ENVIRONMENTAL ENTERIC DYSFUNCTION, AFLATOXIN EXPOSURE, AND POOR GROWTH OUTCOMES DURING THE FIRST 1,000 DAYS IN UGANDA

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Dedication

To my Ugandan brothers and sisters who researched alongside me in the Pearl of Africa,

To my parents, for everything, and

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Abstract

Background: Stunting remains a pervasive form of undernutrition globally, affecting ~155 million children < 5 years of age. Growth failure frequently begins in utero and continues until at least 24 months of life (called the "first 1,000 days"), after which time its effects are largely irreversible. While inadequate diets and diarrheal disease are commonly cited as immediate determinants, they fail to explain much of the burden. Meanwhile, less is known about the role of chronic, asymptomatic gastrointestinal inflammation (environmental enteric dysfunction, or EED) and/or aflatoxin (AF) exposure during the first 1,000 days and their implications for poor child growth.

Objectives: The goal of this PhD thesis was to examine the associations among EED, AF exposure, and poor growth outcomes during the first 1,000 days in Uganda. Specifically, the objective of aim #1 was to examine the associations among poor household drinking water quality, EED, and poor growth outcomes in children 12-16 months of age living in rural southwestern Uganda. The objective of aims #2 and #3 were to examine the association between EED biomarkers (aim #2) and AF exposure (aim #3) in pregnant women aged 18-45 and subsequent adverse birth outcomes in Mukono District, Uganda.

Methods: For aim #1, we conducted a cross-sectional, observational study within a longitudinal birth cohort study. A lactulose: mannitol (L:M) test was performed on 385 children ages 12-16 months living in 7 sub-counties of southwestern Uganda. Water quality data were obtained using a compartment bag test, and safe water was defined as water lacking *E. coli* contamination. Data on child anthropometry and covariates were extracted from an existing birth cohort study. For aims #2 and #3, we conducted a prospective cohort study in Mukono, Uganda with a sample of 258 pregnant women who were enrolled at their first prenatal visit. We measured maternal EED biomarkers, namely L:M ratios and serum anti-flagellin and anti-lipopolysaccharide (LPS) antibodies, as well as maternal aflatoxin B1 (AFB1) concentrations. Data on maternal anthropometry and covariates were obtained at enrollment and 3 weeks prior to participants' estimated date of delivery. For all aims, statistical analyses were conducted using STATA 15 software and results were obtained from multivariate linear regression models.

Results: Aim #1: Children from households with safe drinking water had significantly lower mean ln L:M ratios (0.23-point difference, 95% CI: 0.09, 0.38) and significantly higher length-for-age (LAZ) (β: 0.34, 95% CI: 0.07, 0.61) and weight-for-age (WAZ) (β: 0.24, 95% CI: 0.10, 0.38) Z-scores at 12-16 months of age. Additionally, children with higher LAZ at 6 and 9 months had significantly lower ln L:M ratios at 12-16 months of age (β: -0.06, 95% CI: -0.13, 0.00 and β: -0.05, 95% CI: -0.10, -0.001, respectively). **Aim #2 and #3:** Higher concentrations of maternal anti-flagellin IgG and anti-LPS IgG were significantly associated with shorter length of gestation [(β: -1.35, 95% CI: -2.64, -0.07) and (β: -0.89 [95% CI: -1.52, -0.25)] and with lower length at birth [(β: -0.97, 95% CI: -1.79, -0.14) and (β: -0.45, 95% CI: -0.87, -0.02)] and lower LAZ at in infants at birth [(β -0.52, 95% CI: -0.95, -0.08) and (β: -0.25, 95% CI: -0.48, -0.03)]. Furthermore, elevations in maternal ln AFB1 levels were associated with lower weight (β: -0.07; 95% CI: -0.13, -0.002), lower WAZ (β: -0.16; 95% CI: -0.30, -0.03), smaller head circumference (β: -0.27; 95% CI: -0.51, -0.03), and lower head circumference-for-age Z-score (HCZ) (β: -0.23; 95% CI: -0.41, -0.04) in infants at birth.

Conclusions: Maternal and child EED and AF exposure were associated with significant decreases in growth at birth, infancy, and early childhood. Efforts to improve nutrition and reduce stunting may be enhanced by simultaneously addressing EED and AF exposure.

