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PhD Thesis

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The gut in childhood stunting: a randomised controlled trial in Uganda

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Cover photo: Photo by Author. Fun and games on a MAGNUS household visit, Eastern Uganda.

1 Preface

I moved to Denmark from Australia more than 8 years ago, keen to support the work being done all over the world in alleviating child malnutrition. I was offered an opportunity to work as a student assistant and then a PhD in the Paediatric and International Nutrition research group under Prof Henrik Friis. I am grateful for all I have learned along the way.

There are many people I would like to thank, who have made this journey possible. First, thank-you to Henrik and Benedikte for giving me this opportunity and for all the work you do and have done together with others to improve the treatment of child malnutrition. To Henrik, thank-you for always making time and for keeping a positive outlook. A big thanks to Benedikte for your support and for the wealth of experiential knowledge you have shared along the way. This trial would not have taken place without the efforts of Joseph and Rolland, my two PhD colleagues in Uganda. Thanks to you both for your effort and determination to see that this trial was completed to the highest possible standard. Following from this, I want to thank the many staff who worked tirelessly on the MAGNUS trial, those who took it to completion despite challenging circumstances during Covid-19. To Jack, it has been fun working together here and in Uganda. I am extremely grateful for your contributions to this study. Thanks to Mamane, for having faith in me and for making this PhD possible. To Pernille Kaestel for taking a chance on me all those years ago. To my dear friend Hanne Hauger, for your encouragement and selflessness. To my sister Ambs, thanks for walking the journey with me. Finally, to my wonderful husband Bo, your support and encouragement has made this possible. Thank-you for carrying everything that I could not. To my daughter Hallie, thank-you for your beautiful nature, for keeping life simple and sweet and for making me laugh every single day.

This PhD thesis is a culmination of work on the MAGNUS trial among children with stunting in Eastern Uganda.

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2 Included papers

Paper I

Pesu H, Mutumba R, Mbabazi J, Olsen M.F, Mølgaard C, Michaelsen K.F, Ritz C, Filteau S, Briend A, Mupere E, Friis H, Grenov B. The role of milk protein and whey permeate in lipid-based nutrient supplements on the growth and development of stunted children in Uganda: A randomized trial protocol (MAGNUS). *Current Developments in Nutrition* 2021; 5.

Paper II

Pesu H, Mbabazi J, Mutumba R, Savolainen O, Olsen M.F, Mølgaard C, Michaelsen K.F, Ritz C, Filteau S, Briend A, Mupere E, Friis H, Grenov B. Correlates of plasma citrulline as a marker of enterocyte mass among children with stunting: a cross-sectional study in Uganda *(Manuscript prepared for submission)*

Paper III

Pesu H, Mbabazi J, Mutumba R, Savolainen O, Frøkiær H, Olsen M.F, Mølgaard C, Michaelsen K.F, Ritz C, Filteau S, Briend A, Mupere E, Friis H, Grenov B. Effects of lipid-based nutrient supplements on gut markers: exploratory outcomes from a randomised trial *(Manuscript prepared for submission)*

3 List of abbreviations

CRF	Case-report forms
EED	Environmental enteric dysfunction
ELISA	Enzyme linked immunosorbent assays
f-AAT/AAT	Faecal/ α_1-antitrypsin
f-MPO/MPO	Faecal/ myeloperoxidase
f-NEO/NEO	Faecal/ neopterin
GCP	Good clinical practice
IBD	Inflammatory bowel disease
IF	Intrinsic factor
INF-γ	Interferon-gamma
HAZ	Height or length-for-age Z-score
LAZ	Length-for-age Z-score (aged <24 months)
LMIC	Low and middle-income countries
L:M	Lactulose to mannitol ratio
LNS	Lipid-based nutrient supplements
s-AGP/AGP	Serum/ α_1-acid glycoprotein
s-CRP/CRP	Serum/ C-reactive protein
s-IGF1/ IGF-1	Serum/Insulin-like growth factor-1
MAGNUS	Milk affecting growth, cognition and the gut in child stunting
MAM	Moderate acute malnutrition
MP	Milk protein
MUAC	Mid-upper arm circumference
NO	Nitric oxide
p-cit	Plasma citrulline
p-cobalamin	Plasma cobalamin
p-MMA	Plasma methylmalonic acid
SOPs	Standard operating procedures
UHPLC	Ultra-high performance liquid chromatography
UNCST	Ugandan National Council for Science and Technology
WASH	Water, sanitation and hygiene
WHO	World Health Organisation
WHZ	Weight-for-height Z-score
WP	Whey permeate

4 Summary

Background: Environmental enteric dysfunction (EED), a subclinical inflammatory state of the intestinal mucosa, is thought to play a role in stunting. Stunting affects one in every fifth child under five years and is associated with impaired early development and later risk of chronic disease. EED is widespread among children living in settings where basic water, sanitation and hygiene (WASH) are lacking and is thought to result from a high pathogen burden that increases scarcely met nutrient requirements. The resultant cycle of epithelial damage and inflammation is thought to create or worsen nutrient deficiencies. Recent trials have combined WASH and small-quantity lipid-based nutrient supplement (LNS) interventions but have had little impact on growth. Small-quantity LNS may be insufficient to meet the increased nutrient demands for growth alongside chronic immune activation and intestinal disrepair. An array of biomarkers are currently being explored to explain the functional implications of EED and its role in stunting. Among them are plasma citrulline (p-cit), a marker of small intestinal enterocyte mass and faecal myeloperoxidase (f-MPO) a marker of intestinal inflammation.

Objectives: The main objective of this PhD thesis was to explore environmental enteric dysfunction (EED) using selected biomarkers and investigate the interaction between nutrition intervention and EED in children with stunting. Specific objectives were to write a protocol in order to test the effects of a large-quantity LNS on growth in child stunting. To explore the correlates of p-cit and to examine the effects of LNS containing milk protein (MP) and whey permeate (WP) on p-cit and f-MPO in the study population. Finally, to explore whether the state of the gut status modifies the effects of LNS on outcomes of growth and micronutrient status.

Methods: Paper I describes the MAGNUS trial protocol. A community based 2x2 factorial trial in Eastern Uganda, among 750 stunted children aged 12-59 months. Children were randomized to LNS (100 g/d for 12 weeks) containing MP and/or WP or to no supplementation. In a cross-sectional study (**Paper II)** linear regression was used to explore baseline correlates of p-cit. The influence of covariates age, fasting and systemic inflammation were explored as well as associations with socioeconomics, diet, micronutrient status and WASH characteristics. In **Paper III**, linear mixed-effects models were used to explore p-cit and f-MPO as outcomes and modifiers of the LNS intervention effect.

Results: All children (N=750) were included between February and September 2020. The mean ±SD age was 32 ±11.7 months and the mean height-for-age Z-score (HAZ) was -3.02 ±0.74. In

Paper II, the mean p-cit at baseline (n=730) differed according to the duration of fasting and was 20.7 ±8.9, 22.3 ±10.6 and 24.2 ±13.1 µmol/L if fasted <2, 2-5 and >5 hours, respectively. Positive correlates of p-cit were age and log10 serum insulin-like growth factor-1 (s-IGF1) and negative correlates included serum C-reactive protein, serum α1-acid glycoprotein and anaemia as well as environmental factors; food insecurity, inadequate housing materials, the wet season, an unimproved toilet and lack of soap for handwashing. Many associations attenuated after accounting for the effects of systemic inflammation. Neither MP nor WP had an effect on p-cit or f-MPO in **Paper III**. The LNS intervention had no effect on p-cit, though there was an 82% (95%CI: 12; 196) greater increase in f-MPO in this group compared to the unsupplemented group. In the sensitivity analysis, individuals with recent diarrhoea (n=245) were removed and the effect of LNS on f-MPO disappeared. The effect of LNS on cobalamin (B12) status was reduced in children with p-cit <20 µmol/L. The change in plasma cobalamin at 12 weeks was 20% less and the increase in plasma methylmalonic acid was 59% greater in those with low p-cit at baseline.

Discussion and Conclusion: The factors found to correlate with p-cit are characteristic of environments with a high prevalence of EED. Systemic inflammation was strongly associated with p-cit and is implicated in the pathogenesis of EED and stunting. This study highlights the complex interplay that exists between p-cit and systemic inflammation. With adjustment for systemic inflammation many associations were attenuated including WASH related factors indicating that these are likely pathways of pathogen exposure and infection. There was no effect of ingredients MP and/or WP in the LNS on EED markers and no effect of LNS on enterocyte mass, although a negative effect of the LNS on intestinal inflammation appeared in those with recent diarrhoea. Finally, the beneficial effect of the LNS on cobalamin (B12) status was reduced in those with low enterocyte mass at baseline. These findings demonstrate that even large quantity LNS interventions are insufficient in themselves to improve EED. Instead, there was indication that EED may be compromising the beneficial effect of nutrition interventions, possibly through malabsorption or nutrient sequestration. To reverse EED, it seems necessary to take a combined approach of community wide WASH interventions together with improved nutrient access. A sustainable reduction in the pathogen burden would allow for intestinal repair and improve the effectiveness of nutrition interventions.

5 Resumé (Summary in Danish)

Baggrund: Environmental enteric dysfunction (EED) er en subklinisk betændelsestilstand i tarmslimhinden og menes at spille en rolle i stunting. Stunting rammer et ud af fem børn under fem år og er forbundet med nedsat tidlig udvikling og senere risiko for kroniske sygdomme. EED er udbredt blandt børn, der bor i omgivelser hvor grundlæggende behov til vand, sanitet og hygiejne (WASH) ikke er opfyldt, og menes at stamme fra en høj patogenbyrde, som øger ligeledes uopfyldte næringsbehov. Den resulterende cyklus af beskadigelse af epitelet og betændelse menes at skabe eller forværre næringsstofmangel. Nylige studier har kombineret WASH og små mængder af lipid-baserede næringstilskud (LNS), men dette havde kun en mindre påvirkning af væksten. Små mængder LNS dækker muligvis de øgede næringsbehov for vækst utilstrækkeligt, under et kronisk aktivt immunforsvar og en beskadiget tarmslimhinde. En række biomarkører bliver i øjeblikket undersøgt med det formål at finde forklaringer på de funktionelle implikationer EED medfører og hvilen rolle EED har i forhold til stunting. Blandt disse markører er plasmacitrullin (p-cit), en markør for tyndtarms enterocytmasse og fækal myeloperoxidase (f-MPO), en markør for tarmbetændelse.

Formål: Hovedformålet med denne ph.d.-afhandling var at studere environmental enteric dysfunction (EED) ved hjælp af udvalgte biomarkører og undersøge interaktionen mellem ernæringsintervention og EED hos børn med stunting. Specifikke delformål var at skrive en protokol for at kunne undersøge virkningerne af en stor mængde LNS på væksten af børn med stunting. At undersøge korrelater til p-cit og at undersøge virkningerne af LNS indeholdende mælkeprotein (MP) og vallepermeat (WP) på p-cit og f-MPO i undersøgelsespopulationen. Endeligt, var det et delformål at undersøge om tarmstatus ændrer virkningerne af LNS på vækst og tilstedeværelsen af mikronæringsstoffer.

Metoder: Paper I beskriver MAGNUS forsøgsprotokollen. Et 2x2 faktorielt forsøg i flere lokalsamfund i det østlige Uganda, blandt 750 stuntede børn i alderen 12-59 måneder. Børnene blev randomiseret til LNS (100 g/d i 12 uger) indeholdende MP og/eller WP eller intet næringstilskud. Paper II rapporterer om et tværsnitsstudie, hvor der bruges lineær regression til at undersøge ”baseline” korrelater til p-cit. Påvirkningen af kovariaterne alder, faste og systemisk inflammation blev undersøgt samt sammenhænge med socioøkonomi, kost, mikronæringsstofstatus og WASH-status. I Paper III blev lineære modeller med tilfældige effekter brugt til at undersøge p-cit og f-MPO som respons og modifikatorer af LNS-interventionseffekten.

Resultater: Alle børn (N=750) blev inkluderet i studiet mellem februar og september 2020. Gennemsnitsalderen ±SD var 32 ±11,7 måneder, og den gennemsnitlige Z-score (HAZ) var -3,02 ±0,74. I Paper II afveg den gennemsnitlige p-cit ved "baseline" (n=730) afhængigt af fasteperioden og var 20,7 ±8,9; 22,3 ±10,6 og 24,2 ±13,1 µmol/L, når der blev fastet henholdsvis <2, 2-5 og >5 timer. Positive korrelater til p-cit var alder og log10 serum insulinlignende vækstfaktor-1 (s-IGF1), og negative korrelater omfattede serum C reaktivt protein, serum α1-acid glycoprotein og anæmi samt miljøfaktorer: fødevareusikkerhed, utilstrækkelige boligmaterialer, regntid, ikke-sanitære toiletforhold og mangel på sæbe til håndvask. Mange sammenhænge var mindre tydelige efter at der blev taget højde for effekterne af systemisk inflammation. Hverken MP eller WP havde en effekt på p-cit eller f-MPO i Paper III. LNS-interventionen havde ingen effekt på p-cit, dog var forøgelsen af f-MPO 82 % (95 % CI: 12; 196) større i denne gruppe sammenlignet med den gruppe der ikke modtog næringstilskud. I sensitivitetsanalysen blev personer med nylig diarré (n=245) fjernet, og effekten af LNS på f-MPO forsvandt. Effekten af LNS på cobalamin (B12) status blev reduceret hos børn med p-cit <20 µmol/L. Ændringen i plasma cobalamin efter 12 uger var 20 % mindre, og stigningen i plasma methylmalonsyre var 59 % større hos dem med lav p-cit ved "baseline".

Diskussion og konklusion: De faktorer som korrelerer med p-cit, kan forventes i miljøer med en høj forekomst af EED. Systemisk inflammation var stærkt associeret med p-cit og er impliceret i patogenesen af EED og stunting. Ved at tage højde for systemisk inflammation blev mange sammenhænge svækket, herunder WASH-relaterede faktorer, "pathways" for patogeneksponering og infektion. Dette afspejler enten konfundering, mediation eller måske en kombination af de to og fremhæver det komplekse samspil mellem p-cit og systemisk inflammation. Der var ingen effekt af ingredienser MP og/eller WP i LNS på EED-markørerne og ingen effekt af LNS på enterocytmassen. Der var dog indikationer på en negativ effekt af LNS på tarmbetændelse hos børn med nylig diarré. Desuden blev den gavnlige effekt af LNS på cobalaminstatus reduceret hos dem med lav enterocytmasse ved "baseline". Disse resultater understreger, at selv store mængder LNS-interventioner ikke alene er tilstrækkeligt til at lindre EED. For at forbedre effektiviteten af ernæringsinterventioner og give mulighed for bedring af tarmen, kan det være nødvendigt at give ernæringsinterventioner sammen med vedvarende WASH-interventioner, der reducerer patogenbyrden på tværs af lokalsamfund.

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6 Introduction

Stunting affects more than one in every fifth child under five years, hindering the developmental potential of millions globally [1]. The factors contributing to stunting are numerous and complex. Among the most well-known of these are low socioeconomic status [2], infections and inadequate diet [3]. However, high quality nutrition interventions have had disappointingly little effect on growth [4]. Indeed, there is an interplay between contributing factors that is less well understood, and has led to a new body of research exploring the role of environmental enteric dysfunction (EED) in childhood stunting.

Environmental enteric dysfunction is an asymptomatic inflammatory disorder of the small intestinal mucosa [5] and appears to affect a considerable proportion of children living in low-income settings [6]. The main drivers of EED are thought to be poor nutrition and a high enteropathogen burden [7–9]. In food insecure settings the diet is often deficient in nutrients that are essential for growth and/or intestinal repair [10]. Concurrent infections, widespread where basic water, sanitation and hygiene (WASH) are lacking, then increase nutrient requirements and perpetuate a cycle of epithelial damage and inflammation [11, 12]. In this way, the compounding effects of nutrition and infection are likely to exacerbate or create micronutrient deficiencies.

To prevent stunting, recent trials have targeted diet with small-quantity lipid-based nutrient supplements (LNS) and enteropathogen exposure through improved WASH. Still, they found no effect of WASH and only modest effects of LNS on linear growth [13, 14]. To explain the functional implications of EED and its role in stunting, an array of biomarkers are currently being explored [15]. Many markers have been adopted for use from clinical research in intestinal diseases such as celiac disease, short bowel syndrome and inflammatory bowel disease (IBD). Currently, there is no accepted set of biomarkers to identify children with EED [16]. As such, there is a need to further explore the role of EED in stunting using candidate biomarkers. Small-quantity LNS interventions may be insufficient to meet the increased nutrient requirements for growth and intestinal repair in children, particularly when EED may compromise nutrient absorption. A need remains therefore, to explore whether nutrition can improve EED and equally, whether EED impairs the effectiveness of nutrition interventions.

7 Objectives

The main objective of this PhD thesis was to explore EED using selected biomarkers and to investigate the interaction between nutrition intervention and EED in children with stunting.

Specific objectives were:

- To plan and develop the trial protocol for a randomised controlled trial in children with stunting which explored the effects of LNS containing milk protein (MP) and whey permeate (WP) on growth, child development and exploratory outcomes including gut function (**Paper I**).
- To explore socioeconomic factors, diet, inflammation, micronutrient status, household WASH characteristics and other factors as correlates of plasma citrulline (p-cit), a marker of enterocyte mass, in children with stunting (**Paper II**).
- To explore the role of fasting status and inflammation on correlates of p-cit in children with stunting (**Paper II**).
- To examine the effects of 12 weeks of supplementation with large-quantity LNS containing MP and/or WP and the LNS itself on p-cit and faecal myeloperoxidase (f-MPO), a marker of intestinal inflammation, in children with stunting (**Paper III**).
- To explore whether the state of the gut, as measured by p-cit and f-MPO, modifies the effects of large-quantity LNS on growth and micronutrient status (**Paper III**).

8 Background

8.1 Stunting

8.1.1 Prevalence and consequences

Globally, stunting is the most prevalent form of malnutrition, estimated to affect 149 million children (22%) under five years [1]. Stunting has become a global health priority and in the last decade some countries have made progress in reducing the burden [17, 18]. Across Africa however, absolute numbers of stunting are increasing due to population growth [19]; the prevalence across the region remains higher than the global average [1]. A child is stunted when length or height-for-age Z-score (HAZ) is two or more standard deviations below the reference median, according to the World Health Organization (WHO) growth standards [20]. In a healthy population, about 2.5% of children would be stunted, whereas in populations with a high prevalence of stunting, the entire HAZ distribution has shifted to the left. This means that a much larger proportion of children are affected by varying degrees of growth faltering; a failure to meet one's growth potential [21]. When growth faltering is so widespread, it often goes unnoticed in communities and is not routinely assessed in primary health care settings [19]. Yet, stunting may have important consequences for child health and development. It has been associated with reduced neurodevelopmental and cognitive function, lower educational attainment and increased infections and mortality during childhood [19, 22, 23]. In adulthood, stunting has been associated with increased risk of chronic disease [24] and reduced earnings, with consequences for development and economic productivity [25, 26].

8.1.2 Risk factors

Stunting results from a chronic lack of access to nutrients that are essential for growth and function [27]. Nutrient access is directly influenced by nutritional intake and by the frequency and severity of infections [23]. Underlying these is an array of contextual factors which at the household level include dietary practices, socioeconomic and cultural factors, health seeking behaviours as well as environmental conditions including access to WASH. These factors are enabled by systems of healthcare, agriculture, food distribution and water and waste management [23]. In addition, transgenerational factors have also been associated with impaired growth, including maternal short stature, maternal infections, younger age, and poor nutritional status during pregnancy [28–30]. This complex array of factors is based on a conceptual framework first developed by UNICEF [31] which has since been adapted by others [18, 23].

8.1.3 Growth phases and the window for prevention

There are four main growth phases during the lifecycle; in utero, infancy, childhood and puberty [22]. Growth faltering can already begin in utero and is estimated to contribute 20% to overall stunting [32]. A study using data from 54 developing countries showed that at birth, length-for-age (LAZ) was already -0.5 SD from the median of the WHO growth standards, and subsequently declined to almost -2 SD by the age of two years [33]. This characteristic pattern of early life growth faltering forms the foundation for the well-established critical period of 1000 days, spanning from conception to two years, in which efforts can be made to prevent stunting [33, 34]. The period from birth to three years is one of rapid brain growth and development [19, 35]. During early infancy, children experience maximal growth velocity when nutrient needs are at their highest, however these needs can be adequately compensated through exclusive breastfeeding [22]. The period from 6 to 24 months is when most growth faltering occurs and is due to a manifold increase in environmental exposures, together with poor complementary feeding behaviours [22, 33].

Interventions to prevent stunting have targeted maternal nutrition and infant feeding practices including exclusive breastfeeding until 6 months, continued breastfeeding for two plus years and appropriate complementary feeding behaviours such as; the timing of food introduction, dietary diversity and feeding frequency [23]. It has been proposed that growth continues to falter beyond two years of age and that other windows to address stunting may exist [36], however, to what extent catch-up growth can be achieved in already stunted children is largely unknown [19]. Small quantity LNS have been introduced as a method to fortify home prepared meals (20g, ~100kcal/d) and to provide essential nutrients that are unlikely to be available in adequate amounts from the diet [23, 37]. However, interventions have been inadequate to significantly reduce the global burden of stunting [34] and its pathogenesis remains poorly understood [22]. It is thought that environmental enteric dysfunction (EED) may play a role in limiting the effectiveness of nutrition interventions [38].

8.2 Environmental enteric dysfunction

Environmental enteric dysfunction is a subclinical inflammatory disorder of the small intestinal mucosa characterised by changes to mucosal structure and function [6]. It has been found to be widespread among populations living in low and middle-income country (LMIC) settings [39] and has been associated with increased susceptibility to infection [40], poor oral vaccine response [41, 42], and seems to play a role in stunting [16, 43].

The research surrounding EED now spans more than 50 years and provides an overview of our current understanding of the condition. In the late 1960s, mucosal abnormalities were described in seemingly healthy Ugandan adults [44]. Tropical enteropathy was first described in the 1970s, when small intestinal biopsies collected from adult Peace Corps volunteers on placement in India and Pakistan, showed abnormal morphology of the mucosa. These changes then normalised after having returned to the United States for one to two years [45]. In the 1990s, a similar condition was described as prevalent among children in The Gambia [46] and was found to be associated with stunting in many LMIC settings [5, 47–50]. These earlier studies demonstrate some important characteristics of EED; that it is subclinical, it is widespread among adults and children in LMIC settings, it can be acquired, even during infancy, it is associated with stunting and may be improved with change of environmental factors [39].

8.2.1 Intestinal barrier function

The mucosal barrier of the small intestine plays an essential role in innate immune function. It comprises a complex set of mechanical, antimicrobial (ex. mucins), immunological (ex. mucosal immune cells) and ecological barriers (intestinal microbiota) that are maintained to achieve homeostasis [51]. The mucosal layer is made up of villi, finger-like projections that are the functional unit of the small intestine and serve to amplify absorptive surface area. The villi are lined with a single layer of columnar epithelial cells joined by tight junctions and adherins that form a physical barrier to the external milieu (Figure 8.1) [51]. The intestinal epithelium is one of the most rapid and continuously proliferating tissues in the human body [51]. At the base of the villus crypt, stem cells give rise to different intestinal epithelial cells that migrate up the crypt-villus axis as they develop absorptive, endocrine or immune functions. The resultant cells are enterocytes, goblet cells, paneth cells, enteroendocrine cells, tuft cells, or M cells. The enterocyte is the most abundant epithelial cell with a unique duel function; to absorb nutrients and maintain intestinal barrier function. Enterocytes are replaced every 3-5 days, undergoing apoptosis and extrusion into the intestinal lumen at the villus tip. These processes of cell differentiation, migration and proliferation are tightly regulated at various levels and are highly responsive to changes in the luminal environment, including for example, the presence or absence of nutrients, growth factors and pathogens [52, 53].

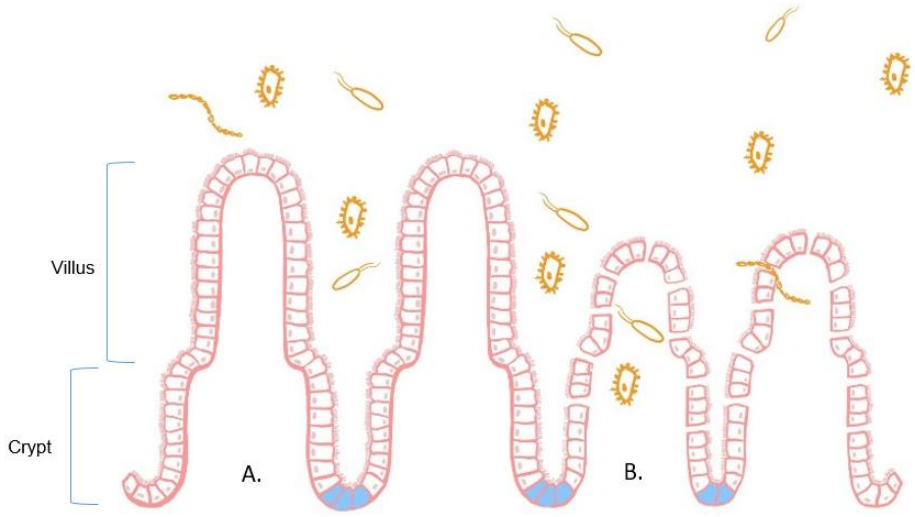

Figure 8.1: Mucosal barrier function. Adapted with permission from Cowardin et al., 2022, pg.3 [54]. A. In conditions of mucosal barrier homeostasis and dietary sufficiency; intact epithelium, microbiota-host symbiosis and nutrient absorption. B. Mucosal barrier disruption; inflammation, microbial translocation, dysbiosis, impaired epithelial repair, altered crypt-villus architecture, loss of enterocyte mass, malabsorption.

8.2.2 A conceptual framework of the role of EED in stunting

Two factors in particular are thought to drive EED; inadequate nutrition and repeated exposure to enteric pathogens [16]. The interplay between poor nutrition and exposure to pathogens seems to perpetuate a vicious cycle of worsening nutritional status and increased susceptibility to further infection [55]. EED is thought to contribute to growth faltering through several mechanisms which overlap with pathways of dysbiosis of the microbiome and systemic inflammation [56]. In Figure 8.2, a conceptual framework of the role of EED in stunting presents all three interacting pathways within the context of EED.

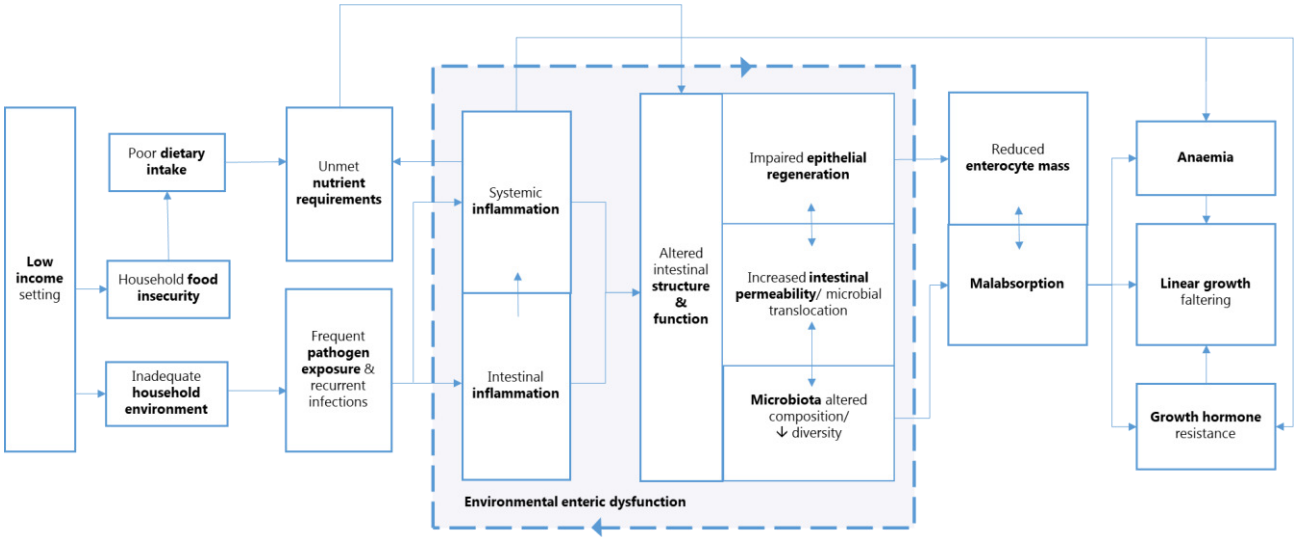

Figure 8.2: Conceptual framework. The role of environmental enteric dysfunction in linear growth faltering and nutrient malabsorption. Adapted from Prendergast et al., 2015, pg.s727 [57]

8.2.3 Role of nutrient intake

Food insecure households live with restricted access to safe, sufficient and nutritious food. Often the diet is subject to what can be grown locally and lacks both variety and animal source foods. Being largely plant based, the diet contains high amounts of antinutrients including phytates (and polyphenols) that can hinder the bioavailability of nutrients such as zinc [58]. About 40 micronutrients are essential for health and must to be present in adequate amounts in the diet for normal growth and physiological function [59]. These nutrients have been broadly classified by Golden (2009) as type I, related to immune or function, and type II essential for growth and tissue repair [59]. In food insecure settings, the diet may be lacking nutrients such as amino acids [60], zinc and vitamin A that are essential for intestinal barrier repair and function [61].

8.2.4 The cycle of malnutrition, infection and immune dysfunction

Evidence suggests that children living in LMIC settings have near universal asymptomatic carriage of multiple enteric pathogens [6]. Under poor WASH conditions, children are frequently exposed to a high pathogen burden which is thought to cause intestinal inflammation and damage the epithelium [8, 16]. Failure to repair the epithelial damage contributes to persistence of barrier dysfunction, allowing bacterial translocation [6]. This cycle of epithelial damage, (impaired barrier function) and bacterial translocation increases susceptibility to further infection [62] and promotes a dysfunctional state of chronic intestinal inflammation [6, 8]. Infection and the resultant state of systemic inflammation increase nutrient requirements through reduced appetite, increased catabolism, impaired intestinal absorption, and redirection of nutrients away from growth and towards the immune response [19, 63]. Furthermore, systemic inflammation is known to suppress the growth hormone axis and therefore growth [64]. Normally, children will experience accelerated growth after episodes of illness so as to reduce the weight or height deficit accumulated [21]. However, EED may worsen or create micronutrient deficiencies through nutrient malabsorption, nutrient sequestration, increased requirements or some combination of these [12, 61]. Thus when the diet is insufficient to replace the nutrient deficits, this can result in a loss of catch-up growth [59].

