.24	0.83	3.80	20.52	0.85

Aflatoxin	Lysine						Tryptophan					
	Individual models			Joint model			Individual models			Joint model		
	Estimate	SE	P-value	Estimate	SE	P-value	Estimate	SE	P-value	Estimate	SE	P-value
AFB1-lysine	-70.07	64.44	0.28				-10.25	25.90	0.69			
AFB1	-1191.01	688.09	0.09	-772.05	761.91	0.31	358.39	276.33	0.20	835.41	297.83	0.006
AFB2	-1540.33	977.46	0.12	-1574.30	1178.51	0.18	-1191.73	388.74	0.003	-1762.41	460.30	0.0002
AFG1	2247.66	3217.94	0.49	2583.35	3300.23	0.43	-158.25	1284.54	0.90	408.98	1284.44	0.75
AFG2	606.54	2014.48	0.76	1923.96	2220.26	0.39	-835.06	825.50	0.31	298.95	881.82	0.74
AFM1	1709.14	1159.02	0.14	1525.81	1168.37	0.19	-228.19	466.23	0.63	-187.73	454.82	0.68

[1].Response variable was natural log-transformed prior to analysis. Individual models are controlled for child sex, child age, time of assessment (pre- or post-harvest), intervention arm, inflammation (CRP: C-reactive protein and AGP: α-1-glycoprotein, both natural log-transformed). Joint models are controlled for all aflatoxins except AFB1-lys, child sex, child age, time of assessment (pre- or post-harvest), intervention arm, inflammation (AGP and CRP, both natural log-transformed).

Table 6. Associations between aflatoxin exposures and linear growth among Ethiopian children. The quantitative effect of each aflatoxin or aflatoxin biomarker on indicators of linear growth was individually assessed ("individual models") and assessed in a joint model ("joint model").

Aflatoxin	Height-for-age Z-score (HAZ)						Height-for-age difference (HAD)					
	Individual models			Joint model			Individual models			Joint model		
	Estimate	SE	*P*-value	Estimate	SE	*P*-value	Estimate	SE	*P*-value	Estimate	SE	*P*-value
AFB1-lys	2.25	1.50	0.14				6.04	4.52	0.18			
AFB1	8.91	16.76	0.60	20.30	18.76	0.28	-11.53	50.57	0.82	36.56	56.47	0.52
AFB2	-12.60	25.31	0.62	-21.49	28.60	0.45	-99.00	76.06	0.20	-109.54	85.95	0.21
AFG1	24.63	73.13	0.74	41.36	76.68	0.59	37.27	219.87	0.87	115.77	230.14	0.62
AFG2	-58.37	63.15	0.36	-63.72	67.13	0.34	-225.68	193.63	0.25	-206.90	205.74	0.32
AFM1	46.99	26.32	0.08	46.79	26.79	0.08	143.76	79.10	0.07	137.81	80.23	0.09

Individual models are controlled for child sex, child age, time of assessment (pre- or post-harvest), intervention arm, inflammation (CRP: C-reactive protein and AGP: α-1-glycoprotein, both natural log-transformed), household wealth tertile, and incidence of diarrhea in the preceding 14 days. Joint models are controlled for all aflatoxins except AFB1-lys, child sex, child age, time of assessment (pre- or post-harvest), intervention arm, inflammation (AGP and CRP, both natural log-transformed), household wealth tertile, and incidence of diarrhea in the preceding 14 days.

In the pre-harvest season, complementary foods consumed by children were mostly prepared from foods produced and stored on household farms. The higher prevalence of aflatoxins detected in the pre-harvest season could have been caused by poor storage conditions over a long period of time, leading to fungal proliferation and mycotoxin contamination of grain used to prepare complementary foods. Research on sources of mycotoxin exposure, including the effects of crop storage technologies, practices, and duration on mycotoxin contamination, is needed to develop effective mitigation strategies.

Exposure to fumonisins was low in our study population. To our knowledge, exposure to fumonisins has never been measured before in human serum. A study *in vitro* showed that fumonisins might have the potential to cause severe hepatotoxic, nephrotoxic, hepatocarcinogenic, and other cytotoxic effects in mammals [39]. Fumonisin B1 is the most toxic fumonisin [36] but a very low fumonisin B1 exposure (2%) was found in the post-harvest season and was not detectable in the pre-harvest season. Fumonisins are the second most important mycotoxins found on maize and in a variety of maize-based human foods, particularly when grown in warmer regions [40,41]. Fumonisin exposure from maize and other cereal-based foods has been reported in many developing countries [42] but not in serum. The low level of exposure to fumonisins in our population might be because fumonisins are poorly absorbed and they are excreted largely via the fecal route [43]. The other reason could be a low level of contamination in complementary foods in our study area. Despite the low fumonisin levels detected in this study, the authors strongly suggest continuing to study the fumonisin exposure in Ethiopia as our study was conducted in only a small sample not representative of the entire country. However, future studies should take other matrixes, such as feces or hair, to assess better fumonisin exposure.

Exposure to AFB1 was inversely correlated with serum IGF-1, while AFB2 exposure was inversely correlated with serum tryptophan in the post-harvest season. However, the correlation between AFB1 and IGF-1 disappeared in regression models adjusted for confounders. Exposure to individual aflatoxins including the AFB1-lys biomarker in serum was not associated with linear growth of children after adjusting for confounders.

Similar to our study, studies from East Africa [22-24] and Nepal [25] did not find an association between AFB1-lys exposure and linear growth of children. Some observational- and cross-sectional studies in West Africa found an inverse relationship between AFB1-lys biomarkers and child growth [16,18-21]. Similarly, an observational study from Mexico showed that the level of exposure to aflatoxins was inversely associated with linear growth [17]. A recent randomized controlled trial among Kenyan children found that in households that had access to aflatoxin-free maize, children had reduced serum aflatoxin concentrations, but linear growth of children was not affected [22].

There are a number of differences between our study and previous studies, most notably in the methods for the aflatoxin analyses and study setting. The LC-MS/MS method used in our analyses is a more specific and sensitive technique [21] than ELISA [44]. Also, most prior studies measured a single aflatoxin as a marker of exposure. The large variability in aflatoxins in serum and in linear growth suggests further studies with larger sample sizes may be required to detect associations or causal linkages between aflatoxin exposure and growth of children. Even without a biological mechanism between aflatoxin exposure and linear growth faltering, aflatoxin exposure remains a critical concern, given its carcinogenicity and other potential implications for health as well as international trade.

When trying to elucidate factors that could explain a relationship between exposure to aflatoxins and growth, Smith et al. hypothesized that aflatoxin exposure may inhibit protein synthesis and increase inflammation or infection [26]. We therefore investigated this hypothesis but found, contrary to our expectation, no significant association between any aflatoxin biomarkers measured in serum and inflammation biomarkers or protein status biomarkers such as serum transthyretin. Serum concentrations of tryptophan and lysine may mediate protein synthesis because these are the two most limiting amino acids in maize-based diets. Exposure to aflatoxins was not associated directly with these serum amino acids; however, AFB2 was inversely associated with serum tryptophan.

In our study, the lack of association between aflatoxin biomarkers in serum and selected biomarkers of protein synthesis may have been due to relatively low serum concentrations of the aflatoxin biomarkers, even though the prevalence of exposure was high. Furthermore, our sample size was small, which limited the statistical power to detect smaller effects from low-level aflatoxin exposure. The lack of association between aflatoxin exposure and biomarkers of protein synthesis and inflammation may suggest the need for further study to better understand biological mechanisms that could lead to linear growth faltering of children.

Conclusions

Children from this study area in rural Ethiopia had a high prevalence of aflatoxin exposure. Biological mechanisms by which aflatoxins affect linear growth were not clear from our study. A further longitudinal study with a larger sample size is needed to evaluate causal linkages between aflatoxin exposure and linear growth in children. Small sample size was a limitation of our study, since aflatoxins could only be assessed for a subset of 102 children, meaning that our study may have been underpowered. Another limitation was the use of observational (nonexperimental) data, which does not allow us to infer causality. Analyses, however, adjusted for key known confounders. The high levels of aflatoxin exposure warrant further research to identify sources of exposure and interventions to mitigate that exposure.

Author Contributions

Conceived and designed the experiments: MT NSG IDB HDG TB EJM BJS. Performed the experiments: MT AK MDB AVC. Analyzed the data: MT NSG HDG IDB. Contributed reagents/materials/analysis tools: MDB AVC. Wrote the first draft of the manuscript: MT HDG IDB NSG. Wrote the paper: MT IDB NSG HDG TB BJS. Agreed with manuscript results and conclusions: MT IDB NSG HDG AK MDB AVC TB EJM BJS. ICMJE criteria for authorship read and met: MT IDB NSG HDG TB EJM BJS MDB AVC AK.

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Ethics approval and consent to participate

The Ethiopian Public Health Institute Scientific and Ethical Review Committee (SERO-006-02-2015) approved this study. Prior to any data collection, research staff obtained written informed consent from study participants in their homes.

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Appendix

Appendix 1

Tex 1. Materials and reagents, and Sample preparation

Materials and reagents

The individual mycotoxin liquid (1000 µg/mL) calibration standards of aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), M1 (AFM1) and $^{13}C_{17}$-aflatoxin B1 ($^{13}C_{17}$-AFB1) (internal standard) were obtained from Sigma Aldrich (Bornem, Belgium). AFB1-lysine standards were kindly supplied by Carleton University, Ottawa, Canada. The working solutions of AFB1, AFB2, AFG1, AFG2, AFM1, AFB1-lysine and $^{13}C_{17}$-AFB1 (10 µg/mL) were prepared in methanol, stored at -18°C, and renewed monthly. Water was obtained from a Milli-Q® SP Reagent water system (Millipore Corp, Brussels, Belgium). Methanol (LC-MS grade) was purchased from BioSolve (Valkenswaard, the Netherlands), while acetonitrile (Analar Normapur) and ammonium acetate were obtained from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100 %) was supplied by Merck (Darmstadt, Germany). Formic acid analytical grade (98–100%) and sodium chloride (>99.5%) were from Merck (Darmstadt, Germany). Ultrafree®-MC centrifugal filter devices (0.22 µm) were obtained from Millipore (Bredford, MA, USA).

Sample preparation for measurement of aflatoxins and fumonisins

A protein precipitation method with few variations was used to prepare samples for analysis [32]. Serum samples were thawed and measured for aflatoxins and fumonisins. Acetonitrile (A_CN) was used for protein precipitation. One hundred µL of A_CN and 100µL of serum were quantitatively poured in eppendorf tubes. The centrifugation (4,000 g, 15 min) of the mixture created two layers: a large aqueous layer on top and a circular flake of proteins at the bottom. An aliquot (160 µL) of the supernatant was carefully transferred to glass tubes and evaporated using the TurboVap 40°C). The samples were reconstituted with 80µL of injection solvent and transferred to the centrifugal filter tubes. We spiked the injection vials with 5µL of an internal standard prior to analysis.

Finally, the samples were transferred from tubes to the injection vials. Serum samples were analyzed in five. For each batch, six standards and one blank calibrator were prepared for the calibration curve. The blank contained 80µL of internal standard ($^{13}C_{17}$-AFB_1) and 120µL injection solvent.

Appendix 2

Mycotoxins	Precursor ion (*m/z*)	Product ions (*m/z*)	Collision energy (V)	Mode	Cone voltage (V)	Retention time (min)
AFB1	313.1	269.1/285.1	30/20	+	30	7.5
AFB2	315.1	259.1/287.1	26/23	+	40	6.8
AFG1	329.1	243.1/283.1	25/25	+	30	6.1
AFG2	329.1	243.1/283.1	25/25	+	30	5.7
AFM1	329.1	259.1/273.1	25/22	+	30	5.9
AFB1-lysine	457.3	310.9/394.2	33/20	+	30	4.5
$^{13}C_{17}$–AFB1	330.0	285.0/301.0	26/22	+	40	7.9

The optimized LC-ESI-MS/MS parameters for the confirmation and quantification of analyzed mycotoxins and internal standards used: aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin M1 (AFM1), aflatoxin B1-lysine (AFB1-lysine) and aflatoxin B1 (13C17 –AFB1).

Appendix 3

Validation of aflatoxin biomarkers

Method Validation parameters	AFB1	AFB2	AFG1	AFG2	AFM1	AFB1-lys
range (µg/L)	0.015-2.00	0.015-2.00	0.015-2.00	0.015-2.00	0.015-2.00	
cut-off (x)	0.5	0.5	0.5	0.5	0.5	
LOD (µg/L)	0.017	0.005	0.005	0.0002	0.004	0.015
LOQ (µg/L)	0.064	0.022	0.018	0.007	0.011	0.035
MU (0.25x) (%)	121.2	119.9	114.3	109.5	115.9	
MU (0.5x) (%)	117.4	115.1	111.5	109.9	118.7	
MU (1x) (%)	113.9	114.1	112.3	114.8	114.4	
MU (2x) (%)	104.8	106.7	107.1	103.9	106.9	
MU (4x) (%)	106.7	107.9	103.1	99.7	107.2	

Appendix 4

Spearman's correlations among aflatoxins, inflammation, protein biomarkers and growth in children during the pre-harvest season.