Acronyms and abbreviations

AAT: Alpha 1-Antitrypsin
AGA: Adequate size-for-Gestational Age
AGP: Alpha-1-Acid Glycoprotein
AF: Aflatoxin
AFB1: Aflatoxin B1
Alb: Albumin
BMI: Body Mass Index
CBT: Compartment Bag Test
CFS: Complimentary Food Supplement
CRP: C-Reactive Protein
E. coli: *Escherichia coli*
EED: Environmental Enteric Dysfunction
ELISA: Enzyme-Linked Immunosorbent Assay
EndoCAb: Endotoxin-Core Antibody
FGR: Fetal Growth Restriction
HAZ: Height-for-Age Z-Score
Hb: Hemoglobin
HCZ: Head Circumference Z-Score
HPLC: High-Performance Liquid Chromatography
HR: Hazard Ratio
IBD: Inflammatory Bowel Disease
Ig: Immunoglobulin
IRB: Institutional Review Board
LAZ: Length-for-Age Z-Score
LBW: Low Birth Weight
LC-MSMS: Liquid Chromatography Tandem-Mass Spectrometry
L:M: Lactulose: Mannitol (test)
LMICs: Low- and Middle-Income Countries
LPS: Lipopolysaccharide

Lys: Lysine

MHC IV: Mukono Health Center IV

MPN: Most Probable Number

MPO: Myeloperoxidase

MUAC: Mid-Upper Arm Circumference

NEO: Neopterin

ODK: Open Data Kit

OR: Odds Ratio

SD: Standard Deviation

SGA: Small-for-Gestational Age

SUN: Scale Up Nutrition

UBCS: Uganda Birth Cohort Study

USAID: United States Agency for International Development

UNICEF: United Nations Children's Fund

WASH: Water, Sanitation, and Hygiene

WAZ: Weight-for-Age Z-Score

WHA: World Health Assembly

WHO: World Health Organization

WLZ: Weight-for-length Z-Score

WHZ: Weight-for-height Z-Score

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Chapter 1: Introduction

Background and significance

Despite global progress in recent decades, undernutrition continues to underlie 3.1 million child deaths annually, representing 45% of all childhood mortality.[1] Worldwide, stunting, or a length- or height-for-age Z-score (LAZ or HAZ) < -2 relative to the World Health Organization (WHO) growth standard median,[2] affects 155 million (22.9%) children < 5 years of age according to the most recent estimates.[3] Today, Africa, which has over one-third of the world's stunted children, is the only region of the world where stunting has increased, from 50.4 million children in 2000 to 59.0 million children in 2016 (24.0 million of these children are in Eastern Africa).[3]

Stunting is often recognized as a syndrome[4] whereby short-for-age children are at an increased risk for morbidity and mortality, impaired neurodevelopment and cognitive ability, lower economic productivity and earnings, and chronic diseases later in life.[5-7] Growth failure frequently begins in utero and continues until at least the first 24 months of life (called the "first 1,000 days") after which time its effects are largely irreversible.[8] During this period, children in low- and middle-income countries (LMICs) are highly vulnerable to a number of nutritional insults.[6] In addition to inadequate diets and feeding practices, exposure to environmental pathogens and toxins, both in utero and during early postnatal life, have increasingly been linked to poor child growth.

Environmental enteric dysfunction (EED) is an incompletely defined, asymptomatic syndrome characterized by reduced barrier function, reduced absorptive capacity, and inflammation in the small intestine.[9] EED is highly prevalent in LMICs and is hypothesized to result from chronic exposure to enteropathogens as the result of living

in poor water, sanitation, and hygiene (WASH) conditions.[10,11] Furthermore, it is hypothesized to impact nutritional outcomes via several pathways, including (1) increased permeability, (2) reduced absorptive capacity, and (3) chronic intestinal and systemic inflammation.[12,13,14] To date, several studies have linked EED to poor growth outcomes, mainly linear growth faltering (i.e. stunting) in young children.[13-16] However, our current understanding of EED, particularly regarding its underlying causes and nutritional consequences, remains limited.