The microbiota is increasingly recognised to play a role in the regulation of immune function and metabolic activity. The composition and relative diversity of the microbiota is established by around three years of age and is greatly influenced by environmental factors including mode of delivery, dietary exposures, antibiotics and infection. It has been proposed that EED reflects a dysbiosis of the microbiota [54, 65, 66]. This interaction between diet, pathogens, EED and the microbiota was elegantly demonstrated using murine models, whereby mice fed a suboptimal diet developed shifts in the microbiota but retained normal intestinal histopathology. If a bacterial cocktail was also given, the mice developed villus blunting and intestinal inflammation[7]. However, the specific mechanisms by which the microbiota contributes to stunting or EED are beyond the scope of this thesis.

8.2.5 Identifying children with EED

EED is difficult to diagnose because carriers have no apparent clinical symptoms. Currently EED is diagnosed using endoscopy and small intestinal biopsy. It is characterised by derangements of the epithelial cells of the small intestinal mucosa including consistent villous blunting, crypt hypertrophy and the presence of lymphocytic inflammatory cells in the lamina propria (Figure 8.3) [8]. In addition, recent examinations of histological features identified a

reduction in the number of goblet and paneth cells in the intestinal crypts of subjects with EED [67]. These key histological features are not unique to EED, but have been likened to a mild form of celiac disease [6, 68]. There is currently no universally accepted case definition or specific diagnostic criteria for EED [6].

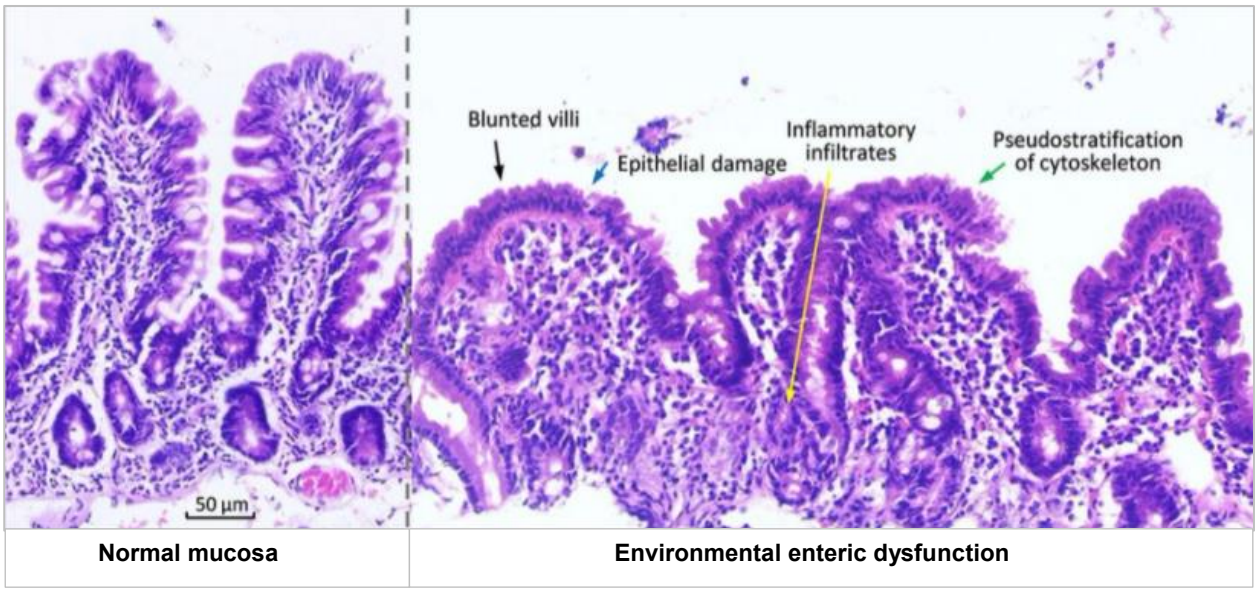

Figure 8.3: Histological photographs of damaged intestinal mucosa. An example of the structural changes seen in environmental enteric dysfunction. Permissions for use from Trehan et al., 2016, pg.742 [69]

While biopsies are the gold standard, the procedure is invasive, costly and difficult to do at scale in LMIC settings. Since the structural changes are also evidence of impairment to function [6], biomarkers are being explored as an alternative means to identify children with EED [39]. Already a wide range of relevant biomarkers are used in diagnosing or monitoring clinical conditions such as celiac disease, inflammatory bowel diseases and short bowel syndrome. A set of functional domains have been proposed as a means to identify EED according to specific components along the hypothesised causal pathway. These domains include; intestinal inflammation, increased mucosal permeability, loss of enterocyte mass or intestinal injury and repair, microbial translocation, microbial dysbiosis, systemic inflammation, and malabsorption [16, 70–72]. There is currently no consensus on which biomarkers should be used to assess EED [73, 74]. A selection of the markers proposed in the literature are presented in Table 8.1.

Table 8.1: Selected biomarkers identified from the literature that are currently being explored in the context of environmental enteric dysfunction

Domain	Biomarker	Rationale	X	Method	Refs.
Altered mucosal structure	Citrulline	▪ Synthesis in mature enterocytes at villus tips ▪ Precursor for arginine synthesis in kidney ▪ ↓ ~ reduced enterocyte mass	P	UHPLC-MS	(15, 72)
	Intestinal fatty acid binding protein	▪ Cytosolic protein of enterocytes at villus tips. ▪ Role fatty acid uptake and metabolism ▪ ↑ ~ epithelial damage	P	ELISA	(72, 75)
Impaired barrier regeneration	Regenerating Gene 1b	▪ ↑ ~ Upregulated to preserve and renew intestinal structure	F	ELISA	(63, 72, 76–78)
Intestinal inflammation	Myeloperoxidase	▪ ↑ ~ Neutrophil activity in inflammatory process ▪ Antimicrobial ▪ Tissue injury	F	ELISA	(9, 72)
	Neopterin	▪ Produced by macrophages and monocytes. ▪ Marker of Th1 cell activity	F	ELISA	(9, 72)
	Calprotectin	▪ Neutrophil/monocyte release upon cell death	F	ELISA	(63, 79)
Intestinal permeability	Lactulose: Mannitol (L:M)*	▪ L and M saccharides with different molecular weights are consumed ▪ ↓ recovery of L (large molecule) in urine ~ ↑ permeability ▪ ↑ recovery of M (small molecule) ~↓ absorptive surface area	U	UHPLC-MS	(72, 79, 80)
	α_1 antitrypsin*	▪ Protein wasting ▪ Endogenous AA leakage	FS	ELISA	(9, 72)
Microbial translocation	Lipopolysaccharide (LPS) binding protein	▪ LPS major structural component of bacteria ▪ Normally in intestinal lumen	P	ELISA	(79, 81)
	Soluble CD14	▪ Marker of monocyte activation ▪ LPS is a potent activator.	P	ELISA	(72)
Dysbiosis	Microbiome immaturity score	▪ Relative abundance and diversity of bacterial taxa	S	16S-r RNA	(66)
	Small intestinal bacterial overgrowth	▪ Hydrogen breath test or cultured aspirate ▪ Overgrowth when small intestine colonised with >10^5 CFU/ml of any bacterial species.	B A	13C-SBT	(74, 82–84)
Systemic sequelae					
Systemic inflammation	α_1-acid glycoprotein	▪ Acute phase protein	S	ELISA	(56, 61, 85)
	C-Reactive protein	▪ Acute phase protein	S	ELISA	(56, 72)
	Kynurenine to trypophan ratio (Kyn:Trp)	▪ Under increased inflammatory activity ~ ↑ IDO enzyme metabolism of Trp to Kyn	P	UHPLC-MS	(72, 86)
Growth hormone resistance	Insulin-like growth factor-1 (IGF-1)	▪ Key mediator of growth hormone effects at the growth plate of bones	SP	RAIS	(64, 72)

*Also possible markers of malabsorption. ST: Sample type; P: Plasma; F: Faeces; S: Serum; B: Breath; A: Aspirate; U: Urine; UHPLC: Ultra-High-Performance Liquid Chromatography- Mass Spectrometry; IDO: indoleamine 2,3dioxygenase; 16S-r RNA: 16s-ribosomal; RNA; RAIS: Random access immunoassay system; SBT: Sucrose breath test. Adapted from Mutasa et al., 2021 [72].

8.3 Selected biomarkers

When selecting biomarkers, several factors were considered. First, it was important to identify markers from different functional domains along the hypothesised causal pathway and to consider the evidence of biomarker validity. Second, the cost of analysis and the available expertise was an important factor. Third, attention was given to the feasibility of sample collection in low-resource settings. The four markers selected for this study are described here; p-cit, myeloperoxidase (MPO), neopterin (NEO) and α1-antitrypsin (AAT). While the lactulose to mannitol ratio (L:M) was not selected for this study, it is briefly highlighted here since it is to date the most frequently used biomarker for EED.

8.3.1 L:M ratio

The lactulose: mannitol (L:M) dual-sugar absorption test is a widely used EED biomarker and is considered by some to be a "gold standard" test of intestinal permeability [80]. This test has previously been used to indicate permeability in coeliac disesase and IBD [87]. It is thought to measure paracellular permeability with increased passage of large lactulose molecules and villous atrophy with reduced passage of small mannitol molecules. It is measured when a known concentration of these saccharides are recovered from the urine. They are first consumed and pass through the intestine, being absorbed to varying degrees before filtration and excretion by the kidney. However, the L:M has several limitations. First, the test requires 2–5 hour collection of all urine passed; a considerable burden and challenging to manage with small children. In addition, the procedure requires extensive experience to implement as there is a high risk of contamination [80]. Finally, in recent years the utility and interpretation of the L:M test has been challenged [88]. Though the timing of collection has been shown to greatly influence the recovery ratios [87], standardised methods for measurement have not been established [80, 87]. As a means to improve interpretation, it has been suggested that the recovery of both lactulose and mannitol be described separately in addition to reporting the L:M ratio [88, 89]. Though the L:M ratio is often used to measure permeability in EED, for the reasons described, this marker was not selected for inclusion in the study.

8.3.2 Citrulline

Citrulline is a non-protein amino acid that is synthesised de novo by mature enterocytes in the mid-to-upper parts of the villi and is considered a marker of enterocyte mass [16, 90, 91]. It is produced in the proximal small intestine (duodenum and jejunum) [92], the same region that is affected in EED [10]. Citrulline is synthesized indirectly via the enzyme ornithine

carbamoyltransferase from dietary and endogenous sources of arginine, glutamine and proline (Figure 8.4) [92], though the relative contributions of each precursor to citrulline synthesis remains controversial [90]. Once released from the enterocytes, citrulline enters the portal vein, bypassing liver metabolism and entering systemic circulation. Circulating citrulline is then taken up by the kidneys and converted to arginine by enzymes arginosuccinate synthase and arginosuccinate lyase [90, 92]. This pathway is referred to as the intestinal-renal axis [93]. Citrulline is also an intermediate in two other important pathways; the nitric oxide cycle in nitric oxide (NO) producing tissues throughout the body, and the urea cycle, for removing ammonia after protein catabolism in the liver (Figure 8.5). However, the citrulline produced along these pathways is not thought to contribute to plasma citrulline concentrations[93]. When kidney function is normal, the citrulline measured in the plasma is largely derived from the amount synthesised in the intestine [90, 92].

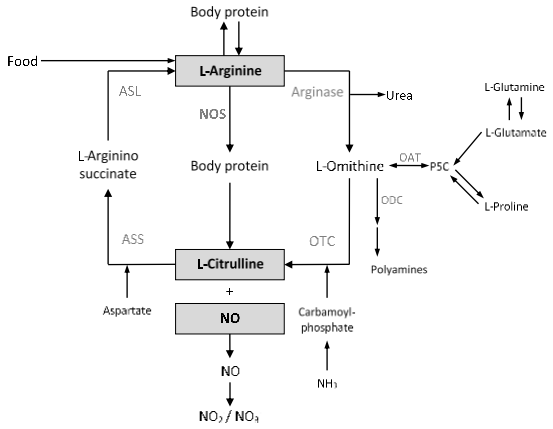

Figure 8.4: Citrulline and arginine metabolism. ASS: arginosuccinate synthase; ASL: arginosuccinate lyase; NOS: nitric oxide synthase; NO: nitric oxide; OTC:ornithine carbamoyltransferase; ODC: ornithine decarboxylase; OAT: ornithine aminotransferase. Adapted from Luiking et al., 2014, Chapter 35 in Modern Nutrition and Disease pg.478 [94]

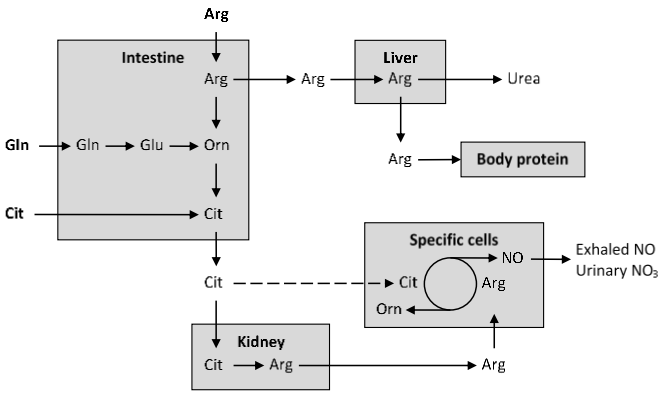

Figure 8.5: Citrulline is an intermediate in three pathways. The pathways are the nitric oxide cycle in specific cells, the intestinal renal axis and the urea cycle in the liver. Arg: arginine; NO: nitric oxide; Orn: ornithine; Gln:glutamine; Glu:glutamate. Adapted from Luiking et al., 2014, Chapter 35 in Modern Nutrition and Disease pg.479 [94]

Lower levels of plasma citrulline in systemic circulation are thus hypothesised to result from damage or loss of enterocytes [16]. Indeed, citrulline has been shown to correlate with loss of enterocyte mass in short bowel syndrome [90, 95–97], and with disease severity score in celiac disease [90, 98]. Citrulline has previously been explored as a marker of EED [15, 72, 84, 99] and has been found to be associated with L:M ratio [100] and shown in some studies to be associated with linear growth [15, 100, 101]. Since this research group has some experience with citrulline from a

previous study in children with severe acute malnutrition [102] and citrulline analysis requires only a small plasma sample, this marker was included in this study.

8.3.3 Myeloperoxidase, neopterin and α1-antitrypsin

At the time of selection, three faecal markers AAT, MPO and NEO were (and still are) widely used in the context of EED. Part of the appeal of these markers is that they require minimal primary processing and analysis can be done at relatively low cost [103]. Moreover, the increased use of these markers creates an opportunity to compare between studies. Myeloperoxidase is a lysosomal protein that is released into the phagosome of neutrophils during the degranulation process. There, MPO reacts to form highly cytotoxic products which are released from the cell to destroy pathogens. It is now understood that these toxic agents can also damage normal tissue and contribute to inflammation [104]. In line with this, a high expression of MPO has been associated with chronic inflammatory conditions such as IBD [105, 106]. Several studies exploring MPO in the context of EED have also found an inverse association with linear growth [15, 103, 107–110]. Neopterin (NEO) is also a marker of intestinal inflammation. It is produced by macrophages and dendritic cells upon stimulation by interferon gamma (INF-γ). Interferon-γ is an important immunoregulatory cytokine that is released by activated Th1 lymphocytes to promote the elimination of harmful pathogens. As such, neopterin is thought to be an indicator of Th1 cell activity as well as macrophage proliferation during the pro-inflammatory response [16]. High recovery of neopterin has also been associated with growth faltering in earlier studies [48]. Finally, AAT is a protease inhibitor released during the acute phase inflammatory response to protect cells from proteolytic enzymes (such as MPO). When the intestinal barrier is dysfunctional, AAT which is not produced in the gut, can appear in increased amounts in the stool. This is an indication of protein loss whereby AAT proteins have leaked out into the gut lumen. In low-resource settings, this marker has also been found to be inversely associated with linear growth [109, 111] and to be in agreement with the L:M score [111]. It appears that these markers confirm widespread EED among children in LMIC settings. A study in Bangladesh examined stool samples of 222 children over 3 time points from 7 to 24 months and found that overall 97%, 71%, and 58% of stool samples were above values considered normal for NEO, MPO and AAT, respectively [112]. It is expected that by including markers from several domains, a more comprehensive picture of EED can be drawn to explain linear growth faltering [16, 103]. As such, these markers have also been developed into a "composite score" for EED [103] which was also to be included in this study.

8.3.4 An updated conceptual framework

An updated conceptual framework of the role of EED in growth faltering is presented in (Figure 8.6). The framework now includes relevant biomarkers and other characteristics measured as part of this study.

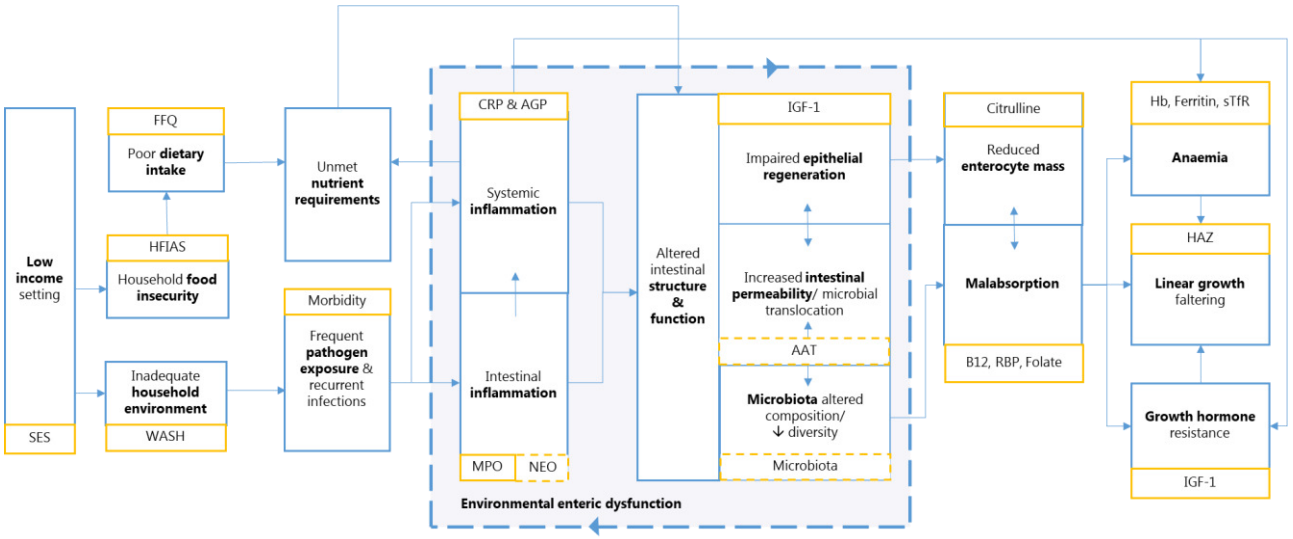

Figure 8.6: Updated conceptual framework. The role of environmental enteric dysfunction in linear growth faltering and nutrient malabsorption, including factors measured in the MAGNUS trial. Adapted from Prendergast et al., 2015, pg.s727 [57]. Factors measured but not included in this thesis are indicated with a dashed yellow line. SES: Socio-economic status; FFQ: Food frequency questionnaire; HFIAS; Household food insecurity access scale; WASH: Water, sanitation and hygiene; CRP: C-reactive protein; AGP: α1-acid glycoprotein; MPO: Myeloperoxidase; NEO: Neopterin; IGF-1: Insulin-like growth factor 1; AAT: α1-antitrypsin ; B12: Cobalamin; RBP: Retinol binding protein; Hb: Haemoglobin; sTfR:Soluble transferrin receptor; HAZ: height-for-age Z-score

9 Methods

This chapter provides an overview of the 'Milk affecting growth, cognition and the gut in child stunting' (MAGNUS) trial. All papers in this thesis are based on the MAGNUS trial. ***Paper I*** *is the protocol. It is a detailed overview of the trial design and was informed by the trial preparation.* ***Paper II*** *and* ***Paper III*** *are based on tertiary outcomes related to EED and were informed by trial implementation and data analysis as described. Further detail of methods are provided in each of the papers.*

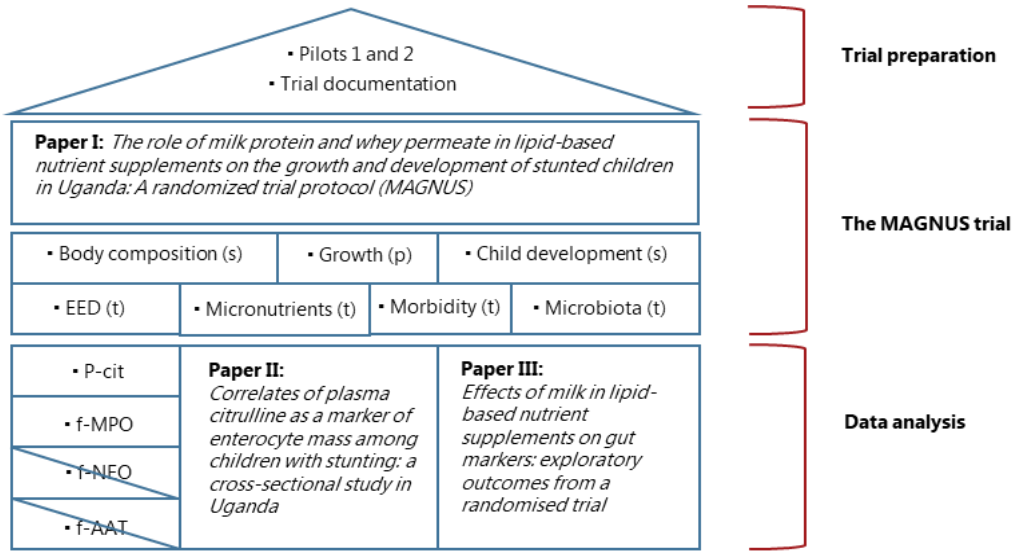

Figure 9.1: Overview of the papers included in this thesis The trial preparation culminated in the development of Paper I, the protocol. The protocol describes the trial design and methods. EED was one of approximately seven research areas within the MAGNUS trial related to primary (p), secondary (s) and tertiary (t) outcomes. Outcomes related to growth, micronutrients and morbidity were explored as exposures or outcomes in relation to EED markers; tertiary outcomes that are the main focus of this thesis. Data from two of the planned EED markers (f-NEO and f-AAT) were excluded during data analysis. The two remaining EED markers were included as outcomes in Paper II and Paper III . EED: Environmental enteric dysfunction; p-cit: plasma citrulline; f-MPO: faecal myeloperoxidase; f-NEO: faecal neopterin; f-AAT: faecal α1-antitrypsin

9.1 Trial preparation

The trial preparation phase was an iterative process. Initially, the trial was planned to include children with moderate malnutrition (MAM), classified as having a weight-for-height Z-score (WHZ) <-2 and ≥-3 and/or a mid-upper arm circumference (MUAC) <125 and ≥115 mm. Children younger than 24 months were to be excluded because high breastfeeding rates were expected to confound results of a milk based intervention [113]. The trial was to take place in the Busoga Sub-region in Eastern Uganda. At the time, the demographic health survey indicated that 3.6% of children under 5 years in the region had MAM [114] .This particular region was chosen

for accessibility to the capital city, Kampala (80 km) and because the population was expected to have residential stability. In 2019, two pilot studies were conducted in preparation for the trial. The first pilot was instrumental in redefining the study population. The second in defining resource requirements and limitations within the trial setting.

9.1.1 Pilot 1

The main objective of the first pilot was to assess the prevalence of malnutrition and breastfeeding in communities within the Busoga Sub-region and secondarily, to test methods of community recruitment and screening. With permissions from local authorities, two teams visited a total of 17 villages and with permission from caregivers, collected data on age, sex, breastfeeding and anthropometrics including length, height, weight and MUAC on screened children (N=1029). A report of findings is provided in Appendix 14.1. Less than half of the population were still breastfed at 12- 24 months. But more importantly, as seen from figure #, the median WHZ in this population was close to that of the WHO standard growth curve. This pilot demonstrated that it was infeasible to recruit 750 children with MAM from within these communities. On the other hand, the HAZ was left-skewed, indicating that the vast majority of children in this population were experiencing linear growth faltering, as described in section 8.1.1. Since there was increasing global research interest in stunting and all of the planned outcomes in the trial were relevant for children with stunting, whilst at the same time, the definition of MAM was up for debate, it made sense to shift focus to stunting. Thus, the outcome of this pilot was a modification of our eligibility criteria to include children with an HAZ <-2 and aged 12-59 months.

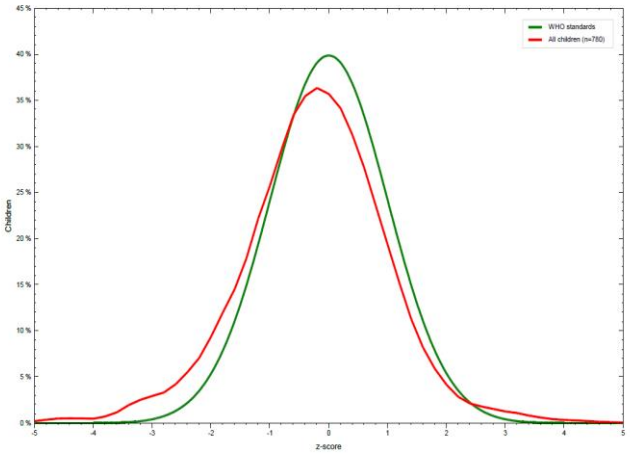

Figure 9.2: Weight for Height Z-score of the sample population (n=792) compared to the WHO standard curve (2006)

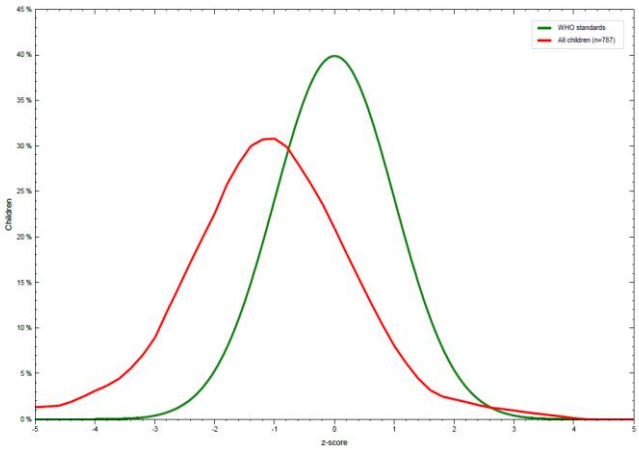

Figure 9.3: Height for age Z-score of the sample population (n=792) compared to the WHO standard curve (2006)

9.1.2 Pilot 2

The objective of the second pilot was to select site locations, identify trial resource requirements, to assess feasibility of an electronic CRF and check comprehension of our standard operating procedures (SOPs). Two sites in the district of Jinja were selected; Buwenge and Walukuba health centres (Figure 9.6). Neither site had electricity nor immediate access to running water. These sites were selected for the following reasons: i) available space for study operations, ii) an easily identifiable location within the community, iii) ease of access to public transport, iv) population density in the surrounding area and v) an adequate distance between the two sites (27km) to avoid intervention spill-over. In consultation with caregivers, laundry soap was identified as the preferred gift for those not receiving supplements. A site flow plan was put in place to account for the many on- and off-site activities and to ensure that baseline and follow-up activities could operate simultaneously (Figure 9.4). From this pilot, it was concluded that electronic data capture was not possible due to limited internet and access to electricity. All data in this trial was therefore collected on paper and double entered. The REDCap electronic data capture system was incorporated into the trial however for appointment scheduling, distance monitoring and reporting of adverse events.

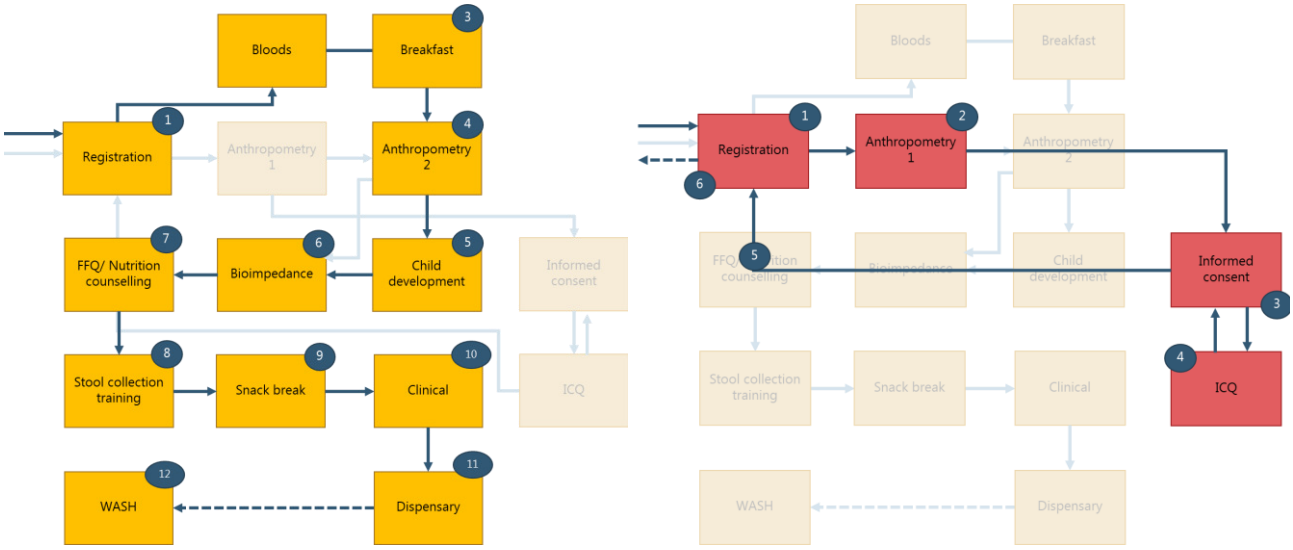

Figure 9.4: Site flow at screening and at baseline visits. Each brick is a different activity that took place at a separate station but allowed for overlap of different visits. Anthropometry 1 collected length, weight and arm circumference data, anthropometry 2 collected knee-heel and skin fold data. The household water, sanitation and hygiene assessment (WASH) took place at the participant home.