		HAD	HAZ	AFB1 (µg/L)	AFB2 (µg/L)	AFG1 (µg/L)	AFG2 (µg/L)	AFM1 (µg/L)	AFB1lys (µg/L)	AGP (gram/L)	CRP (mg/L)	Serum transthyretin (gram/L)	Serum IGF-1 (ng/mL)	Serum tryptophan (µmol/L)
HAZ	r	.952**												
	sig	.000												
AFB1 (µg/L)	r	.129	.142											
	sig	.198	.154											
AFB2 (µg/L)	r	.040	.071	.633**										
	sig	.692	.481	.000										
AFG1 (µg/L)	r	.021	.042	-.008	.273**									
	sig	.837	.676	.933	.005									
AFG2 (µg/L)	r	.070	.077	.407**	.559**	.375**								
	sig	.483	.439	.000	.000	.000								
AFM1 (µg/L)	r	.104	.110	-.125	-.072	.089	-.078							
	sig	.297	.270	.211	.475	.376	.434							
AFB1lys (µg/L)	r	.112	.103	.068	.010	-.084	-.004	.037						
	sig	.261	.305	.498	.920	.400	.969	.715						
AGP (gram/L)	r	-.131	-.126	-.054	-.133	-.054	-.166	-.061	-.045					
	sig	.190	.207	.590	.181	.589	.096	.545	.655					
CRP (mg/L)	r	-.031	-.040	-.034	-.041	.025	.040	-.135	.099	.498**				
	sig	.759	.691	.736	.682	.802	.690	.176	.324	.000				
Serum transthyretin (gram/L)	r	.078	.125	-.026	.066	-.014	.124	.007	.080	-.301**	-.327**			
	sig	.434	.210	.792	.507	.889	.215	.943	.424	.002	.001			
Serum IGF-1 (ng/mL)	r	.064	.080	.106	.046	.007	.090	-.009	.106	-.247*	-.022	.363**		
	sig	.522	.424	.289	.646	.947	.366	.929	.288	.012	.829	.000		
Serum tryptophan (µmol/L)	r	.209*	.150	.104	.003	-.018	.108	.011	-.075	-.228*	.000	.048	-.058	
	sig	.035	.133	.298	.975	.858	.280	.916	.455	.021	.997	.632	.563	
Serum lysine (µmol/L)	r	.065	.052	-.107	-.081	.073	.072	.120	-.059	-.058	.085	.043	-.045	.441**
	sig	.518	.605	.285	.420	.469	.474	.228	.559	.564	.397	.669	.652	.000

***: p < 0.001, **: p < 0.01, * p < 0.05. HAZ: Height-for-age Z-score; HAD: Height-for-age difference. IGF-1: Insulin-like growth factor 1; AFB1-lys: Aaflatoxin B1-lysine; AFB1: Aflatoxin B1; AFB2: Aflatoxin B2; AFG1: Aflatoxin G1; AFG2:Aflatoxin G2; AFM1:Aflatoxin M1; CRP: C-reactive protein; AGP: α-1-glycoprotein.

Appendix 5: Spearman's correlations among aflatoxins, inflammation, protein biomarkers, and growth in children during the post-harvest season.

		HAD	HAZ	AFB1 (µg/L)	AFB2 (µg/L)	AFG1 (µg/L)	AFG2 (µg/L)	AFM1 (µg/L)	AFB1lys (µg/L)	AGP (gram/L)	CRP (mg/L)	Serum transthyretin (gram/L)	Serum IGF-1 (ng/mL)	Serum (µmol/L)	tryptophan
HAZ	r	.977**													
	sig	.000													
AFB1 (µg/L)	r	-.120	-.126												
	sig	.231	.206												
AFB2 (µg/L)	r	-.186	-.193	.427**											
	sig	.061	.052	.000											
AFG1 (µg/L)	r	.078	.051	.245*	.132										
	sig	.435	.609	.013	.188										
AFG2 (µg/L)	r	-.069	-.093	.485**	.651**	.121									
	sig	.491	.353	.000	.000	.227									
AFM1 (µg/L)	r	.000	.012	.044	.120	-.046	.054								
	sig	.997	.903	.663	.228	.647	.589								
AFB1lys (µg/L)	r	.085	.055	.200*	.128	.098	.194	.272**							
	sig	.398	.581	.044	.200	.329	.051	.006							
AGP (gram/L)	r	-.059	-.032	-.078	-.108	.067	-.087	-.051	.042						
	sig	.553	.749	.437	.279	.505	.384	.612	.672						
CRP (mg/L)	r	.083	.070	-.162	-.131	-.119	-.170	-.026	-.040	.517**					
	sig	.405	.487	.103	.189	.235	.088	.794	.692	.000					
Serum transthyretin (gram/L)	r	.066	.069	.088	-.039	.044	.029	-.071	.057	-.389**	-.456**				
	sig	.512	.488	.378	.700	.664	.773	.481	.567	.000	.000				
Serum IGF-1 (ng/mL)	r	.146	.147	-.257**	-.091	-.131	-.147	.074	-.039	-.148	-.056	.166			
	sig	.144	.140	.009	.361	.189	.142	.459	.700	.138	.575	.095			
Serum tryptophan (µmol/L)	r	.278**	.231*	-.001	-.334**	.024	-.174	-.139	-.003	-.248*	-.120	.327**	.204*		
	sig	.005	.020	.993	.001	.813	.080	.165	.978	.012	.231	.001	.039		
serum lysine (µmol/L)	r	.176	.124	-.155	-.148	-.041	-.105	-.099	-.108	-.106	.064	.022	.277**	.417**	
	sig	.077	.215	.120	.137	.685	.295	.320	.282	.287	.523	.828	.005	.000	

***: p < 0.001, **: p < 0.01, * p < 0.05. HAZ: Height-for-age Z-score; HAD: Height-for-age difference. IGF-1: Insulin-like growth factor 1; AFB1-lys: Aflatoxin B1-lysine; AFB1: Aflatoxin B1; AFB2: Aflatoxin B2; AFG1: Aflatoxin G1; AFG2:Aflatoxin G2; AFM1:Aflatoxin M1; CRP: C-reactive protein; AGP: α-1-glycoprotein.

Chapter 5

Soil zinc is associated with serum zinc but not with linear growth of children in Ethiopia

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Abstract

To our knowledge, the relationships among soil zinc, serum zinc and children's linear growth have not been studied geographically or at a national level in any country. We use data from the cross-sectional, nationally representative Ethiopian National Micronutrient Survey (ENMS) (n=1776), which provided anthropometric and serum zinc (n=1171) data on children aged 6–59 months. Soil zinc levels were extracted for each child from the digital soil map of Ethiopia, developed by the Africa Soil Information Service. Children's linear growth was computed using length/height and age converted into Z-scores for height-for-age. Multi-level mixed linear regression models were used for the analysis. Nationally, 28% of children aged 6–59 months were zinc deficient (24% when adjusted for inflammation) and 38% were stunted. Twenty percent of households in the ENMS were located on zinc-deficient soils. Soil zinc (in mg/kg) was positively associated with serum zinc (in µg/dL) (b = 0.9, p = 0.020) and weight-for-height-Z-score (b = 0.05, p = 0.045) but linear growth was not associated with soil zinc (p = 0.604) or serum zinc (p = 0.506) among Ethiopian preschool children. Intervention studies are needed to determine whether there are causal links between soil and human zinc status.

Background

Zinc is an essential micronutrient for both plants and animals, including humans. It is estimated that about 17% of the global population has inadequate zinc intake [1] and zinc deficiency is widespread in developing countries [2]. Zinc supports normal growth and development during pregnancy and childhood and it is required for the catalytic activity of approximately 100 enzymes; it plays a role in immune function, protein synthesis, wound healing, DNA synthesis and cell division [3–5]. Zinc deficiency is caused mainly by insufficient intake or inadequate absorption of zinc in the body [6]. Human zinc deficiency is highly prevalent in sub-Saharan Africa, where diets are typically high in cereals and low in animal source products and contain low levels of bioavailable zinc [7,8]. Studies on the effect of zinc supplementation on linear growth of children showed conflicting results [9–11]. For instance, a review in developing countries found that zinc supplementation has a significant effect on linear growth of children [9]. However, another review found that zinc supplementation did not have a significant effect [11]. A recent systematic review published in Cochrane suggested that zinc supplementation resulted only in a marginal improvement in linear growth of children [10]. An earlier study conducted with Ethiopian preschool children found that zinc supplementation significantly improved linear growth of stunted children [12]. The recent Ethiopian National Food Consumption Survey demonstrated that over half of preschool children in Ethiopia are estimated to have low dietary zinc intake [13].

Zinc is also important for plants, including food crops. Soils with insufficient zinc for optimal crop growth are classified as zinc deficient. Zinc deficiency in agricultural soils is a global problem reported in many countries [14,15]. Most soils in sub-Saharan Africa are affected by zinc deficiency [16,17]. Soil zinc deficiency has a major effect on food security and human health by limiting the yields and the grain zinc concentrations of staple crops grown on zinc-deficient soils [10,15], especially in Africa [16–20]. Several studies have shown that zinc fertilizers, applied to the soil or through the foliar application, improved both the zinc content and

yield of grains [16,21–24]. The relationships among soil zinc, serum zinc and linear growth of children are poorly understood [25].

To our knowledge, to date, no study has established a quantitative relationship among soil zinc, human serum zinc status and linear growth of children from nationally representative data. We hypothesize that a lower soil zinc level is associated with lower grain zinc levels and lower zinc levels in the diet, resulting in higher prevalence of linear growth failure among preschool children, mediated by lower serum zinc status (Fig. 1). This would support emerging efforts to improve human zinc status by improving soil zinc status [16]. Improved soil zinc could also increase crop productivity and production, which in turn could improve children's growth through higher incomes, lower food insecurity, reduced inflammation through improvements in the health environment and increased resources for child feeding and caregiving (Fig. 1). Using data from two nationally representative cross-sectional surveys on soils and children's nutritional status, this is the first study that assesses the geographical distribution of poor zinc soils, poor serum zinc status and poor linear growth and their relationship among Ethiopian preschool children.

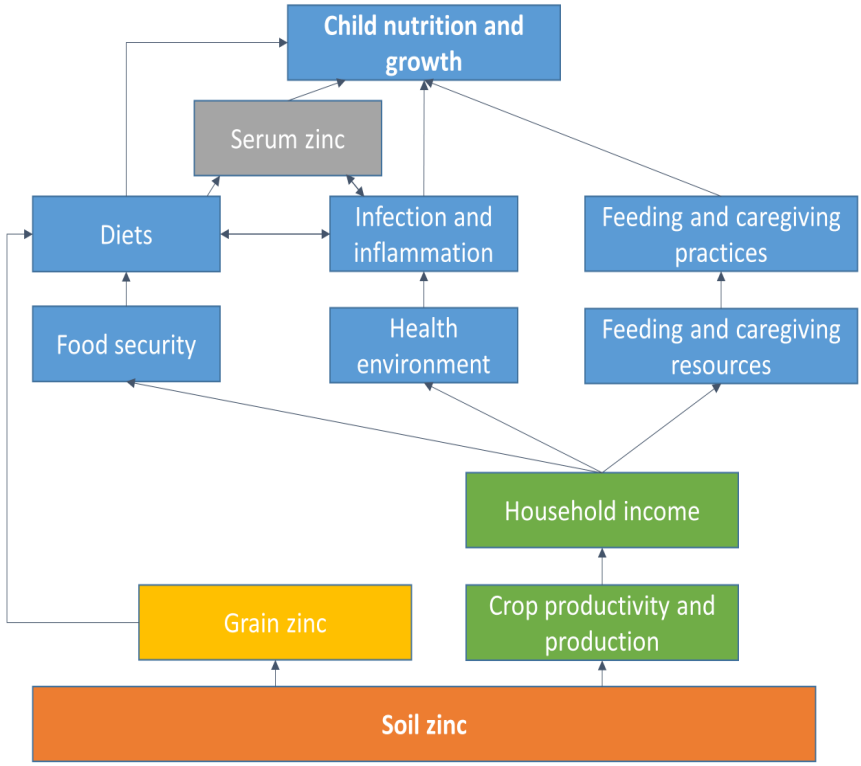

Fig. 1. A conceptual framework describing the relationships among soil zinc, serum zinc status and linear growth of children (based on a framework to achieve optimum child nutrition and development from Black et al [26]).

Materials and Methods

Study Design and Study Population

We merged two datasets: the Ethiopian National Micronutrient Survey (ENMS), which provided serum zinc and anthropometric data from children in georeferenced households and the Africa Soil Information Service (AfSIS) soil map, which provided soil zinc levels as raster data [27]. The ENMS was designed as a regionally- and nationally-representative cross-sectional survey of children (6–59 months) and was

conducted between March and July 2015. Ethiopia is administratively sub-divided into nine regional states and two city administrations (Addis Ababa and Dire Dawa) [28]. The ENMS enumeration areas (EAs) or clusters are geographic areas defined by the Central Statistics Agency (CSA) for the Ethiopia Population and Housing Census [28]. EAs contain on average 181 households (150 to 200) and are subsets of the regions [28].

In the ENMS, 366 clusters were first randomly selected from all region or city administrations with probability proportional to size. Prior to the actual survey, all households within the boundary of each selected cluster were listed and a census was conducted of the people living in the households. In the next stage, within each selected cluster (or segment of clusters), 11 households were randomly selected. In the final stage, all preschool children aged 6 to 59 months in the 11 households were selected for the actual survey. If eligible occupants of a house were not present, two return visits with a written appointment were made. If no eligible respondents were available during the two visits, the household was recorded as refusing to participate and was not replaced. The children's mother or caretaker responded to the questionnaires on behalf of the children. In addition to blood samples, anthropometric measurements of the children were collected. Further, information relating to the household's demographic and socioeconomic characteristics and their geographic coordinates were obtained. A total of 4026 households in 366 clusters across the nine regions and the two administrative cites were selected; about 92% (n = 3700) of households in 353 clusters gave their consent and were included in the study. In the consenting households, 1776 preschool children (on average about 5–6 children per EA) were eligible for blood collection and adequate blood samples for serum zinc were collected from 1171 children in 316 clusters. Anthropometric data were collected from 1673 children. To account for the multistage sampling employed in the ENMS, sample weights were calculated from the 2007 census [28] and used to estimate regional and national prevalence of households on zinc-deficient soils and of serum zinc deficiency and stunting among children under five years of age [29].

Ethical clearance was obtained from the National Research Ethical Review Committee of the Ethiopian Science and Technology Ministry (number 3.10/433/06). Informed consent was obtained from all adults who were interviewed, specifically the household head and caregiver.

Data Collection and Analysis

Collection, Processing and Analysis of Biochemical Samples

In the ENMS, non-fasting venous blood was collected aseptically in the morning by experienced phlebotomists from the left arm by venepuncture using vacutainer trace element-free tubes (Royal Blue top tube, 6.0 mL). The samples were collected at the household, placed in cold boxes containing frozen gel packs (<8 °C) and transported as soon as possible after collection to the centralized temporary field laboratory sites. Within ~1 hour of collection, the blood was allowed to clot for 30 min and centrifuged at 3000 rotations per minute (rpm) for 10 min. An aliquot was separated based on the recommended procedures of the International Zinc Nutrition Consultative Group [30].