Aflatoxins are the toxic metabolites of *Aspergillus* molds and are widespread in the staple food supply, particularly in LMICs where it is estimated that 4.5 billion people are chronically exposed.[17] Aflatoxin B_1 (AFB_1), the most prevalent and toxic variety,[18] is a known human liver carcinogen,[19] and, more recently, has been linked to poor child growth in LMICs.[20-22] Furthermore, AFB_1 is capable of crossing the placental barrier,[23] and a limited number of human studies have demonstrated an association between maternal aflatoxin exposure and both low birth weight (LBW)[24,25] and infant growth faltering throughout the first year of life.[26]

While the mechanistic pathways by which both EED and AF exposure affect growth outcomes are still unclear, a link between the two has been suggested. A recent paper by Smith et al.[27] proposes that AF exposure can mediate certain enteropathies, including pathology that resembles the intestinal changes seen in EED. According to the authors, experimental animal models have implicated four pathways by which AF exposure can lead to intestinal damage, including (1) inhibition of protein synthesis, (2) increased local and systemic proinflammatory cytokines, (3) inhibition of ceramide synthase, and (4) tight junction protein expression.

As part of their global nutrition targets, the World Health Assembly (WHA) aims to reduce the number of children < 5 who are stunted by 40% by 2025.[28] Achieving this goal requires a more comprehensive understanding of the nutritional insults faced by children in LMICs during the first 1,000 days. The goal of this PhD thesis is to examine two such insults - environmental enteric dysfunction and aflatoxin exposure - and their association with poor child growth outcomes during the first 1,000 days in Uganda.

Specific aims and hypotheses

Aim 1: To examine the associations among poor WASH conditions, specifically poor household drinking water quality, EED, and growth outcomes in children aged 12-16 months living in rural southwestern Uganda.

Hypothesis 1a: Unsafe water quality, evaluated at 6 months, will be associated with elevated L:M ratios and poorer growth outcomes at 12-16 months.

Hypothesis 1b: Poorer growth outcomes, measured at 6 and 9 months, will be positively associated with L:M ratios at 12-16 months.

Aim 2: To examine the association between EED biomarkers in pregnant women aged 18-45 years and subsequent adverse birth outcomes in Mukono District, Uganda.

Hypothesis 2: Elevated EED biomarkers, measured in mid-gestation, will be positively associated with adverse birth outcomes, including shorter gestational age, lower birth weight and length, and smaller head circumference.

Aim 3: To examine the association between aflatoxin exposure in pregnant women aged 18-45 years and subsequent adverse birth outcomes in Mukono District, Uganda.

Hypothesis 3: Elevated maternal aflatoxin exposure, measured in mid-gestation, will be positively associated with adverse birth outcomes, including shorter gestational age, lower birth weight and length, and smaller head circumference.

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Chapter 2: Review of the Literature

Introduction

Across LMICs, 3.1 million, or 45%, of deaths among children < 5 years of age are linked to various forms of undernutrition, including fetal growth restriction (FGR), stunting, wasting, micronutrient deficiencies, and suboptimum breastfeeding.[1] Stunting refers to infants and children who are too short for their age, measured using recumbent length for children < 2 years old and standing height for children ≥ 2 years old. By definition, those with a LAZ/HAZ > 2 SD below the median, determined using the WHO growth standards, are considered "stunted."[2] However, the health and economic consequences of the condition, including an increased risk of morbidity and mortality and poor cognitive development, exist along a LAZ/HAZ continuum (i.e. without a notable inflection point at -2 SD).[3]

Despite the enormous global burden, certain mechanisms underlying linear growth failure are not well understood. According to the Lancet's 2013 series on Maternal and Child Undernutrition, interventions to resolve stunting implemented at 90% coverage would only avert 20% of the global burden, leaving most of the problem unaddressed.[4] Two underexplored domains contributing to the burden are chronic, asymptomatic gastrointestinal inflammation (i.e. EED) and AF exposure, although their relative contributions are largely unknown.