9.1.3 Documentation

Trial documents were prepared according to international standards of good clinical practice (GCP). These documents included the trial protocol, informed consent forms, product labelling and case-report forms (CRF). These documents and revisions were submitted to and approved by

The School of Medicine Research and Ethics Committee (SOMREC) and the Uganda National Council for Science and Technology (UNCST) prior to study start. Standard operating procedures (SOPs) were also prepared in line with GCP and a list of these can be found in Appendix 14.2.

9.2 The MAGNUS trial

9.2.1 Study location

The district of Jinja, is located on the northern shores of Lake Victoria, within the Busoga Sub-region, Eastern Central Uganda (Figure 9.5). The region experiences two rainy seasons each year (mid-August to December and March to July); the dry seasons are often associated with increased food insecurity. A majority of livelihoods in the district are derived from subsistence farming, where maize and millet are main staple crops [115]. Besides subsistence farming, most private land is hired out for sugar cane production and thus land shortage is a major hindrance to the development of commercial food agriculture systems in the area [115]. Food availability is not generally a limiting factor in most regions of Uganda, however, due to a limited supply chain, food prices have increased so that many low-income households do not have access to adequate amounts and quality of food. In the period leading up to 2020, it was estimated that 37% of the region experienced chronic food insecurity, whereby quality foods were lacking and household livelihoods were unsustainable. Of these, 12% were likely to experience severe seasonal deficits for extended periods of the year [116]. The Covid-19 pandemic that began in March 2020, limited movement and trading and may have worsened food insecurity, although data on this is lacking.

Figure 9.5: The district of Jinja, Eastern Uganda. Use licensed under the Creative Commons.

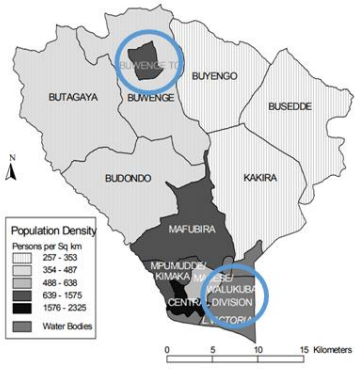

Figure 9.6: Locations of Walukuba and Buwenge health centres in the district of Jinja (27km apart). Adapted from Uganda Bureau of Statistics, 2017 (117)

9.2.2 Study population

Mobile teams travelled to different villages in the vicinity of the study sites to screen for children aged 12 to 59 months with a HAZ <-2. Those identified were referred to the nearest site for additional screening while children with severe acute malnutrition (SAM) according to WHO criteria [118] were referred for treatment. Inclusion was conditional upon meeting the following eligibility criteria;

Inclusion

- Living in the catchment area
- Aged 12 to 59 months
- Height-for-age z-score (HAZ) of <-2 according to WHO growth standards [20]
- Provision of caregiver written informed consent

Exclusion

- Severe acute malnutrition (WHZ <-3, bilateral pitting oedema or MUAC <115mm)
- Complications requiring hospitalisation
- History of allergy to peanuts or milk
- Disability impeding eating or measurement
- Plans to relocate from the area
- Any child in the household had previously been enrolled in the study

9.2.3 Design

MAGNUS was a randomised, double-blind, two-by-two factorial trial that tested the effects of large-quantity LNS containing milk protein (MP) and whey permeate (WP) on growth and development in stunted children in the community. The comparators for MP and WP were soy protein isolate and maltodextrin, respectively. The LNS was developed and manufactured by Nutriset (Malaunay, France). A total of 750 children who met eligibility criteria were randomised to one of four formulations of LNS (100g/510-530 kcal) to be taken as a daily supplement, or to no supplement for a period of 12 weeks. An unsupplemented group was included to assess the overall effect of LNS (Figure 9.7). Individual nutrition counselling was given to all caregivers at baseline. Throughout the intervention period, participants were followed-up on site at two week intervals to be supplied with rations of LNS or laundry soap if not supplemented. The original trial protocol is available online for further reference: ISRCTN13093195.

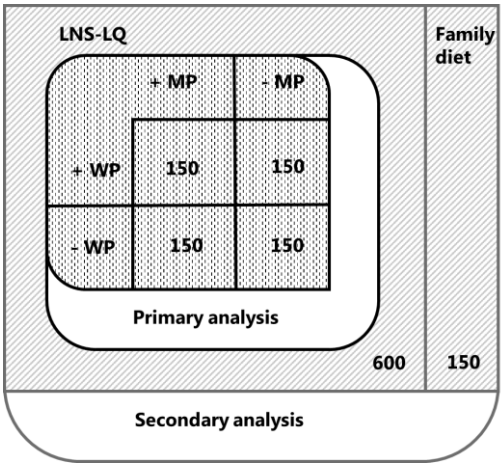

Figure 9.7: 2x2 factorial trial design including an unsupplemented group (family diet). Pesu et al., 2021 [119]. MP: milk protein; WP: whey permeate.

9.2.4 Outcomes

The **primary outcomes** were change in knee-heel length and total length/height from baseline to 12 weeks**. Secondary outcomes** were child development, body composition, anthropometric indicators (MUAC, weight, head-circumference, weight-for-age, HAZ and WHZ) and haemoglobin. **Tertiary outcomes** were; growth factors [serum insulin-like growth factor-1 (s-IGF1) and insulin], markers of micronutrient status; iron [serum ferritin (s-ferritin), serum soluble transferrin receptor (s-sTfR), serum folate (s-folate), vitamin B12 [plasma cobalamin (p-cobalamin), plasma methylmalonic acid (p-MMA)] and serum retinol binding protein (s-RBP), markers of systemic inflammation [serum C-reactive protein (s-CRP) and serum α1-acid glycoprotein (s-AGP)], and a marker of enterocyte mass (p-cit). Stool samples were analysed for markers of intestinal inflammation [f-MPO, faecal neopterin (f-NEO) and function (faecal α1-antitrypsin (f-AAT)) and the gut microbiota.

The main outcomes explored in this thesis were baseline p-cit (µmol/L)**(Paper II)**. The change in p-cit (µmol/L) and f-MPO (ng/mL) from baseline to 12 weeks in main and subgroup analysis **(Paper III).** Subgroup analysis of baseline p-cit and f-MPO as modifiers of the intervention effect on growth and micronutrient-related outcomes from baseline to 12 weeks (**Paper III**).

9.2.5 Data collection

An overview of data collection at each visit is provided in Figure 9.8. In this section, a description of the household WASH assessment is provided. Following this, collection of EED outcomes relevant for this thesis are described. Details of related growth and micronutrient outcomes are described in **Paper III**.

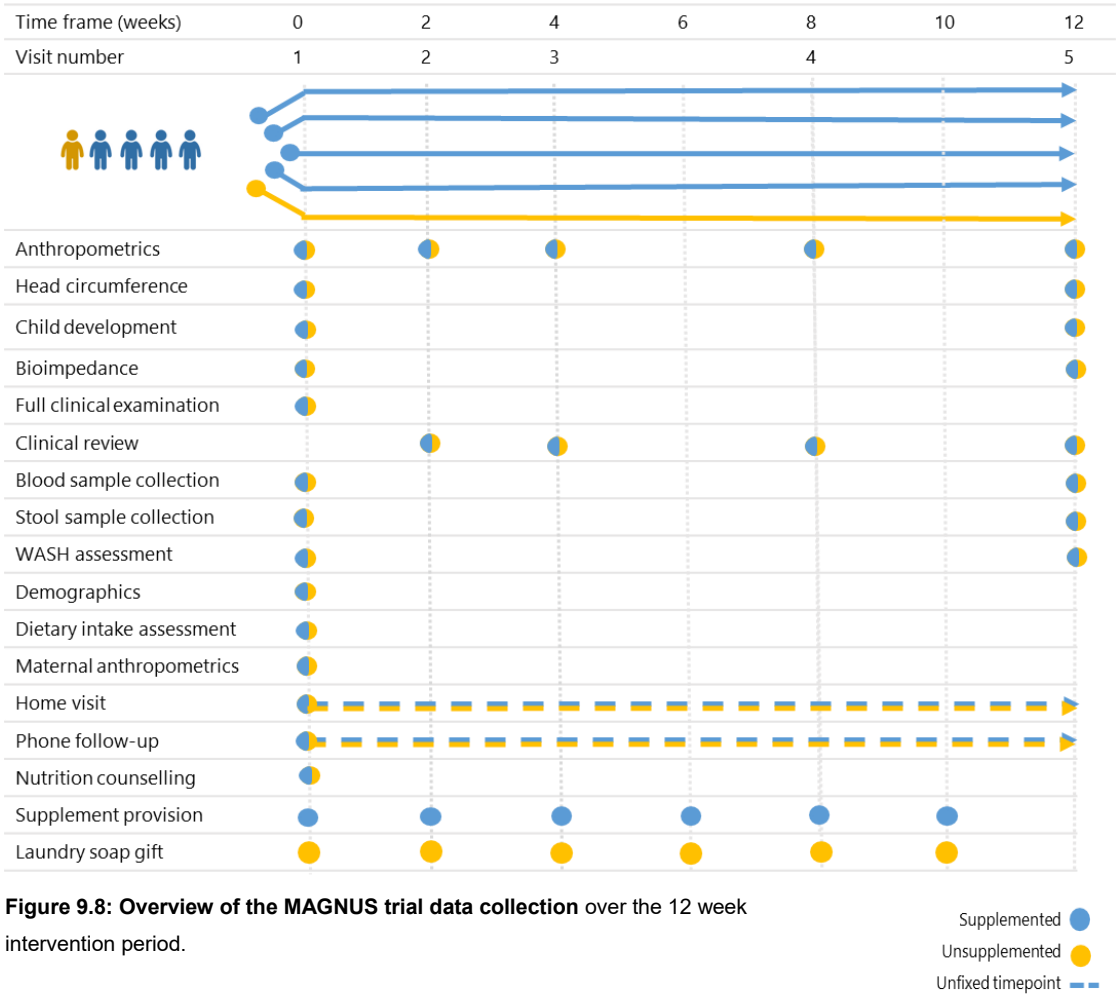

Figure 9.8: Overview of the MAGNUS trial data collection over the 12 week intervention period.

9.2.6 WASH assessments

The WASH assessment allowed for collection of important household level data on environmental exposures. Furthermore, collection of GPS coordinates (with permission) provided a means to follow-up. With permission granted, trained staff visited households together with the caregiver and child at baseline to assess conditions of water, sanitation and hygiene.

This visit included observation of;

i) The type of materials used for the home walls and floor,
ii) The availability of clean and accessible water in the home,
iii) Basic sanitation services, such as the type of latrine used,
iv) Hygiene practices demonstrated by the availability of soap and water to wash hands

The observed assessment was supported by an interviewer-administered questionnaire about toilet sharing, animal ownership and water access and treatment. The Joint Monitoring Programme for Water Supply, Sanitation and Hygiene Household Wash Strategy informed the WASH assessment and its classifications for drinking water source and toilet type [120].

9.2.7 Biological sample collection and processing

Blood and stool samples were collected at baseline and 12 weeks. Details of blood collection are provided in **Paper II**. A stool sample collection kit was assembled at the study lab where sample tubes were weighed to the nearest 0.01g prior to and after sample collection. The kit contained a 1 mL plastic spoon, a free standing screw-top tube containing 4ml of StayRNA (A&A Biotechnology, Gdynia, Poland) or RNAlater (Sigma-Aldrich, Darmstadt, Germany), a support frame for the sample container (to prevent spills) and a pictogram to aid stool collection (Appendix 14.3). Caregivers were provided with kits at baseline and at week 10 for home collection of stool. With individual instruction and a demonstration (using peanut butter), caregivers were asked to collect approximately 1 g of stool into the tube without displacing the buffer and to return the tube containing the sample soon after collection. Stool samples were collected and returned within two days of the baseline and week 12 visits. In the lab, samples were homogenised using a vortex mixer with the aid of glass beads and the slurry was transferred to cryotubes for storage at -20°C. Samples were transferred weekly to a storage facility at -80°C.

9.2.8 Citrulline

Plasma citrulline was measured in plasma samples at Chalmers University of Technology, Gothenburg, Sweden using a Siex QTRAP 6500+ system (AB Sciex) with a Nexera Ultra-High-Performance Liquid Chromatography (UHPLC) system (Shimadzu) [121]. Details are provided in **Paper II**.

9.2.9 Myeloperoxidase, neopterin and α_1-antitrypsin

Markers f-MPO, f-NEO and f-AAT were analysed at the Faculty of Health and Medical Sciences, University of Copenhagen. Commercially available sandwich enzyme linked

immunosorbent assay (ELISA) kits were used to analyse f-MPO (Human Myeloperoxidase DuoSet, R&D systems, Minneapolis, MN) and f-AAT (Human Serpin A1 DuoSet, R&D systems, Minneapolis, MN) according to the manufacturer's instructions. The ELISA technique uses antibodies to detect the presence of target antigens in the sample. The sandwich ELISA technique involves two antibodies that immobilise and promote detection of the antigen. Analysis of f-NEO was done using a competitive ELISA (Neopterin ELISA kit, Human GenWay Biotech, San Diego, CA), according to the manufacturer's instructions. In the competitive assay, the sample antigen is detected when it competes with a reference antigen to bind to a specific amount of labelled antibody. Briefly, stool samples were taken from freezer storage (-140° C) and thawed (4° C) overnight. Once thawed, the samples were transferred to Eppendorf tubes and centrifuged (15000 RCF, 4°C) for 10 minutes, thereafter the supernatant was collected for analysis (150-200 µl). The supernatants were diluted 1:100 and 1:500 for the analysis of f-MPO and 1:1000 and 1:5000 for the analysis of f-AAT. Samples which could not be analysed at these dilutions were diluted more or less, respectively. Samples used for NEO analysis required no further dilution.

9.3 Data analysis

9.3.1 Data management

Data sets were cleaned using R (R Core Team, 2021). Five stool samples were excluded due to labelling errors and a total of 22 blood samples were excluded. The blood samples were excluded for the following reasons; clear outliers (n=1), unspecified technical reasons during analysis (n=9) and labelling errors (n=12). For the stool samples that had a value below the limit of detection after further dilution (n=17 MPO, n=63 AAT, n=41 NEO), this limit was given as an absolute value. During data analysis, it became evident that stool weight data could not be used for sample analysis due to large discrepancies in the recorded weights (Figure 9.10). This was likely to have resulted from spillage or overfilling of the sample container during collection, however the descriptive data was not available to confirm this. Furthermore, the mean values of all three stool markers were found to be significantly lower than other studies in similar populations. Upon closer examination of the results from analysis and further lab tests, we found that by collecting samples in StayRNA or RNA-later instead of using raw stool, there was a significant decrease in the amount of AAT, NEO and perhaps MPO that was extracted and detected. Due to the uncertainty concerning the validity of both AAT (Figure 9.9) and NEO data, it was decided to remove both markers from further data analysis. Reanalysis was discussed but

was hindered by financial and time constraints. The MPO samples had a wide variation however, this marker also showed wide variations between samples in other studies [109]. Thus MPO was included for analysis. The statistical methods used are described further in **Paper II** and **Paper III**.

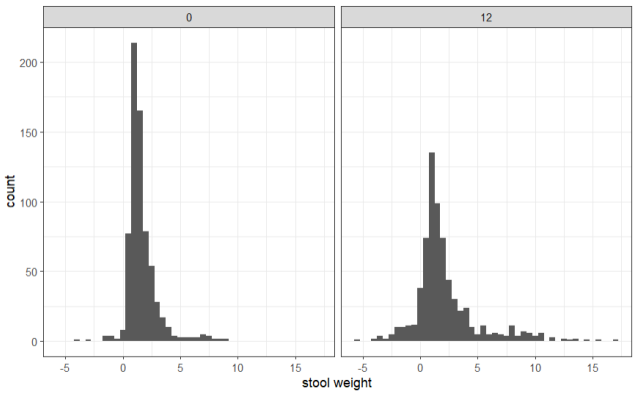

Figure 9.9: Stool weights (g) with large variation at baseline and more so at week 12.

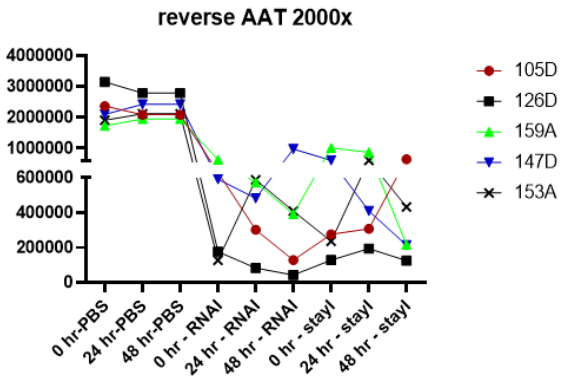

Figure 9.10: Test analysis of raw stool samples collected in different buffers; PBS solution, RNA-later and StayRNA.

9.3.2 Good clinical practice and quality assurance

A study initiation visit was performed from 6th January to 18th February 2020. During this time all staff involved in data collection were trained in GCP and SOPs relevant to their position. Sites were setup, site and trial master files were prepared and data collection and monitoring procedures put in place. The trial was initiated on the 13th of February 2020 with close monitoring of all activities. Recruitment was completed on the 17th of September 2020 and the last participant completed the 12 week visit on the 18th of December 2020. Due to strict national lock-downs during the Covid-19 pandemic, trial recruitment was paused between 1st March and 1st June 2020. All planned in person monitoring of the study activities was no longer possible. However, online monitoring of data and recruitment was maintained throughout the study. Recruitment activities resumed on June 1st with Covid-19 safety precautions in place. An external monitor visited the sites and retraining was performed as needed during the study. The UNCST inspected the study sites in November 2020, they had no major comments and were generally satisfied with their observations. There were no serious adverse events during the study.

10 Results

Presented here is a brief summary of the design and main findings from the three papers included in this thesis. Each of the papers are included as a subsection of this chapter.

Paper I

The MAGNUS trial protocol. A randomised, double-blind, two-by-two factorial trial, that tested the effects of MP and WP in large-quantity LNS (100g/510-530 kcal), with inclusion of an unsupplemented group to assess the overall effect of the LNS.

Paper II

This was a cross-sectional analysis of baseline correlates of p-cit using univariate and multivariate linear regression models. A number of environmental exposures were associated with lower p-cit. These included food insecurity, the wet seasons and selected WASH characteristics including the use of a non-improved toilet system and the lack of soap for handwashing. Morbidity factors that were associated with lower p-cit included malaria, diarrhoea, systemic inflammation, low s-IGF1 and anaemia. Most associations attenuated with additional adjustment for systemic inflammation. The exceptions were socio-economic factors, the wet seasons and low s-IGF1, which were partially reduced. Many of the correlates of p-cit are characteristic for populations with a high EED prevalence.

Paper III

Linear mixed-effects models were used to explore f-MPO and p-cit as outcomes and modifiers of the intervention effect. There was no effect of the LNS on p-cit, but there was a greater increase in f-MPO among those receiving 12 weeks of supplementation. Compared to the comparators used, there was no effect of MP or WP on intestinal markers p-cit and f-MPO. Low p-cit at baseline seemed to modify the effect of the LNS intervention on cobalamin status. Comparing those with high to low p-cit, the latter experienced a 20% reduced effect of the LNS on p-cobalamin and a 59% relative increase in p-MMA.

10.1 Paper I

Hannah Pesu, Rolland Mutumba, Joseph Mbabazi, Mette F Olsen, Christian Mølgaard, Kim F Michaelsen, Christian Ritz, Suzanne Filteau, André Briend, Ezekiel Mupere, Henrik Friis, and Benedikte Grenov

The Role of Milk Protein and Whey Permeate in Lipid-based Nutrient Supplements on the Growth and Development of Stunted Children in Uganda:
A Randomized Trial Protocol (MAGNUS)

Current Developments in Nutrition 2021; 5.

RESEARCH METHODOLOGY/STUDY DESIGN

CURRENT DEVELOPMENTS IN NUTRITION

Maternal and Pediatric Nutrition

The Role of Milk Protein and Whey Permeate in Lipid-based Nutrient Supplements on the Growth and Development of Stunted Children in Uganda: A Randomized Trial Protocol (MAGNUS)

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ABSTRACT

Stunting is associated with cognitive impairment and later chronic disease. Previous trials to prevent stunting have had little effect, and no trials seem to have provided larger amounts of energy and high-quality proteins to already stunted children. We aimed to assess the effects of milk protein (MP) and whey permeate (WP) in large-quantity lipid-based nutrient supplements (LNS-LQ), among stunted children, on linear growth and child development. This was a randomized, double-blind, 2-by-2 factorial trial. Stunted children aged 12–59 mo from eastern Uganda ($n = 750$) were randomly assigned to receive 100 g LNS-LQ with or without MP and WP ($n = 4 \times 150$) or no supplement ($n = 150$) for 3 mo. The primary outcomes were change in knee-heel and total length. Secondary outcomes included child development, body composition, anthropometry, and hemoglobin. Micronutrient status, intestinal function, and microbiota were also assessed. Our findings will contribute to an understanding of the role of milk ingredients and LNS in linear catch-up growth. This trial was registered at www.isrctn.com as ISRCTN13093195. *Curr Dev Nutr* 2021;5:nzab067.

Keywords: stunting, linear growth, lipid-based nutrient supplement (LNS), milk protein, whey permeate, child development, body composition, gut

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Abbreviations used: DIAAS, Digestible Indispensable Amino Acid Score; EED, environmental enteric dysfunction; FFM, fat-free mass; FM, fat mass; HAZ, height-for-age *z* score; IGF-I, insulin-like growth factor I; LNS, lipid-based nutrient supplement(s); LNS-LQ, large-quantity lipid-based nutrient supplement(s); LNS-SQ, small-quantity lipid-based nutrient supplement(s); MAGNUS, Milk Affecting Growth, Cognition, and the Gut in Child Stunting; MDAT, Malawi Development Assessment Tool; MP, milk protein; MPI, milk protein isolate; MUAC, midupper arm circumference; SAM, severe acute malnutrition; WASH, water, sanitation, and hygiene; WAZ, weight-for-age *z* score; WHZ, weight-for-height *z* score; WP, whey permeate.

Introduction

Globally, 144 million children under the age of 5 y are classified as stunted, having a length- or height-for-age *z* score (HAZ) of less than −2 (1). Stunting is associated with adverse short- and long-term health outcomes. It is associated with delayed cognitive development, increased morbidity and mortality (2), poor schooling performance (3, 4), and later with reduced economic productivity (4) and risk of chronic disease (5, 6). Stunting also contributes to an intergenerational cycle of malnutrition and poverty, whereby a child born to a stunted mother is more likely to be stunted themselves (7).

In the east African region, close to 1 in 3 children under the age of 5 are stunted (1). High stunting prevalence is experienced in many low- and middle-income countries and is indicative of exposure to environments of inadequate care, suboptimal nutrition, and recurrent infections (3, 5, 6). The majority of growth faltering occurs from 3 to 24 mo of age (8). Nutrition interventions to reduce the risk of stunting have therefore focused on prevention through optimizing maternal and early infant nutrition (9, 10). These interventions, however, have had little impact on linear growth. This was summarized in a recent meta-analysis, whereby complementary feeding interventions, in food-insecure settings, improved HAZ by a mere 0.08 overall (10). The lack of effect on linear growth has been attributed, at least in part, to environmental enteric dysfunction (EED) (11, 12). The premise is that frequent exposure to pathogens in environments with unsafe water and inadequate sanitation and hygiene [water, sanitation, and hygiene (WASH)] encourages a

state of systemic and intestinal inflammation, as well as morphological and functional changes to the intestine, which can, in turn, exacerbate nutrient deficiencies. However, large trials combining comprehensive WASH interventions with small-quantity lipid-based nutrient supplements (LNS-SQ) reported no effects from the WASH interventions and only minimal effects from the LNS-SQ (13–17).

While there have been many studies aiming to prevent stunting in young children, or to improve linear growth in wasted children, this is to our knowledge, the first trial which provided large-quantity LNS (LNS-LQ) to children recruited on the basis of stunting. There have been concerns that supplementation in stunted children will lead to excessive accretion of fat rather than lean tissue, and therefore increase the subsequent risk of chronic disease. These concerns, however, are not substantiated by the evidence. Recent supplementation studies among children with moderate (18) and severe acute (19) malnutrition have shown that even those who are also stunted predominantly gain fat-free mass (20). There is a gap in the evidence, however, as to the extent that an LNS-LQ or one containing milk protein (MP) will encourage catch-up growth in already stunted children, and to what extent this impacts body composition (21–23), mitigates vulnerability to illness, and improves child development and other functional outcomes (23, 24). However, it is possible that nutritional support to stunted children could have beneficial effects even in the absence of linear catch-up growth. We now know that the co-existence of wasting (low weight-for-height) and linear growth faltering increases a child's risk of morbidity and mortality (25, 26). Moreover, new evidence from a large 40-y cohort study in The Gambia suggests that stunting not only develops as a chronic condition but also develops interactively with episodes of wasting as a short-term adaptation (27, 28).

Previous nutrition interventions may have been limited by an inadequate supply of energy and high-quality proteins. Considering this, and the recent evidence demonstrating that even short children with wasting predominantly gain fat-free mass (20), there is sufficient justification to assess the effects of an LNS-LQ among stunted children.

Milk intake has long been associated with linear growth (29, 30) and is suggested to have a stronger effect in low-income compared with high-income countries (29). However, a new review based on studies from predominantly high-income countries was not able to confirm an effect of milk intake on linear growth (31). Several studies have shown that the addition of milk in supplements to treat acute malnutrition has had positive effects on body composition, weight gain, recovery, and anemia (32–37), but limited (18) or no effect in encouraging linear catch-up growth (32, 35, 38). In studies from low- and high-income countries, milk intake in children has been associated with improved lean mass deposition (31), bone-mineral composition (39), and cognitive function (40, 41), benefits that may be experienced to a greater extent in children exposed to growth-deficient environments (31, 42). Furthermore, the different components of milk may provide unique health benefits (43). MPs have a complete amino acid profile (31) and are thought to promote growth by stimulating the growth factors insulin-like growth factor-I (IGF-I) and insulin (44). On the other hand, whey permeate (WP) is predominantly composed of lactose and bioavailable minerals, which may have prebiotic effects (45) as well as a role in bone mineralization and fat-free mass accretion (46).

In this study, we aimed to assess the individual and combined effects of MP and WP, provided as part of an LNS-LQ, using a 2 × 2 factorial design, among stunted children. The primary outcome was linear growth. Secondary outcomes were child development, body composition, HAZ, weight-for-age (WAZ), and weight-for-height *z* scores (WHZ), weight, midupper arm circumference (MUAC), head circumference, and hemoglobin. In addition, we assessed the main effect of LNS on these outcomes, irrespective of milk ingredients, as well as the role of the gut as mediator or modifier of effects.

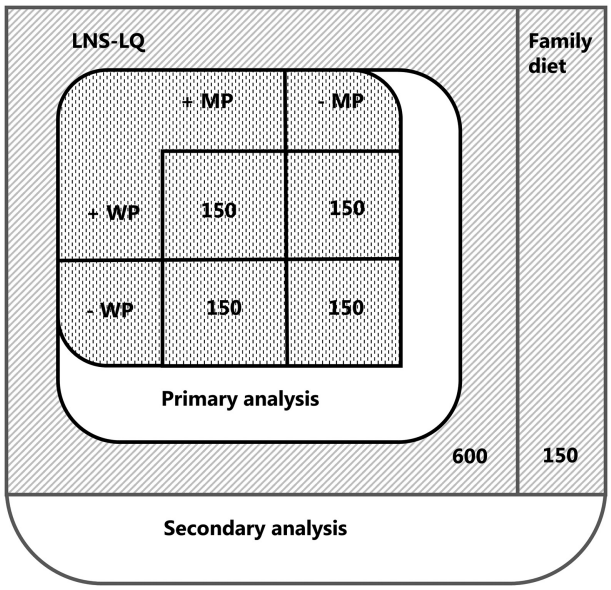

FIGURE 1 The primary analysis will compare LNS-LQ with and without MP and WP in a 2-by-2 factorial design with 150 participants in each given combination. Secondary analysis will compare all LNS-LQ interventions ($n = 600$) with the reference group (family diet, $n = 150$). LNS-LQ, large-quantity lipid-based nutrient supplements; MP, milk protein; WP, whey permeate.

Methods

The reporting of this protocol followed the Standard Protocol Items: Recommendations for International Trials (SPIRIT) 2013 checklist.