The zinc status of under five children was assessed by serum zinc, which is the recommended biomarker to estimate zinc status [31]. Serum zinc concentration was measured using Shimadzu Flame Atomic Absorption Spectroscopy (AA 6800 Japan model) with an air-acetylene flame at a wavelength of 213.9 nm and a slit width of 0.7 nm. Serum zinc deficiency was defined as concentration < 65 µg/dL for children [30]. Staff serum samples were used as a control during analysis of every 30 samples and intra-assay CV was 4.3%. Inflammation was measured using C-reactive protein (CRP) and α-1-glycoprotein protein concentration (AGP). CRP and AGP concentrations were determined using the fully automated Cobas 6000 immune-turbidimetry method using Roche kits (Roche Diagnostics, GmbH, Mannheim, Germany) [32]. Further, diarrhea was defined as three watery or loose stools in any 24 hours period during those two weeks and measured by asking the caregiver to recall diarrhea incidence in the two weeks prior to the survey.

Serum zinc concentration was adjusted for inflammation using the biomarkers CRP and AGP, according to the regression correction method proposed by the BRINDA Working Group [33]. Reference concentrations (maximum of lowest decile) for serum CRP and AGP were used to avoid over-adjusting serum zinc among preschool children with low levels of inflammation [32,34]. Acute or chronic inflammation were defined by serum CRP>5 mg/L or AGP >1 gram/L, respectively [35]. Stages of inflammation were categorized as no inflammation (CRP ≤5 mg/L and AGP ≤1 gram/L); incubation (CRP >5 mg/L and AGP <1 gram/L); early convalescence (CRP >5 mg/L and AGP >1 gram/L); late convalescence (CRP ≤5 mg/L and AGP >1 gram/L) [35]. The analyses of serum zinc and inflammation biomarkers were conducted at the Ethiopian Public Health Institute laboratory, certified by the Ethiopian National Accreditation Office in accordance with the requirements of ISO 17025:2005 and ISO 15189:2012.

Demographic and Socioeconomic Characteristics

A three-week training course was provided for the ENMS data collectors and supervisors on data collection and overall quality control, followed by one week of field pilot testing in a cluster not selected for the survey. After the pilot testing, the questionnaires were revised before the actual survey. Demographic characteristics collected included family size and sex and age of children; socioeconomic characteristics included household assets, household food insecurity status and education level of the children's caretaker. In addition, data were collected on the foods (animal and plant-based) consumed by children in the last 24 hours [36]. Household food insecurity status was measured using the validated three-month food insecurity experience scale (FIES) [37].

Anthropometric Data

Anthropometrics (i.e., weight and height or recumbent length) were collected on the selected children. Their weight was measured with light clothing and without shoes to the nearest 100g using a standard UNICEF SECA 874 U digital scale (UNICEF Supply Division, Copenhagen, Denmark). The scale was calibrated using a standard weight after moving

from one household to the next. The length of younger children (6–23 months) was measured in a recumbent position to the nearest 0.1 cm using a UNICEF measuring board (UNICEF Supply Division, Copenhagen, Denmark) with an upright wooden base and a movable headpiece. The height of children older than 23 months of age was measured in a standing position to the nearest 0.1 cm. All anthropometric measurements were taken twice (or three times if the measurements differed between the first and the second reading) and the average values were taken. The age of the children was calculated based on the date of birth and the date of the interview. The weight and height/length of the children were converted into Z-scores for height-for-age (HAZ) and weight-for-height (WHZ) according to 2006 WHO child growth standards, using WHO Anthro software [38]. Linear growth failure was computed using HAZ; HAZ scores less than two standard deviations below median values were considered indicative of stunting.

Collection and Analysis of Soil Zinc

The soil zinc map of Ethiopia was obtained from AfSIS. The map provides a grid of 1 km^2 and for each grid cell a soil zinc level in mg/kg. The methodology used to obtain soil zinc data and derive the map have been described elsewhere [27,39]. Soil zinc levels were extracted for all households based on their geographic coordinates and merged with the ENMS data. Zinc-deficient soils were defined as having zinc levels lower than 1.5 mg/kg [40].

Statistical Analysis

Statistical analyses were conducted with SAS version 9.3 (SAS Institute, Cary, North Carolina, USA). A wealth index was created using the first principal component [41] constructed with the following household assets (binary variables): grid electricity, watch, radio, television, mobile telephone, landline telephone, refrigerator, solar panel, bicycle, motorcycle, animal-drawn cart, car and motorboat. Using this index, households were assigned to wealth tertiles.

Spearman's rank correlation was used to investigate correlations among soil zinc, child's serum zinc, child's growth (HAZ and WHZ scores), child's inflammation markers (CRP and AGP) and household food insecurity. To examine the associations among soil zinc, serum zinc and linear growth of children, a multi-level mixed linear model with a random intercept was fitted with restricted maximum likelihood estimation using the SAS procedure "proc mixed." Clusters were used as a random intercept. Independent variables in the model were those known or suspected to be biologically important predictors of child growth or serum zinc (Fig. 1). Model diagnostics were checked to ensure assumptions of normality of error terms and homogeneity of error variance were met.

Results

Population Characteristics

The median (25th, 75th percentile) age of the 1776 children in the ENMS was 36 (24, 48) months and 48% were female (Table 1). One in seven children had diarrhea in the two weeks prior to the study. About one-third of the children with diarrhea received medication during diarrheal illness. Consumption of meat and meat products was minimal (11%) in the 24 hours preceding the survey. The median (25th, 75th percentile) serum zinc concentration was 74 μg/dL (63.4, 87.4) indicating relatively low serum zinc values in this population.

A substantial proportion of households living in the ENMS were found to be located on zinc-deficient soils (20%). The prevalence of households living on zinc-deficient soils varied between the administrative regions and in general was higher in the lowlands of Ethiopia and in sparsely populated regions. Among the populous regions in the highlands of Ethiopia, more households were located on zinc deficient soils in Tigray (50%) and Amhara (25%). However, the prevalence of households on zinc deficient soils was lower in Southern Nations, Nationalities and Peoples' Region (SNNPR) (2%) and Oromia (17%).

Table 1. Characteristics of study participants (children under five years) from the Ethiopian National Micronutrient Survey (ENMS).

Indicators	*N*	Median (25th, 75th percentiles or %)
Age in months	1776	36 (24, 48)
Age categories		
Age (6–11 months)	118	7%
Age (12–23 months)	288	16%
Age (24–59 months)	1370	77%
Sex (female)	1776	48%
Child had diarrhea in preceding two weeks	1776	15%
Child received medication during the diarrheal episode	1776	5%
Child consumed meat or meat products in the last 24 hours	1776	11%
Unadjusted serum zinc (µg/dL)	1171	74.1 (63.4, 87.4)
AGP (gram/L)	1180	0.95 (0.75, 1.20)
CRP (mg/L)	1164	0.64 (0.25, 2.20)

AGP: α-1-glycoprotein concentration and CRP: C-reactive protein concentration.

The prevalence of serum zinc deficiency was comparable across age and sex groups (20–25%) (Table 2). The highest adjusted prevalence of serum zinc deficiency was found in Afar (34%), Tigray (29%), Amhara and Harari (28%); and the lowest in Gambella (11%) and Benishangul (16%). Adjustment for inflammation decreased the overall prevalence of zinc deficiency from 28% to 24% (Table 2).

The national prevalence of stunting was 38%, being higher in rural areas (39%), in boys (41%), in Tigray (44%) and in Amhara (42%) and lower in Addis Ababa (16%) and Gambella (21%). Stunting prevalence was higher in older age groups (Table 2). The geographic distribution of stunting, poor zinc soil and poor serum zinc status is indicated in Fig. 2. Linear growth failure and zinc deficiency in preschool children were prevalent in all regions. Further, most lowland regions of Ethiopia were affected by low soil zinc status (Fig. 2).

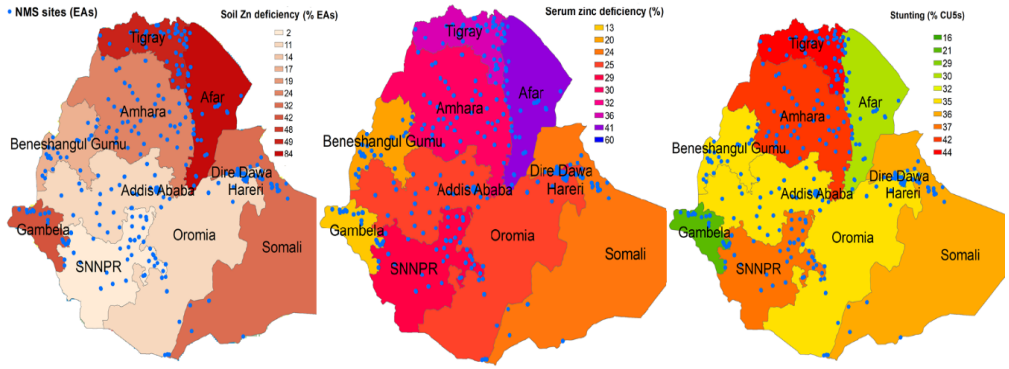

Fig. 2. Geographical distribution of poor soil zinc, poor serum zinc status and poor linear growth of children in Ethiopia. NMS=National Micronutrient Survey; EAs = Enumeration Areas; Serum zinc deficiency (%) = Percent of under five children who are deficient as measured by serum zinc; CU5s = children under five.

Correlations Among Soil Zinc, Serum Zinc, Inflammation and Children's Growth

Soil zinc level was significantly correlated with serum zinc level (r = 0.09, p = 0.006), WHZ (r = 0.08, p = 0.001), inflammation biomarkers: CRP (r = 0.07, p = 0.017) and AGP (r = 0.12, p < 0.001) and diarrhea (r = 0.06, p = 0.027). Serum zinc concentration was significantly negatively correlated with inflammation (AGP) (r = −0.08, p = 0.010). WHZ was significantly negatively correlated with household food insecurity (r = −0.07, p = 0.009) and diarrheal illness (r = −0.07, p = 0.005). HAZ was not correlated with soil zinc level or serum zinc status (Table 3).

Associations among Soil Zinc, Serum Zinc and Child Growth

The Association between Soil Zinc and Serum Zinc

Using a multi-level mixed linear regression model to predict serum zinc (n = 1171), we found that soil zinc level was positively associated with child's serum zinc concentration (b = 0.9 µg/dL, p = 0.019), indicating that a 1 mg/kg increase in soil zinc content was associated with an increase in child serum zinc level of 0.9 µg/dL (Table 4). A quadratic effect

of soil zinc on serum zinc was not significant (result not shown). Other factors that significantly negatively affected serum zinc status were inflammation level (CRP and AGP) (b = −6.8, p = 0.0008) and time since most recent meal (b = −6.3, p = 0.057) (Table 4). We have conducted subgroup analyses by age of children (≥24 months and ≤23 months) and residence (rural and urban). Our subgroup analyses by age group found that soil zinc was significantly and positively associated with serum zinc in children ≥24 months (b = 0.8 µg/dL, p = 0.043) (Appendix, Table S1), who consume more foods and are less dependent on breastmilk.

However, soil zinc was not significantly associated with serum zinc in children ≤23 months (b = 1.8 µg/dL, p = 0.099) (Appendix, Table S1). We also found that soil zinc was significantly and positively associated with serum zinc from children in both urban and rural areas (Appendix, Table S3).

The association between soil zinc and child growth

The results of the multi-level mixed linear regression models to predict linear growth of children (HAZ) show that soil zinc level was not associated with HAZ (p > 0.05) (Table 5, model 1).

Table 2. Prevalence and geographical distribution of soil zinc deficiency, child serum zinc deficiency and child stunting in Ethiopia by region.

	Percentage of households on zinc-deficient soils (<1.5 mg/kg), *n* = 1298	**Prevalence serum zinc deficiency [1] (<65 µg/dL), *n* = 1171**		**Prevalence of stunting (HAZ < −2.0), *n* = 1673**
		Unadjusted	**Adjusted [2,3]**	
Region				
Tigray	50	36	29	44
Afar	87	40	34	31
Amhara	25	30	28	42
Oromia	17	25	22	35
Somali	33	24	22	36
Benishangul	15	20	16	36
SNNPR	2	29	22	37
Gambella	42	13	11	21
Harari	46	32	28	29
Addis Ababa	25	60	60	16
Dire Dawa	20	29	29	32
Age group				
Age (6–11 months)	31	28	20	6
Age (12–23 months)	24	26	24	34
Age (24–59 months)	18	28	24	41
Sex				
Boys	20	26	23	41
Girls	20	29	25	34
Residence				
Urban	24	32	25	26
Rural	20	27	24	39
National prevalence [4]	20	28	24	38

[1] All subjects were non-fasting. [2] BRINDA internal regression correction approach, which accounts for both CRP and AGP, was applied to calculate the adjusted prevalence of zinc deficiency [32]. [3] Adjusted for inflammation = exp(unadjusted ln serum zinc−(regression coefficient for CRP) × (CRP−(maximum of lowest decile for CRP))−(regression coefficient for AGP) × (AGP−(maximum of lowest decile for AGP))). [4] Prevalence of soil zinc deficiency, serum zinc deficiency and stunting was weighted using a regional weight factor.

Table 3. Spearman's rank correlations between soil zinc, serum zinc, inflammation biomarkers child's growth, incidence of diarrhea in children and household food insecurity.

Indicators	Soil zinc	Serum zinc	AGP	CRP	HAZ	WHZ	Diarrhoea
Serum zinc (µg/dL)	0.09 **						
AGP (gram/L)	0.12 **	−0.08 *					
CRP (mg/L)	0.07 *	-0.05	0.55**				
HAZ	−0.03	0.02	0.02	−0.03			
WHZ	0.08 **	0.02	−0.01	−0.0006	−0.05*		
Diarrhoea	0.06 **	−0.05	0.08**	−0.001	−0.01	−0.07 **	
FIES	−0.03	0.0001	0.04	−0.02	−0.02	−0.07 *	0.09 **

*p < 0.05; **p < 0.01. AGP: α-1-glycoprotein protein concentration. CRP: C-reactive protein concentration. HAZ: Height-for-age Z-score. WHZ: Weight-for-height-Z-score. FIES: The Food Insecurity Experience Scale.