Stunting

Burden

Stunting remains an extremely pervasive form of undernutrition in LMICs, particularly in infants and young children in Asia and Africa. Based on the 2016

UNICEF-WHO-World Bank joint child malnutrition estimates, approximately 155 million (22.9%) children < 5 years of age are stunted.[5] In Uganda, specifically, stunting continues to affect 29% of children < 5 years of age, with the highest prevalence rates observed in the north (Karamoja and West Nile) and the southwest.[6] Currently, the country is off-track to meet both the WHA target by 2025 and its government's own target to reduce stunting to 25% by 2019/20 and to 0% by 2040 as outlined in their Second National Development Plan 2015/16-2019/202.[7] According to a recent report, undernutrition is estimated to cost Uganda ~900 million USD annually, or 5.6% of GDP.[8]

In recent decades, addressing the burden of stunting in LMICs has become a major global health priority, and, consequently, has been the center of several high-profile initiatives, including Scaling Up Nutrition (SUN), the Zero Hunger Challenge, and the Nutrition for Growth Summit. Furthermore, in 2012 the World Health Assembly set a target to reduce by 40% by the year 2025 the number of children < 5 years of age who are stunted. However, despite these initiatives, progress toward reducing stunting rates has been both slow and inconsistent across regions of the world. In Asia, for example, the proportion of stunted children fell from 38% to 24% between 2000 and 2016 whereas in Africa it fell from 38% to only 31% during this time. Furthermore, owing to population growth, Africa is the only region in the world where the number of stunted children < 5 years has risen, from 50.4 million to 59.0 million over the years 2000-2016.[5]

Etiology

Although height is an heritable trait, the > 200 genes involved explain only ~10% of the variation in adult height, while environmental factors make up the remaining observable differences.[9,10] Furthermore, under ideal circumstances, infants and young children from different geographical areas have been shown to grow at very similar rates during fetal life and throughout the first few years of postnatal life.[11,12] However, infants and young children in LMICs face numerous assaults during this time, such as poor maternal nutritional status, inadequate feeding practices, poor WASH conditions, limited access to healthcare, and frequent infections, which limit growth, often irreversibly.[13]

Stunting frequently begins in utero and continues until at least the first 24 months of life, after which time its effects are considered largely irreversible. Numerous factors, such as maternal undernutrition and infections, anemia, tobacco use, and indoor air pollution, are known to restrict fetal growth and result in LBW (and other adverse birth outcomes) and stunting from birth which accumulates through childhood.[14] According to Victora et al.,[15] the average LAZ among newborns at birth in LMICs is ~ -0.5 SD and continues to decline after birth to reach ~ -2.0 SD by 18-24 months. Using data from 19 longitudinal cohorts, Christian et al.[16] concluded that approximately 20% of cases of stunting begin in utero, most often as the result of premature birth, FGR, or both. According to the authors' findings, relative to adequate size-for-gestational age (AGA) and term, the odds ratio (OR) (95% confidence interval) for stunting associated with AGA and preterm, small-for-gestation-age (SGA) and term, and SGA and preterm were 1.93 (1.71, 2.18), 2.43 (2.22, 2.66) and 4.51 (3.42, 5.93), respectively.

During early postnatal life, infants and young children face further insults to their linear growth, although certain aspects of the pathogenesis remain poorly understood. Chronic nutrient deficiencies (due to suboptimal breastfeeding and complementary feeding practices), recurrent infections, and/or chronic inflammation are established proximal determinants;[1] however, their relative contributions remain unknown. As a result, effective interventions to promote healthy growth also remain unclear.[17] This was highlighted in a 2008 systematic review by Dewey et al.[18] which concluded that complementary feeding inventions for malnutrition in children 6-24 months had only moderate benefits. Furthermore, according to Bhutta et al.,[4] the known interventions to resolve stunting implemented at 90% coverage would only avert 20% of the global burden, leaving most of the problem unaddressed.

Consequences

Addressing the burden of stunting has become a major global health priority in recent years, mainly due to the condition's demonstrated association with an array of health and economic consequences. As a result of these findings, stunting is now viewed as an easily measurable indicator of a "syndrome," which has short- medium- and long-term implications at both the individual and societal levels.[13] Stunting is associated with increases in morbidity and mortality such that severe stunting (LAZ/HAZ < -3) in early childhood is associated with a 4 times greater risk of mortality before age 5.[19] In a pooled analysis involving infants and children 1 week to 59 months old in 10 prospective studies in Africa, Asia, and South America, Olofin et al.[20] found that childhood stunting was associated with morbidity in a dose response relationship. Children with a LAZ/HAZ

between -2 and < -1 SD had an elevated risk of respiratory infections (HR 1.55, 95% CI: 1.02–2.37) and diarrhea (HR 1.67, 95% CI: 1.20–2.30), and severely stunted children had a much greater risk of the two infections (HR 6.39, 95% CI: 4.19–9.75 and 6.33, CI: 4.64–8.65, respectively).