Trial overview and design

The MAGNUS study (Milk Affecting Growth, Cognition, and the Gut in Child Stunting) was a randomized, double-blind, 2-by-2 factorial trial testing the effects of MP and WP in LNS-LQ. An unsupplemented group was included as a reference. For a 12-wk period between February and December 2020, 750 Ugandan children classified as stunted received 1 of 4 formulations of LNS-LQ as a daily supplement ($n = 4 \times 150$) or continued with the family diet ($n = 150$) (see **Figure 1**). All caregivers received individual nutrition counseling at baseline. All participants were followed up at the same intervals throughout the intervention period (see **Figure 2**). This design will allow us to assess the individual and combined effects of MP and WP among the 600 children allocated to LNS, based on the factorial design: If the effects are independent, then we can compare the 300 given LNS with MP to the 300 given LNS without milk. And likewise, we can compare the 300 given LNS with WP to

			Time-point (weeks)							
Activity			**S**	**T_0**	**T_2**	**T_4**	**T_6**	**T_8**	**T_{10}**	**T_{12}**
Referral		Screening for referral	▪							
Enrollment		Screening for eligibility		▪						
		Informed consent		▪						
		Nutrition counselling		▪						
		Allocation		▪						
Data collection	Primary outcomes	Knee-heel length		▪	▪	▪		▪		▪
		Height	▪	▪	▪	▪		▪		▪
	Secondary outcomes	Weight	▪	▪	▪	▪		▪		▪
		Mid-upper arm circumference	▪	▪	▪	▪		▪		▪
		Head circumference		▪						▪
		Family care indicators		▪						
		Child development assessment		▪						▪
		Bioimpedance		▪						▪
		Skinfolds		▪	▪	▪		▪		▪
	Tertiary outcomes	Blood sample collection		▪						▪
		Stool sample collection		▪						▪
		Clinical examination		▪						
		Clinical review			▪	▪		▪		▪
	Baseline	Demographics		▪						
		WASH assessment		▪						
		Dietary intake assessment		▪						
		Maternal anthropometrics		▪						
Intervention		Supplement provision		▪	▪	▪	▪	▪	▪	
		Reference group gift allocation		▪	▪	▪	▪	▪	▪	
Adherence		Empty sachet collection			▪	▪	▪	▪	▪	▪
		Phone follow-up		▪	—	—	—	—	—	▪
		Home visit		▪	—	—	—	—	—	▪

FIGURE 2 MAGNUS data collection time points and visits. Timeline and visit overview for participants enrolled in the study. Phone follow-up and home visits were carried out as required and thus were unfixed time points. All participants were invited to the same follow-up visits. Time points were considered valid if taken within ±7 d of baseline, week 2 and week 4, and ±14 d from all other time points. Hemoglobin was a secondary outcome. LNS-LQ, large-quantity lipid-based nutrient supplement; MAGNUS, Milk Affecting Growth, Cognition, and the Gut in Child Stunting; MUAC, midupper arm circumference; S, screening in village for referral; T, week of visit from baseline (T0) to discharge (T12); WASH, water, sanitation, and hygiene.

the 300 given LNS without WP. If the effects are not independent, then we will compare each of the 4 combinations pairwise. In addition, we will be able to assess the effect of LNS by comparing the 600 given LNS, irrespective of milk ingredients, to the 150 given no supplements.

The intervention

LNS are fortified lipid-based pastes that are well adapted for use in resource-limited settings; they are produced to a high safety standard, do not require refrigeration or preparation, and are packaged in standard portion sizes.

Our LNS-LQ supplements, manufactured by Nutriset (Malaunay, France), varied with respect to the incorporation of WP and MP isolate (MPI). The MPI contained casein and whey proteins in the same proportions as milk; the lactose and mineral components were removed so that the MPI was close to 90% protein by weight with a Digestible Indispensable Amino Acid Score (DIAAS) of 120 (47). As a comparator to MPI, soy protein isolate, a high-quality plant protein with a DIAAS of 84, was used (47). WP contained 80–85% lactose and minerals (potassium, phosphorus, magnesium, calcium, sodium, and to a lesser extent zinc). As a comparator to WP, maltodextrin, a standard ingredient used in LNS products was used. All formulations were standardized to contain similar proportions of energy, protein, and carbohydrates. The supplements contained a mineral and vitamin mix to improve micronutrient content, and in 2 of the formulations the milk minerals provided by WP were in addition to the standard amount provided in all formulations (**Table 1**).

The 600 participants randomly assigned to LNS-LQ received one 100-g sachet (530–535 kcal)/d for 12 wk, distributed every 14 d. Those randomly assigned to the family diet received laundry soap at each visit.

Participant recruitment and enrollment

The study was conducted from 2 local community health centers in Walukuba and Buwenge. All participants were recruited from within the surrounding district of Jinja, in the Busoga Subregion, eastern Uganda. Here, the prevalence of child stunting is estimated to be 29%, similar

TABLE 1 Nutrient composition for 4 formulations of LNS-LQ supplied to 1- to 5-y-old stunted children[1]

	Milk protein and whey permeate LNS-LQ	Milk protein and no whey permeate LNS-LQ	Soy protein and whey permeate LNS-LQ	Soy protein and no whey permeate LNS-LQ
Macronutrients (components per 100 g)				
Energy, kcal	531	535	530	534
Carbohydrates, g	42	43	42	43
Lactose, g	15.7	0.4	15.3	0
Proteins, g	13.9	13.5	13.9	13.5
Milk proteins, g	7.15	6.75	0.40	0
Vegetable proteins, g	6.75	6.75	13.50	13.50
Lipids, g	33.7	33.7	33.7	33.7
Linoleic acid (C18:2), g	3.0	3.0	3.0	3.0
Linolenic acid (C18:3), g	0.5	0.5	0.5	0.5
Minerals				
Calcium, mg	691	594	691	594
Copper, mg	1.65	1.65	1.65	1.65
Iron, mg	12	12	12	12
Iodine, μg	127	113	127	113
Magnesium, mg	199.2	175.8	199.2	175.8
Manganese, mg	1.8	1.8	1.8	1.8
Phosphorus, mg	661	539	661	539
Potassium, mg	1315	985	1315	985
Sodium,[2] mg	84	7	156	79
Selenium, μg	30	30	30	30
Zinc, mg	12.5	12.5	12.5	12.5
Vitamins				
Vitamin A, mg	619	619	619	619
Vitamin B-1, mg	1.2	1.1	1.2	1.1
Vitamin B-12, μg	3.2	3.0	3.2	3.0
Vitamin B-2, mg	3.1	2.8	2.7	2.4
Niacin, mg	14.9	14.6	14.9	14.6
Pantothenic acid, mg	5.7	4.5	5.7	4.5
Vitamin B-6, mg	2.1	2.0	2.1	2.0
Biotin, μg	74.1	67.6	74.1	67.6
Folic acid, μg	223	223	223	223
Vitamin C, mg	67.9	67.6	67.9	67.6
Vitamin D, μg	16.9	16.9	16.9	16.9
Vitamin E, mg	18	18	18	18
Vitamin K, μg	30	30	30	30

[1]The same amount of micronutrient premix was used in all formulations. The additional micronutrients provided are from the other ingredients used. LNS-LQ, large-quantity lipid-based nutrient supplement.
[2]Soy protein isolate and whey permeate contribute additional sodium.

to the national average (48). To identify stunted children, communities within the district of Jinja were mobilized by Village Health Teams for an initial screening for referral. Study staff screened children in the community for age, stunting, and severe acute malnutrition (SAM). All children identified as having SAM were referred for appropriate treatment; others who met the inclusion criteria for stunting and age were invited to one of the study sites for eligibility screening.

At the study sites, children were considered eligible if they were aged between 12 and 59 mo and had an HAZ of less than −2, according to the WHO growth standards (49). Children <12 mo old were not eligible to avoid interfering with breastfeeding. Caregivers had to be living in the catchment area and willing to return for follow-up visits, and able to provide written informed consent and agree to both phone follow-up (if a phone contact was available) and home visits. Children were excluded if they were identified with SAM according to the WHO classification (50), had medical complications requiring hospitalization, a history of allergy to peanuts or milk, obvious disability that impeded eating capacity, or a disability that impeded the measurement of length or height. Children were also excluded if they were participating in another study, if the family planned to move away from the catchment area within 6 mo, if previously enrolled in the MAGNUS study, or if another child from the same household was already included.

Informed consent

If all eligibility criteria were met, trained staff took the caregiver through the informed-consent information individually, using the most appropriate of 3 commonly spoken languages in the region (English, Lusoga, or Luganda). The same information was given verbally and in writing. Caregivers were also taken through a short verbal questionnaire to ensure that the information provided was adequately understood. After necessary clarifications were given, the caregiver consented on behalf of the participant. If illiterate, a literate witness was present dur-

ing the informed-consent process. Consenting caregivers were asked for permission to store 1–2 mL of blood and stool samples from the participant for future use; this was independent of trial consent. The caregiver could opt to withdraw consent at any time.

Blinding, randomization, and assignment of interventions

The sachets of LNS-LQ were labeled with a unique 3-letter code that corresponded to the different formulations. Two unique codes were given to each of the 4 formulations and a further 2 codes were created for the reference group so that 10 unique codes were used in the allocation sequence list. Only the manufacturer (Nutriset) had access to the blinding code. Two allocation sequence lists, one for each study site, were computer generated using R (R Foundation for Statistical Computing). These were generated and sealed by a member of staff at the University of Copenhagen, Denmark, who was otherwise not involved in the study. Site-stratified, block randomization, with variable block sizes of 10 and 20 were used to allocate the sequential list of ID numbers to the 10 unique codes.

Upon inclusion, administrative staff allocated a unique ID from a sequentially ordered list. After completion of baseline activities, the study pharmacist allocated the intervention according to a hard-copy random allocation list. Only the pharmacist had access to the allocation list, which was checked for each participant, at each visit. Using QR codes, the pharmacist recorded the code of what was distributed in a spreadsheet, which was regularly monitored by an independent assessor in Copenhagen. Hard copies of the allocation lists were kept securely in sealed envelopes at the University of Copenhagen.

Outcome assessors and data analysts were blinded both with respect to the allocation of the intervention and to the type of ingredients contained in differently coded LNS sachets. Caregivers were blinded with respect to the type of LNS allocated, since the taste, smell, and appearance of all 4 products were indistinguishable. Caregivers were not, however, blinded with respect to receiving LNS or not. Only the Data Safety Monitoring Board, which operated independently of the study, could choose to break the blinding in order to monitor safety parameters.

Adherence

The LNS was distributed in packs of 14 sachets. To counteract the likelihood of sharing, an additional pack of the same LNS product code was distributed every 2 wk to caregivers with other children aged between 6 and 59 mo living in the same household. The additional stock provided to the household increased the likelihood that the participating child had access to the required daily quota. When collecting new sachets, the caregiver was requested to return any empty and unused sachets from the previous 2-wk supply, including those from the additional pack.

Outcomes

Primary outcomes.

The primary outcomes were changes in knee-heel length (mm) and total length/height (cm) from baseline to 12 wk.

Secondary outcomes.

All secondary outcomes were measured over time from baseline to 12 wk. Child development was assessed at baseline and at discharge using a locally adapted version of the Malawi Development Assessment Tool (MDAT). Anthropometric indices HAZ, WAZ, and WHZ were assessed as well as weight (g), MUAC (cm), and head circumference (mm). Body composition was assessed using bioimpedance and the triceps and subscapular skinfold thicknesses (mm). The raw data from bioimpedance were used to calculate the fat mass (FM) (kg), fat-free mass (FFM) (kg), fat mass index (kg/m^2), and fat-free mass index (kg/m^2). Hemoglobin concentration was assessed from blood samples collected at baseline and 12 wk.

Tertiary outcomes

Biological samples.

Blood and stool samples were collected at baseline and at week 12. Blood samples will be analyzed for growth factors (IGF-I and insulin), markers of micronutrient status [i.e., iron (ferritin, soluble transferrin receptor), folate (serum folate), vitamin B-12 (cobalamin, methylmalonic acid), and vitamin A (retinol binding protein)], markers of systemic inflammation [C-reactive protein and α1-acid glycoprotein (AGP)], and markers of intestinal function (citrulline), together with other amino acids. Stool samples will be analyzed for markers of intestinal inflammation [myeloperoxidase (MPO), neopterin (NEO)] and function (α1-antitrypsin (AAT)] and the gut microbiota.

Safety, morbidity, and loss to follow-up.

Data will be reported on the proportion of children who, during the intervention period, deteriorated to moderate acute malnutrition or SAM according to the WHO classifications (49). The proportion of participants who died during the study period will be reported, as well as the number of morbidity episodes including the duration and severity of the illness. Finally, the number of children who were lost to follow-up will be reported. Caregivers were called with reminders to attend upcoming or missed appointments. Loss to follow-up was defined as those who had not returned for the 12-wk follow-up visit by 14 wk post-inclusion.

Baseline participant characteristics.

Additional information collected at baseline included demographics, a dietary intake assessment, and a WASH assessment taken at the initial home visit.

Measurements

Time points for each measurement are shown in Figure 2.

Anthropometrics.

Knee-heel length was measured using a digital caliper with a resolution of 0.01 mm (Mitutoyo) mounted with knee and heel caps, cast in hard plastic. The distance between the knee (from the lateral condyle) and the heel (calcaneus) was measured 5 times consecutively on the left leg while the child was seated with both legs hanging over the edge of a table or the caregiver's lap. All other anthropometric measurements were repeated in triplicate. Participant length and height measurements were taken using a wooden Shorrboard (Weight and Measure), ensuring 4 points of contact with repositioning between measurements. Maternal height was measured using a fixed wall stadiometer (SECA 206). The weights of the mother and participant were measured using an electronic double-weighing scale (SECA 876). Head circumference, MUAC, and skinfold thickness were measured using a windowed, nonelastic head cir-

cumference tape (SECA 212); a nonelastic MUAC tape (UNICEF SD); and a Harpenden skinfold caliper (Baty International), respectively. Height or length, weight, MUAC, and head circumference were measured according to accepted international standards for anthropometric measurement (51). Skinfold thicknesses were measured on the left side, according to the manufacturer's instructions. For referral and inclusion, *z* scores were calculated using the WHO field growth charts. The WHO Anthro program will be used to calculate *z* scores for data analysis (52).

Bioimpedance.
Bioimpedance was measured using the Bodystat 500 (50 kHz) and in accordance with the manufacturer's instructions (Bodystat Ltd.). Measurements were taken while the child was lying on his/her back, with limbs spread apart, preferably at rest and with removal of wet or soiled diapers. A measure was repeated a minimum of 2 times but up to 3 times if the child's positioning or movement rated poorly. Measurements for impedance, resistance, reactance, and phase angle were recorded. Using an equation, the raw data will be used to calculate FM and FFM.

Child development.
Child development officers, trained in use of the MDAT (Manual V06, March 2018), took the participant through a series of activities adapted for the Ugandan context. The activities were related to 4 domains of development: gross motor, fine motor, language, and social development (53). The participant was graded as to whether or not he/she could complete each task successfully. The assessment continued until the child had failed to complete 6 tasks consecutively. At baseline, an interviewer-administered questionnaire was also used to gather information from the caregiver about household and family indicators for the support of child development (54).

Clinical assessment.
A thorough clinical examination was carried out at baseline. It included rapid tests for HIV and malaria, a thorough medical history with questions related to signs and symptoms of wasting or hospitalization due to SAM, and assessment of vital signs (pulse, blood pressure, and respiratory rate). At follow-up visits, a short review was conducted, assessing the most recent medical history, milk intake, and where applicable, monitoring of adverse events. To maintain blinding, the pharmacist distributing LNS inquired about adherence and if the caregiver had experienced problems with the LNS.

Biological sample collection.
Stool samples were collected at 2 time points and stored for later analysis of markers of gut function and microbiota. If not collected on site, a sample collection kit was given to caregivers along with specific instruction on stool sample collection at home. Collection vials contained StayRNA (A&A Biotechnology), allowing samples to be stored at room temperature for up to 5 d after collection. A maximum of 6.0 mL of venous blood was collected on site at 2 time points. A small amount was used for rapid tests: HIV status, malaria, and hemoglobin status. The remaining sample was processed and stored within hours for later analysis of selected markers. All biological samples were stored at −20°C until delivery to the main storage site in Kampala where they were stored at −80°C until they were shipped on dry ice to the University of Copenhagen, Denmark, for additional analyses.

Other measurements, baseline questionnaires, and WASH assessment.
At baseline, the child's age and birth weight were recorded, wherever possible, using a birth information card. Information on sociodemographic characteristics, breastfeeding status, food frequency, and diet diversity was collected via interviewer-administered questionnaires. At the baseline home visit, GPS coordinates of the home site location were collected to facilitate later follow-up. In addition, trained staff conducted a short assessment of observed household WASH characteristics, including water source, access to basic sanitation, and the use of soap. To minimize response bias, local study staff with a good knowledge of the language and the culture were trained in asking questions to get as clear and precise answers as possible.

Participant retention, reimbursement, referral, and withdrawal

If visits were missed and phone contact was unsuccessful, attempts were made to visit the caregiver's home. To facilitate attendance, a travel reimbursement was provided at each visit to cover the cost of return transport and food while at the clinic visit. Any participants requiring hospital attention were referred for treatment. If a caregiver requested for their child to stop receiving LNS, this was permitted; however, all included participants continued to be followed up for the remainder of the 12-wk intervention period. In case of participant withdrawal, all available data up to the point of withdrawal were used in data analysis.

Data management

Participant data were collected in a paper case report form and were double entered using Epidata software (https://www.epidata.dk/) with inbuilt range checks. The secure electronic data collection platform REDCap (Open Source; Vanderbilt University) was used to monitor participant registration and visits but not for primary data collection. All source data will be kept securely on file for a minimum of 5 y after completion of the study. Adverse and serious adverse events occurring during the intervention period were recorded and reported to the sponsor and the institutional review board. Events occurring after a subject was discontinued from the study were not reported unless the investigator suspected that the event was related to the LNS-LQ intervention.

Sample size calculation

To detect a 0.35-SD or greater difference between any 2 groups, with 5% significance and 80% power, 129 children were required in each group. To allow for 10% loss to follow-up, 150 children were included in each group, based on the 4 combinations of MP and WP. If there were no interactions between the 2 experimental interventions, 2 groups of 300 children could be compared, enabling differences of 0.24 SD to be detected. In the Treatfood trial (18), the SD of knee-heel length at baseline was 18.1 mm (18), so that a 0.24-SD difference corresponded to 4.3 mm. In secondary analysis, to assess the effect of LNS, 600 supplemented children were compared with 150 unsupplemented children, with the ability to detect a 0.27-SD difference, corresponding to 4.9 mm.

Statistical methods

Primary and secondary outcomes will be analyzed using linear mixed models that account for the correlation between repeated measurements from the same participant, whereas tertiary outcomes will be analyzed using ordinary ANCOVA models. In all of these ANCOVA models, the baseline value will be included as a covariate. Additional covariates may be included as appropriate. Results will be reported as estimated differences with corresponding 95% CIs and *P* values. A statistical analysis plan was prepared before unblinding of the trial and uploaded to the ISRCTN registry.

This is an effectiveness trial. Therefore, the primary statistical analysis will be carried out as intention to treat. In subsequent per-protocol statistical analysis, participants with major protocol deviations or violations are excluded.

Ethics approval and consent to participate

The study was conducted in accordance with the ethical principles set forth in the current version of the Declaration of Helsinki and all applicable local regulatory requirements. The study was approved by the School of Medicine Research Ethics Committee at Makerere University and The Ugandan National Council of Science and Technology. The study also received consultative approval from the Danish National Committee on Biomedical Research Ethics. The study was initiated only after approval was given by all aforementioned authorities. Written informed consent was obtained from all caregivers who consented to study participation of the child in their care. The rights, safety, and well-being of the children involved in the study prevailed over science and society. Before participant recruitment, the study was registered at www.isrctn.com as ISRCTN13093195.

Discussion

The findings from the MAGNUS trial will help to clarify to what extent MP and WP, given in LNS, or the LNS per se, play a role in linear catch-up growth and benefit functional outcomes such as cognition and the gut. In this, we will explore to what extent functional benefits are possible with or without effects on linear growth. Our results will also contribute to current knowledge on whether stunted children will predominantly gain lean mass when supplemented with LNS-LQ. Since our study population is aged between 12 and 59 mo, we will also be exploring the potential for catch-up growth in children beyond 2 y of age.

The gut is thought to play a role in the pathogenesis of stunting, but studies aiming to minimize environmental pathogen exposures, and so reduce the risk of EED, have not seen improvements in linear growth. It may be that, once damaged, the gut requires larger quantities of essential nutrients in order to repair and facilitate nutrient absorption. We will explore whether milk components provided in LNS-LQ can improve reparation of the gut in already stunted children and to what extent gut function and inflammation act as mediators and effect modifiers of the effect of LNS-LQ on linear growth.

The high lactose content in WP may have positive effects on the microbiota and growth. If this is demonstrated in our study, it may have implications on the future development of LNS, since WP has the potential to be used as a nutritious substitute for maltodextrin or sugar.

This is the first randomized controlled trial we are aware of that explores the effects of LNS-LQ supplementation in already stunted children. The strengths of this study are the randomized 2 × 2 factorial design, which allows us to assess both the individual and combined effects of the milk ingredients, as well as the unsupplemented reference group, allowing us to assess the main effects of LNS per se. It is also a strength that the study includes several secondary functional outcomes alongside anthropometrics and body composition, as well as tertiary mechanistic outcomes. It is a limitation that we are unable to include a longer follow-up period. A follow-up study of the cohort would be of great benefit to measure the benefits and chronic disease risks associated with the 12-wk LNS-LQ supplementation.

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10.2 Paper II

Hannah Pesu, Joseph Mbabazi, Rolland Mutumba1, Otto Savolainen, Mette F. Olsen, Christian Mølgaard, Kim F. Michaelsen, Christian Ritz, Suzanne Filteau, André Briend, Ezekiel Mupere, Henrik Friis, Benedikte Grenov

Correlates of plasma citrulline as a marker of enterocyte mass among children with stunting: a cross-sectional study in Uganda

(Manuscript prepared for submission)

Correlates of plasma citrulline as a marker of enterocyte mass among children with stunting: a cross-sectional study in Uganda

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Running title

Correlates of citrulline in stunted children

What is new?

We explore correlates of plasma citrulline (p-cit) as a marker of enterocyte mass in stunted children. We demonstrate that p-cit is associated with many factors that would be expected in populations with a high prevalence of environmental enteric dysfunction. We propose that p-cit may be a useful marker of mucosal regeneration in low-income settings. Furthermore, we show that p-cit is affected by fasting status and that systemic inflammation is an important mediator or confounder in many relationships which should be taken into consideration for the correct interpretation of p-cit.

List of abbreviations

EED	Environmental enteric dysfunction
HAZ	Height or length-for-age Z-score
s-AGP	Serum α1-acid glycoprotein
s-CRP	Serum C-reactive protein
s-IGF1	Serum insulin-like-growth factor-1
MAGNUS	Milk affecting growth, cognition and the gut in child stunting
MDD	Minimum dietary diversity
p-cit	Plasma citrulline
WASH	Water, sanitation and hygiene

Abstract

Background: Environmental enteric dysfunction (EED) is associated with stunting. Citrulline, produced in mature enterocytes, may be a valuable biomarker of small intestinal enterocyte mass in the context of EED.

Objective: To explore the correlates of plasma citrulline (p-cit) in children with stunting.

Methods: In a cross-sectional study using baseline data from the community-based MAGNUS trial (ISRCTN13093195), we explored potential correlates of p-cit in stunted Ugandan children aged 12-59 months. Using linear regression in univariate and multivariate models, we explored associations with socioeconomics, diet, micronutrient status and WASH characteristics. The influence of covariates age, fasting and systemic inflammation were also explored.

Results: Of 750 children, mean ±SD age was 32 ±11.7 months and height-for-age Z-score (HAZ) was -3.02 ±0.74. P-cit, available for 730 children, differed according to time fasted and was 20.7 ±8.9, 22.3 ±10.6 and 24.2 ±13.1 µmol/L if fasted <2, 2-5 and >5 hours, respectively. Positive correlates were age (0.07, 95%CI: 0.001; 0.15 µmol/L) and $\log_{10}$ serum insulin-like growth factor-1 (s-IGF1) (8.88, 95%CI: 5.09; 12.67 µmol/L). With adjustment for systemic inflammation, the association with s-IGF1 reduced (4.98, 95%CI: 0.94; 9.03). Negative correlates included food insecurity, wet season (-3.12, 95%CI: -4.97; -1.26 µmol/L), serum C-reactive protein (-0.15, 95%CI: -0.20; -0.10 µmol/L), serum α_1-acid glycoprotein (-5.34, 95%CI: -6.98; -3.70µmol/L) and anaemia (-1.95, 95%CI: -3.72; -0.18 µmol/L). Among the negatively correlated WASH characteristics was lack of soap for handwashing (-2.53, 95%CI: -4.82; -0.25µmol/L). Many associations attenuated with adjustment for inflammation.

Conclusions: Many of the correlates of p-cit are characteristic for populations with a high EED prevalence. Systemic inflammation was strongly associated with p-cit and is implicated in EED and stunting. Adjustment for systemic inflammation attenuated many associations, reflecting either confounding, mediation or both. This study highlights the complex interplay between p-cit and systemic inflammation.

Keywords

stunting, environmental enteric dysfunction, citrulline, inflammation, water sanitation and hygiene, children

Introduction

Stunting is associated with poor childhood survival, developmental delays and lower educational attainment (1). Globally, it affects an estimated 149 million children under five years (2). Multiple factors are thought to contribute to the stunting, including low socioeconomic status (3), repeated infections and inadequate diet (4). The interplay between these factors is still poorly understood.

Environmental enteric dysfunction (EED), a subclinical inflammatory condition of the small intestine, is pervasive among low-income settings and has been proposed as an important contributor to stunting (5). Biopsies taken from adults and children living in these settings indicate morphological changes to the small intestinal mucosa that are considered characteristic of EED: flattened villi, crypt hyperplasia and evidence of submucosal inflammation (6). It is widely believed that EED results from exposure to pathogens and toxins through inadequate water, sanitation and hygiene (WASH). This perpetual state of inflammation damages tissues and leads to a loss of epithelial cells, barrier integrity and possibly absorptive function (7), thus exacerbating or creating nutrient deficiencies (8).

To elucidate the functional implications of EED and its role in stunting, a range of biomarkers are currently being explored (9). One candidate marker is citrulline, a non-protein amino acid produced in the small intestinal by enterocytes at the villus tips. In a number of intestinal disease states, plasma citrulline (p-cit) has been shown to be a marker of enterocyte mass (10). In short bowel syndrome, p-cit has been positively correlated with intestinal length (11) and in celiac disease, negatively correlated with the grade of disease severity (12). Given its use as a marker in other enteropathies, p-cit may be relevant in the context of EED and stunting (8). The objective of this study was to explore correlates of p-cit; socioeconomic factors, diet, inflammation, micronutrient status, household WASH characteristics and other factors, in children with stunting.

Methods

Study design and ethics

This cross-sectional study used baseline data from the 'Milk affecting growth, cognition and the gut in child stunting' trial (MAGNUS). The trial aimed to assess the effects of large-quantity lipid-based nutrient supplements (LNS-LQ) containing milk protein and/or whey permeate on the growth and development of children with stunting. The trial protocol (13) and main results are published elsewhere (Mbabazi et al., 2022, under review). The study protocol was approved by the School of Medicine Research Ethics Committee at Makerere University and The Ugandan National Council of Science and Technology (UNCST). Consultative approval was given by the Danish National Committee on Biomedical Research Ethics. Prior to enrolment, caregivers provided written informed consent whereby understanding was assessed using a verbal questionnaire. A literate witness was present if the caregiver was illiterate.

Study sites and population

The district of Jinja of the Busoga sub-region is located on the northern shores of Lake Victoria. An estimated 29% of children in the region are stunted (14). Much of the agricultural land is used for commercial production of sugar cane, however, the majority of livelihoods are derived from subsistence farming, where maize is the staple crop (15).

During recruitment, mobile teams screened the villages surrounding the study sites at Buwenge and Walukuba health centres. Those living in the catchment area, aged between 12 and 59 months, with a height-for-age Z-score (HAZ) of less than -2 according to WHO growth standards (16), were invited to a study site for eligibility screening. Those identified with severe acute malnutrition (SAM) according to WHO criteria (17), were excluded and referred for treatment. Additional criteria for exclusion were medical complications requiring hospitalisation, obvious disability and a history of allergy to peanuts or milk. If the family planned to move from the catchment area within six months or if the household had previously been enrolled in MAGNUS or in another study, the child was excluded.

Data collection

Baseline data was collected at inclusion. Trained nutritionists used interviewer-administered questionnaires to enquire about diet including breastfeeding status, meal variety and frequency during the past 24 hours. The diet was classified according to the minimum dietary diversity score

(MDD), where at least five of eight food groups should be consumed for the diet to be considered adequately diverse (18). The same MDD cut-offs were applied irrespective of age group. A medical doctor conducted a clinical assessment which included a 14-day history of diarrhoeal symptoms and antibiotic use. Height and weight were measured in triplicate and the median value used. Height was measured to the nearest 0.1 cm, while standing with 5 body points in contact with a wooden height board (Weigh and Measure LLC, USA). For participants aged less than 24 months, length was measured. Bodyweight was measured to the nearest 100 g using a digital double weighing scale (SECA 876, Hamburg, Germany). The Z-scores were calculated twice; first for inclusion using WHO field growth charts and subsequently for analysis using STATA software; Igrowup (UNICEF, Data & Analytics, 2019).

Home visit and WASH assessment

With permission, study staff accompanied the caregiver and participating child home to conduct a WASH assessment. Dwellings were classified urban if the township exceeded 2000 persons. Staff used interviewer-administered questionnaires to enquire about family characteristics, education, income and household food insecurity. Food insecurity during the previous 30 days was assessed using the Household Food Insecurity Access Scale (HFIAS) and responses were later classified according to severity (19). Caregivers also indicated the proportion of income spent on food by separating a pile of dried beans into groups. Characteristics evaluated as part of the WASH assessment were; building materials used for the dwelling walls and floor, the availability of clean and accessible water, basic sanitation services and hygiene practices. The assessment was supported by an interviewer-administered questionnaire about toilet use, animal ownership and water access and treatment. Where possible, questionnaire responses were confirmed through observation. The Joint Monitoring Programme for Water Supply, Sanitation and Hygiene Household Wash Strategy informed the WASH assessment and its classifications for drinking water source and toilet type (20). Building materials used for the walls were classified as either brick/cement or other; sheet metal, thatch and mud. For the floors, building materials were classified as cement or other; wooden planks, mud, mud mixed with dung or dirt. Brick and cement were considered improved materials. Water treatment was adequate when reported as boiling or using appropriate chemical treatment. Treatment was inadequate if not reported or if reported as filtering through a cloth or leaving in the sun. Sharing was indicated when more than one household shared the same toilet facility. Handwashing was assessed by the presence of a handwashing station with water and/or soap.