Other factors such as inflammation level, wealth status and food security status were also not associated with HAZ (p > 0.05). Further, adjustment for serum zinc had no significant effect on the relationship between soil zinc and child growth (Table 5, model 2). Soil zinc was not significantly associated with height-for-age in either age group (Appendix, Table S2).

A similar model, now with WHZ as the dependent variable, found WHZ to be positively associated with soil zinc (b = 0.05, p = 0.026); the association remained when serum zinc was included in the model (Table 6, models 1 and 2). The children with lower wealth status had lower WHZ than children from relatively wealthier households (b = −0.40, p = 0.001) and this association remained after adjusting for serum zinc status (b = −0.43, p = 0.0002).

Discussion

We found that the risk of zinc deficiency as measured of low serum zinc concentrations was high among Ethiopian preschool children. This underlines the fact that zinc deficiency in Ethiopia is a public health problem. Soil zinc was positively associated with children's serum zinc. The associations persist even when controlling for other factors. To the

best of our knowledge, this is the first study to investigate quantitatively the association between soil zinc level, serum zinc status and linear growth in Ethiopian children using data from nationally representative studies.

Table 4. Multi-level mixed linear regression model predicting serum zinc (n = 1171).

Fixed effects	**Estimate** [1]	**SE**	***P***
Soil zinc (mg/kg)	0.9	0.4	0.020
Diarrhea in past two weeks	−1.9	1.8	0.284
CRP (mg/L) and AGP (gram/L) (ref = normal)			
Elevated CRP only (mg /L)	−4.4	6.5	0.503
Elevated AGP only (gram/L)	−4.1	1.4	0.003
Elevated AGP (gram/L) and CRP (mg/L)	−6.8	2.0	0.0008
Age in months (Ref = 6–11 months)			
Age category 2 (12–23 months)	−1.5	3.5	0.655
Age category 3 (24–59 months)	−1.2	3.1	0.702
Sex of child (female)	−1.9	1.2	0.127
Wealth status (Ref = wealthier)			
Wealth status (poorer)	1.8	1.8	0.301
Wealth status (medium)	2.6	1.6	0.111
Time since most recent meal (hr)	−6.3	3.3	0.057
FIES	−0.1	0.2	0.693
Consumption of meat or meat products in the last 24 hours	−3.0	2.0	0.136
Random effects			
Intercept (cluster)	29.7	11.5	0.0049

[1] Models adjusted for regions as fixed effects. The restricted maximum likelihood (REML) method was used to estimate the parameters. FIES: The Food Insecurity Experience Scale. AGP: α-1-glycoprotein protein concentration; and CRP: C-reactive protein concentration.

A high proportion of ENMS households were located on zinc-deficient soils, although this varied among regions. We found that in sparsely populated regions such as Gambela, many households lived on soils deficient in zinc. Those low altitude areas are mostly pastoral or semi-agrarian. Furthermore, both soil and serum zinc deficiencies were high in populous regions such as Tigray and Amhara but other populous regions such as Oromia and SNNPR had low levels of soil zinc deficiency. This may suggest that soil in agrarian areas in Ethiopia is more fertile or nutrient-

rich than pastoral or non-agrarian regions. Furthermore, these findings suggest that high prevalence of soil zinc deficiency in some regions such as Tigray and Amhara may contribute to lower agricultural productivity and food insecurity, which can result in high zinc deficiency and poor child growth.

Table 5. Multi-level mixed linear regression models predicting height-for-age Z-score [1] (*n* = 1673).

	Model 1 [2]			**Model 2** [2]		
Fixed effects	**Estimate**	**SE**	***P***	**Estimate**	**SE**	***p***
Soil zinc (mg/kg)	0.02	0.03	0.6035	0.02	0.03	0.522
Serum zinc (µg/dL)				−0.002	0.003	0.506
Diarrhea in past two weeks	−0.30	0.16	0.0654	−0.3	0.2	0.091
CRP (mg/L) and AGP (gram/L) (Ref = normal)						
Elevated CRP only (mg/L)	0.01	0.57	0.9842	−0.11	0.60	0.848
Elevated AGP only (gram/L)	−0.09	0.13	0.4751	−0.04	0.13	0.753
Elevated CRP (mg/L) and AGP (gram/L)	−0.10	0.18	0.5839	−0.05	0.19	0.781
Age in months (Ref = 6–11 months)						
Age category 2 (12–23 months)	−0.40	0.3	0.199	−38	0.32	0.235
Age category 3 (24–59 months)	−1.1	0.3	<0.0001	−1.1	0.28	<0.001
Sex (female)	0.2	0.1	0.054	0.2	0.12	0.036
Wealth status (Ref = wealthier)						
Wealth status (poorer)	−0.2	0.2	0.214	−0.13	0.16	0.435
Wealth status (medium)	−0.2	0.1	0.269	−0.11	0.15	0.451
FIES	−0.02	0.02	0.286	−0.03	0.02	0.175
Random effects						
Intercept(cluster)	0.2	0.1	0.007	0.2	0.08	0.009

[1] Models adjusted for regions as fixed effects. The restricted maximum likelihood (REML) method was used to estimate the parameters. [2] Model 1 adjusted for soil zinc, Model 2 adjusted for soil zinc and serum zinc. FIES: The Food Insecurity Experience Scale.

The results support our hypothesis of a dietary mechanism in which people grow crops on soils deficient in zinc or with sufficient zinc and consume these crops, affecting serum zinc either negatively or positively (Fig. 1). Specifically, the present study may suggest that low soil zinc lowers production or crop yield and the zinc content of grain, which in turn lowers zinc intake and serum zinc levels. Low soil zinc levels have been

shown to decrease yields of major crops and therefore reduce agricultural production [24].

Table 6. Multi-level mixed linear regression models predicting weight-for-height-Z-score[1] (*n* = 1673).

	Model 1 [2]			Model 2 [2]		
Fixed effects	**Estimate**	**SE**	***P***	**Estimate**	**SE**	***p***
Soil zinc (mg/kg)	0.05	0.02	0.026	0.05	0.023	0.045
Serum zinc (µg/dL)				0.002	0.002	0.488
Diarrhea in past two weeks	−0.16	0.12	0.162	−0.184	0.118	0.121
CRP (mg/L) and AGP (gram/L) (Ref = normal)						
Elevated CRP only (mg/L)	−0.14	0.40	0.737	−0.104	0.426	0.808
Elevated AGP only (g/L)	0.04	0.09	0.694	0.037	0.093	0.687
Elevated CRP (mg/L) and AGP (gram/L)	−0.01	0.13	0.916	−0.0002	0.134	0.999
Age in months (Ref = 6–11 months)						
Age category 2(12–23 months)	−0.04	0.23	0.846	−0.020	0.228	0.931
Age category 3 (24–59 months)	0.07	0.20	0.734	0.044	0.201	0.827
Sex (female)	−0.03	0.08	0.717	−0.031	0.082	0.704
Wealth status (Ref = wealthier)						
Wealth status (poorer)	−0.40	0.11	0.001	−0.434	0.117	0.0002
Wealth status (medium)	−0.09	0.10	0.366	−0.141	0.107	0.187
FIES	0.01	0.02	0.703	0.008	0.015	0.604
Random effects						
Intercept(cluster)	0.12	0.05	0.005	0.124	0.048	0.005

[1] Models adjusted for regions as fixed effects. The restricted maximum likelihood (REML) method was used to estimate the parameters. [2] Model 1 adjusted for soil zinc, Model 2 adjusted for soil zinc and serum zinc. FIES: The Food Insecurity Experience Scale.

An earlier review covering 10 African countries demonstrated that the application of zinc in soil or by foliar fertilization increased the median Zn concentration of maize, rice and wheat grain [16]. Other developing countries in Africa are experiencing lower food production per capita as a result of unhealthy soils and a loss of soil nutrients [17,18]. A recent review showed that zinc deficiency in agricultural soils limit crop production, with yield losses up to 40% [40]. Further, a recent study from China showed that agronomic zinc biofortification (zinc fertilizer on the soil) of wheat greatly increased grain zinc content and improved the zinc bioavailability in grain and flour [19]. The current plan and initiative by the government of Ethiopia to address soil nutrient deficiency with

blended fertilizers should be implemented as soon as possible [42]. This is likely to increase crop yields as well as the micronutrient content of crops grown on zinc-deficient soils, with the potential of reducing human zinc deficiency among the people living in those areas.

Linear growth of children in our study was not associated with soil zinc level and serum zinc status. Therefore, we were unable to detect a mediation effect of serum zinc on the relation between soil zinc and children's growth. In contrast, an intervention study in Ethiopian preschool children demonstrated that zinc supplementation significantly improved linear growth of stunted children [12]. Several national surveys in other countries showing that serum zinc was not associated with linear growth in children [43–45]. The causative mechanisms for childhood linear growth failure are still poorly understood [46] but existing evidence shows that the cause of linear growth failure is multifactorial [46]. Our findings suggest the need for a longitudinal and interventional study to understand causal linkages between soil zinc, serum zinc and linear growth in children.

We found that the risk of zinc deficiency based on low serum zinc concentrations was high, with high variability among regions. Nationally, 28% of children were deficient in zinc, reducing to 24% when adjusted for inflammation, confirming that inflammation causes an overestimation of the prevalence of zinc deficiency [32]. While the correlation between serum zinc and soil zinc was significant, it was relatively low ($r = 0.09$), indicating that other determinants affect serum zinc, including inflammation and phytate content of complementary foods. The relationship between serum zinc and inflammation has not been widely studied [31]. In this study, the correlation between inflammation and serum zinc was also relatively low ($r = -0.08$ with AGP). However, our adjusted regression analysis showed that the effect of inflammation on serum zinc was high (Table 4). We included other factors in these models, such as meat consumption and time since the last meal, along with child and household characteristics but these factors did not have significant effects. Earlier studies, both experimental studies with animal models and human studies of infected and non-infected adults, indicate that systemic infections producing an acute phase response cause the plasma zinc

concentration to fall [47]; this is in line with the findings in our current study. Therefore, to determine zinc status in populations, inflammation should be taken into account, which will likely lead to a substantial decrease in the estimates of zinc deficiency prevalence. Health promotion and disease prevention programs may be considered as complementary strategies to reduce zinc deficiency in Ethiopia. Another factor is the effect of phytate on zinc bioavailability, which has not been addressed in our study. However, the existing evidence in Ethiopia and other developing countries show that phytate concentrations are high in cereals-based complementary foods and may inhibit zinc absorption [48]. The Ethiopian National Food Consumption Survey indicated that phytate intake from children's complementary foods was high, with low variability at the sub-national level [13]. Strategies to reduce phytate in children's complementary foods may therefore also be considered as a strategy to reduce zinc deficiency in Ethiopia.

In the absence of a gold standard biomarker for zinc status, plasma or serum zinc is endorsed to be the best available biomarker of zinc status [49] for both zinc exposure and the risk of clinical deficiency [50]. Serum zinc is associated with dietary zinc intake, responds consistently to zinc supplementation and decreases with very low zinc intakes [50]. It is suggested that in nutritionally deficient children like in our study population, a higher sequestration rate of zinc by tissues in need of zinc may lead to a higher functional response [51]. However, there are limitations in using serum zinc to assess zinc status: serum zinc responds less to additional zinc provided in food than to a supplement administered between meals, serum zinc seems to predict functional responses to supplementation only when the initial serum zinc concentration is very low, there is large interindividual variability in serum zinc with changes in dietary zinc, and serum zinc is influenced by recent meal consumption, the time of the day, inflammation and certain drugs and hormones [50]. Further research is needed to evaluate potentially useful biomarkers such as hair, nail, or urinary zinc.

Strengths of our study include large population coverage based on nationally representative samples. We used advanced analysis methods to elucidate the association among soil zinc level, serum zinc status and

linear growth of children in Ethiopia. Exposure (to poor soil) and outcomes (serum zinc and child growth) were measured on the same individuals or intrapolated from a grid based on their georeference and used to analyze relationships among these variables while adjusting for potential confounders. Despite the strengths of the current study, it also has limitations; in particular, it did not measure all factors that may be important for children's linear growth. Further, because of the cross-sectional design of our study, we were unable to draw conclusions about the causal effect that low soil zinc might have on lower serum zinc and poor linear growth in children.

Conclusion

In conclusion, low agricultural soil zinc was found to be a predictor of lower serum zinc and lower weight-for-height among Ethiopian preschool children but neither soil zinc nor serum zinc were associated with linear growth of preschool children in Ethiopia. The relationship between soil and serum suggests that interventions to improve soil zinc fertility could benefit children with zinc deficiency in rural areas, especially those reliant on subsistence agriculture. This could be an alternative or complementary strategy to supplementation or fortification, which often face difficulties reaching rural children. An intervention strategy such as agronomic biofortification with zinc may need to be combined with other interventions to realize improvements in child nutritional status. Intervention studies are needed to determine whether there are causal links between soil and human zinc status.

Author Contributions

M.T. designed the study protocol, performed the statistical analyses, composed the draft manuscript and was responsible for the final content of the manuscript. H.D.G., N.S.G. and I.D.B. designed the study protocol, supervised the statistical analysis and manuscript preparation and approved the final version of the manuscript. E.J.M.F. and T.B. participated in study protocol development and read the final manuscript.

D.Z., A.B. and Y.D. carried out ENMS data and specimen collection and all laboratory work. All authors read and approved the final manuscript.

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Ethical approval and consent to participate

Ethical clearance was obtained from the National Research Ethical Review Committee of the Ethiopian Science and Technology Ministry (number 3.10/433/06). Informed consent was obtained from all adults who were interviewed, specifically the household head and caregiver.

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Appendix

Table S1. Multi-level mixed linear regression model predicting serum zinc: subgroup analyses by age[1].