Biological sample collection

Using a vacutainer butterfly kit, 6 mL of venous blood was collected into 4 mL serum, lithium-heparin and ethylene diamine-tetra acetic acid (EDTA) vacutainers. Caregivers were encouraged (not required) to bring the child in a fasted state, attending the visit in the early morning before feeding. At the time of blood draw, phlebotomists recorded the estimated time interval since the child had last fed. All children received porridge after blood sampling. Samples were transported for processing at room temperature (20-25°C). Approximately 200 µL of whole blood was used for rapid diagnostic tests (RDT) for malaria (SD bioline malaria Ag Pf, Abbott, USA), human immunodeficiency virus (HIV) using serial RDT (Determine HIV-1/2 [Abbott], STAT-PAK [Chembio Diagnostics], and SD Bioline HIV-1/2 [Standard Diagnostics]), and haemoglobin concentration (Hb201+, HemoCue, Sweden) according to the manufacturer's instructions. Haemoglobin (Hb) <110 g/L was used as a definition of anaemia (21) and results were used to direct treatment where appropriate. Remaining blood was centrifuged (EBA200, Hettich, Germany) at 3500 rpm for 10 minutes before the serum and plasma aliquots were transferred to cryotubes for storage at -20°C. Serum and plasma samples were transferred within 7 days to the Integrated Biorepository (IBRH3AU) in Kampala, for storage at -80°C. Samples were shipped on dry ice to the University of Copenhagen and subsequently to labs in Sweden, Germany and Denmark for analysis.

Analysis of plasma citrulline

Plasma citrulline was assayed by ultrahigh-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) at Chalmers University of Technology, Gothenburg, Sweden. To prepare the samples for analysis, 20 µL of 10% sulfosalicylic acid containing 400µmol/L norleucine was added to 80 µL of each plasma sample. Samples were vortex mixed and centrifuged before 10 µL of the supernatant was mixed with 40 µL of labelling buffer containing norvaline. From this, 10 µL of supernatant was recovered and 5 µL of diluted aTRAQ Δ8-reagent was added and subsequently mixed before incubation at room temperature for 30 minutes. Thereafter, 5 µL of hydroxylamine was added and the solution was incubated for a further 15 min at room temperature. A volume of 32 µL of reconstituted aTRAQ Internal Standard solution and 400 µL of water was added to the reaction mixture. This solution was mixed and transferred into vials for analysis by UHPLC-MS/MS. A quality control plasma sample was used to assess within and between batch precision. This control sample was injected at the beginning, at every tenth sample and at the end of each batch.

Samples were analysed using a Siex QTRAP 6500+ system (AB Sciex) with a Nexera UHPLC system (Shimadzu, Japan). A volume of 2 µL of sample was separated on a Waters BEH C18 column (150 x 2.1 mm, 1.7 µm) held at 50°C by using the following gradient: 0 min 2% B, 0-2.5 min 2-40% B, 2.5-3.9 min 40% B, 3.9-4.2 min 40-90% B, 4.2-6.0 min 90% B. Mobile phases were with water (A) and methanol (B), both containing 0.1% formic and 0.01% heptafluorobutyric acids, as mobile phases (total flow 0.4 mL/min). The mass spectrometer was set to monitor the transitions 324.2 to 121.1 m/z for citrulline and 316.2 to 113.1 m/z for the citrulline internal standard. The following ion source parameters were used: CUR 30, CAD MED, IS 5500, TEM 500, GS1 60 and GS2 50 and compound parameters: DP 30, EP 10, CE 30 and CXP 5.

Other biomarkers

Both serum C-reactive protein (s-CRP) and serum α1-acid glycoprotein (s-AGP) are upregulated during acute systemic inflammation. While s-CRP increases and decreases rapidly in response to stimuli, s-AGP tends to remain elevated for an extended duration and may be an indicator of chronic, sub-clinical inflammation. The somewhat arbitrary cut-offs >5 mg/L, >10 mg/L and >20 mg/L for s-CRP indicate increasing grades of acute systemic inflammation, while for s-AGP cut-offs of 0.8 - 1.2 g/L and ≥1.2 g/L indicate marginal and active systemic inflammation, respectively. Serum ferritin (s-ferritin) <12 µg/L and serum soluble transferrin receptor (s-sTfR) >8.3 mg/L, were indicators of depleted iron stores and peripheral iron deficiency, respectively. Serum retinol binding protein (s-RBP) <0.7 µmol/L was a proxy of low vitamin A status. The aforementioned were analysed by VitMin labs in Willstaedt, Germany, using sandwich-ELISA (22). Cobalamin was analysed from plasma samples using the Advia Centaur CP Immunoassay System (Siemens, Germany) at the Department of Clinical Medicine, Aarhus University Hospital, Denmark. Plasma cobalamin (p-cobalamin) of <148 pmol/L and 148-221 pmol/L indicated low and marginal levels, respectively (23). Insulin-like growth factor-1 (s-IGF1) was determined from serum with the Immulite2000 (Siemens, Germany) at the Department of Nutrition, Exercise and Sports, University of Copenhagen, Denmark and was log10 transformed for analysis.

Data handling and statistical methods

Data was double entered using EpiData Software (v3.1). Statistical analyses were made using R version 4.1.2 (R-Core team, 2021). We assessed associations between potential correlates and p-cit using multiple linear regression. The correlates explored were: age, sex, rural residence, diet (breastfeeding status, animal source foods, diet diversity and meal frequency), food insecurity (income spent on food and HFIAS classification), anthropometrics (HAZ, Weight-for-height Z-

score and weight-for-age Z-score), clinical and para-clinical factors (s-IGF1, history of diarrhoea and antibiotics, positive malaria RDT, s-CRP, s-AGP), household WASH characteristics (materials used for dwelling roof and floor, drinking water source, water treated, toilet improved, toilet shared, handwashing practices and animals owned and/or observed close to living quarters) and micronutrient status (p-cobalamin, s-RBP, s-ferritin, haemoglobin, s-sTfR and iron deficiency anaemia). S-ferritin and s-RBP were corrected for inflammation as described by Cichon et al. (24). We fit four models: a univariate model and multivariate models adjusted for age and sex (model 2), age, sex and fasting (model 3) and finally, age, sex, fasting and inflammation (model 4). Covariates included in what was considered the main analysis were; age, sex and fasting (model 3). To adjust for fasting, the duration since the child's last feed according to caregiver reports, was classified into three groups; <2, 2-5 and >5 hours since the child's last feed. We included an additional adjustment using continuous data from inflammatory markers s-AGP and s-CRP to account for the differences in citrulline that may be mediated through the inflammatory process (model 4). Finally, in a post-hoc sub-analysis we used a Pearson's chi-square test to determine whether there were changes in household handwashing practices before and after community level Covid-19 restrictions were enforced in Uganda. Due to the explorative nature of this study, we did not adjust for multiplicity and no imputations were made. Data was presented as β coefficients with 95% confidence intervals and p-values. A p-value of <0.05 was considered significant.

Results

Between February and September 2020, 7611 children were screened for stunting; 1112 were referred for eligibility screening and of these 750 were enrolled. The mean ±SD age was 32.0 ±11.7 months with 30% (222) below 24 months. The mean ±SD HAZ was -3.02 ±0.74 **(Table 1)**. Eight children (1%) had positive serology for HIV. Thirteen percent (n=95) were breastfed and for those no longer breastfeeding, the mean ±SD age at cessation was 15.8 ±6.2 months. Dietary diversity was inadequate among 74% (n=552) and was more prevalent among those above compared to below 24 months (81% vs 56%, P <0.001). Forty-five percent (n=333) lived in rural areas, mean ±SD household size was 5.4 ±2.1 and 41% (311) owned agricultural land.

Plasma citrulline was determined from 730 (97%) of the children enrolled. The 20 children without p-cit data had similar baseline characteristics, except that the majority were from rural areas (95%). Mean ±SD p-cit was 23.0 ±11.7 µmol/L with 44% (324) and 5% (39) of p-cit values below 20 µmol/L and 10 µmol/L, respectively. Compared with children who had fasted for more than 5 hours, those who fasted <2 and 2-5 hours were associated with a 3.24 (95%CI: -1.14; 7.62) and 2.04

(95%CI: 0.29; 3.79) µmol/L lower p-cit, respectively.

Increasing age in months, was associated with a 0.08 (95%CI: 0.004; 0.15) µmol/L increase in p-cit, while female sex was not associated (1.46 µmol/L, 95%CI:-0.25; 3.16). After adjustment for age, sex and fasting, rural residence was associated with a 2.33 (95%CI: 0.18; 4.49) µmol/L lower p-cit **(Table 2**, model 3**)**, but the association disappeared with adjustment for inflammation (model 4). Food insecurity was a correlate of p-cit but only became statistically significant in those who were moderately food insecure (-5.42 µmol/L, 95%CI: -9.69; -1.14). Neither breastfeeding nor any other dietary factors including dietary diversity and consumption of animal source foods were associated with p-cit.

Of the WASH characteristics, use of building materials other than brick and cement was associated with lower p-cit (**Table 3**, model 3), and these associations were only slightly attenuated with further adjustment for inflammation (model 4). The use of a non-improved toilet system was associated with 1.93 (95%CI 0.17; 3.69) µmol/L lower p-cit, but the association disappeared with adjustment for inflammation. There was no difference according to toilet sharing, the source of drinking water or water treatment methods. Compared to households with both soap and water available for handwashing, lacking one or both was associated with lower p-cit (model 3), but this disappeared with adjustment for inflammation. There was a pause in study recruitment during April and May 2020 due to the implementation of national Covid-19 restrictions across Uganda. Our sub-analysis of the handwashing practices of enrolled households showed that a greater proportion were using soap for handwashing after Covid-19 restrictions were enforced (9% vs 30%, P <0.001) (**Supplementary Table 1**). Compared to the months after these restrictions were implemented, those included before had a 3.81 (95%CI: 1.53; 6.09) µmol/L lower p-cit **(Supplementary Table 2**, model 3), which was reduced to 2.72 (95%CI: 0.45; 5.00) µmol/L after accounting for inflammation (model 4). Likewise, recruitment during the wet season was associated with 3.12 (95%CI: 1.26; 4.97) µmol/L lower p-cit, which reduced to 2.13 (95%CI: 0.29; 3.98) µmol/L with adjustment for inflammation. Keeping animals was not associated (1.25 µmol/L, 95%CI: -0.52; 3.02), but after adjustment for inflammation the estimate increased to 2.15 (95%CI: 0.42; 3.89) µmol/L higher p-cit. Observation of animals close to the residence was not associated.

History of diarrhoea and positive malaria RDT were associated with lower p-cit (model 3), but both associations attenuated with additional adjustment for inflammation (**Table 4**, model 4). With adjustment for age, sex and fasting, height-for-age Z-score was not associated with p-cit. However, an inverse association appeared with adjustment for inflammation; a 1.23 (95%CI: 0.07; 2.39)

µmol/L lower p-cit per unit increase in HAZ, or a 2.15 (95%CI: 0.44; 3.86) µmol/L higher p-cit in children with severe compared to moderate stunting (model 4**)**. Neither wasting nor underweight were associated with p-cit. There was a positive association with s-IGF1. For each log10 increase in s-IGF1 there was an 8.88 (95%CI: 5.09; 12.67) µmol/L higher p-cit **(Table 4)**.This association remained after adjustment for inflammation, albeit somewhat reduced (4.98 µmol/L, 95%CI: 0.94; 9.03).

Anaemia was associated with 1.95 (95%CI: -0.18; 3.72) µmol/L lower p-cit (**Table 5,** model 3), but this disappeared after adjustment for inflammation (model 4). None of the other markers used to define micronutrient deficiencies were associated with p-Cit.

Discussion

We found that food insecurity, malaria, diarrhoea, systemic inflammation, low s-IGF1, anaemia, the wet seasons and selected WASH characteristics including the use of a non-improved toilet system and the lack of soap for handwashing were associated with lower p-cit. Most associations attenuated however with additional adjustment for systemic inflammation. The exceptions were socio-economic factors, wet seasons and low s-IGF1, which were partially reduced.

We found that fewer hours fasted was associated with lower p-cit. Those who had fasted for less than 2 and 2-5 hours had lower levels compared to those fasted for more than 5 hours. In line with this, others have observed a transient postprandial decline in p-cit that returned to basal levels after three to four hours (25,26). Ideally, p-cit should be measured in the fasted state (25). Yet, few studies among children in low-income settings require fasting, as it may not be feasible. To account for fasting in this study we adjusted for the approximate number of hours since the last meal.

An increase in p-cit with age has been reported previously (27,28), by some accounts reaching a plateau at around five years (27). The age dependence is thought to reflect maturation of the intestinal-renal axis and the timed expression of the enzymes involved (29). However, no true reference intervals for citrulline exist and no cut-offs have been established to indicate reduced enterocyte mass in children under 5 years. The most readily used cut-off is 20.0 µmol/L (10). This cut-off has been shown to correlate with lower grades of histopathological damage in paediatric patients with celiac disease (12), but also to predict the severity of short bowel syndrome in adults by distinguishing between permanent and transient intestinal failure (25). On the other hand, some report that a threshold of 15.0 µmol/L in children (30) and adults (31) indicates a loss of function, while others use thresholds below 10.0 µmol/L (32,33). In this study, 44% of the children had p-cit

below 20.0 µmol/L and just over five percent below 10 µmol/L, indicating that this population is experiencing some form of reduced enterocyte mass. A recent study among children aged 2-5 years in Madagascar and the Central African Republic reported that in stunted children, 2.5% and 8.8% respectively, had p-cit below 7 µmol/L (32). Another study in Lao among younger children aged 6-24 months, 37% of whom were stunted, reported that 5.3% had p-cit below 17 µmol/L (34). In Burkina Faso, 70% of children aged 6-24 months had p-cit below 14 µmol/L (35). With such variation in both the cut-offs used and the proportions reported, it is clear that validation studies against biopsies would be necessary to understand which of these cut-offs most reflect reduced enterocyte mass in different populations with EED. In the research context, p-cit may be used together with functional tests to better understand malabsorption and mucosal repair in the context of EED.

There was a strong negative association between markers of systemic inflammation (s-CRP and s-AGP) and p-cit that warrants further consideration. Similar negative associations have been reported in studies among children who are both stunted or at risk of stunting (32,34). In addition, studies of other inflammatory conditions widely report the same inverse association between markers of systemic inflammation and p-cit (36–38), with few not finding an association (31,39). Citrulline and arginine are closely related and this may partly explain the association with systemic inflammation. Citrulline is the main precursor for endogenous arginine synthesis. Arginine, a conditionally essential amino acid, has a complex metabolism but plays an important immune modulating role in many tissues, as the main precursor in nitric-oxide synthesis. It has been suggested that for critically ill patients, low p-cit may result from increased requirements for arginine to produce nitric oxide during the inflammatory process (40) and in such cases, may not be a clear indication of low enterocyte mass. On the other hand, requirements for nutrients such as arginine increase during a state of chronic intestinal inflammation and/or growth and if they remain unmet, this can cause damage the intestinal mucosa (41). Given the complex relationship that exists between citrulline and systemic inflammation, we chose to include an additional adjustment to account for the differences in p-cit that may be confounded by or mediated through systemic inflammation. When analyzing p-cit, Wessels et al. chose to exclude children with s-CRP >10 mg/L (34). However, this risks excluding a proportion of children with intestinal damage and concurrent systemic inflammation. Low plasma citrulline could result from increased use of arginine in the nitric oxide cycle or loss of enterocyte mass in a state of nutrient deficiency, inflammation or a combination of these.

After adjustment for inflammation, associations with malaria and diarrhoea disappeared. This was expected as these effects are known to be mediated by systemic inflammation. Nevertheless, it is also possible that both malaria and diarrhoea have a direct effect on the gut via inflammation and exposure to intestinal pathogens, as reported by others (41). We found that associations related to minimising infection, such as handwashing with soap and improved toilet facilities were attenuated after accounting for the effects of inflammation. In the context of EED, the most widely accepted pathway of damage to the enterocytes is via exposure to pathogens, that cause local (42) and subsequent systemic inflammation (5). These WASH associations could be a reflection of this pathway. Interestingly, a greater proportion began handwashing with soap after Covid-19 lock-downs and this was associated with increased p-cit. The effect was not completely explained by inflammation however and seasonality may have played a role; where lower p-cit was associated with the wetter seasons. This is in line with what others have reported on seasonality for different EED markers (43).

We found a positive association between the growth factor s-IGF1 and p-cit. Linear growth and IGF-1 are both known to be downregulated in the presence of inflammation (44). Thus, we expected attenuation of the association in the inflammation-adjusted model. While partially reduced, the association with s-IGF1 remained significant. In contrast, we did not find an association between HAZ and p-cit, which was contrary to some studies (32,45), but not all (46,47). Surprisingly after adjusting for inflammation, a negative association appeared between HAZ and p-cit. This may be a chance finding, but would certainly be better explained had we included a non-stunted comparison group. Still, a positive association remained between s-IGF1 and p-cit. While no causal inferences can be made, it is at least biologically plausible that increases in s-IGF1 may encourage enterocyte proliferation and rejuvenation of intestinal crypts (48), and thus increase p-cit.

Dietary diversity was inadequate among a large proportion of children, and while poor dietary intake is thought to play a role in EED, dietary factors were not associated with p-cit. Of the micronutrient deficiencies explored, it was surprising that low serum cobalamin was not associated with p-cit, given its role in DNA synthesis and cell division (49). However, low serum cobalamin was not highly prevalent in this cohort. Still, we found a positive association between haemoglobin and p-cit. A similar finding had been reported in a recent study including both stunted and not stunted children (32), however, we added to this by showing that the relationship was driven by systemic inflammation. There was a tendency for socioeconomic factors such as food insecurity and poorer living standards to be associated with lower p-cit, even after adjustment for inflammation, supporting the notion that poverty is associated with EED (8).

The strengths of this study were its large sample size, high quality citrulline analysis, the near complete dataset, as well as the novel use of adjustments for fasting and inflammation to improve the interpretation of p-cit as a marker of enterocyte mass. On the other hand, the cross-sectional design limits any causal inference. Furthermore, we made many comparisons which could have resulted in chance findings. The lack of non-stunted controls was a limitation as well as the lack of other functional EED markers.

In conclusion, we found that a number of characteristics expected in populations with EED, were correlated with lower p-cit. These correlates included food insecurity, systemic inflammation, low s-IGF1, low haemoglobin and some WASH characteristics including a lack of soap for handwashing. Both younger age and fewer hours fasted were associated with lower p-cit and so should be accounted for in analysis. Finally, adjustment for systemic inflammation attenuated many associations, reflecting confounding, mediation or both. We propose that p-cit is a useful marker of mucosal regeneration in low-income settings. However, this study highlights the complex interplay between p-cit and systemic inflammation that warrants further investigation so as to improve the use and validity of p-cit for future research.

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H.P was part of research design, analyzed data and wrote the paper. J.M conducted the research and wrote the paper, R.M conducted the research and wrote the paper, O.S conducted the research and wrote the paper, M.F.O wrote the paper, C.M designed the research and wrote the paper, K.F.M designed the research and wrote the paper, C.R designed the research and wrote the paper, S.F wrote the paper, A.B wrote the paper, E.M wrote the paper, H.F designed the research and wrote the paper and B.G. designed the research, wrote the paper and had primary responsibility for final content. All authors have read and approved the manuscript.

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Table 1: Baseline characteristics of children with stunting[a]

Age, months	32 ±11.7	750
Sex, female	45% (338)	750
Anthropometrics		
Height-for-age Z-score	-3.02 ±0.74	750
Weight-for-height Z-score	-0.36 ±0.99	749
Weight-for-age Z-score	-1.93 ±0.85	749
Diet		
Currently breastfeeding	13% (95)	741
Inadequate dietary diversity[c]		
12 -23 months	59% (130)	222
24 -59 months	81% (422)	524
Household		
Rural residence	45% (333)	750
Own agricultural land	41% (311)	750
Own animals	50% (379)	750
Food secure[d]	4% (33)	750
Maternal age, years	26.4 ±6.1	692
Maternal education, primary or less	61% (444)	726
Paraclinical		
Malaria positive (rapid test)	40% (292)	737
Anaemia[b]	65% (479)	743
Insulin-like growth factor-1, ng/mL	37.4 (24.2; 53.3)	740
C-reactive protein, mg/L	1.56 (0.33; 8.25)	741
α1-acid glycoprotein, g/L	1.20 (0.88; 1.61)	741
Outcome		
Citrulline, µmol/L	23.0 ±11.7	730
Fasted <2 hrs	20.7 ±8.9	30
2-5 hrs	22.3 ±10.6	404
5+ hrs	24.2 ±13.1	296

[a]Data are presented as mean ±standard deviation, median (interquartile range) or % (n) and N. [b] Haemoglobin <110 g/L. [c]Minimum dietary diversity score. [d]Household food insecurity access scale

Table 2: Dietary and socioeconomic correlates of plasma citrulline (μmol/l) in children with stunting (n=730)

		Model 1 (Unadjusted)			Model 2 (Model 1 +age and sex)			Model 3[a] (Model 2 + fasting)			Model 4[b] (Model 3 + inflammation)		
	n	β	95% CI	P	β	95% CI	P	β	95% CI	P	β	95% CI	P
Residence	730												
Urban	396	Ref			Ref			Ref			Ref		
Rural	334	-2.24	-3.94; -0.55	0.01	-2.56	-4.28; -0.84	0.003	-2.33	-4.49; -0.18	0.03	-0.20	-2.39; 2.00	0.86
Household food insecurity[d]	730												
Food secure	33	Ref			Ref			Ref			Ref		
Mildly insecure	26	-4.35	-10.35; 1.64	0.15	-4.53	-10.51; 1.46	0.14	-4.64	-10.62; 1.33	0.13	-3.49	-9.30; 2.32	0.24
Moderately insecure	208	-5.02	-9.30; -0.73	0.02	-5.50	-9.79; -1.22	0.01	-5.42	-9.69; -1.14	0.01	-4.41	-8.57; -0.25	0.04
Severely insecure	463	-3.35	-7.46; 0.77	0.11	-3.73	-7.85; 0.39	0.08	-3.76	-7.87; 0.35	0.07	-2.68	-6.69; 1.34	0.19
Income spent on food	683												
<75%	565	Ref			Ref			Ref			Ref		
75% +	118	2.03	-0.30; 4.36	0.09	2.16	-0.17; 4.48	0.07	1.61	-0.79; 4.01	0.19	0.73	-1.63; 3.10	0.54
Diet													
Breastfeeding	726												
Yes	92	Ref			Ref			Ref			Ref		
No	634	2.46	-0.08; 5.01	0.06	1.50	-1.36; 4.35	0.30	1.37	-1.49; 4.23	0.35	2.34	-0.46; 5.15	0.10
Diet includes	730												
Meat, eggs or dairy	425	Ref			Ref			Ref			Ref		
No meat, eggs or dairy	305	0.31	-1.41; 2.03	0.72	0.07	-1.65; 1.80	0.93	0.42	-1.33; 2.17	0.64	1.03	-0.70; 2.75	0.24
Dietary diversity[c]	726												
Adequate	185	Ref			Ref			Ref			Ref		
Inadequate	541	0.75	-1.20; 2.70	0.45	0.35	-1.63; 2.33	0.73	0.45	-1.54; 2.43	0.66	1.16	-0.78; 3.10	0.24
Meal frequency	728												
3 +	668	Ref			Ref			Ref			Ref		
<3	60	0.37	-2.72; 3.46	0.82	0.35	-2.73; 3.44	0.82	0.42	-2.65; 3.50	0.79	0.78	-2.23; 3.79	0.61

[a]Adjustment for fasting based on categorical variable <2hrs, 2-5hrs and 5+hrs after a meal. [b]Inflammation: serum α1-acid glycoprotein and serum C-reactive protein. [c]Positive score is minimum 5/8 food groups (including breastmilk) eaten in past 24hrs [d] Based on household food insecurity access scale

Table 3: Household WASH correlates of plasma citrulline (µmol/l) in children with stunting (n=730)

		Model 1 (Unadjusted)			Model 2 (Model 1 +age and sex)			Model 3[a] (Model 2 + fasting)			Model 4[b] (Model 3 + inflammation)		
	n[c]	β	95% CI	P	β	95% CI	P	β	95% CI	P	β	95% CI	P
Dwelling materials													
Walls	730												
Cement or brick	488	Ref			Ref			Ref			Ref		
Thatch and other[d]	242	-1.89	-3.68; -0.09	0.04	-1.78	-3.58; 0.01	0.051	-2.31	-4.15; -0.47	0.01	-2.21	-4.00; -0.42	0.02
Floor	730												
Cement	280	Ref			Ref			Ref			Ref		
Dirt or mud[d]	450	-2.92	-4.65; -1.18	0.001	-3.14	-4.88; -1.40	<0.001	-2.88	-4.66; -1.09	0.002	-1.70	-3.50; 0.09	0.06
Drinking water													
Source	730												
Private tap	27	Ref			Ref			Ref			Ref		
Public tap	323	-3.02	-7.61; 1.56	0.20	-2.72	-7.29; 1.86	0.24	-2.86	-7.45; 1.72	0.22	-1.54	-6.02; 2.94	0.50
Public borehole	380	-4.22	-8.77; 0.34	0.07	-4.24	-8.79; 0.32	0.07	-3.75	-8.36; 0.85	0.11	-0.89	-5.44; 3.66	0.70
Treated	730												
Yes[e]	213	Ref			Ref			Ref			Ref		
No	517	-0.89	-2.75; 0.98	0.35	-1.11	-2.98; 0.75	0.24	-0.82	-2.72; 1.08	0.40	-0.06	-1.93; 1.81	0.95
Toilet[f]	728												
Improved	409	Ref			Ref			Ref			Ref		
Not improved	319	-2.09	-3.80; -0.39	0.02	-2.27	-3.98; -0.55	0.01	-1.93	-3.69; -0.17	0.03	-0.74	-2.49; 1.02	0.41
Toilet shared	713												
No	332	Ref			Ref			Ref			Ref		
Yes[g]	381	1.38	-0.35; 3.11	0.12	1.51	-0.22; 3.25	0.09	1.01	-0.89; 2.91	0.30	-0.11	-1.98; 1.76	0.91
Handwashing station	730												
Soap and water	186	Ref			Ref			Ref			Ref		
Water only	222	-2.59	-4.86; -0.32	0.03	-2.67	-4.92; -0.41	0.02	-2.53	-4.82; -0.25	0.03	-1.45	-3.71; 0.81	0.21
Neither soap nor water	322	-3.08	-5.18; -0.98	0.004	-3.24	-5.34; -1.13	0.003	-2.87	-5.17; -0.57	0.01	-0.64	-2.99; 1.72	0.60
Animals[h]	727												
Not kept, not observed	91	Ref			Ref			Ref			Ref		
Kept, not observed	138	1.11	-1.98; 4.20	0.48	0.95	-2.14; 4.04	0.55	1.21	-1.88; 4.31	0.44	2.59	-0.45; 5.63	0.09
Not kept, observed	241	2.02	-0.79; 4.84	0.16	1.98	-0.83; 4.79	0.17	1.48	-1.44; 4.41	0.32	0.80	-2.06; 3.66	0.58
Kept, observed	257	3.16	0.37; 5.95	0.03	2.84	0.04; 5.64	0.047	2.72	-0.08; 5.53	0.06	2.72	-0.01; 5.45	0.051

[a]Adjustment for fasting based on categorical variable <2hrs, 2-5hrs and 5+hrs after a meal. [b]Inflammation: serum α1 -acid glycoprotein + serum C-reactive protein as continuous variables. [d]Building materials sheet metal, thatch, wood planks, dirt and mud classified as other materials. [e]Yes: treatment by boiling or chemicals. [f]Improved: flush/pour flush toilets and pit latrines with slabs, Unimproved: pit latrines no slab, hanging or bucket latrines and open defecation. [g]Toilet facility shared with at least one other household. [h]Animals kept by household and/or observed roaming/tied up close to the living quarters.

Table 4: Anthropometric and clinical correlates of plasma citrulline (µmol/l) in children with stunting (n=730)

		Model 1 (Unadjusted)			Model 2 (Model 1 + age and sex)			Model 3[a] (Model 2 + fasting)			Model 4[b] (Model 3 + inflammation)		
	n[c]	β	95% CI	P	β	95% CI	P	β	95% CI	P	β	95% CI	P
Anthropometrics													
Height-for-age, Z-score[d]	730	0.03	-1.12; 1.17	0.96	-0.20	-1.36; 0.95	0.73	-0.33	-1.49; 0.83	0.57	-1.23	-2.39; -0.07	0.04
<-2 to -3	424	Ref			Ref			Ref			Ref		
<-3	306	0.73	-0.99; 2.45	0.41	1.01	-0.72; 2.73	0.25	1.17	-0.55; 2.90	0.18	2.15	0.44; 3.86	0.01
Weight-for-height, Z-score[d]	729	0.37	-0.48; 1.23	0.39	0.21	-0.65; 1.08	0.63	0.32	-0.54; 1.19	0.46	0.26	-0.58; 1.11	0.54
≥-2	692	Ref			Ref			Ref			Ref		
<-2	37	-1.42	-5.29; 2.45	0.47	-0.95	-4.83; 2.93	0.63	-1.15	-5.02; 2.73	0.56	-1.23	-4.98; 2.53	0.52
Weight-for-age, Z-score[d]	729	0.22	-0.78; 1.22	0.66	0.13	-0.86; 1.13	0.79	0.18	-0.82; 1.17	0.73	-0.26	-1.25; 0.72	0.60
≥-2	408	Ref			Ref			Ref			Ref		
<-2	621	-0.93	-2.64; 0.78	0.29	-0.79	-2.50; 0.92	0.36	-0.86	-2.56; 0.85	0.32	-0.15	-1.83; 1.52	0.86
Clinical and para-clinical													
Diarrhoea[e]	730												
No	531	Ref			Ref			Ref			Ref		
Yes	199	-1.90	-3.80; -0.004	0.049	-1.67	-3.58; 0.25	0.09	-1.91	-3.82; 0.01	0.051	-1.00	-2.89; 0.89	0.30
Antibiotics[e]	468												
No	256	Ref			Ref			Ref			Ref		
Yes	212	-0.38	-2.48; 1.73	0.73	-0.31	-2.42; 1.80	0.77	-0.53	-2.72; 1.67	0.64	-1.46	-3.62; 0.70	0.18
Malaria rapid test	720												
Negative	434	Ref			Ref			Ref			Ref		
Positive	286	-2.95	-4.67; -1.23	<0.001	-3.22	-4.95; -1.49	<0.001	-2.95	-4.73; -1.18	0.001	-0.59	-2.47; 1.30	0.54
Serum CRP, mg/L	724												
< 5	479	Ref			Ref			Ref			-	-	-
5 - 10	86	-2.21	-4.83; 0.41	0.10	-2.16	-4.77; 0.46	0.11	-1.91	-4.54; 0.71	0.15			
10 - 20	60	-5.66	-8.73; -2.59	<0.001	-5.76	-8.82; -2.70	<0.001	-5.64	-8.70; -2.58	<0.001	-	-	-
20 +	99	-6.93	-9.40; -4.46	<0.001	-7.07	-9.54; -4.60	<0.001	-6.93	-9.41; -4.45	<0.001			
Serum AGP, g/L	724												
< 0.8	135	Ref			Ref			Ref			-	-	-
0.8 - 1.2	232	-3.26	-5.69; -0.83	0.01	-3.37	-5.80; -0.95	0.01	-3.46	-5.89; -1.02	0.01			
1.2 +	357	-6.63	-8.90; -4.37	<0.001	-6.56	-8.83; -4.30	<0.001	-6.47	-8.77; -4.18	<0.001	-	-	-
Log_{10} (Serum IGF-1), ng/mL	730	9.08	5.63; 12.53	<0.001	8.66	4.86; 12.47	<0.001	8.88	5.09; 12.67	<0.001	4.98	0.94; 9.03	0.02

[a]Adjustment for fasting based on categorical variable <2hrs, 2-5hrs and 5+hrs after a meal. [b]Inflammation: serum α1 -acid glycoprotein and serum C-reactive protein as continuous variables. [c]May not sum up to 730 due to missing data. [d]Continuous variable [e]History in the 14 days prior. AGP: α1 -acid glycoprotein; CRP: C-reactive protein; IGF-1: Insulin-like-growth factor-1.

Table 5: Micronutrient correlates of plasma citrulline (µmol/l) in children with stunting (n=730)

		Model 1 (Unadjusted)			Model 2 (Model 1 +age and sex)			Model 3[a] (Model 2 + fasting)			Model 4[b] (Model 3 + inflammation)		
	n[c]	β	95% CI	P	β	95% CI	P	β	95% CI	P	β	95% CI	P
Haemoglobin, g/L	727												
110 +	256	Ref			Ref			Ref			Ref		
<110	471	-2.40	-4.15; -0.65	0.01	-2.16	-3.92; -0.39	0.02	-1.95	-3.72; -0.18	0.03	-0.33	-2.12; 1.45	0.71
Serum ferritin, µg/L[d]	724												
25+	113	Ref			Ref			Ref			Ref		
12-24	230	-0.10	-2.74; 2.54	0.94	0.10	-2.54; 2.74	0.94	0.24	-2.40; 2.87	0.86	1.08	-1.47; 3.64	0.41
<12	381	-0.71	-3.17; 1.75	0.57	0.07	-2.50; 2.64	0.96	-0.21	-2.79; 2.36	0.87	0.72	-1.79; 3.22	0.57
Serum sTfR, mg/L	724												
<= 8.3	272	Ref			Ref			Ref			Ref		
>8.3	452	-2.00	-3.76; -0.25	0.03	-1.48	-3.33; 0.36	0.11	-1.49	-3.33; 0.35	0.11	-0.59	-2.40; 1.21	0.52
Plasma cobalamin, pmol/L	706												
222+	539	Ref			Ref			Ref			Ref		
148-221	142	0.33	-1.84; 2.50	0.76	0.43	-1.73; 2.59	0.70	0.84	-1.34; 3.02	0.45	1.29	-0.86; 3.43	0.24
<148	25	1.92	-2.78; 6.63	0.42	2.11	-2.57; 6.80	0.38	2.69	-2.01; 7.38	0.26	3.13	-1.46; 7.72	0.18
Serum RBP, µmol/L[d]	724												
0.7+	597	Ref			Ref			Ref			Ref		
<0.7	127	-0.99	-3.23; 1.25	0.39	-0.96	-3.20; 1.27	0.40	-0.89	-3.13; 1.34	0.43	1.21	-3.37; 0.96	0.27

[a]Adjustment for fasting based on categorical variable <2hrs, 2-5hrs and 5+hrs after a meal. [b]Inflammation: serum α1 -acid glycoprotein + serum C-reactive protein as continuous variables.
[c]May not sum up to 730 due to missing data. [d]Corrected for inflammation according to Cichon et al. (2017) . RBP: Retinol binding protein; sTfR: Soluble transferrin receptor

Table S1: Handwashing in relation to Covid19 restrictions before and after pause in recruitment

	Pre-covid (n=153)	During covid (n=577)	95% CI	p-value
None	48% (74)	43% (248)	-0.04; 0.14	0.30
Soap only	1% (1)	1% (4)	-0.01; 0.01	1.0
Water only	41% (64)	26% (153)	0.07; 0.25	<0.001
Water + Soap	9% (14)	30% (172)	-0.27; -0.15	<0.001

Table S2: Season and time of recruitment as correlates of plasma citrulline (µmol/L) in children with stunting (n=730)

		Model 1 (Unadjusted)			Model 2 (Model 1 +age and sex)			Model 3[a] (Model 2 + fasting)			Model 4[b] (Model 3 + inflammation)		
	n[b]	β	95% CI	p-value	β	95% CI	p-value	β	95% CI	p	β	95% CI	p
Recruited during	730												
Dry season	488	Ref			Ref			Ref			Ref		
Wet season[c]	242	-2.41	-4.20; -0.61	0.01	-2.29	-4.09; -0.50	0.01	-3.12	-4.97; -1.26	0.001	-2.13	-3.98; -0.29	0.02
Recruited	730												
February	35	Ref			Ref			Ref			Ref		
March	118	2.89	-1.49; 7.27	0.20	2.62	-1.76; 7	0.24	3.08	-1.28; 7.44	0.17	2.82	-1.47; 7.12	0.20
June	105	2.41	-2.04; 6.85	0.29	2.07	-2.39; 6.54	0.36	4.18	-0.41; 8.77	0.07	2.44	-2.09; 6.97	0.29
July	219	5.15	1.00; 9.29	0.01	4.83	0.67; 8.99	0.02	6.81	2.53; 11.08	0.002	5.38	1.15; 9.6	0.01
August	164	5.61	1.38; 9.85	0.01	5.42	1.18; 9.66	0.01	7.47	3.10; 11.84	<0.001	6.30	1.99; 10.61	0.004
September	89	2.44	-2.10; 6.98	0.29	2.37	-2.18; 6.92	0.31	4.62	-0.06; 9.31	0.053	3.91	-0.71; 8.54	0.10
COVID19 lock-downs	730												
Feb-March	153	-2.13	-4.21; -0.06	0.04	-2.11	-4.19; -0.03	0.047	-3.81	-6.09; -1.53	0.001	-2.72	-5.00; -0.45	0.02
June-September	577	Ref			Ref			Ref			Ref		

[a.] Adjustment for fasting based on categorical variable <2hrs, 2-5hrs and 5+hrs after a meal. [b.] Inflammation adjustment with serum α1 -acid glycoprotein and serum C-reactive protein as continuous variables. [c.] May not sum up to 730 due to missing data. [d.] Wet season months were end February, March and September and dry season months were June to August

10.3 Paper III

Hannah Pesu, Joseph Mbabazi, Rolland Mutumba, Otto Savolainen, Hanne Frøkiær, Peter Riber Johnsen, Mette Frahm Olsen, Christian Mølgaard, Kim F. Michaelsen, Christian Ritz, Suzanne Filteau, André Briend, Ezekiel Mupere, Henrik Friis, Benedikte Grenov

Effects of lipid-based nutrient supplements on gut markers: exploratory outcomes from a randomised trial

(Manuscript prepared for submission)

Effects of lipid-based nutrient supplements on gut markers: exploratory outcomes from a randomised trial

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Conflicts of Interest and Source of Funding

HF and CM received research grants from ARLA Food for Health Centre, HF, CM and BG received research grants from the Danish Dairy Research Foundation, CM and KFM from Arla Foods Amba. HF, BG, CM, KFM and SF have research collaborations with Nutriset, a producer of LNS products, and patent owner; HP received a research grant from Nutriset. Other authors declare no financial relationships with any organisations that might have an interest in the submitted work in the previous three years, and declare no other relationships or activities that could appear to have influenced the submitted work. The trial was supported by Arla Food for Health, Danish Dairy Research Foundation, Augustinusfonden, Læge Sofus Carl Emil Friis og Hustru Olga Doris Friis' Legat, A. P. Møller Fonden til Lægevidenskabens Fremme and Nutriset.

Trial registration

ISRCTN13093195

List of abbreviations

EED	Environmental enteric dysfunction
ELISA	Enzyme Linked Immunosorbent Assay
f-MPO	Faecal myeloperoxidase
HAZ	Height or length-for-age z-score
IF	Intrinsic factor
LNS	Lipid-based nutrient supplements
MAGNUS	Milk affecting growth, cognition and the gut in child stunting
MP	Milk protein isolate
p-cit	Plasma citrulline
p-cobalamin	Plasma cobalamin
p-folate	Plasma folate
p-MMA	Plasma methylmalonic acid
s-AGP	Serum α_1-acid glycoprotein
s-CRP	Serum C-reactive protein
s-ferritin	Serum ferritin
s-IGF1	Serum insulin-like-growth factor-1
s-RBP	Serum retinol binding protein
s-sTfR	Serum soluble transferrin receptor
WASH	Water, sanitation and hygiene
WP	Whey permeate

Keywords

Environmental enteric dysfunction, malabsorption, cobalamin, citrulline

What is known?

- Current nutrition interventions seem inadequate to encourage normal growth in children; environmental enteric dysfunction (EED) is thought to play a role.
- EED may create or exacerbate problems of malabsorption and micronutrient deficiency.

What is new?

- There was no effect of lipid-based nutrient supplements (LNS), or ingredients milk protein and whey permeate on plasma citrulline, a marker of enterocyte mass. There was a greater increase in faecal myeloperoxidase, a marker of intestinal inflammation, among those supplemented with LNS, compared to no supplementation.
- Our data suggests that low enterocyte mass reduces the beneficial effect of LNS on B12 status.

Abstract

Objectives: To examine the effects of lipid-based nutrient supplements (LNS) containing milk protein (MP) and whey permeate (WP) on markers of intestinal inflammation and enterocyte mass and to explore whether gut status modifies the effects of LNS on growth and micronutrient markers.

Methods: In a 2x2 factorial trial among stunted children aged 12-59 months in Uganda, children were randomized to LNS (100 g/d for 12 weeks) containing MP or soy protein and WP or maltodextrin or to no supplementation. Linear mixed-effects models were used to explore faecal myeloperoxidase (f-MPO) and plasma citrulline (p-cit) as outcomes and modifiers of the intervention effect (ISRCTN13093195).

Results: Of 750 children, mean±SD age was 32.0±11.7 months and height-for-age Z-score was -3.02±0.74. Neither MP nor WP had effects on p-cit or f-MPO. Compared to the unsupplemented group, LNS had no effect on p-cit (1.30 μmol/L, 95%CI: -1.03; 3.64) but produced an 82% (95%CI: 12; 196) greater increase in f-MPO. In sensitivity analysis, 245 individuals with recent diarrhoea were removed and the effect of LNS on f-MPO disappeared (20%, 95%CI: -29; 112).

The effect of LNS on cobalamin (B12) status was reduced in children with p-cit <20 μmol/L; whereby there was a lesser change in plasma cobalamin (0.80, 95%CI: 0.65; 0.98) and a greater change in plasma methylmalonic acid (1.59, 95%CI: 1.13; 2.25).

Conclusion: We found no effect of LNS on enterocyte mass and a possible negative effect of LNS on intestinal inflammation. The effect of LNS on cobalamin status was lessened in those with low enterocyte mass.

Introduction

More than one in every fifth child under five years is stunted [1]. Stunting is associated with increased risk of infection, developmental delays and poor educational attainment as well as chronic disease and reduced working capacity later in life [1]. Environmental enteric dysfunction (EED) may be an important factor contributing to growth faltering [2]. EED is an asymptomatic inflammatory disorder of the small intestinal mucosa, characterised by villous atrophy, crypt hyperplasia and microbial translocation [3]. Data from biopsies and biomarkers alike suggests that EED affects a substantial proportion of children living in low-income settings [4]. Two factors in particular may be driving EED [5–7]; poor nutrition and a high enteropathogen burden. In food insecure settings the diet often lacks quality and variety; limiting access to the nutrients that are essential for growth, development and/or intestinal repair [8]. Concurrent infections increase nutrient requirements and promote a cycle of epithelial damage and inflammation [9, 10], that likely exacerbates or creates micronutrient deficiencies.

Recent trials that aimed to prevent stunting by targeting enteropathogen exposure through improved water, sanitation and hygiene (WASH) and diet with small-quantity lipid-based nutrient supplements (LNS), found no effect of WASH and only small effects of LNS on linear growth [11, 12]. It was concluded that transformative WASH approaches would be necessary to sufficiently reduce the pathogen burden in these settings [13]. In a similar way, perhaps small-quantity LNS interventions have been insufficient to meet the nutrient requirements for growth and intestinal repair in a population with EED. Consequently, there is a need to explore the interacting and possibly compounding effects of diet and enteropathy.

We examined the effects of 12 weeks of supplementation with large-quantity LNS containing milk protein (MP) and/or whey permeate (WP) on plasma citrulline (p-cit), a marker of enterocyte mass, and faecal myeloperoxidase (f-MPO), a marker of intestinal inflammation, in children with stunting. Furthermore, we explored whether the state of the gut, as measured by these markers, modified the effects of LNS on growth and micronutrient status.

Methods

Study design

This intervention study examined exploratory outcomes as part of the 'Milk affecting growth, cognition and the gut in child stunting' trial (MAGNUS) [14]. Briefly, MAGNUS was a randomised, double-blind, two-by-two factorial trial that aimed to assess the effects of large-quantity LNS

containing MP and/or WP on growth and development in stunted children. The primary outcomes were height and knee-heel length, secondary outcomes included cognitive development, and results are published elsewhere (Mbabazi et al., Under review). Ethical approval was obtained from Makerere University, The Ugandan National Council of Science and Technology and consultative approval obtained from the Danish National Committee on Biomedical Research Ethics. All caregivers gave written informed consent and a literate witness was present when there were indications of illiteracy.

Study sites and population

Between February and September 2020, mobile teams screened for stunting in the villages surrounding chosen study sites Buwenge and Walukuba health centres, Jinja, East Central Uganda. Children living in the catchment area, aged 12 to 59 months with a height-for-age z-score (HAZ) of <-2 according to WHO growth standards [15], were invited for eligibility screening. Those with severe acute malnutrition according to WHO criteria [16], were excluded and referred for treatment. Additional exclusion criteria were complications requiring hospitalisation, history of allergy to peanuts or milk, disability that impeded eating or measurement, plans to move from the area, previous enrollment of any child in the household or enrollment in another study at the time.

Intervention

Participants were randomly allocated to one of five arms (1:1:1:1:1); either to one of the four LNS: +MP+WP, +MP-WP, -MP+WP, -MP-WP, or to no supplement (4:1). The comparators were soy protein isolate for MP and maltodextrin for WP. The LNS was produced by Nutriset (Malaunay, France) as individually packaged 100 g (530-535 kcal) sachets of fortified paste based on peanuts. Rations of one sachet per day were supplied to caregivers fortnightly for 12 weeks. Those not receiving LNS received laundry soap at these intervals. The intervention composition is described in **Supplemental Digital Content #1** and details of randomisation and blinding are reported in **Supplemental Digital Content #4**.

Data collection

At baseline, information was collected on socio-demographics, breastfeeding, minimum dietary diversity score, household food insecurity access scale, and 14-day morbidity recall, using interviewer-administered questionnaires. At this time, trained staff visited households to assess water, sanitation and hygiene characteristics as described in detail elsewhere [14].

Outcomes

The main effects of LNS containing MP and/or WP as well as related subgroup effects were assessed on exploratory markers p-cit and f-MPO. Subgroup analysis of baseline p-cit and f-MPO as modifiers of effects was assessed for the following growth and micronutrient-related outcomes: height, weight, knee-heel length, HAZ, weight-for-height Z-score and serum insulin-like growth factor-1 (s-IGF1), haemoglobin, serum ferritin (s-ferritin), plasma folate (p-folate), serum soluble transferrin receptor (s-sTfR), plasma cobalamin (p-cobalamin), plasma methylmalonic acid (p-MMA) and serum retinol binding protein (s-RBP). For these outcomes, the effect estimates are the differences in change between intervention groups from baseline to week 12

Height and weight were measured in triplicate, and the median value was used. Height was measured while standing on a wooden height board (Weigh and Measure LLC, USA) to the nearest 0.1 cm; for those <24 months, length was measured. A knee-heel caliper (Mitutoyo, Neuss, Germany), was used to collect five consecutive knee-heel length measurements, of which the median was used. Body weight was measured to the nearest 100 g using a digital double weighing scale (SECA 876, Hamburg, Germany).

Biological sample collection and analysis

Blood and stool samples were collected at baseline and 12 weeks. The estimated time interval since the child had last fed was recorded to indicate fasting status. A lab technician performed rapid diagnostic tests for malaria (SD bioline malaria Ag Pf, Abbott, USA), human immunodeficiency virus using serial rapid tests (Determine HIV-1/2 [Abbott], STAT-PAK [Chembio Diagnostics], and SD Bioline HIV-1/2 [Standard Diagnostics]), and haemoglobin concentration (Hb201+, HemoCue, Sweden) according to the manufacturers' instructions. Haemoglobin <110 g/L indicated anaemia. Stool collection kits and individual instruction on their use was given to caregivers. All samples were returned within 2 days of the scheduled visit. Approximately 1g of stool was collected in a screw-top tube containing 4ml of StayRNA (A&A Biotechnology, Gdynia, Poland) or RNAlater (Sigma-Aldrich, Darmstadt, Germany). Stool sample consistency and history of diarrhoea were recorded with the aid of a modified Bristol Stool Scale

Plasma citrulline was measured at Chalmers University of Technology, Gothenburg, Sweden using a Siex QTRAP 6500+ system (AB Sciex) with a Nexera Ultra-High-Performance Liquid Chromatography (UHPLC) system (Shimadzu). Sample preparation and analysis was based on the commercial aTRAQ kit for analysis of amino acids in physiological fluids (AB Sciex) [17], with certain modifications as reported elsewhere (Pesu et al., Unpublished). F-MPO was analysed at the

University of Copenhagen using a commercially available sandwich Enzyme Linked Immunosorbent Assay (ELISA) kit according to the manufacturer's instructions (Human Myeloperoxidase DuoSet, R&D systems, Minneapolis, MN). The following biological markers were analysed by VitMin labs in Willstaedt, Germany, using sandwich-ELISA [18]: serum C-reactive protein (s-CRP) and serum α_1-acid glycoprotein (s-AGP), both markers of systemic inflammation, s-ferritin a marker of iron stores, s-sTfR, indicating peripheral iron deficiency and serum retinol binding protein (s-RBP), an indicator of vitamin A status. Both s-ferritin and s-RBP were corrected for inflammation using the method described by Cichon et al [19]. P-cobalamin, p-MMA and p-folate were analysed using the Advia Centaur CP Immunoassay System (Siemens, Germany) at the Department of Clinical Medicine, Aarhus University Hospital, Denmark [20]. Together, p-cobalamin and p-MMA are markers of cobalamin (vitamin B12) status. S-IGF1 was determined using the Immulite2000 (Siemens, Germany) at the University of Copenhagen, Denmark. Further description of sample collection and processing are described in **Supplemental Digital Content #5.**

Statistical methods

Participant data was collected using paper case report forms and double entered using EpiData Software (Epidata Association, Odense, Denmark). Z-scores were calculated using STATA software; Igrowup (UNICEF, Data & Analytics, 2019). Statistical analyses were made using R version 4.1.2 (R-Core team, 2021) according to an intention-to-treat (ITT) approach. Analyses were adjusted for the baseline level of the outcome, age, sex, season and random effects; id, site (and batch number for citrulline only). Missing values were not imputed. For right-skewed outcomes a logarithm transformation was applied, implying that results should be understood in terms of fold changes or ratios of the fold changes for stratum specific values over time. P-cit was corrected for fasting status and inflammation as previously described [19], with further detail in **Supplemental Digital Content #6.** A p-value of <0.05 was considered significant.

The effect of milk in LNS on EED markers

Linear mixed models were used to assess the effects of LNS containing MP or WP on the change in p-cit and f-MPO from baseline to 12 weeks. First we tested for interactions between MP vs WP. In case of no interaction, we tested for effects of +MP vs -MP and +WP vs -WP on p-cit and f-MPO. Additionally, we compared LNS irrespective of ingredient against the unsupplemented group. Sensitivity analysis was conducted to evaluate whether exclusion of those with a history of diarrhoea impacted the estimated effect of LNS on p-cit and f-MPO.

Subgroup analyses

We assessed for effect modification using linear mixed models with interaction between LNS containing MP or WP and then in LNS irrespective of ingredient and i) sex, ii) breastfeeding status, iii) severity of stunting being moderate (≥-3 HAZ) or severe (<-3 HAZ), grade of systemic inflammation being high for iv) s-CRP (>10 mg/L) or v) s-AGP (≥1.2 g/L), vi) recent diarrhoea and vii) stool sample classified as diarrhoea. Additionally, we explored whether baseline p-cit <20µmol/L and f-MPO >2000 ng/L modified the effects of LNS on growth and micronutrient status.

Results

Between February and September 2020, 7611 children were screened for stunting; 1112 were referred for eligibility screening and 750 were included in the trial **(Supplemental Digital Content #2)**. Of those included, 55% (n=412) were male, the mean ±SD age was 32.0 ±11.7 months and HAZ was -3.02 ±0.74. Half of children (n=368) had s-AGP >1.2 g/L, 22% (n=163) had s-CRP >10 mg/L and 64% (n=479) had anaemia. Twenty-six percent (n=194) met dietary diversity requirements. The majority had access to an improved water source (n=748), but only 4% (n=29) to a private tap. The randomization resulted in baseline equivalence (**Table 1)**.

The effect of milk in LNS

There was no interaction between MP and WP for p-cit or f-MPO (p >0.2), hence the main effect of each intervention was assessed independently. Neither MP nor WP had an effect on p-cit (MP: -0.97 µmol/L, 95%CI: -2.87; 0.93; WP: 1.07 µmol/L, 95%CI: -0.83; 2.97) or f-MPO (MP: 1.03, 95%CI: 0.67; 1.60; WP: 1.03 µmol/L, 95%CI: 0.66; 1.59). Over the 12 weeks, p-cit decreased in both the supplemented and unsupplemented groups, and there was no effect of the LNS on p-cit (1.30µmol/L, 95%CI: -1.03; 3.64) **(Table 2**). However, the LNS was associated with an 82% (95%CI: 12; 196) greater increase in f-MPO; there was no change in those who were supplemented (0.99, 95%CI: 0.79; 1.23) but a decrease over time in the control group (0.54, 95%CI: 0.35; 0.84). There were no differences between the unadjusted and adjusted models for both outcomes. In sensitivity analysis where individuals with a history of diarrhoea in the past two weeks were excluded (removed baseline n=199, endpoint n=36), the effect estimate remained unchanged for p-cit. However, the estimate for f-MPO was reduced to 20% (95%CI: -29; 112) after removing those with a history of diarrhoea (baseline n=207, endpoint n=38), and there was no longer a difference between the supplemented and unsupplemented groups.

Subgroup effects

Among those receiving LNS, MP was associated with a 4.69 (95%CI: 0.88; 8.50) µmol/L greater increase in p-cit for girls (data not shown). There was no difference in the effect of LNS according to systemic inflammation indicated by s-CRP and s-AGP **(Table 3)**. However, the LNS increased f-MPO in those with recent or current diarrhoea, but not in those without diarrhoea (interaction, p <0.04). There were no other subgroup effects of LNS or LNS containing MP or WP on p-cit and f-MPO (data not shown).

Gut as a modifier of the effect of LNS

At baseline, 26% (n=194) had f-MPO >2000 ng/mL and 15% (n= 109) had p-cit <20 µmol/L. Neither p-cit nor f-MPO modified the effects of the LNS intervention on growth outcomes **(Supplemental Digital Content #3)**. In those receiving LNS, p-cobalamin increased over the 12 weeks, as reported elsewhere (Mutumba et al., Unpublished). However, the effect of LNS on both markers of cobalamin status was modified by baseline p-cit (interaction p≤0.03). There was a reduced effect of LNS on p-cobalamin (-20%, 95%CI: -2; -35) and an increased effect on p-MMA (59%, 95%CI: 13; 125) in those with low baseline p-cit **(Table 4)**. Likewise, f-MPO >2000 ng/mL tended to reduce the effects of LNS on markers of cobalamin status, but was not significant (interaction p>0.07).

Discussion

There was no effect of the LNS on p-cit, but there was a greater increase in f-MPO among those receiving 12 weeks of supplementation. Compared to the comparators used, we found no effect of MP or WP on intestinal markers p-cit and f-MPO. We found evidence that enterocyte mass modifies the effect of the LNS intervention on cobalamin status. Comparing those with high to low p-cit at baseline, the latter experienced a 20% reduced effect of the LNS on p-cobalamin and a 59% relative increase in p-MMA. A similar reduction in the effect of LNS on p-cobalamin and an increase in p-MMA was reflected in those with high f-MPO, albeit to a lesser extent.

Although we provided a large-quantity LNS, the lack of effect on EED markers is not dissimilar to previous studies providing small-quantity LNS. Two large birth cohort trials in Zimbabwe and Bangladesh in which children were randomised to small-quantity LNS from 6 to 18 months, concluded that LNS did not impact any EED biomarkers, including p-cit [21] and f-MPO [21, 22]. Likewise, a trial in Bangladesh providing daily small- to medium-quantity LNS for children from 6 months, reported at 18 months that EED markers including f-MPO were not improved [23]. In this

study, f-MPO decreased over time to a greater extent in the unsupplemented group. However, with removal of diarrhoeal samples in sensitivity analysis, a difference could no longer be seen between groups. Interestingly, we found a greater increase in f-MPO among those receiving LNS who had current or recent diarrhoea and this may be an indicator that LNS has an inflammatory effect, which has also been reported by others [24].

This large-quantity LNS was in itself inadequate to improve markers of enterocyte mass and intestinal inflammation. In-vivo models have demonstrated that neither an insufficient diet nor exposure to bacteria in themselves are a cause of enteropathy, but it is the interaction between these two factors that seems to drive EED [5]. For populations living in conditions of frequent pathogen exposure, chronic inflammation and perhaps malabsorption, it is unclear whether recommended nutrient intakes would be sufficient. It is perhaps only with a transformative WASH approach combined with quality nutrition interventions, that we will see improvements in EED.

Interestingly, the results of two markers, p-cobalamin and p-MMA indicate that the beneficial effect of LNS on cobalamin status is reduced in those with low baseline p-cit. Cobalamin is absorbed as a complex with intrinsic factor (IF) in the terminal ileum, part of the intestine reportedly unaffected by EED [8]. Therefore, rather than low enterocyte mass *per se* being responsible for the reduced effect on cobalamin status, it is more likely driven by other factors associated with low enterocyte mass. These factors could be for example, the excessive presence of bacteria or parasites in the small intestine [25].

Children living in low-income settings are predisposed to small intestinal bacterial overgrowth [26] and intestinal parasitic infections. Bacterial overgrowth has consistently been associated with stunting [9, 27, 28] and has been implicated in EED [26, 29]. Although the role of bacterial overgrowth in EED remains unclear [28, 30], it is hypothesised to drive local inflammation, epithelial damage and impaired absorptive function [31–33]. Indeed, bacterial overgrowth is a well-established cause of cobalamin deficiency. Bacteria in the upper small intestine outcompete host IF and sequester cobalamin, thus reducing the amount of IF-bound cobalamin that reaches the terminal ilium for absorption [34]. Likewise, small intestinal helminths (eg *Ascaris lumbricoides* and hookworm) are associated with EED [35], and likely compete for host cobalamin. Although data on the association between endemic intestinal helminths and cobalamin status is lacking, evidence suggests that the fish tapeworm (*Diphyllobothrium latum*) can cause severe deficiency by competing for cobalamin in the small intestine [36]. In addition, intestinal protozoa are associated with compromised intestinal barrier function [37] and may also compete for cobalamin. In both this study (Mutumba et al.

Unpublished) and a study among young children in Burkina Faso, we found an association between a positive malaria test and low serum cobalamin status [38]. We speculate that the relationship is due to an uptake of cobalamin from the blood by the plasmodium parasite, known to express cobalamin-dependent methionine synthase [39]. These findings may be reflecting an association between reduced enterocyte mass and cobalamin-sequestering pathogens in the proximal small intestine.

Still, some malabsorption of cobalamin may be directly related to loss of enterocyte mass. Patients with untreated coeliac disease commonly experience villous atrophy as a result of chronic intestinal inflammation and a greater proportion are reported to have insufficient cobalamin status compared to the normal population [40, 41]. Furthermore, these insufficiencies have been shown to improve with transition to a gluten-free diet and thus reparation of the intestinal mucosa [42]. Since cobalamin is an essential nutrient for DNA synthesis and cell division, malabsorption has implications for rapidly dividing cells, including the small intestinal enterocytes. As such, malabsorption of cobalamin may be contributing to the pathogenesis of EED, perhaps also worsening malabsorption.

The strengths of this study were the factorial randomised design, the relatively large and near complete dataset and the inclusion of a non-supplemented control group. It was a strength that we had data on both p-cobalamin and p-MMA that confirm cobalamin status. While unlikely, we cannot rule out that the laundry soap given to families of unsupplemented children conferred an effect and that this would have led to an underestimation of the true effect of the LNS intervention. Another important limitation was that stool samples were collected in fixative (RNAlater and StayRNA), and this is likely the reason for larger variation/standard deviations in f-MPO results. Finally, the differences between groups may be underestimated since we did not measure f-MPO according to the dry weight of the sample or exclude diarrhoeal samples from stool analysis. Diarrhoea can dilute the sample and so reduce the amount of stool markers measured in the sample.

In conclusion, there was no effect of MP or WP contained in large-quantity LNS, on markers of intestinal inflammation or enterocyte mass. There was no effect of the LNS itself on enterocyte mass but a possible negative effect of LNS on intestinal inflammation. Interestingly, the beneficial effect of LNS on markers of cobalamin status was reduced in those with low enterocyte mass, and this may be related to cobalamin-sequestering by pathogens. Cobalamin malabsorption may be contributing to the pathogenesis of EED. There is a need to understand the interplay between EED and malabsorption.