	Model 1 (Children ≥ 24 months)			Model 2 (Children ≤ 23 months)		
Fixed effects	**Estimate**	**SE**	**P**	**Estimate**	**SE**	**P**
Soil zinc (mg/kg)	0.8	0.4	0.043	1.8	1.1	0.099
Diarrhea in past two weeks	-0.8	2.1	0.692	-6.1	3.8	0.107
CRP (mg/L) and AGP (gram/L) (ref=normal)						
Elevated CRP only (mg /L)	-1.6	7.4	0.827	-11.5	15.1	0.446
Elevated AGP only (gram/L)	-4.8	1.5	0.002	-0.1	3.5	0.972
Elevated AGP (gram/L) and CRP (mg/L)	-7.0	2.3	0.002	-6.8	5.0	0.175
Sex of child (female)	-2.1	1.4	0.127	0.3	3.2	0.918
Wealth status (Ref = wealthier)						
Wealth status (poorer)	1.6	1.9	0.402	4.3	4.1	0.293
Wealth status (medium)	2.2	1.8	0.223	7.7	4.0	0.052
Time since most recent meal (hr)	-4.9	3.6	0.173	-18.2	8.8	0.040
FIES	-0.2	0.3	0.363	0.6	0.6	0.343
Consumption of meat or meat products in the last 24 hours	-2.0	2.1	0.345	-17.2	6.9	0.014
Random effects						
Intercept (cluster)	32.4	13.4	0.008	0.0	--	--

1. Models adjusted for regions as fixed effects. The restricted maximum likelihood (REML) method was used to estimate the parameters. FIES: The Food Insecurity Experience Scale. AGP: α-1-glycoprotein protein concentration; and CRP: C-reactive protein concentration.

Table S2. Multi-level mixed linear regression models predicting height-for-age Z-score: subgroup analyses by age[1].

	Model 1 (Children ≥ 24 months)			Model 2 (Children ≤ 23 months)		
Fixed effects	**Estimate**	**SE**	**P**	**Estimate**	**SE**	**P**
Soil zinc (mg/kg)	0.017	0.034	0.612	0.056	0.121	0.645
Serum zinc (µg/dL)	0.000	0.003	0.941	-0.008	0.008	0.295
Diarrhea in past two weeks	-0.423	0.191	0.027	-0.158	0.375	0.675
CRP (mg/L) and AGP (gram/L) (ref=normal)						
Elevated CRP only (mg /L)	-0.247	0.670	0.713	-0.503	1.481	0.735
Elevated AGP only (gram/L)	-0.044	0.141	0.755	0.041	0.345	0.907
Elevated AGP (gram/L) and CRP (mg/L)	-0.050	0.207	0.808	0.130	0.453	0.774
Sex of child (female)	0.220	0.125	0.078	0.428	0.298	0.153
Wealth status (Ref = wealthier)						
Wealth status (poorer)	-0.072	0.177	0.683	-0.252	0.409	0.538
Wealth status (medium)	-0.055	0.161	0.734	-0.384	0.385	0.319
FIES	-0.023	0.023	0.329	-0.056	0.059	0.345

1. Models adjusted for regions as fixed effects. The restricted maximum likelihood (REML) method was used to estimate the parameters. FIES: The Food Insecurity Experience Scale. AGP: α-1-glycoprotein protein concentration; and CRP: C-reactive protein concentration.

Table S3. Multi-level mixed linear regression model predicting serum zinc: subgroup analyses by residence[1].

	Model 1 (Urban)			Model 2 (Rural)		
Fixed effects	**Estimate**	**SE**	**P**	**Estimate**	**SE**	**P**
Soil zinc (mg/kg)	2.7	1.0	0.008	0.9	0.3	0.006
Diarrhea in past two weeks	-0.3	5.8	0.957	-1.1	1.9	0.580
CRP (mg/L) and AGP (gram/L) (ref=normal)						
Elevated CRP only (mg /L)	-14.6	20.2	0.472	-2.5	7.0	0.719
Elevated AGP only (gram/L)	-10.9	3.7	0.004	-2.7	1.5	0.079
Elevated AGP (gram/L) and CRP (mg/L)	-9.7	5.6	0.087	-5.3	2.2	0.015
Sex of child (female)	-3.7	3.5	0.293	-2.0	1.4	0.139
Wealth status (Ref = wealthier)						
Wealth status (poorer)	6.1	7.0	0.385	2.7	2.0	0.190
Wealth status (medium)	-2.7	6.2	0.658	3.5	1.9	0.062
Age in months (Ref=6–11 months)						
Age category 2 (12–23 months)	-10.1	9.5	0.291	-0.2	3.7	0.947
Age category 3 (24–59 months)	-10.7	8.5	0.208	0.4	3.3	0.910
Time since most recent meal (hr)	-18.5	8.6	0.034	-2.6	3.5	0.464
FIES	0.2	0.7	0.786	0.0	0.3	0.870
Consumption of meat or meat products in the last 24 hours	-0.6	4.5	0.894	-3.3	2.2	0.143

[1].As region and urban/rural designation are closely related in Ethiopia, region was omitted from model. The restricted maximum likelihood (REML) method was used to estimate the parameters. FIES: The Food Insecurity Experience Scale. AGP: α-1-glycoprotein protein concentration; and CRP: C-reactive concentration.

Chapter 6

General Discussion

The primary aim of this thesis was to contribute to the understanding of the aetiology of poor linear growth and stunting in rural Ethiopia. Following the conceptual framework presented in Fig. 1, this thesis focused on the role played by protein, zinc, and mycotoxins in and on the effect of quality protein maize (QPM) promotion and consumption on linear growth of Ethiopian children. In this chapter, a summary of the main findings is presented, methodological issues are discussed, the key findings are discussed and interpreted, and policy implications and suggestions for future research are given.

Main findings

The main findings of this thesis are presented in Table 1 and briefly described below. The frequency of QPM consumption was increased in both intervention groups (AE: adoption encouragement, and AE + CE: AE plus a consumption encouragement) during the high consumption season and decreased afterward. However, overall, the intake of QPM in both intervention groups was low. We found that encouraging farming households to adopt and feed QPM to infants and young children in a real-life setting had no effect on serum transthyretin (TTR), serum lysine, serum tryptophan, or insulin-like growth factor-1(IGF-1) nor on linear growth of children (chapter 2). In the cross-sectional study carried out before intervention, the daily intake of tryptophan and protein, and the serum level of TTR were positively associated with linear growth of children. Over a third of children had inflammation, about half of them had intestinal parasites, and most children were energy deficient (chapter 3). One in five children was exposed to aflatoxin B1 (AFB1) and one in three was exposed to aflatoxin B2 (AFB2) in both pre-harvest and post-harvest seasons. In the pre-harvest season, exposure to aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) and aflatoxin M1 (AFM1) was higher than in the post-harvest season.

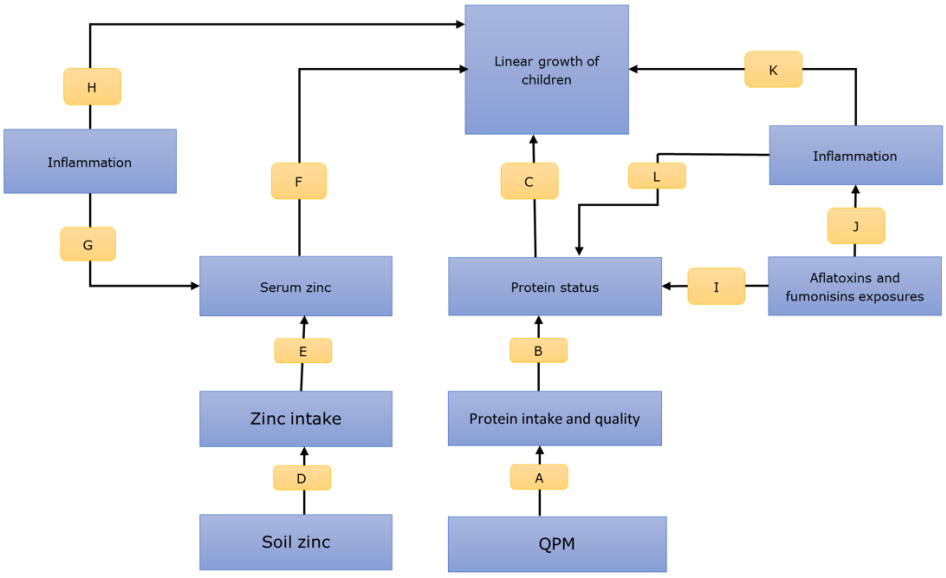

Fig.1. Conceptual framework showing the aetiology of poor linear growth: the role of quality protein maize, protein, soil zinc, serum zinc and mycotoxin exposure in linear growth of children in Ethiopia.

Legend: A, B and C: Protein and amino acids play a biological role in linear growth of children [1,2]. Consumption of QPM could improve quality protein intake, thereby improving protein and amino acids status, which may, in turn, improve linear growth of children [3]. D, E and F: Low soil zinc may lower grain zinc levels, thereby reducing dietary zinc intake, resulting in a higher prevalence of linear growth failure [4-6]. I and C: Exposure to aflatoxins and fumonisins may inhibit protein synthesis which in turn reduces growth [7]. J and K: Exposure to aflatoxins and fumonisins may increase infection and inflammation, which in turn reduces children's the growth [7]. K and H: Inflammation may affect linear growth of children [8]. G and L: Inflammation may also affect zinc status [9,10] and protein status [11-13].

Exposure to any aflatoxin was not associated with protein status or linear growth nor with inflammation (chapter 4). In chapter 5, we found that nationally in Ethiopia 24 percent of children were zinc deficient and 38 percent were stunted, while 20 percent of households were located on zinc-deficient soils. Soil zinc status was positively associated with serum zinc however, neither soil zinc status nor serum zinc were associated with linear growth of children.

Methodological considerations

This section addresses methodological issues that may potentially affect the validity and interpretation of work presented in this thesis. The methodological issues discussed in this chapter are: study design, sampling bias, information bias, and confounding.

Study design

The study design determines the strength of the evidence provided [14,15]. In the area of experimental designs, a randomized controlled trial (RCT) is considered as the gold standard study design as it permits assessing a cause and effect relationship between an intervention and an outcome with minimal influence of bias and confounding factors [14]. In chapter 2, we used an RCT design that allowed us to measure the effect of QPM promotion and consumption on protein status and children's linear growth. In the other chapters, we used an observational or cross-sectional design (chapters 3 and 5), or nested case-control design (chapter 4). A major limitation of these designs was that we could not draw conclusions on the causal relationships between exposure and linear growth of children but could only show associations [16,17]. In addition, seventeen percent of children had a missing value in the outcome variable in chapter 5 and were left out from analysis, while in chapter 4, the sample size was small, both resulting in low ability to find associations (chapters 3 and 5), or nested case-control design (chapter 4). A major limitation of these designs was that we could not draw conclusions on the causal relationships between exposure and linear growth of children but could only show associations [16,17]. In addition, seventeen percent of children had a missing value in the outcome variable in chapter 5 and were left out from analysis, while in chapter 4, the sample size was small, both resulting in low ability to find associations.

Table.1. Summary of the main findings

Objectives	Main results
Chapter 2 Type: randomized controlled trial Population: children aged 6-35 months in rural Ethiopia	
• Effect of QPM promotion and consumption on protein status and linear growth of children in a real-life setting.	• Daily intake of QPM: 27 gram/day and of conventional maize: 80 gram/day. • QPM contributed to 5 percent of total protein, 12 percent lysine and 15 percent of tryptophan daily intakes. • No significant differences in change in lysine intake, tryptophan intake, serum TTR , serum lysine, serum tryptophan, or IGF-1 in interventions vs. control (p>0.05). • No significant effect on linear growth of either intervention: AE (p=0.10) and AE + CE (p=0.97) compared to control.
Chapter 3 Type: cross-sectional Population: children aged 6-35 months in rural Ethiopia	
• Assess protein intake and status, and associations with linear growth of children.	• 11 percent dietary protein deficient; 31 percent lysine deficient; 76 percent with energy intake below EAR. • 35 percent inflammation; 48 percent one or more intestinal parasites. • Tryptophan intake (b=0.01, p=0.001), protein intake (b=0.01, p=0.01), serum TTR (b=2.6, p=0.04) and IGF-1 (b=0.01, p=0.003) positively associated with linear growth of children.
Chapter 4 Type: longitudinal case-control Population: children aged 6-35 months in rural Ethiopia	
• Assess exposure to aflatoxins and fumonisins in serum during post-harvest and pre-harvest seasons. • Assess associations among aflatoxin exposure, serum protein and linear growth of children.	• Exposure to any aflatoxins higher in pre-harvest (51 percent) than post-harvest season (41 percent). Exposure to fumonisins 0-11 percent, depending on the type of fumonisins. • Exposure to any aflatoxins not associated with inflammation, serum TTR or IGF-1 or with linear growth of children (p>0.05).
Chapter 5 Type: cross-sectional Population: children aged 6-59 months in Ethiopia	
• Geographical distribution of poor zinc soils, poor zinc status and poor linear growth among Ethiopian preschool children. • Establish a quantitative relationship between soil zinc content, human serum zinc levels, and linear growth of children.	• 20 percent of households lived on zinc-deficient soils; 24 percent of children zinc deficient. • Soil zinc level positively associated with serum zinc (p=0.020); no association of linear growth with soil zinc (p=0.60) or serum zinc (p=0.50).

Abbreviations: IGF-1: insulin-like growth factor-1; QPM: quality protein maize; TTR: serum transthyretin; EAR: estimated average requirement. AE: Adoption encouragement, and CE: Consumption encouragement.

Sampling bias

Any systematic differences between comparison or intervention groups caused by the way study participants are recruited can lead to selection bias [18,19]. Selection bias may lead to spurious conclusions [18]. Selection bias could occur as a result of non-random selection from the target population, of refusals (non-response) to participate and of self-selection by volunteers [18]. None of the studies reported in chapters 2, 3, and 4 were based on self-selection. Farmers attended the field demonstration days as part of the community agricultural extension services in Ethiopia. A sampling frame was constructed by a development agent (which is a government extension officer for agriculture and rural development) assisted by research coordinators in the respective village (kebele) (chapter 2). In the selected villages, this sampling frame consisted of a list of all households who participated in the field demonstration days and who were eligible for inclusion in the study. These households were randomly assigned to the interventions and control groups, and all eligible households consented to participate in the study. Analyses in chapters 3 and 4 were based on data from the baseline and follow-up of the study reported in chapter 2. Therefore, we do not expect selection bias to be present in the studies reported in these chapters. In chapter 5, 17 percent of households refused to give serum samples and besides, of those who consented, about 20 percent of children's serum samples were not analyzed due to an insufficient amount of serum obtained. Hence, for more than one-third of the participating children, we did not analyze serum sample. This could have been a potential source of bias: if the reasons for refusal or limited serum samples had been related to the exposure or outcome of the study, this may have hampered the generalizability of results [16,17]. Further, we checked for differences in socioeconomic status (educational, and/or household assets, ownership of land and livestock and demographic characteristics of the children) between those who did and those who did not have a serum sample analyzed and we did not find any differences. Therefore, the risk of selection bias in chapter 5 was considered minimal.

A systematic difference between intervention groups in dropouts and compliance with the interventions is another potential source of bias [15,20]. In chapter 2, the total dropout rate was relatively low (5 percent) with no difference between intervention arms, hence a systematic bias was unlikely. One strategy for eliminating this form of bias is by conducting an "intention to treat" analysis, a method for analyzing results in a prospective randomized study where all participants who are randomized are included in the statistical analysis and analyzed according to the group they were originally assigned, regardless of what treatment they received [16]. It also ignores the withdrawal of the subjects during the follow-up. This way, all participants included in the statistical analysis were part of the groups to which they were randomly assigned regardless of whether they completed the study or not.

Information bias

Information bias occurs when there are either random or systematic errors in the measurement of exposures, outcomes and possible confounders or when some respondents provide inaccurate information intentionally (social desirability bias) or unintentionally (recall bias) [16]. Common sources of information bias discussed in the following section are measurement bias (including recall bias), and non-blinding of intervention allocation and outcome assessment.

Measurement bias

Respondent bias is a form of bias caused by participants not giving accurate or truthful responses [21]. This bias is highly prevalent in studies that involve self-reports and interviews. Data collection methods that rely on memory may cause a respondent bias. In chapters 2 and 3, the method used for dietary assessment (24hr recall or one-week food frequency) relied on the caregivers' ability to recall foods consumed in the previous 24 hours or past week. Recall bias in foods reported and in estimation of the amounts of food consumed particularly snacks and main staples foods, has been reported with the use of the 24 hr recall method which often results in an underestimation of nutrient intake [22]. To reduce this recall bias, we used multiple pass techniques, in which

caretakers' memory was supported by asking them to mention step-by-step what food and drinks they had given to the child. In addition, in the final step, we specifically asked for out-of-home consumed food such as fruits and snacks well known to be prone to recall bias [23]. Further, we asked caretakers to use household measures from their own home to estimate quantities and portion sizes, and when food ingredients were available, actual weights were taken to avoid mistakes in the estimation of the amount of foods consumed [23,24].

In chapters 2, 3, 4 and 5, biomarkers for protein status (TTR, and IGF-1) and amino acid status (serum level of lysine and tryptophan), mycotoxin exposure (serum level of aflatoxins and fumonisins), and zinc status (serum zinc concentration) were measured. Every biochemical analysis has its own variability and validity. Quality control is an important way of reducing bias in the measurement of biomarkers. Coefficients of variation (CV) (inter-assay) are important indicators of the quality of the measurement of biomarkers [25]. TTR concentration was measured by immune-turbidimetry using Cobas 6000; serum zinc was measured using Shimadzu Flame Atomic Absorption Spectroscopy; serum mycotoxins were measured by LC-MS/MS; and the analysis of serum amino acids was conducted using Biochrom 30 amino acid analyzer. The CV's for the measurement of these biomarkers were below 5 percent which is considered as acceptable [26]. IGF-1 concentration was measured by ELISA assays that have higher variability and are acceptable when CV is below 20 percent. In our analysis, quality control indicated that variability was generally acceptable with a CV for IGF-1 of 17 percent [27,28].

In the absence of a gold standard biomarker to measure zinc status, serum zinc is recommended by WHO/UNICEF/IAEA/IZiNCG as a valid indicator of zinc status at population level [29,30]. Evidence suggests that the interpretation of serum zinc could be affected by the time of serum collection, the time of consumption of the previous meal, the time of centrifugate and by the level of inflammation [29]. At the time of the blood sample collection in the study described in chapter 5, all subjects were in a non-fasting state and the blood samples were centrifuged within one hour after blood collection as per the recommendation of the International Zinc Nutrition Consultative Group [31]. Inflammation and

micronutrient status are closely related, and the concentration of nutritional biomarkers in blood such as serum zinc is altered in the presence of inflammation [32,33]. During the inflammatory process, there are rapid and large changes in the concentrations of serum zinc being a negative acute-phase protein (APP) [34]. Two of the most commonly used APPs to reflect an individual's inflammatory response are C-reactive protein (CRP), which rises rapidly and acutely in response to an inflammatory stimulus, and α-1-acid glycoprotein (AGP), that has a slower and longer response [35,36]. To account for the inflammation effect on micronutrient status, previous studies used fixed cutoffs for CRP (>5 mg/l) and AGP (>1 gram/l) irrespective of the magnitude and stage of the inflammatory response [37]. The adjustment for inflammation suggested by the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project used in chapter 5 was based on a regression method as an alternative best approach to avoid over-adjusting of zinc status in settings with a high prevalence of inflammation. The BRINDA method uses the reference cutoffs for CRP and AGP adjustment for each defined by the maximum of the lowest decile for their distribution in the study population [38,39].

The data for chapter 5 were derived from two different datasets, one providing soil data and the other one providing serum zinc and other nutritional data. These two datasets were merged using Geographic Information System coordinates. Unfortunately, soil zinc and serum zinc were measured in a different year; soil data were collected in 2012, while serum zinc was collected in 2015. Soil zinc could be affected by soil pH, organic matter content, clay and calcium carbonate, by microbial activity in the rhizosphere, soil moisture status, concentrations of other trace elements and macro-nutrients, especially phosphorus and by climate [40]. Although these factors were not studied in our thesis, they may cause soil zinc alteration over time. Furthermore, evidence suggests that serum zinc concentration decreases or increases with dietary zinc intake [41]. The dietary zinc intake and zinc status varied by agricultural seasons in Ethiopia [42]. Thus, the difference in year and season between the assessment of soil zinc and serum zinc might have reduced our ability to find associations between soil zinc and serum zinc in our study.

Blinding of intervention treatments and biomarker assessment

In intervention studies, blinding is an important design feature to protect against bias that may occur through knowledge of the nature of the allocated intervention, and also through the preference of the researcher for a particular outcome [18,19]. It would have been preferable to carry out the study reported in chapter 2, 3 and 4 with triple-blindness, i.e., subjects, research staff, and data analysts are kept unaware of interventions or control groups. However, in the study reported in chapter 2, blinding of subjects and research staff was virtually impossible to maintain throughout the study due to the fact that the intervention needed the active participation of study participants (farmers) who knew whether they had been planting QPM or not. However, the biochemical analysis was conducted blindly, as laboratory personnel who analyzed the serum samples were not aware of the trial nor of the intervention groups.

Confounding

Confounding occurs when the effect of an exposure is mixed with the effect of another variable leading to results that do not reflect the actual relationship between exposure and outcome [16]. Confounding is best controlled at the design stage by procedures such as randomization as used in chapter 2 (see study design on page 37). Suspected confounding can also be controlled at the data analysis stage. In chapter 3, the possible confounders that may have affected the association between protein intake, protein status and linear growth of children were inflammation, age and sex of the child, and household wealth. In chapter 4, while investigating the association between mycotoxins and linear growth, we took age and sex of the child, inflammation and morbidity, time of treatment intervention, and household wealth status into account as potential confounders. In chapter 5, to assess the association between zinc status and child linear growth, we adjusted for inflammation and morbidity as inflammation and infection can cause a decrease in serum zinc being a negative acute-phase reactant (see also page 203). As the data were nationally and sub-nationally representative in chapter 5, to

count for variability among clusters at sub-national levels, the analyses were also adjusted for clusters at sub-national levels. In chapters 3, 4, and 5, although we adjusted for measured potential confounders, there might be a possibility of residual confounders we did not know and did not measure and could not adjust for. However, we belief that most known confounders were included and adjusted our results for those.

Discussion of main findings

This section of the thesis discusses the key findings in relation to literature as well as to what extent they are generalizable to different settings.

Role of protein, amino acids and QPM intake on protein status and linear growth

Fig.1 describes a conceptual framework depicting pathways showing how protein and amino acid intake (Fig.1: pathway B, C, and L, K), and household-level QPM consumption could improve linear growth of children (Fig.1: pathway A, B, C). Lysine and tryptophan are precursors for several neurotransmitters, regulators of metabolic pathways and building blocks in protein synthesis [43,44]. In our cross-sectional study in chapter 3, we found a statistically significant positive association between protein and tryptophan intake with the biomarkers of protein status (TTR and IGF-1) and between both intake and protein status with linear growth of children. A study by Semba et al. (2016) showed that stunted children had a lower essential amino acid status, suggesting that stunted children consumed less essential amino acids and protein [45]. Similarly, a recent systematic review showed that protein-based interventions had a greater effect on linear growth of children age ≥ 2 years compared with micronutrient-based interventions [46]. Protein or energy restriction in children could also reduce serum IGF concentration, which may result in growth restriction [47]. Our results suggest that protein may contribute to the improvement of children's linear growth, providing justification for an intervention study.

As consumption of lysine-fortified foods was shown to improve the growth of school-age children in previous studies [48,49], it was expected

that, theoretically, in areas where maize is a staple food, the consumption of QPM, with almost double the content of lysine and tryptophan compared to conventional maize, could improve the daily intake of lysine and tryptophan and hence, improve linear growth [50]. Also, a previous controlled study in Ethiopia found that consumption of QPM instead of conventional maize led to a greater weight increase in children [51]. In addition, a meta-analysis of data from nine different studies also found that QPM consumption was associated with a 12 percent increased rate of weight gain and a nine percent greater rate of height gain. However, the individual studies included in the meta-analysis showed several limitations: methodologies were not sufficiently described; only two studies were peer-reviewed and the rest were from technical reports, and there was lack of clarity on randomization [52]. Furthermore, none of the previous studies measure the actual QPM intake, protein and energy intake, or the biomarkers of protein and amino acid status, nor did they take into account inflammation. We studied the effect of QPM consumption in a setting where households decided themselves whether or not to give QPM to their children and, to our knowledge, there are no similar studies on the effect of QPM on the protein status or linear growth of children in real-life settings.

However, we found that encouragement to adopt and feed QPM to infants and young children in a real-life setting did not have a significant effect on children's protein biomarkers or on linear growth of children in rural Ethiopia (chapter 2). The lack of a positive effect may have been caused by several reasons; the first and probably most important reason could be that the consumption of QPM in our study was not sufficient to improve serum biomarkers and linear growth. Children were fed QPM, but the QPM intake on average was low (27 gram/day) for both intervention groups. Other studies have suggested that the lowest estimated intake to bring about biologically meaningful changes in children's lysine intake should be higher than 100 gram/day of QPM [50,53]. In our study, households did not substitute the QPM for conventional maize and most also remained consuming conventional maize (80 gram/day). Biofortified crops, QPM and derived foods may be treated as a new product by our population rather than used to replace the conventional food or food

products. If the QPM promotion and consumption interventions had continued for a longer period (into subsequent seasons), consumption may have increased as participants would have become more familiar with the biofortified crop. A second explanation, related to the above mentioned low intake, might be related to the role of poor appetite. Poor appetite is a common response to infection or inflammation [8] and for example worms, impaired absorption, and altered metabolism might be major causes of low QPM intake and low energy intake in our children [54,55].

Thirdly, the pathogenesis of linear growth failure is still poorly understood. Linear growth faltering begins in utero (where about 20 percent of growth faltering occurs) [56], and linear growth failure may result from a complex interaction of household, environmental, socioeconomic and cultural influences [57]. Effective combinations of interventions to reduce stunting or improve linear growth of children remain unclear [58], but it is now increasingly realized that dietary interventions alone have minimal effect on linear growth of children [59]. Just improving lysine or tryptophan intake through QPM may not be sufficient to reduce linear growth faltering and additional interventions may be needed.

Lastly, most of our study children were deficient in their daily energy intake. This may have interfered with the effect of the increased protein intake. Energy deficit in children may lead to growth retardation in itself, and increased morbidity and mortality [60]. Previous evidence has shown that both energy and protein restriction in children reduces IGF-1 concentration, a hormone that has a regulatory role in growth [47]. In addition, when energy intake is limited, extra dietary protein is used to produce energy, potentially diverting it from other uses [61]. An interventional study among Indian children found that an energy-rich low-protein supplement improved linear growth [62]. Thus, energy deficiency may be a major factor limiting child growth in our study population.

Presently, protein and amino acid-based interventions are not included in strategies for reducing linear growth of children in developing countries [63-65]. Between the 1950s and 1970s, protein malnutrition

was the major focus of the global nutrition agenda [63], however, it was addressed in isolation without considering low energy intake and inflammation or infection [66]. Later it was shown that a high protein feeding was not successful in improving linear growth due to lack of sufficient energy [67], and that protein malnutrition resulted mainly from energy deficiency [68]. This caused reduced attention to protein deficiency, also because recent studies, as well as our study, showed that total protein intake in child complementary foods is largely adequate in developing countries, according to the prevailing protein recommendations [68], while most children have low energy intake. However, it is increasingly felt that the recommendation for adequate protein intake for children who have low energy intake and repeated infections should be revised [70]. Firstly, frequent illness and other stress may increase the amount of protein that the child needs to grow, through affecting ability of the gut to absorb nutrients. Secondly, when the energy intake is too low, it could be aggravated by the loss of nitrogen caused by infections and several metabolic changes [61]. Early evidence has shown that adults with an energy deficit need additional protein; even a modest energy deficit (5 percent) increases protein needs by about 10 percent [61]. Furthermore, a study conducted in India found that children's lysine requirement increased by 20 percent in children with intestinal parasitic infections [12]. Other studies suggested that bacterial infection increases protein requirements by about 30 percent [69] and lysine requirements by 50 percent [13] in malnourished children in India. In our study population also, intestinal parasites and inflammation were prevalent. This may suggest that protein and amino acids deficiency may be prevalent and need to be reconsidered in nutrition strategies in developing countries such as Ethiopia.