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Table 1: Baseline characteristics of 750 children with stunting randomised to lipid-based nutrient supplements (LNS) with or without milk protein or whey permeate or to no supplement[a]

	LNS											
	Milk protein[b]				Whey permeate[b]				LNS		No supplement	
	n	+	n	-	n	+	n	-	n	+	n	-
Age, months	299	32.4 ±11.5	301	31.5 ±12.0	301	32.6 ±12.1	299	31.2 ±11.4	600	31.9 ±11.8	150	32.3 ±11.7
Sex, male	299	52% (156)	301	57% (172)	301	53% (159)	299	56% (169)	600	55% (328)	150	56% (84)
Anthropometrics												
Height-for-age	299	-3.02 ±0.74	301	-3.04 ±0.73	301	-3.06 ±0.75	299	-2.99 ±0.72	600	-3.03 ±0.73	150	-2.99 ±0.75
Weight-for-height	299	-0.27 ±1.03	300	-0.42 ±0.93	301	-0.34 ±0.95	298	-0.35 ±1.01	599	-0.35 ±0.98	150	-0.43 ±1.03
Weight-for-age	299	-1.87 ±0.91	300	-1.97 ±0.79	301	-1.94 ±0.84	298	-1.90 ±0.87	599	-1.92 ±0.86	150	-1.97 ±0.83
Clinical												
Diarrhoea in 2 weeks prior	299	24% (73)	301	29% (88)	301	24% (71)	299	30% (90)	600	27% (161)	150	32% (48)
Serum C-reactive protein, mg/L	294	1.45 [7.54]	297	1.74 [9.12]	296	1.51 [7.65]	295	1.51 [8.93]	591	1.51 [8.24]	150	1.64 [5.55]
Serum α1-acid glycoprotein, g/L	294	1.24 [0.77]	297	1.18 [0.67]	296	1.20 [0.67]	295	1.22 [0.76]	591	1.20 [0.72]	150	1.16 [0.80]
Haemoglobin, g/L	296	103.7 ±14.7	297	103.4 ±14.3	297	103.4 ±15.3	296	103.6 ±13.7	593	103.5 ±14.5	150	104.6 ±15.4
Diet												
Currently breastfed	298	14% (42)	300	13% (38)	299	16% (48)	299	11% (32)	598	13% (80)	148	10% (15)
Diet includes milk, meat or eggs	299	57% (172)	301	59% (177)	301	52% (157)	299	64% (192)	600	58% (349)	150	61% (91)
Minimum dietary diversity[c]	298	27% (80)	300	25% (76)	299	26% (77)	299	26% (79)	598	26% (156)	148	26% (38)
Household												
Urban residence	299	55% (166)	301	56% (170)	301	57% (172)	299	55% (164)	600	56% (336)	150	53% (79)
Maternal age, years	273	26.8 ±6.3	282	25.9 ±5.7	278	26.4 ±5.8	277	26.2 ±6.3	555	26.3 ±6.0	137	26.6 ±6.3
Mother education, primary or less	289	62% (178)	292	62% (182)	296	67% (197)	285	57% (163)	581	62% (360)	145	58% (84)
Food secure[d]	299	5% (16)	301	5% (14)	301	4% (11)	299	6% (19)	600	5% (30)	150	2% (3)
WASH characteristics												
Drinking water not treated	299	71% (213)	301	68% (204)	301	70% (212)	299	69% (205)	600	69% (417)	150	65% (98)
Soap available for hand washing	299	29% (86)	301	24% (72)	301	27% (82)	299	25% (76)	600	26% (158)	150	25% (38)

[a]Data are mean ±standard deviation, median [interquartile range] or % (n). [b]Milk protein and comparator soy protein isolate, whey permeate and comparator maltodextrin. [c]Positive score is minimum 5 of 8 food groups eaten in past 24hrs [(122)]. [d]Household food insecurity access scale (USAID, FANTA v.3). [e]Corrected for fasted state and systemic inflammation.

Table 2: The effect of 12 weeks of supplementation with vs without milk protein (MP) and with vs without whey permeate (WP) and the effect of lipid-based nutrient supplements (LNS) compared with no supplementation on markers plasma citrulline (p-cit) and faecal myeloperoxidase (f-MPO) in children with stunting

	Supplement		N	Baseline	N	12 weeks	Change	95% CI	Difference in change[a]		
Outcome				Mean ±SD		Mean ±SD			Δ	95% CI	p
Plasma citrulline, µmol/L[b]	LNS	- MP	291	28.0 ±9.4	287	25.8 ±7.8	-1.38	-1.38; -3.29	-	-	-
		+ MP	287	28.9 ±10.6	289	25.9 ±8.2	-2.34	-4.28; -0.41	-0.97	-2.87; 0.93	0.32
	LNS	- WP	288	28.2 ±10.2	284	25.1 ±7.4	-2.77	-4.68; -0.86	-	-	-
		+ WP	290	28.6 ±9.8	292	26.6 ±8.5	-1.70	-3.61; 0.22	1.07	-0.83; 2.97	0.27
	None	-	146	30.0 ±15.7	145	26.0 ±8.0	-3.92	-6.19; -1.65	-	-	-
	LNS	LNS	578	28.4 ±10.0	576	25.7±8.0	-2.61	-3.98; -1.25	1.3	-1.03; 3.64	0.27
									0		
				Median (IQR)		Median (IQR)					
Faecal myeloperoxidase, ng/mL[c]	LNS	- MP	298	436 (135; 1663)	292	394 (121; 1407)	1.10	0.75; 1.61	-	-	-
		+ MP	297	535 (200; 2171)	292	494 (200; 1813)	1.13	0.77; 1.66	1.03	0.67; 1.60	0.89
	LNS	- WP	297	457 (140; 2029)	292	451 (155; 1488)	1.25	0.85; 1.82	-	-	-
		+ WP	298	530 (179; 1687)	292	453 (159; 1541)	1.28	0.87; 1.87	1.03	0.66; 1.59	0.91
	None	-	149	536 (170; 2674)	148	305 (116; 1167)	0.54	0.35; 0.84	-	-	-
	LNS	LNS	595	497 (159; 1951)	584	452 (157; 1498)	0.99	0.79; 1.23	1.82	1.12; 2.96	0.016

[a]Data are presented as mean difference in change (p-cit) or the ratio of change (f-MPO) between the intervention and comparison group, with 95% confidence intervals and p-values based on linear mixed effects models adjusted for baseline values, age, sex, season and random effects; site, ID and batch number (p-cit only). The estimation of change determined by the model is not always equal to the difference in means calculated on raw data. All p-values for interaction between p-cit or f-MPO and MP and WP were >0.2. [b]Corrected for fasted state and systemic inflammation. [c]Log-transformed.

Table 3: Interaction between 12 weeks of supplementation with lipid-based nutrient supplements and systemic inflammation or recent diarrhoea on markers plasma citrulline (p-cit) and faecal myeloperoxidase (f-MPO) in children with stunting[a].

	Inflammation s-CRP ≤10 mg/L >10 mg/L			Inflammation s-AGP ≤1.2 g/L >1.2 g/L			Recent history of diarrhoea[b] No diarrhoea Diarrhoea			Diarrhoeal stool sample No diarrhoea Diarrhoea		
Outcome	p		95% CI	p		95% CI	p		95% CI	p		95% CI
p-cit,	0.56	1.77	-0.81; 4.36	0.92	1.69	-1.46; 4.83	0.88	0.27	-2.35; 2.89	0.82	1.46	-1.20; 4.12
µmol/L[c]		-0.21	-6.34; 5.92		1.44	-2.23; 5.11		1.01	-7.88; 9.90		0.76	-4.50; 6.01
f-MPO,	0.20	2.06	1.20; 3.53	0.83	1.91	0.99; 3.68	0.04	1.22	0.71; 2.11	0.02	1.34	0.78; 2.33
ng/mL[d]		0.86	0.26; 2.91		1.71	0.80; 3.62		7.53	1.40; 40.47		5.65	1.97; 16.20

[a]Analysed using a linear mixed effects models adjusted for the baseline value, age, sex, season, and random effects; site, ID and batch (p-cit only). Data is p value for interaction for the mean difference in change (p-cit) or the ratio of the fold change (f-MPO) over time between stratum specific values and 95% confidence intervals. [b]Diarrhoea in last 14 days. [c]Corrected for fasted state and systemic inflammation, p-cit at baseline (n=724) and endpoint (n=721). [d]Log transformed, f-MPO at baseline (n=744) and endpoint (n=732). s-CRP: Serum C-reactive protein; s-AGP: serum α1-acid glycoprotein

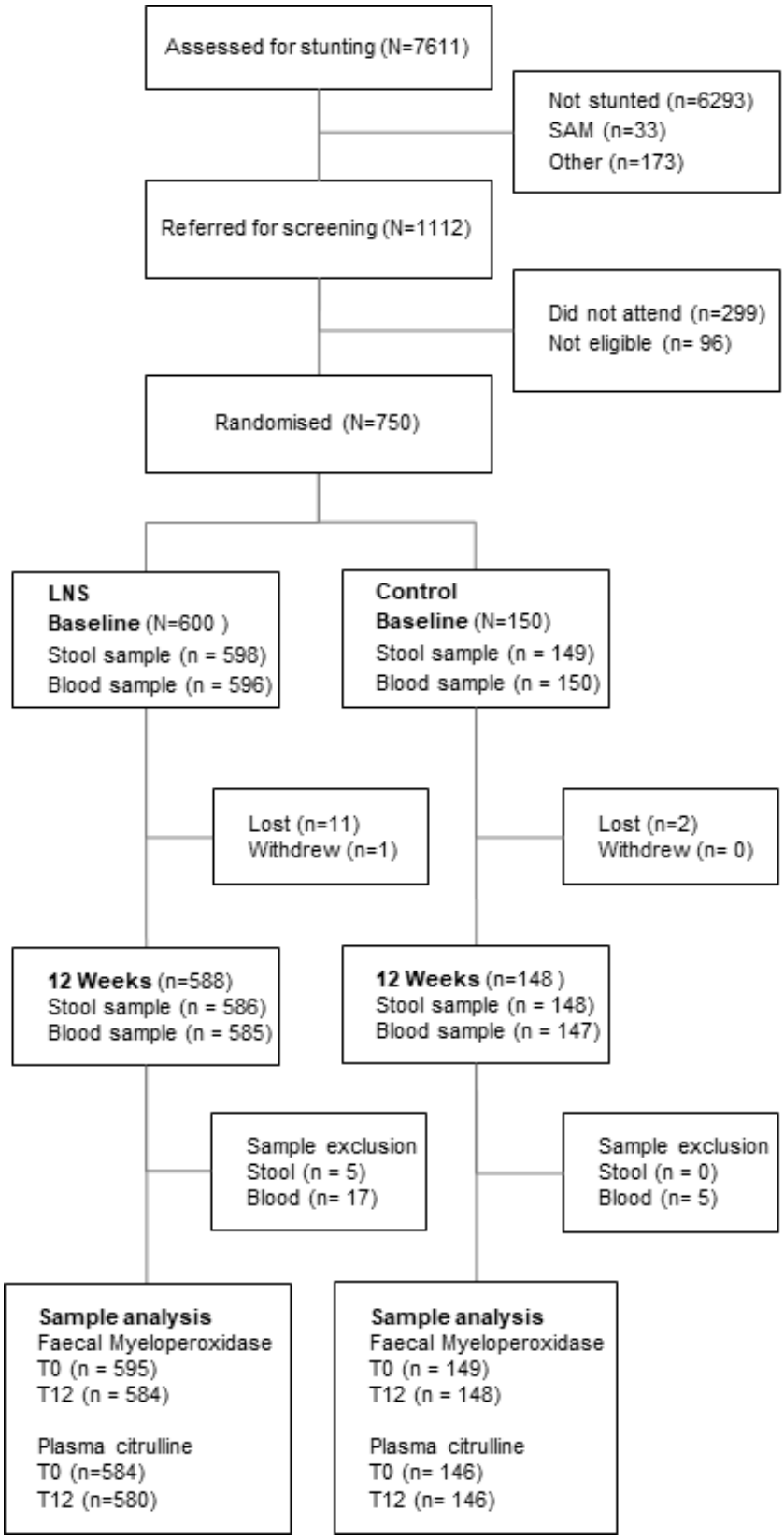
Table 4: Baseline plasma citrulline (p-cit) <20 µmol/L and faecal myeloperoxidase (f-MPO) >2000 ng/mL as modifiers of the effects of 12 weeks of supplementation with lipid-based nutrient supplements on micronutrient status[a]

		p-cit low[bc]				**f-MPO high**		
		≥ 20 µmol/L				≤ 2000 ng/mL		
	Baseline	< 20 µmol/L			Baseline	>2000 ng/mL		
Outcome	n[d]	p		95% CI	n[e]	p		95% CI
Plasma cobalamin[f], pmol/L	700	0.03	1.51	1.41; 1.61	713	0.07	1.51	1.40; 1.63
			1.20	0.99; 1.46			1.33	1.18; 1.49
Plasma methylmalonic acid[f],	714	0.008	0.62	0.55; 0.70	727	0.10	0.63	0.55; 0.71
nmol/L			0.99	0.71; 1.37			0.77	0.63; 0.94
Serum ferritin[cf], µg/L	724	0.41	1.40	1.20; 1.63	735	0.57	1.47	1.23; 1.74
			1.70	1.10; 2.61			1.34	1.03; 1.74
Haemoglobin[g], g/L	721	0.56	4.07	1.25; 6.89	737	0.18	3.33	0.17; 6.48
			6.52	-1.32; 14.36			7.25	2.43; 12.06
Plasma folate[g], nmol/L	675	0.86	9.51	6.93; 12.1	686	0.42	9.17	6.30; 12.03
			10.23	2.82; 17.65			11.33	6.96; 15.69
Serum soluble transferrin	724	0.21	0.87	0.79; 0.97	735	0.10	0.81	0.72; 0.90
receptor[f] mg/L			0.72	0.55; 0.95			0.96	0.81; 1.14
Serum retinol binding	724	0.32	-0.07	-0.13; -0.01	735	0.56	-0.07	-0.13; -0.005
protein[cg], µmol/L			0.02	-0.14; 0.18			-0.03	-0.13; 0.06

[a]Produced from linear mixed effects models adjusted for the baseline value, age, sex, season, and random effects; site and ID. [b]Corrected for fasted state. [c]Corrected for systemic inflammation. [d] p-cit <20 µmol/L ~15% of this value. [e]f-MPO >2000 ng/mL ~26%. [f]Outcome log transformed and data presented as p-value for interaction for the ratio of the fold change between stratum specific values and 95% confidence intervals. [g]Data is p-value for interaction for the mean difference in change between stratum specific values and 95% confidence intervals.

Table, Supplemental Digital Content 1: Composition of each lipid-based nutrient supplement (LNS)					
Name		**LNS with or without milk protein[1]**		**LNS with or without soy protein[2]**	
Whey permeate[3]		**+**	**-**	**+**	**-**
Digestible indispensable amino acid score (DIAAS)[4]					
Age 6 months to 3 years		0.93		0.78	
Age above 3 years		1.10		0.93	
Macronutrients					
Components	unit	Per 100g			
Calories	Kcal	531	535	530	534
Carbohydrates	g	42	43	42	43
Lactose	g	15.7	0.4	15.3	0
Proteins	g	13.9	13.5	13.9	13.5
Milk proteins	g	7.15	6.75	0.40	0
Vegetable proteins	g	6.75	6.75	13.50	13.50
Lipids	g	33.7			
Linoleic acid C18:2	g	3.0			
Linoleic acid C18:3	g	0.5			
Minerals					
Calcium	mg	691	594	691	594
Copper	mg	1.65			
Iron	mg	12			
Iodine	mg	127	113	127	113
Magnesium	mg	199.2	175.8	199.2	175.8
Manganese	mg	1.8			
Phosphorous	mg	661	539	661	539
potassium	mg	1315	985	1315	985
Sodium	mg	84	7	156	79
Selenium	µg	30			
Zinc	mg	12.5			
Vitamins[5]					
Vitamin A	mg	619			
Vitamin B1	mg	1.2	1.1	1.2	1.1
Vitamin B12	µg	3.2	3.0	3.2	3.0
Vitamin B2	mg	3.1	2.8	2.7	2.4
Niacin	mg	14.9	14.6	14.9	14.6
Pantothenic acid	mg	5.7	4.5	5.7	4.5
Vitamin B6	mg	2.1	2.0	2.1	2.0
Biotin	µg	74.1	67.6	74.1	67.6
Folic acid	µg	223			
Vitamin C	mg	67.9	67.6	67.9	67.6
Vitamin D	µg	16.9			
Vitamin E	mg	18			
Vitamin K	µg	30			

[1)] Milk protein isolate (casein + whey protein) [2)] Soy protein isolate [3)] Whey permeate was replaced with maltodextrin in formulae without this milk ingredient [4)] DIAAS calculation was performed according to FAO, 2011 (40). For peanut, ileal digestibility from rats was used (41) and for soy and milk protein isolates, ileal digestibility values from pigs were used (42). Whey permeate, which only accounted for 0.5% of protein was not taken into account because data were not available [5)] Target values by the end of the products' shelf life



Figure, Supplemental Digital Content 2: Flow diagram for collection of biological material and analysis of gut markers in the MAGNUS trial. SAM: Severe acute malnutrition; LNS: Lipid-based nutrient supplements

Table, Supplemental Digital Content 3: Baseline plasma citrulline (p-cit) <20 µmol/L and faecal myeloperoxidase (f-MPO) >2000 ng/mL as modifiers of the effects of 12 weeks of supplementation with lipid-based nutrient supplements on growth[a]

Overall Δ		**P-cit low**[b]				**f-MPO high**		
Strata		≥ 20 µmol/L				≤ 2000 ng/mL		
	Baseline	< 20 µmol/L			Baseline	>2000 ng/mL		
Outcome	n[c]	p		95% CI	n[d]	p		95% CI
Height[e], cm	724	0.72	0.56	0.37; 0.75	744	0.36	0.64	0.43; 0.86
			0.67	0.13; 1.21			0.46	0.13; 0.79
Knee-heel length, mm[e]	723	0.92	2.05	1.34; 2.76	742	0.65	2.01	1.21; 2.81
			1.94	-0.05; 3.92			1.67	0.45; 2.89
Height-for-age Z-score[e]	724	0.54	0.17	0.11; 0.22	743	0.87	0.17	0.11; 0.23
			0.22	0.06; 0.37			0.16	0.07; 0.25
Weight[e], kg	723	0.51	0.21	0.12; 0.31	743	0.79	0.22	0.11; 0.33
			0.12	-0.15; 0.39			0.20	0.03; 0.36
Weight-for-height Z-score[e]	723	0.80	0.06	-0.04; 0.17	743	0.97	0.07	-0.05; 0.19
			0.02	-0.28; 0.32			0.07	-0.11; 0.24
Serum insulin-like-growth factor-1[f], ng/ml	718	0.73	1.07	0.99; 1.17	734	0.89	1.07	0.98; 1.17
			1.03	0.82; 1.29			1.06	0.92; 1.21

[a]Produced from linear mixed effects models adjusted for the baseline value, age, sex, season, and random effects; site and ID. [b]Corrected for fasted state and systemic inflammation. [c]Baseline p-cit <20 µmol/L ~15%. [d]Baseline f-MPO >2000 ng/mL ~26%.[e]Data is p-value for the interaction for the mean difference in change between stratum specific values and 95% confidence intervals. [f]Outcome was log transformed, data presented as p-value for interaction for the ratio of the fold change between stratum specific values and 95% confidence intervals

10.3.1 Supplementary text for Paper III

Supplemental Digital Content #4.

Intervention

The LNS formulations were standardised to contain similar proportions of energy, protein and carbohydrates but they differed in whether they contained approximately 7% milk protein isolate (MP) and/or 15% whey permeate (WP). Where MP and WP were absent, similar amounts of soy protein isolate (SPI) and maltodextrin were included as comparators, respectively. To improve micronutrient content, all supplements contained a mineral and vitamin mix. For two of the formulations however, the milk minerals supplied in the whey permeate provided additional nutrients (**Supplemental Digital Content #1**). All caregivers received individual nutrition counselling at baseline, lunch was provided for the caregiver and child at baseline and week 12 visits and all transport costs were reimbursed. Supplies of LNS-LQ or laundry soap were collected from the pharmacist on site every 14 days and empty sachets were returned and counted.

Randomisation and blinding

Site-stratified block randomisation with variable block sizes of 10 and 20 were used to generate the allocation sequence list in R (R-Core team, 2019). One of ten unique three-letter codes were assigned to IDs 001 to 999 where two codes corresponded to each of the five arms. Study ID's were assigned sequentially upon inclusion when after completion of all data collection activities, the study pharmacist, with sole access to the random allocation list, distributed the intervention to caregiver and child. Outcome assessors were blinded to the allocation of the intervention. Caregivers were blinded to the formulation but not to allocation to supplementation. The blinding code was only accessible to the manufacturer Nutriset (Malaunay, France) until initial data analysis was complete. To minimise sharing of the daily quota, an additional 14 sachets with the same code were provided to households with other children aged between 6 and 59 months. The caregiver returned with empty and unused sachets at each collection visit.

Supplemental Digital Content #5.

Biological sample collection

Blood and stool samples were collected at baseline and 12 weeks. At the time of blood draw, the estimated time interval since the child had last fed was recorded. Samples were kept at room temperature (20-25°C) and transported to the lab for processing. Approximately 200 µL of the whole blood collected in EDTA was used for rapid diagnostic tests. Remaining bloods were centrifuged (EBA200, Hettich, Germany) at 3500 rpm for 10 minutes before serum and plasma aliquots were transferred to cryotubes for storage at -20°C. Approximately 1g of stool was collected in a free standing screw-top tube. Samples were homogenised using a vortex mixer and glass beads and the slurry transferred to cryotubes for storage at -20°C. Samples were transferred within 7 days to the Integrated Biorepository (IBRH3AU) in Kampala, for storage at -80°C. After study completion, samples were shipped on dry ice to the University of Copenhagen and subsequently to labs in Sweden, Germany and Denmark for analysis.

Supplemental Digital Content #6.

Correction of p-cit for fasting and inflammation

Citrulline values were corrected for both fasting status and inflammation as described previously [19]. The duration since the child's last feed, according to caregiver reports, was classified into three groups; less than two hours, two to five hours and more than five hours since last feed. In the case that the child had eaten within 5 hours, the original citrulline value was corrected. An additional correction was made for inflammation using continuous data from two acute phase inflammatory markers; serum 1α -acid glycoprotein and serum C-reactive protein.

11 Discussion and Perspectives

*The hypothesised causal pathway of EED in stunting provides a framework (Figure X) from which findings of this thesis are hereafter discussed. The associations found between different factors (environmental, systemic and nutritional) and p-cit, in **Paper II** are explored. Then, attempts to intervene in EED are discussed based on results from **Paper III**. The strengths and limitations of this thesis are highlighted and finally some of the implications for future research are presented.*

11.1 Environmental factors associated with EED

A high pathogen burden is believed to be a main driver of EED [9, 123]. Environmental factors such as poor WASH conditions (unsafe drinking water [124], poor sanitation [125, 126], unhygienic home environments [127]), geophagy [128], exposure to animals [129] and the wet season [74, 110] have been shown to increase the risk of pathogen exposure. Poor environmental and thus living conditions are also closely related to low socioeconomic status, previously shown to be associated with EED and stunting [19, 39]. Several of the aforementioned factors were explored as correlates of p-cit.

11.1.1 Poor WASH conditions

In **Paper II,** an unimproved toilet facility was found to be associated with lower p-cit. Others have previously reported an association with increased markers of intestinal inflammation [125, 130], but not with p-cit [125, 131]. Different to the aforementioned studies which used structured interview based questionnaires without a home visit [125, 130, 131], the MAGNUS study employed objective methods of WASH data collection wherever possible. This was likely to improve the validity of the data collected. Still, there was no difference in p-cit according to water source or water treatment **(Paper II)**. A study in Lao among children aged 6-23 months reported an association between an improved water source and p-cit, although the within group variation in types of access was not reported [131]. In the MAGNUS study, almost every household had access to an improved water source. A study in Western Uganda examined the drinking water of households and found no difference in contamination with *E.coli* bacteria between improved and unimproved water sources. However, safe drinking water was associated with improved L:M and higher LAZ [124]. In MAGNUS objective measures of water safety were not used to differentiate households with safe from those with unsafe drinking water and classification by access and treatment may not be equivalent indicators of safe drinking water. Few in the MAGNUS study

had access to a private water tap, but others have shown that uninterrupted water access on premises is associated with improved markers of intestinal inflammation [125] and improved growth [132]. In **Paper II**, irrespective of having a private tap, the use of water and soap for handwashing was associated with increased p-cit. In other studies, the use of soap has been associated with reduced diarrhoeal prevalence [133] but not with improved EED markers [125, 133, 134] including p-cit [125, 131]. The positive finding in this study is supported by the observational data collection methods used; avoiding the subjectivity of a questionnaire. The WASH associations identified in **Paper II,** reflect a relationship between increased pathogen exposure and loss of enterocyte mass [135]. After accounting for the effects of systemic inflammation however, the associations were attenuated, reflecting the importance of infection in the pathway [136]. Either systemic inflammation is associated with infection apart from EED, or systemic inflammation is mediated by EED and thus associated with a loss of enterocyte mass and lower p-cit. The inclusion of additional markers from other domains might have shed light on these associations.

11.1.2 The wet season

The wet season was associated with lower p-cit (**Paper II**). Seasonal variations have been demonstrated before, both for EED markers [74, 110, 130] and linear growth faltering [137]. A birth cohort study in Kenya found an increase in EED during the wet season and suggested that this was related to the longer duration of food storage together with the associated risk of bacterial and toxin contamination [74]. As mentioned in **Paper II**, the MAGNUS study was initiated in the months before the Covid-19 pandemic and as such, the seasonal changes closely coincided with national lock-downs across Uganda. Sub-analysis of hygiene practices at baseline showed that the proportions using soap for handwashing increased after lock-downs. Though diarrhoea was not an outcome of this thesis, it could be seen in **Paper III** that the rates of diarrhoea had substantially decreased by 12 weeks in both the intervention and control groups. One might surmise that the reduced rate of infection was related to changes in handwashing practices associated with Covid-19. After controlling for the effects of systemic inflammation however, the wet season and the period before Covid-19, remained associated with lower p-cit. Since infection did not fully explain the association, it seems plausible that the wet season is a factor contributing to low enterocyte mass.

11.1.3 Housing and SES

Socioeconomic factors such as food insecurity and poorer living standards remained associated with lower p-cit in **Paper II**, even after adjustment for inflammation. The use of housing

materials other than brick or cement for the walls and/or the floor was associated with lower p-cit. This aligns with a study in Brazil where compared to cement, dirt floors were associated with impaired L:M [125]. In addition, a recent survey of 33 African countries found that inadequate housing materials were associated with adverse child health outcomes including stunting, [138]. The household food insecurity access scale (HFIAS) was also associated with reduced p-cit, though only in those reporting moderate food insecurity **(Paper II)**. The population in MAGNUS presented as more food insecure than previous reports from the region [116] (see also section 9.2.1). Severe food insecurity was likely over-reported in this study. Measures of socioeconomic status are highly contextual and the use of interview administered questionnaires comes with a risk of reporting bias. Several studies have grouped socioeconomic factors such as maternal education, income and assets to develop a wealth index [131, 139]. Wealth indices have been associated with p-cit [131], and have correlated well with EED markers and LAZ between countries [110]. In this study, poor housing materials and food insecurity were associated with low p-cit and this reflects a similar notion to what others have reported, that low socioeconomic status is associated with EED [39].

11.2 Systemic sequelae of EED

Central to the pathogenesis of EED is a chronic state of inflammation. This is hypothesised to lead to various interrelated sequelae including systemic inflammation, growth hormone impairment and malabsorption [39] and each one is implicated in growth faltering.

11.2.1 Systemic inflammation

As discussed in **Paper II**, there was a strong negative association between markers of systemic inflammation and p-cit. This negative association has been widely reported by others [102, 140, 141], including studies involving children who were already stunted or at risk of stunting [101, 131, 135], Still, the association is contested by some [142, 143]. On the one hand, this finding is consistent with current thinking in EED; namely that enterocyte function and systemic immune activation are closely linked via intestinal inflammation [135]. On the other hand, as described in **Paper II**, this association may be explained to some degree by the close metabolic relationship between citrulline and arginine. Citrulline serves as a precursor in arginine synthesis. Arginine is involved in several metabolic pathways including an immune modulating role in many tissues throughout the body, as the main precursor for nitric oxide synthesis. It has been suggested therefore, that in critically ill patients, low p-cit may result from increased requirements for arginine to produce nitric oxide and so low p-cit may not always be a clear indication of loss of enterocyte mass [90,

[144, 145]. To account for the differences in p-cit that may be confounded by or mediated through systemic inflammation, a model adjustment was included in **Paper II** and a correction of p-cit in **Paper III.** In light of acute or even chronic inflammation, it seems that some caution should be taken in the interpretation of p-cit results [90, 146]. The association found between systemic inflammation and low p-cit could indicate loss of enterocyte mass in a state of nutrient deficiency and/or inflammation, or it may result from increased use of arginine in the nitric oxide cycle, or perhaps some combination of these.

11.2.2 Impaired growth

In **Paper II,** there was no association between HAZ and p-cit, though a negative association appeared after adjusting for inflammation. However, the association is likely a spurious finding since it lacks a feasible biological explanation. Associations between p-cit and HAZ have been reported previously in children who were either stunted or at risk of stunting [15, 101, 135], but a lack of association has also been reported [72, 99, 131]. EED is thought to be widespread in communities with high rates of stunting, where the entire HAZ growth curve is shifted to the left. A recent study among 110 children aged 12-18 months in Bangladesh, demonstrated that different histological changes reminiscent of EED were present among >90% of children and were just as prevalent among the half who were growth faltering (LAZ <-1), compared to those who were stunted (LAZ <-2) [147]. To better describe the association between HAZ and p-cit, the MAGNUS study would have benefited from the inclusion of a non-stunted comparison group. Chronic intestinal inflammation is believed to contribute to growth faltering via the growth hormone-IGF-1-axis [148]. In **Paper II**, a positive association was found between s-IGF1 and p-cit. In contrast to this, a study in the Central African Republic, among children aged 2-5 years who were either stunted or at risk of stunting, found an association between HAZ and p-cit, but not s-IGF1 and p-cit [101]. As expected, after adjusting for systemic inflammation in **Paper II**, the effect size of the association with p-cit reduced, as IGF-1 is downregulated in the presence of systemic inflammation [22, 64, 148]. The remaining association between s-IGF1 and p-cit suggests that p-cit was associated with s-IGF1 apart from systemic inflammation. As a growth factor, IGF-1 has been shown to promote the proliferation and regeneration of intestinal epithelial cells in the small intestine [149, 150]. Though most IGF-1 is produced in the liver, other tissues including the gastrointestinal tract are important sources [151]. Though causality cannot be established in a cross sectional design, it is biologically plausible that increased s-IGF1 is associated with increased enterocyte mass and thus increased p-cit.