The role of aflatoxin exposure in linear growth of young children

In Fig. 1 we proposed a pathway to understand the mechanism of how aflatoxins affect children’s linear growth by the inhibition of protein status and increased inflammation (Fig.1: pathway I, C, and J, L, K). Our data suggest that aflatoxin exposure is a public health problem of concern in

our study population. We found one in five children to be exposed to AFB1, and one in three to be exposed to AFB2 irrespective of season (chapter 4). There was a high prevalence of exposure to some aflatoxins during the pre-harvest season compared with the post-harvest season: AFG1 (8 percent vs. 4 percent), AFG2 (33 percent vs. 28 percent), and AFM1 (7 percent vs. 4 percent). Agronomic practices, time of planting and harvesting [71] and temperature and humidity [72] are factors that influence contamination of grains by aflatoxins. Proper storage and grain moisture management have been suggested as critical aflatoxin contamination control interventions [73,74]. Most aflatoxin intervention programs have been focused on post-harvest management [75]. Aflatoxins are highly stable secondary metabolites and thus, even in the pre-harvest season, grains infected by toxigenic strains and contaminated pre-harvest are still at risk during transport, processing, handling, and storage, if environmental conditions favor the growth of the fungus [76]. Good post-harvest handling and storage will prevent further accumulation of aflatoxins but will do nothing to reduce aflatoxin levels already present in food samples before harvest.

AFB1 is the most toxic of the studied mycotoxins because it is a human carcinogen [77,78]. In general, the order of level of carcinogenicity is categorized as AFB1>AFG1>AFM1>AFB2>AFG2 [78]. Aflatoxin M1 is another significant member of the aflatoxin family and is an oxidative form of AFB1. Our study is the first to report exposure to multiple aflatoxins as measured in serum in Ethiopia. Compared to other African studies in Kenya, Tanzania and Mexico, exposure to aflatoxin measured in serum in our population appeared to be low. In those studies, exposure to aflatoxin B1 measured in serum was detected in all study populations [79-81]. Similarly, a study conducted among Gambian children found that exposure to aflatoxin B1 was detected in serum in the majority of study children [82]. In Ethiopia, exposure to aflatoxin in child complementary foods was also relatively low compared with other countries in developing countries [83]. This may suggest that contamination levels in Ethiopia are still low possibly due to local climate conditions not being favorable for the growth of aflatoxigenic molds.

Exposure to any aflatoxins - AFB1, AFB2, AFG1, AFG2 and AFM1 - as measured in serum in our study population was not associated with serum level TTR or IGF-1 nor with linear growth. Smith et al. (2012) suggested that the mechanisms through aflatoxin exposure affected linear growth faltering of children were the inhibition of protein synthesis and increased inflammation; however, this hypothesis has been poorly tested in humans [7]. There is growing concern that linear growth faltering or stunting has not been reducing due to high aflatoxin exposure in developing countries [84]. Several observational and cross-sectional studies have been conducted on the relationship between aflatoxins and growth faltering in developing countries [79,82,85-89] with inconsistent findings. Three studies in West African countries, namely Benin, Gambia, and Nigeria, found an inverse association between AFB1 exposure measured as aflatoxin-albumin in serum and linear growth of children [82,85,87-89]. Similarly, a study from Mexico showed that a low aflatoxin exposure level was associated with greater linear growth [79]. Contrary to these findings, studies in East Africa, namely in Kenya and Tanzania [80,81,90] and Nepal [86] did not find any association between aflatoxin exposure (measured by aflatoxin-albumin or AFB1-lysine/mg albumin) and children's linear growth. A recent intervention study among Kenyan children suggested that improving household access to aflatoxin-free maize reduced level aflatoxin exposure in serum, but showed no effect on linear growth of children [80]. More recently a systematic review of numerous studies showed that aflatoxins were not associated with linear growth [91]. In our study population, we did not observe an association between aflatoxin exposure and protein or amino acid biomarkers nor with inflammation. Our findings support recent research that describes an unclear relationship between aflatoxin exposure and linear growth of children in developing countries, especially in East Africa. However, even though the relationship between aflatoxin exposure and children's linear growth is unclear; aflatoxins have carcinogenic properties and the current exposure is a major public health problem that warrants intervention.

The role of zinc in linear growth of children

A low level of soil zinc could lead to a low zinc intake and low zinc status, which in turn could affect linear growth of children mediated by inflammation (Fig.1: pathway D, E, F and G, H). To our knowledge, there is currently no study that has examined the relationships among soil zinc, serum zinc and children's linear growth using nationally representative data. In our study (chapter 5), zinc deficiency was found in 24 percent of Ethiopian children, indicating that zinc deficiency is a significant public health problem in Ethiopia [92]. Nationally representative studies to assess zinc deficiency are absent in Ethiopia, as in other developing countries, and most studies were conducted in limited settings and not in preschool children. Based on national food supply data from FAO's Food Balance Sheets and level of stunting, it is estimated that over a third of children under five in developing countries are zinc deficient [93]. A recent review of 12 national surveys that used serum zinc as an indicator of zinc status, showed that the prevalence of zinc deficiency varied greatly, with the highest prevalence reported in Cameroon (83 percent) and the lowest in Nigeria (20 percent) [94]. However, most studies reviewed did not adjust for inflammation which could have inflated the prevalence of zinc deficiency [39]. In our study population, inflammation biomarkers were negatively associated with serum zinc levels, and the prevalence of zinc deficiency reduced from 28 percent to 24 percent after adjusting for inflammation. Zinc deficiency was also prevalent in agricultural soils, with about 20 percent of Ethiopian agricultural soils having low zinc levels. Furthermore, our study showed that an increased level of soil zinc was associated with higher serum zinc levels in Ethiopian children.

Plant-based diets are major contributors to the daily zinc intake in Ethiopia and other developing countries [93], where the intake of zinc-rich flesh foods is often low especially in rural areas. Our results suggest that plants grown on zinc-poor soils may have a low zinc concentration, leading to crops with low zinc content, and when consumed leading to a low zinc intake and eventually resulting in zinc deficiency. This may suggest that zinc fertilization of soils and foliar or agronomic

biofortification may be alternative strategies that could contribute to an increased zinc intake in Ethiopia. Previous studies in developing countries have shown that the application of zinc to soils or foliar fertilization increased the median zinc concentration of maize, rice, and wheat grain [6]. However, whether this increased zinc concentration will increase zinc intake, depends, among others factors, on the bioavailability of zinc in the diet [95]. The inhibitory effect of phytate on zinc bioavailability is a key consideration in dietary zinc intake [96]. Plant-based diets often contain high levels of phytic acid and dietary fiber, components known to inhibit the absorption of dietary zinc [97]. The phytate content of foods may vary depending on the location from which it is sourced, due to inherent differences in soil composition, climatic or environmental conditions, crop plant cultivars, and agricultural management practices; the different stages of seed maturation and the use of food processing techniques, such as milling and flour extraction, are additional factors that influence the phytate concentration [98].

Linear growth of children in our study was not associated with soil zinc levels or serum zinc status (chapter 5). Zinc plays an important role in growth; it also interacts with important hormones involved in bone growth such as somatomedin-c, osteocalcin, testosterone, thyroid hormones, and insulin [99,100]. However, results from intervention studies on the effect of zinc supplementation on linear growth of children were not consistent. A meta-analysis on the effect of zinc supplementation on children's linear growth suggested that zinc deficiency leads to the impairment of growth and contributes to a high prevalence of stunting in developing countries [101]. Another systematic review and meta-analysis showed that zinc supplementation had no effect on the growth of children from 1-8 years old [102]. A Cochrane systematic review concluded that zinc supplementation has a small, not clinically meaningful effect on linear growth in children aged from 6 months-12 years [103]. The lack of association between serum zinc and linear growth in our study may be because linear growth could be affected by other nutritional and health factors. Although zinc status was not associated with linear growth of children, interventions to address low serum zinc in Ethiopia are warranted.

Rethinking the use of stunting or linear growth as an outcome measurement in nutrition interventions

Our study was unable to find clear associations between linear growth and factors such as protein intake, protein quality, serum zinc or aflatoxin exposure, and this shows a need to reflect on linear growth faltering or stunting as an outcome of nutrition interventions. Recently, stunting or linear growth retardation has been widely promoted and targets have been set in the health and nutrition programs of Ethiopia and other developing countries. The Sustainable Development Goals (SDGs) targets and countries' specific development agendas have also set stunting reduction as one of the main targets [104-106]. In Ethiopia and in other low and middle-income countries many donor agencies are also interested in supporting interventions that focus on improving linear growth or reducing stunting. Linear growth retardation and stunting are often used interchangeably, but they represent different concepts. Both are markers of inadequacy of the environment and markers of the past and future [107,108]. Linear growth faltering is a failure to reach one's linear growth potential but does not automatically have to lead to stunting. A study conducted by Leroy et al. (2014) found that the number of children in developing countries who are suffering from linear growth retardation is much higher than the number of children who are stunted [109]. Children who are stunted are a subset of those with linear growth retardation. Prevalence of stunting may therefore indicate a shift of the distribution of growth in the whole population towards growth faltering.

Despite the recent attention to stunting and the large number of interventions conducted to reduce stunting or improve children's linear growth, the number of stunted children remains high, especially in sub-Saharan Africa. Numerous interventions, both nutrition-sensitive and nutrition-specific have set their primary outcomes as reduced stunting or improved linear growth. Based on recent evidence, it is unclear whether those interventions were causally linked with linear growth of children [107]. Most nutrition-specific interventions are estimated to have had only a small effect on linear growth or stunting [59,110]. Maternal and child micronutrient-based interventions have also only a small effect or

no effect on stunting or linear growth [111], while the effect of biofortified crop interventions on linear growth remains unclear [52,112]. Social protection interventions have shown little or no effect on linear growth of children [113]. The recent water, sanitation and hygiene plus lipid based nutrient supplement interventions in Zimbabwe, Kenya and Bangladesh also showed no effect on stunting or linear growth [114,115]. The lack of results of the above interventions necessitates rethinking the appropriateness of using linear growth or stunting as a primary outcome in nutrition interventions. There is no biological or clinical base for the –2 SD cutoff for height-for-age (HAZ) to define stunting, and HAZ<-2SD was originally used as an indicator of the general population living and welfare status [107,108].

The misinterpretation of stunting or linear growth as a direct indicator of nutritional status may have shifted focus away from a broad set of environmental and social determinants of child growth to single interventions [108,116]. As a consequence, the use of stunting or linear growth for program outcomes could be leading to good programs to be incorrectly assessed as having failed to achieve their aim [107]. Based on the current evidence, the use of stunting or linear growth as main outcomes in monitoring the effectiveness of health or nutrition program may therefore, be inappropriate. However, as suggested by Leroy and Frongillo (2019), the severity of linear growth retardation and stunting in groups of children can be used to compare between or within countries, and can also be used to monitor the progress of children of the same age distribution over time [107]. To reduce stunting or linear growth failure, interventions should focus on intermediate outcomes. Thus, in our study and also in nutrition intervention programs, measuring intermediate indicators as outcomes to improve linear growth might be a more feasible approach than measuring linear growth or stunting.

Generalizability

Our effectiveness trial was designed to measure the effectiveness of an intervention conducted in a natural setting and with randomly selected participants. The study was conducted with children whose staple diet was

maize or cereal-based. The results of our effectiveness trial may be extrapolated to other biofortified crops and other cereal consuming rural communities especially in Ethiopia.

In chapter 3, the intake and status data were assessed during the pre-harvest season that is characterized by food insecurity. Therefore, the results may not reflect seasonal variation. In chapter 4, randomly selected data have been used from both pre-harvest and post-harvest seasons. The results may not represent the whole country i.e., it may represent only the study areas (rural Jimma and East Wellega zones). In chapter 5, nationally and sub-nationally representative data of serum zinc and soil zinc levels were used. The biological data were collected from one season (pre-harvest). A previous study in Ethiopia showed that the zinc status of the mother and her children varied by season [42]. Our data on zinc status may not represent seasonal variability.

General Conclusions

- Low protein (of low quality) intake, high prevalence of zinc deficiency and high exposure to multiple aflatoxins were public health problems of concern in Ethiopia.
- Children's linear growth was positively associated with protein intake, energy intake, as well as with protein status, but not with zinc soil levels, zinc status or multiple aflatoxin exposure.
- QPM promotion and consumption in a natural setting did not have an effect on protein and amino acid status nor on linear growth of children under five years age, probably due to low QPM consumption.

Implications for Public Health and Future Research

The Ethiopian Food and Nutrition Policy has indicated nutrition-sensitive agricultural interventions, biofortification and food-based approaches as among the main strategies to address nutritional problems. Further, government and other development partners have indicated their commitment to being involved in the delivery of biofortified crops such as

QPM to farmers. The development and promotion of biofortified crops such as QPM would be helpful in addressing malnutrition and food insecurity and also in achieving the SDGs and national goals. Although the beneficial effects of QPM are well-demonstrated in controlled settings, our study has shown no effect on linear growth perhaps due to the low consumption of QPM in a natural setting. Changing household dietary habits may take time. An effort to make households understand why they should prefer QPM to conventional maize is needed; lack of awareness of the nutritional benefits of biofortified crops may be one of the major factors for their low consumption. If our intervention had continued for a longer period i.e., for more than one agricultural season, it may be that farmers would have become more accustomed to QPM and its uptake might have increased. Future research should be done to identify barriers to low consumption in natural settings. Conducting intense behavior change communication alongside the introduction of biofortified crops is also advised [117]. In addition, a formative study on the process and impact pathway may further help to better understanding. Numerous studies have shown the positive effects of other biofortified crops in controlled settings [112]. However, the effect of biofortified crops in real practice has been poorly studied yet [118]. Our study suggests that implementation of biofortified crops in practice may need a longer time and multiple years to change feeding behavior and practices. Future studies over a longer period of time are needed, perhaps continuing over more than one agricultural season.

Despite the lack of a positive effect of QPM compared with conventional maize in our intervention, QPM is an important crop in terms of nutritional benefits [119]. Conventional maize in Ethiopia is the second most cultivated cereal, grown by 66 percent of cereal-farming households and cultivated on 2.1 million hectares [120]. However, conventional maize has a low concentration of essential amino acids such as lysine, and tryptophan. Furthermore, most farming households do not have alternative sources of protein such as legumes or animal products (meat, eggs, and milk) [121,122]. Therefore, taking this into consideration, the Ethiopian Government has aimed to cover 10 percent of the total maize growing area with QPM. The absence of an effect in our study population

may not necessarily mean that QPM is not an important crop nutritionally. QPM has been introduced in some parts of Ethiopia to smallholder farmers and is mostly preferred to conventional maize by most farmers [123]. In Ethiopia, there was also a plan to scale or increase QPM or replace conventional maize with QPM. From a programmatic point of view, the current initiative and plan to replace conventional maize with QPM may need further evidence.

Concerning the implementation of biofortified crops, infection and environmental enteropathy should also be considered. Infection or inflammation can reduce children's appetites [54,55], leading to low intake. In our study and most other studies conducted in Ethiopia, energy intake was low and inflammation was high [124].

The role of protein, particularly from the perspective of essential amino acids in preventing child undernutrition in developing countries has not been addressed in the recent nutrition agenda. Unlike in developed countries, consumption of high-quality protein-rich food by children is minimal in developing countries. Earlier protein research did not recognize the role of energy deficit besides that of essential amino acids, nor did it take into account infection or inflammation. Thus, the current protein research agenda should focus on concurrent low qualities of protein, low energy intake and persistent infection in developing countries. In this thesis, it was indicated in chapter 3, that protein, energy, and tryptophan intakes, protein status, and IGF-1, were positively associated with linear growth. Thus, protein and essential amino acids might have a contribution to make in improving linear growth of children in developing countries. The current estimated adequacy of protein and amino acids for children in developing countries who have an infection and low energy intake may not be valid. Future longitudinal intervention studies on the effect of intake of quality protein and essential amino acid on protein and amino acid status are needed.

Mycotoxin control has not been included in the nutrition policy of Ethiopia. However, due to the recent concern that exposure to aflatoxins has been associated with linear growth or stunting of children, a mycotoxin controlling strategy is included in the recent National Food and

Nutrition Policy. Despite the lack of association with linear growth, aflatoxins are the most carcinogenic substances for humans and interventions to reduce the exposure are needed. Our study revealed that exposure to most aflatoxins was high in pre-harvest season, and good practices are necessary to prevent contamination by aflatoxins in both post and pre-harvest seasons. The current planned policy to implement post-harvest and pre-harvest handling and related food safety activities should be implemented at all levels. Future studies with a large sample size are needed to examine the biological mechanisms between aflatoxin exposure and linear growth and sources of high exposure. In addition, future research needed on the complex and interacting pathophysiology of multiple mycotoxins and exposure management.

Currently, the government of Ethiopia has committed to begin implementing effective soil fertilization on a large scale. In a setting where a plant-based diet is common, the application of zinc fertilizer or agronomic fortification to the soil could potentially increase the grain zinc content. In chapter 5, we investigated whether an increase of zinc in soil was associated with a high zinc status in children. In Ethiopia, most households' food consumption comes from own production [125]; however, crop production on zinc-deficient soils and its effect on humans has not been studied. Therefore, a future longitudinal experimental study on the effects of soil zinc application on crop zinc content and human serum zinc levels may help better understand the relationship. The phytate content of foods may affect zinc bioavailability [98]. Future research may also be needed to study the effect of phytate on the zinc bioavailability of crops grown on using zinc-deficient soil.

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About the author

Masresha Tessema was born on 26 September 1983 in Shone, Ethiopia. He attended his primary school in Shone School at Badawacho district (Hadiya Zone), SNNPRS, and completed his secondary school at Hawassa, Ethiopia. In 2007, he completed his Bachelor of Science degree from School Nutrition and Food Science – Ethiopia. After graduation, he worked as a coordinator of the district (Western Badawacho) Food Security, disaster prevention and management, Hadiya zone, Ethiopia for two years. In December 2009, Masresha went back to Hawassa University to continue his MSc study and received his MSc degree in Applied Human Nutrition in July 2011. His Master thesis focused on Child feeding and stunting in rural Sidama, SNNPR region. After completing his MSc study, he continued working as an assistant researcher in the Ethiopian Public Health Institute, Food Science and Nutrition Directorate, which was used to be the Ethiopian Health and Nutrition Research Institute. In the Institute he served as assistant research, team leader of Nutrition research and director Food Science and Nutrition Research Directorate. He was involved in coordinating and leading several nutrition and health researches, to mention few, National Micronutrient Survey, Community Based Nutrition program evaluation, Validation of Minimum Women dietary diversity data collection method and National Nutrition program End line survey. He served as a chair of the National Nutrition Research, Monitoring and Evaluation steering committee of the National Nutrition Program. He also served as the organizing committee for micronutrient forum 2014, representing Ethiopia. Further, he also served as a chair of the scientific steering committee of 13th International Association of National Public Health Institutes (IANPHI) annual meeting which is hosted by the Ethiopian Public Health Institute in 2019. He is a member of the Food and Nutrition Society of Ethiopia, Ethiopian pediatric association,

and Ethiopian Public Health Association. In 2015, he received a scholarship to pursue his PhD from CIMMYT. In January 2016, he joined Wageningen University & Research, Division of Human Nutrition and Health to pursue his PhD under the supervision of Professor Edith J.M. Feskens and Associate Professor Inge D. Brouwer and Assistant Professor Nilupa S. Gunaratna. His work was on linear growth failure of Ethiopian children. The PhD research project was funded by CIMMYT and Agricultural Technology Adoption Initiative (ATAI). This dissertation presents the results of her PhD study, which also comprises published, peer-reviewed and submitted articles in scientific journals. Masresha also attended several national and international conferences within the education program of the graduate school of VLAG. His ORCID identifier is 0000-0002-7155-4815. He can be contacted on masresha88@gmail.com.

List of publications

Peer reviewed articles

Masresha, Tessema., Laillou, A., Tefera, A., Teklu, Y., Berger, J., & Wieringa, F. T. (2020). Routinely MUAC screening for severe acute malnutrition should consider the gender and age group bias in the Ethiopian non-emergency context. *Plos one*, *15*(4), e0230502.

Masresha, Tessema., De Groote, H., D Brouwer, I., JM Feskens, E., Belachew, T., Zerfu, D., Belay, A., Demelash, Y. and S Gunaratna, N., 2019. Soil zinc is associated with serum zinc but not with linear growth of children in Ethiopia. *Nutrients*, *11*(2), p.221.

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Tesfaye, Hailu., **Masresha, Tessema**., Biniyam Tesfaye., Aweke Kebede., Adamu Belay., Girmay Ayana., Yosef Beyene., Temesgen Awoke., Desalegn Kuche., Andinet Abera., Tsehai Assefa., Dilnesaw Zerfu., Tibebu Moges., Aregash Samuel., Mekonen Tadesse., Tewodros Getachew., Mesret W/Yohanes., Birhanu Wedajo., Mesfin Gose., Barbara Tembo., and Yibeltal Assefa. Effectiveness of Chickpea-Based Ready-to-Use-Supplementary Foods for Management of Moderate Acute Malnutrition in Ethiopia: A Cluster-Randomized Control Trial. EC Nutrition 11.5 (2017): 201-215.

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Masresha, Tessema., Brouwer, D.I., Gunaratna, S.N., Belachew, T., Belayneh, D., Donato, K. and De Groote, H., 2017, January. Protein intake, protein quality, protein status and early childhood linear growth in rural Ethiopia. In *annals of nutrition and metabolism* (vol. 71, pp. 1097-1097). Allschwilerstrasse 10, ch-4009 basel, switzerland: karger.

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Zerfu, D., Tekle, A., Moges, T., Belay, A., Abera, A., Ayana, G., **Masresha, Tessema**., Kuche, D., Kebede, A. and Samuel, A., 2015. The Proportion of Households with Adequately Iodized Salt in Ethiopia. *European Journal of Nutrition & Food Safety*, pp.1120-1120.

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Kennedy, E., **Masresha, Tessema**., Samuel, A., Kassaye, T., Fekadu, H., Hailu, T., Assefa, T., Kuche, D., Ayana, G., Zerfu, D. and Moges, T., 2015. Effects of Governance Structures in Ethiopia on Implementation of Nutrition Interventions. *European Journal of Nutrition & Food Safety*, pp.463-464.

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Zerfu, D., Moges, T., **Masresha Tessema.**, Eshetu, S., Awoke, T., Beyen, Y., Zeru, T., Tekle, A., Belay, A., Kebede, A. and Samuel, A., 2015. The Nutritional Status of Vulnerable Populations in Ethiopia. *European Journal of Nutrition & Food Safety*, pp.1132-1132.

Gameda, Samuel., Tekalign Mamo., Hailu Shiferaw., **Masresha Tessema.**, and Dilnesaw Zerfu. "Potential Linkages between Zinc in

Soils and Human Nutrition." In the 4th International Zinc Symposium: Improving Crop Production and Human Health, p. 18. 2015.

Expected publications

Masresha, Tessema., Hugo De Groote., Inge D. Brouwer., Edith J.M. Feskens., Jessica L. Cohen., Margaret McConnell., Katherine Donato., Demissie Belayneh., Tefera Belachew., and Nilupa S. Gunaratna *(Submitted).* "Effect of quality protein maize on protein status and linear growth of Ethiopian children: a randomized controlled trial."

Masresha Tessema, Hugo De Groote, Inge D. Brouwer, Marthe De Boevre, Arnau Vidal, Barbara J. Stoecker, Edith J.M. Feskens, Tefera Belachew, Anastasia Karakitsou and Nilupa S. Gunaratna (Accepted for publication). "Exposure to aflatoxins and fumonisins and linear growth of children in rural Ethiopia: a longitudinal study."

Conference presentations (Poster and Oral)

Poster presentations

Masresha, Tessema., Brouwer, D.I., Gunaratna, S.N., Belachew, T., Belayneh, D., Donato, K. and De Groote, H., 2017, January. Protein intake, protein quality, protein status and early childhood linear growth in rural Ethiopia at IUNS 21st International Congress of Nutrition, in Buenos Aires, Argentina, 15-20 October, 2017.

Masresha, Tessema., Hugo, De Groote., Inge D. Brouwer., E.J.M Feskens., Tefera Belachew., Dilnesaw Zerfu., Adamu, Belay2., Yoseph, Demelash., and Nilupa S, Gunaratna. Associations Among Soil Zinc, Serum Zinc, and Linear Growth of Children in Ethiopia at the conference organized by American Society for Nutrition (ASN) in Boston, USA, 9-12 June, 2018.

Masresha, Tessema., Hugo De Groote., Inge D. Brouwer., Edith J.M. Feskens., Jessica L. Cohen., Margaret McConnell., Katherine Donato., Demissie Belayneh., Tefera Belachew., and Nilupa S. Gunaratna. Effect of quality protein maize on protein status and linear growth of Ethiopian children: a randomized controlled trial at Agriculture, Nutrition and Health (ANH) Academy, Accra, Ghana, June 25-29, 2018.

Hugo, De Groote., Nilupa, S. Gunaratna., **Masresha, Tessema.**, and Samuel Gameda. Soil zinc, serum zinc, and the potential for agronomic

biofortification to reduce human zinc deficiency in Ethiopia at Agriculture, Nutrition and Health (ANH) Academy, Accra, Ghana, June 25-29, 2018.

Oral presentations

An oral presentation entitled: "**Associations Among Soil Zinc, Serum Zinc, and Linear Growth of Children in Ethiopia**" at the conference organized by American Society for Nutrition (ASN) in Boston, USA, 9-12 June, 2018.

An oral presentation entitled: "**Biomarkers of aflatoxin exposure, diet, climate and children's growth in rural Ethiopia**" at ANEC, Addis Ababa, Ethiopia, October 1-5, 2018.

Overview of completed training activities

Discipline-specific courses	Institute	Year
International post-graduate course on the production and use of food composition data in Nutrition	WUR	2017
Exposure Assessment in Nutrition Research	WUR	2016
Training on Food Consumption Data analysis	WUR	2017
Energy metabolism and body composition in nutrition and health research	WUR	2018
Training on the analysis of serum aflatoxin using LC MS/MS	Ghent University	2018
Comprehensive Systematic Review Training	The Joanna Briggs Institute, Australia	2018
Masterclass Healthy and sustainable diets	WUR	2019
Transforming Nutrition: Ideas, Policies and Outcome	Institute of Development Studies, UK	2019
Discipline-specific conferences and meetings	**Country**	**Year**
21th International Congress of Nutrition	Buenos Aires, Argentina	2017
Agriculture, Nutrition and Health (ANH) Academy	Addis Ababa, Ethiopia	2016
American Nutrition Society meeting	Boston, USA	2016
Agriculture, Nutrition and Health (ANH) Academy	Accra, Ghana	2018
African nutrition epidemiology conference	Addis Ababa, Ethiopia	2018

General Courses	Country	Year
Techniques for writing and presentation a scientific paper	VLAG	2016
Erasmus Summer course on statistical analysis	Erasmus University - NIHES	2016
Reviewing a Scientific Paper	WUR	2016
Information Literacy for PhD including Endnote Introduction	WUR	2016
Data management planning	WUR	2016
Longitudinal data analysis using R	Jimma University	2017
IRB review and Good clinical research practice (GCP) guidance	CDC with EPHI, Ethiopia	2018
Optional courses and activities	**Country**	**Year**
Preparation of PhD research proposal	HNE, Wageningen	2016
Paper Café	HNE, Wageningen	2017
Seminar on Multi-Criteria Decision-Making for Healthy, Affordable and Sustainable diets	HNE, Wageningen	2017
Citing and referencing	WUR	2017
Last stretch	VLAG	2017
PhD study Tour	Canada	2019

Colophon

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