11.2.3 Unmet nutrient requirements

Alongside a high pathogen burden, inadequate nutrition is thought to perpetuate EED. Dietary diversity was inadequate for a majority of the children in this study (**Paper II)**. However, no associations were found between dietary factors such as breastfeeding, dietary diversity or meal frequency and p-cit. This finding was reflected in a study in Lao referred to previously, in which no associations were found between dietary factors and p-cit [131]. Few have examined dietary intake in relation to EED [61, 152, 153]. In MAGNUS, challenges in data quality assurance were pre-empted and thus quantitative nutrient intake data was not collected. Instead, a 24 hour food frequency questionnaire was used to collect qualitative baseline dietary information on food group adequacy and meal frequency. While low intake can be part of the problem, malabsorption is considered an important factor contributing to widespread vitamin A [61, 154], iron [155] and zinc [49, 156] deficiencies in regions where EED is prevalent [10]. Micronutrient deficiencies are common complications of intestinal enteropathies in children. Among others, B12, vitamin A, iron and zinc deficiencies have been demonstrated in children with coeliac disease [157, 158]. However, in **Paper II**, micronutrient status was not found to be associated with p-cit, with the exception of a positive association with haemoglobin, which has been reported by some studies [101], though not consistently [131]. In this study, adjustment for inflammation attenuated the association between haemoglobin and p-cit, showing that the relationship was likely driven by systemic inflammation. In settings of frequent pathogen exposure and heightened immune activation, it is thought that requirements for certain nutrients may increase [27]. Evidence that EED leads to or exacerbates these micronutrient deficiencies however is lacking [10].

11.3 Interventions to improve EED

Interventions targeting EED are aimed at the basic causes; reducing the pathogen burden [40] and improving nutrient intake. However, EED may also compromise nutrient absorption and thus influence the effectiveness of nutrition interventions.

11.3.1 LNS interventions

As described in **Paper III**, there was no effect of large-quantity LNS, or ingredients MP and WP on p-cit. Nor was there an effect of ingredients MP or WP on f-MPO. Compared to no supplementation however, there was a relative increase in f-MPO among those receiving LNS. A larger quantity LNS was provided in this study, yet the lack of effect on EED markers was similar to previous trials using small-quantity LNS. Two recent birth cohort trials provided daily small-quantity LNS to children from 6 to 18 months in Zimbabwe [159] and Bangladesh [134] and

reported no effect on EED markers including f-MPO [134, 159] and p-cit [159]. Similarly, a trial in Bangladesh provided daily small- to medium-quantity LNS from 6 to 18 months and reported that EED markers including f-MPO had not improved [85]. Despite a lack of effect on EED markers, LNS interventions have had a small effect on linear growth [14, 160]. There was no evidence in this study (**Paper III)**, that baseline p-cit or f-MPO modified the effect of the intervention on linear growth outcomes, others have also reported that baseline p-cit [131] and f-MPO [72] did not predict subsequent linear growth. There was surprisingly no effect of WP on intestinal markers. Whey permeate contains approximately 70% lactose and although it is generally understood that young children can tolerate lactose, children with EED may be prone to secondary lactose intolerance [161]. There was evidence elsewhere however, that the LNS did not benefit all recipients equally [37]. Those supplemented with LNS who also had diarrhoea, showed an increase in f-MPO compared to those supplemented without diarrhoea. This indicated a possible negative effect of the LNS on intestinal inflammation which has been described by others [162]. These findings add to the current body of research demonstrating that nutrition interventions have been largely unsuccessful at improving EED.

11.3.2 EED and impaired intervention effects

It is thought that EED impairs the effects of nutrition interventions on linear growth [10, 163].In **Paper III**, subgroup analysis demonstrated that those with low p-cit at baseline experienced a reduced beneficial effect of the LNS intervention on cobalamin (B12) status. This was shown by a 20% reduced effect on p-cobalamin and a 59% relative increase in p-MMA after 12 weeks of supplementation. A similar trend of a reduced LNS effect on p-cobalamin and an increase in p-MMA was also seen in those with high f-MPO at baseline, although to a lesser extent. Cobalamin is an essential nutrient in DNA synthesis and cell division. Thus, malabsorption of cobalamin has implications for rapidly dividing cells in the haematopoietic, nervous and gastrointestinal systems [164] and in this way, may contribute to epithelial disrepair and worsening EED. Plasma MMA accumulates when the supply of cobalamin is reduced, and is thought to be a marker reflecting the adequacy of cobalamin for metabolic function [165]. As discussed in **Paper III**, cobalamin is absorbed as a complex with intrinsic factor (IF) in the terminal ileum. Malabsorption of cobalamin may be directly related to villus atrophy and a loss of enterocyte mass. Though histological changes in EED are thought to predominate in the proximal intestine [10]. Patients with untreated coeliac disease who similarly experience damage to the upper intestine, are reported to have insufficient cobalamin status compared to the normal population [157, 166, 167]. There may also be secondary factors involved that are both related to cobalamin

malabsorption and low-enterocyte mass. For example, an overgrowth of bacteria or parasites in the small intestine can outcompete the host IF to bind cobalamin [168, 169], and therefore reduce the amount of IF-bound cobalamin that reaches the terminal ileum for absorption [170]. Bacterial overgrowth of the small intestine is a well-known cause of cobalamin deficiency [169] and has been associated with stunting in LMIC settings [39, 83, 171]. The overlap between the roles of bacterial overgrowth and EED in stunting however, is unclear [74, 171] as both are thought to contribute to intestinal inflammation, epithelial damage and malabsorption [172–174]. The results of this study provide some evidence that EED, and in particular low enterocyte mass, may be associated with a reduced beneficial effect of the LNS nutrition intervention.

11.3.3 WASH and a combined transformative approach

WASH characteristics such as an unimproved toilet and lack of soap for handwashing were associated with low p-cit as described earlier in the chapter. However, WASH related interventions were not implemented as part of this study. At best, large scale WASH interventions have had transient effects on EED markers [134, 159] and have shown no additional benefit to growth when provided in combination with nutrition interventions [159, 175]. Importantly, animal models have demonstrated that neither inadequate diet nor exposure to bacteria are sufficient in themselves to cause enteropathy. Rather it seems that an interaction between the two promotes mucosal damage [7]. The interventions implemented in the aforementioned WASH trials [134, 159] may not have been sufficient to minimise enteropathogen exposure and infection. Though large-quantity LNS may improve nutrient access, it seems ineffective to improve EED without a combined approach that also reduces the pathogen burden. Sustainable community wide transformative WASH approaches may be necessary to reduce widespread growth faltering [176] but are insufficient without improved access to quality and adequate nutrition.

11.4 Strengths and limitations

11.4.1 Strengths

The strengths of this study were in the randomised factorial design that ensured baseline equivalence and allowed for exploration of several interventions. The inclusion of a non-supplemented group provided a comparison of the effect of the LNS overall. A representative sample of stunted children were included and the high participant retention ensured a near full data set at 12 weeks, minimising selection bias. The study was conducted according to GCP regulation and thus the quality of data collection was to a high standard with comprehensive training and retraining of staff throughout the trial to minimise random measurement error. In addition, the objective baseline WASH assessment at all participant homes improved the accuracy of the data collection and minimised the risk of reporting bias. The use of high-throughput mass spectrometry to analyse p-cit, made it a robust marker to explore. It was a strength that fasting, age and inflammation were accounted for in the analysis of p-cit as each one seems to have a bearing on the validity of results.

11.4.2 Limitations

This thesis also has several limitations. Few EED biomarkers were explored. The compromise to collect stool samples in buffer resulted in the loss of AAT and NEO and may have introduced wide variation in MPO results. The unexpectedly low mean values and large intra sample variation for each of these stool markers could not be resolved within the time frame or budget for this project. It is unlikely that a single marker is as useful as a combination of markers to identify changes in intestinal function that are indicative of EED. In addition, a lack of markers made the study less comparable with others. The analysis of f-MPO would have been improved if measured according to the dry-weight of the sample, as diarrhoea can dilute the stool. The loss of stool weight data impacted the interpretation of stool markers as they could not be analysed per gram of stool. Instead, an approximate dilution factor was applied. Others have removed diarrhoeal samples from analysis when examining EED stool markers [110], but this practice may be less relevant in a population with a high prevalence of diarrhoea.

Paper II would have benefited from the inclusion of a non-stunted comparison group as this would have improved generalisability to the entire population at risk of EED. Importantly, the cross sectional nature of **Paper II** means that the direction of the association cannot be established. For example, it cannot be seen whether increased s-IGF1 resulted in increased p-cit, if the direction was reversed or indeed whether a third unidentified factor was confounding or

mediating the relationship. As no corrections were made for multiple testing in **Paper II**, the likelihood of type I error increases and significant findings without biological plausibility were therefore ignored. Part of the study unavoidably ran during the Covid-19 pandemic, this may have introduced behavioural changes such as handwashing that can influence results. Finally, although p-cit seems to be a good candidate marker for EED in clinical and research settings, it is expensive to analyse and as such is unlikely to be used broadly in LMIC settings.

11.5 Implications for future research

EED is asymptomatic and as such, diagnosis requires biopsy or biomarkers. There is currently no accepted set of biomarkers to identify children with EED [(56)]. The findings of this thesis suggest that p-cit is a suitable candidate marker of EED. Environmental factors such as poor WASH characteristics, the wet-season and low-socioeconomic status, previously shown to be risk factors of EED, were correlated with lower p-cit in **Paper II**. Additionally, s-IGF1, a marker of growth hormone activity, was positively associated with p-cit. In **Paper II,** factors younger age and fewer hours fasted were associated with lower p-cit and should be accounted for in future analysis. In line with other studies, including those exploring EED [(135)], p-cit was negatively associated with markers of systemic inflammation. However, this association is seldom considered in the analysis and interpretation of p-cit [(177)]. These results call for further research to better understand the interaction between systemic inflammation and p-cit so as to improve the interpretation of this biomarker in the context of chronic low-grade or acute inflammation. The appropriate cut-offs for p-cit also need to be established. It has been suggested that decreased plasma citrulline concentrations mainly reflect the extreme ends of the disease spectrum in short bowel syndrome [(143)]. However, a cut-off of 20µmol/L has also been shown to correlate well with atrophy in paediatric patients with coeliac disease [(98)]. To establish the predictive value of p-cit in paediatric EED, validation studies against biopsies would be needed.

It has been proposed that EED limits the effectiveness of nutrition interventions [(38)]. The findings of **Paper III** support this, in that low enterocyte mass at baseline was associated with a reduced beneficial effect of the LNS intervention on cobalamin status. A plausible explanation for this is a secondary (unmeasured) association of cobalamin sequestration in the small intestine as a result of bacterial overgrowth or other pathogens. There is a growing research interest in the overlap between pathogen-induced changes to the small intestinal microbiota, EED, bacterial overgrowth and the implications for stunting [(16, 74)]. A relationship between bacterial overgrowth, EED and stunting was recently demonstrated in animal models, whereby mice developed EED

after being colonized with cultured duodenal strains obtained in Bangladesh from children with EED [178]. Improved techniques to identify bacterial overgrowth, such as hydrogen breath testing are currently being developed and adapted for use in LMIC settings [74, 169]. However, the definition and criteria for diagnosis remain controversial and the incidence of bacterial overgrowth in LMIC settings is unclear [164, 170]. Considering the reported ubiquity of EED, even marginal nutrient malabsorption could have important implications at a population scale [10]. Cobalamin malabsorption may have implications for cognitive function as well as intestinal epithelial repair. As such, future research should explore the relationship between bacterial overgrowth, EED and cobalamin malabsorption in young children in LMIC settings.

12 Conclusion

Overall, these results add to the growing body of literature of EED by identifying associations of p-cit along the hypothesised causal pathway of EED in stunting. P-cit is a marker of enterocyte mass and a promising candidate marker of EED. The associations identified between poor WASH conditions and low p-cit provide evidence that stunted children living in poor environmental conditions have increased systemic inflammation, which may be mediated by low enterocyte mass. After accounting for the effects of systemic inflammation, the wet season, low socioeconomic status and low s-IGF1, an important maker of growth, remained associated with low p-cit. Plasma citrulline seems an eligible candidate marker, but the complex interplay with systemic inflammation warrants further investigation. There was no effect of ingredients MP and/or WP in the LNS intervention on EED markers p-cit and f-MPO. In fact, for those who had recent diarrhoea, there was some indication of a negative effect of the LNS on f-MPO. So far, nutrition interventions have not altered EED markers and have had only modest impacts on growth. It was demonstrated in this study that alone large quantity LNS interventions were insufficient to improve EED. Rather, there was some indication that the beneficial effect of LNS on cobalamin status was compromised in those with low enterocyte mass at baseline. To improve the effectiveness of nutrition interventions, it seems necessary to reduce the frequency of pathogen exposure. Community wide and sustainable WASH approaches are needed together with improved access to essential nutrients, to adequately reduce the pathogen burden, replace nutrient deficits and allow for intestinal repair.

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14 Appendices

14.1 Appendix 1: Report of findings from pilot phase 1

Author: Hannah Pesu, PhD student

Date: March/ April 2019

14.1.1 Purpose

The purpose of this report is to summarise the findings from a three-week first phase pilot for the MAGMAM study.

14.1.2 Background

The pilot took place in the districts of Jinja and Mayuge in the Busoga region of Uganda, from March 19th to April 4th 2019. Screening was carried out in areas that were expected to be representative and in several villages recommended as 'hot spots' for malnutrition.

14.1.3 Objectives

Main objective: To carry out a pre-screening pilot whereby standard operating procedures to recruit children with moderate acute malnutrition (MAM) aged 12-59 months would be tested. This pilot had three main objectives:

- To understand the prevalence of malnutrition in the two districts, particularly moderate acute malnutrition (MAM).
- To gain a clearer understanding of the breastfeeding practices in the area, with a focus on age of cessation, introduction of other foods/liquids as well as patterns of frequency and duration of breastfeeding.
- To identify feasible methods for pre-screening and to test the use of RedCAP in the pre-screening phase.

14.1.4 Pilot study findings

Table 14.1: General parameters

Parameter	N	Mayuge	Jinja
Screened, n (%)	1029	459 (45%)	570 (55%)
Villages screened	17	7	10
Mean number of children screened per site	-	66	57

A total of 683 households were screened, the mean ±sd number of children <5 years in each household was 1.6 ± 0.7 and 2.3% (n=24) were twins. Of those with data available on sex (n=847), 53% (n=447) were male.

Objective 1: Malnutrition

Table 14.2: General trends

Entire screened population	n	(mean ± sd)	Children aged 12-59 months	n	(mean ± sd)
MUAC < 125mm, % (n)	1013	2.5% (25)	MUAC ≥ 125mm, % (n)	849	2.1% (18)
WHZ	785	-0.23 ± 1.47	WHZ (mean ± sd)	662	-0.23 ± 1.32
HAZ	792	-1.16 ± 1.78	HAZ (mean ± sd)	670	-1.33 ± 1.76
WAZ	803	- 0.77 ± 1.19	WAZ (mean ± sd)	670	-0.85 ± 1.13

Table 14.3: Prevalence of acute malnutrition in those screened during the pilot (n =1029)

Moderate acute malnutrition (MAM)	n	Prevalence %(n)
MAM in all children screened	1029	4.5% (46)
MUAC <125 and ≥115mm	1013	2.2% (22)
WHZ <-2 and ≥-3	785	5.2% (41)
MAM in those aged 12-59 mths[a]	853	4.6% (39)
MUAC <125 and ≥115mm	849	2.0% (17)
WHZ <-2 and ≥-3	662	5.1% (34)
MAM in those aged 12-24 mths[a]	185	8.6% (16)
MAM in those aged >24-59 mths[a]	605	3.8% (23)
Severe acute malnutrition (SAM)	n	Prevalence %(n)
SAM in all screened	1029	2.1% (22)
MUAC <115mm	1013	0.3% (3)
WHZ <-3	785	2.3% (18)
Oedema	1024	0.2% (2)
SAM in those aged 12-59 mths	662	2.5% (17)

Table 14.4: Prevalence of stunting (n=792) and underweight (n=802) in those screened during the pilot

Stunting (HAZ <-2)	n	Prevalence %(n)
HAZ <-2 in all screened	792	23.6% (187)
HAZ <-2 in those aged 12-59 mths	670	26.7% (179)
HAZ <-2 and MAM	670	1.9% (13)
HAZ <-2 and SAM	670	1.0% (7)
HAZ <-2 in those aged 12-18 mths	83	12.0% (10)
HAZ <2 in those aged >18 - 24mths	117	30.7% (36)
HAZ <-2 in those aged >24-59 mths	485	27.4% (133)
HAZ <-2 in those aged 12-24 mths	185	24.9% (46)
Underweight (WAZ <-2)	n	Prevalence %(n)
WAZ <-2 in all screened	803	13.4% (108)
WAZ <-2 in those aged 12-59 mths	670	14.7% (100)
WAZ <-2 in those aged 24-59 mths	490	13.1% (64)

As a proportion of the total number screened, children with MAM aged 12-59 months and 24-59 months represent 3.8% and 2.2% of the sample respectively

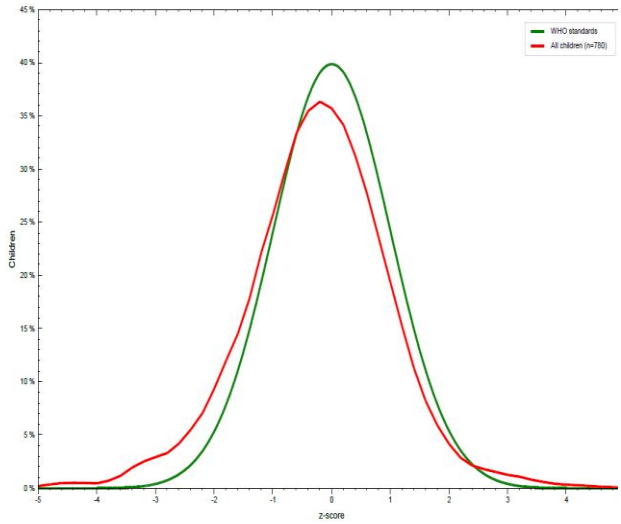

Figure 14.1: Weight-for-height Z-score of the sample population (n=792) compared to the WHO standard curve (2006)

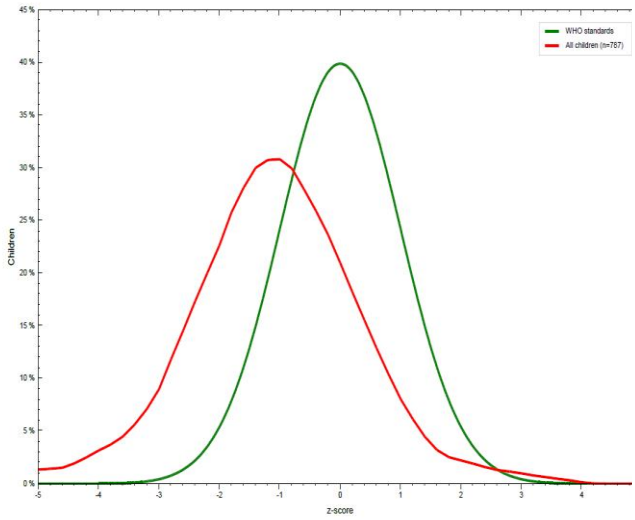

Figure 14.2: Height-for-age Z-score of the sample population (n=792) compared to the WHO standard curve (2006)

Note from Figure 14.1 that weight-for-height follows closely with the median of the reference population. Figure 14.2 on the other hand shows that the entire population has a height-for-age that is left-skewed compared to the reference population.

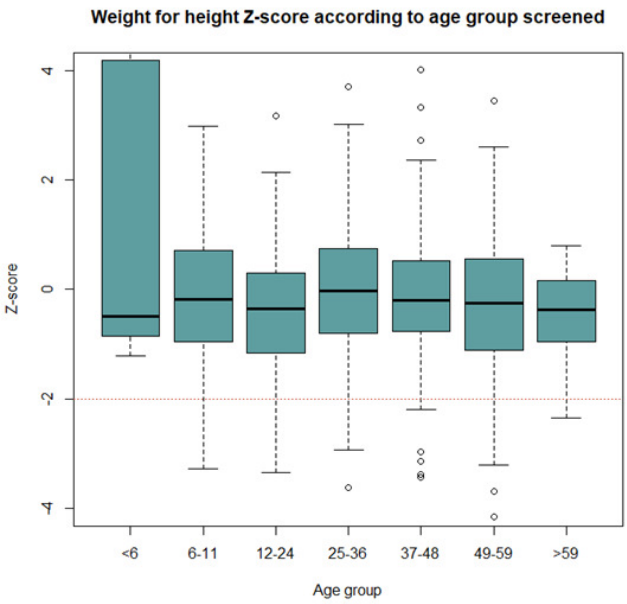

Figure 14.3: Weight-for-height according to age groups in children screened

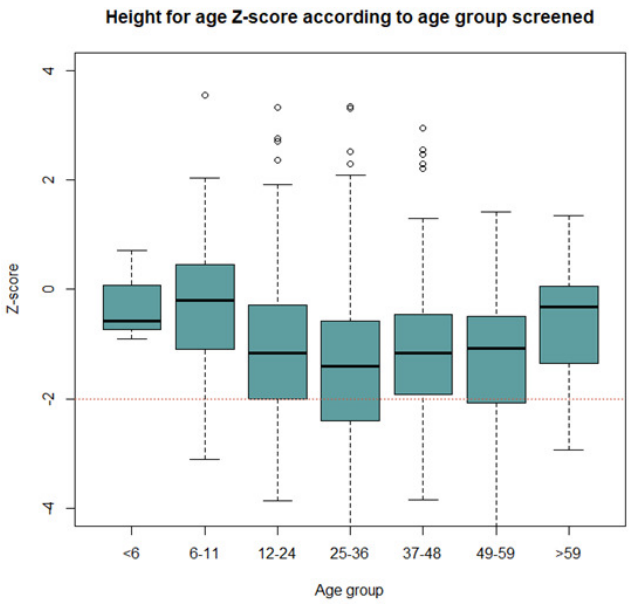

Figure 14.4: Height-for-age according to age groups in children screened

Note from Figure 14.4 that that the Z-score in children younger than 6 months is already -0.5 below the median for the reference population.

Objective 2: Breastfeeding practices

Table 14.5: Age distributions of those screened (n=1025) according to breastfeeding[a]

Age category (months)	Proportion screened, % (n)	Still BF % (n)
<6	<1% (5)	80% (4)
6- 11	14% (139)	91% (126)
12- 24	24% (248)	38% (94)
25 – 36	28% (286)	2% (6)
37 – 48	19% (197)	0.5% (1)
49 – 59	12% (125)	2% (3)
>59	3% (28)	0% (0)

[a]Of children screened, 1025 had breastfeeding information available. Of these, 23% (n=234) were still breastfed.

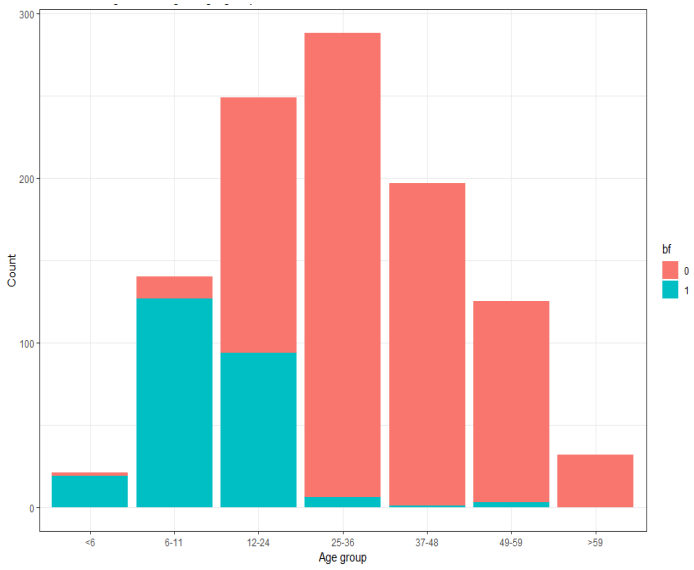

Figure 14.5: Figure 5: Still breastfed according to age groups in children screened, 0= not breastfed, 1 = still breastfed

Table 14.6: Breastfeeding duration, frequency and introduction of other liquids

Average age (months)	n	mean ± sd
Breastfeeding was stopped	282	17.7 ± 6.1
Age other liquids introduced	351	4.0 ± 1.9
24 hour recall of breastfeeding patterns	n	% (n)
Duration of breastfeeding- short (< 10 minutes)	151	49% (74)
Frequency of breastfeeding- often (>10 times/day)[a.]	178	55% (99)

[a].Breastfeeding frequency was most often reported as “too many times to count”

14.1.5 Feasibility and challenges of the pre-screening model

Team composition and competencies

Two teams were deployed to a different village each day to screen within the Mayuge and Jinja districts. Each team included four staff members, at least two of which were trained anthropometrists/nutritionists. Only one out of the eight was fluent in the local Lusoga Language, others spoke Luganda, which I am told is somewhat similar. It was clear that although staff had training in anthropometrics, they would need repeated and thorough training in order to ensure that each person carries out the required measurements correctly and consistently.

Remarks: A higher education level does not necessarily indicate greater reliability in taking anthropometric measurements. Being able to think critically and have a willingness to help are extremely important in this type of field-work. It has been recommended that we recruit by posting job advertisements and interviewing. Having a command of the Lusoga language should be considered an asset.

Involvement of VHTs

VHTs were advised a week prior to the village pre-screening date, of their task to mobilise the village community. Each VHT was invited to a half-day meeting where they were informed of the message to be given to the community and the purpose of the pilot study. The VHTs were given half payment at the meeting and the other half upon adequate completion of their given task. From the pilot study we learned that local VHTs vary in their competencies and willingness to be involved in the pre-screening process. Often times several VHTs show up when only one has been given the task.

Remarks: It could be of benefit to have a VHT on site who is relied upon to support the rest of the team if need be. It could be an asset to have a literate VHT. Therefore, it was decided that we will require one Super-VHT to be attached to each pre-screening team. It will be the responsibility of that S-VHT to mobilise the local VHTs and organise a site and equipment where needed. The S-VHT would be on a "regular payment" while the local VHTs would be facilitated for their work on a one off basis.

Terrain, distances and time

On several occasions, it took 2 to 3 hours to travel by car to and again from the village site. This was due not only to the distances from Jinja to the site but also road accidents, police roadblocks and poor roads/rough terrain. The poor roads are a greater problem in Mayuge and during the rainy season and make the use of a 4x4 necessary. Another challenge was having staff to show up to the meeting point on time for our departure each day. Relying on staff to meet will be an inevitable cause for delay.

Remarks: First, the distances travelled were too great for a pre-screening team to undertake for an extended period. This will be the same challenge for moving as a mobile team to different sites from Jinja each day. This could be overcome by having an office

base in both Mayuge and Jinja and therefore instead have staff based in each of the districts. Second, it is inefficient to wait for people to arrive at a meeting point prior to getting to the site. It would be better if staff were required to meet at the site and therefore travel shorter distances.

Equipment and nutrition education

Local staff had included nutrition education in the pre-screening program they recognise that the caregivers expect to receive something in exchange for showing-up. During the pilot a loud speaker and nutrition education kits were used. We had one height board and weighing scale per team.

> **Remarks:** It is apparent that if we are to have several pre-screening teams working every day, they will need to be mobile using motorbikes (instead of cars). Therefore, I suggest that we purchase a minimum number of motorbikes to make that possible and carry all equipment in bags on a motorbike. This means that at least one of the nutritionists should be able to ride a motorbike. Charts (which are easily transportable) will be used for nutrition education instead of kits. We will need to have spare anthropometric equipment available (in stock) so that teams are able to operate and collect reliable data on a daily basis. Any equipment other than anthropometric equipment, such as tables, chairs and benches will need to be sourced locally and set up by the local and super VHTs prior to the team arrival. This appeared to be a possibility during the pilot.

RedCAP

The RedCAP system does not seem feasible to use at pre-screening sites. First, we will require at least two per team and second, the tablets will be overexposed to dust and rain since adequate cover is not always available.

> **Remarks:** Considering the risks, I propose it is better to use paper log forms for pre-screening.

14.2 Appendix 2: Overview of SOPs (MAGNUS)

	Standard operating procedures (SOPs)	Current version
1.	Adverse events registration	V03-20191118
2.	Anthropometry	V08-20200616
3.	Bioimpedance	V02-20191128
4.	Bioimpedance – validating the controlled method	V01-20191204
5.	Blood sample processing	V03-20200110
6.	Blood sample collection	V02-20191226
7.	Caregiver nutrition counselling	V07-20200119
8.	Child development	V09-20200115
9.	Clinical assessment	V03-20200119
10.	Data entry and management	V03-20200216
11.	Deviations	V01-20191230
12.	Equipment calibration - Lab	V03-20191230
13.	Equipment calibration - Sites	V03-20191230
14.	Informed consent	V06-20200114
15.	LNS distribution	V03-20200218
16.	Pre-screening	V05-20200103
17.	Randomization and blinding	V03-20191204
18.	Registration in REDCap	V01-20191205
19.	Scheduling in REDCap	V01-20191205
20.	Screening and inclusion	V02-20200103
21.	Stool collection	V02-20191230
22.	Stool processing in lab	V03-20200102
23.	Water, sanitation and hygiene (WASH) visit	V02-20191204
24.	COVID-19 IPC	V02-20200618

14.3 Appendix 3: Stool kit visual aid to guide caregiver sample collection.