While undernutrition underlies 45% of all child deaths, morbidity and mortality have been described as merely the "tip of the iceberg" with regard to consequences.[21] Undernutrition also affects areas of the brain involved in cognition, memory, and locomotor skills. As a result, stunted children are more apathetic and display less exploratory behavior in early life, and later on they are less likely to enroll at school or enroll late, achieve lower grades, and have poorer cognitive ability compared to non-stunted children.[13] With regard to long-term effects, Victora et al.[22] reviewed 5 prospective cohort studies (from Brazil, Guatemala, India, Philippines, and South Africa) with long-term follow-up. Overall, the authors found a link between childhood stunting and a number of consequences, including short adult stature, reduced lean body mass, less schooling, diminished IQ, reduced earnings and productivity, and lower birthweight of infants born to women who themselves had been stunted as children.

Environmental Enteric Dysfunction

Background and burden

Previously called "tropical enteropathy" (i.e. jejunitis), EED was first identified in the 1960s in adult populations living in LMICs after intestinal biopsies showed abnormal villi that were blunted and shortened.[23-26] Also during this time, studies of stillborn fetuses reported that EED is not present at birth and therefore is an acquired rather than genetic condition.[27] Meanwhile, studies on expatriates demonstrated that EED symptoms

resolved after repatriation, suggesting that EED is both an environment-dependent as well as reversible condition.[28,29] EED in children was not well recognized until decades later, most notably through a series of studies in the Gambia.[30,31]

These studies in the Gambia, which concluded that EED develops during early infancy and is associated with poor growth outcomes in young children, resulted in the renewed attention to the condition that continues today. While EED remains incompletely-defined, it is characterized by reduced barrier function, reduced absorptive capacity, and inflammation in the small intestine.[32] The condition is prevalent in environments where WASH conditions are poor and is hypothesized to result from chronic exposure to enteropathogens, especially via the fecal-oral transmission route.[33,34] Today, EED is a significant public health concern mainly due to its postulated association with poor growth and development outcomes in young children in LMICs.[31,35-37]

Early investigations involving the use of intestinal biopsies concluded that EED is practically universal among populations living in LMICs [38,39] Today, quantifying the burden of EED is difficult given both the practical and ethical implications of conducting intestinal biopsies and the lack of validated proxy biomarkers. However, studies comparing lactulose: mannitol (L:M) ratios between children in LMICs and age-matched healthy developed country populations, have found the prevalence of EED in children in LMICs to be very high, commonly around 80-90%.[30,37,40,41]

Physiology

The small intestine plays a crucial role in several major functions within the body, including digestion, absorption, barrier function, and immune response, which are all necessary for normal growth and development. Within the small intestine, the intestinal

mucosa, which includes the intestinal epithelium, maintains a barrier between luminal contents and the sterile blood stream. The intestinal epithelium itself is comprised of two major entities-villi and crypts. Villi, which protrude into the lumen as finger-like projections, are made up of mature, absorptive cells. Together with a "brush border" of microvilli, the primary function of villi is to greatly increase the absorptive surface area of the small intestine. Villi are also covered by a single cell layer epithelium with tight junctions between the individual enterocytes that act as an impermeable barrier and prevent microbial translocation. Crypts, on the other hand, are a contiguous pocket of epithelial cells at the base of the villi that are primarily involved in the secretion of enzymes necessary for nutrient digestion and absorption.[42,43]

Currently, there is no universally accepted case definition for EED. Rather, EED is often characterized by nonspecific histological features which resemble celiac disease (CD) and include small intestine villous atrophy (i.e. the blunting or flattening of the villi), crypt hyperplasia (i.e. the elongation of the crypts), and leakiness of the tight junctions between enterocytes.[44] Villous atrophy and crypt hyperplasia result in the reduction of surface area available for the secretion of digestive enzymes and the absorption of nutrients. Meanwhile, leaky tight junctions result in the translocation of pathogens into the blood stream which can trigger an immune response and low-grade, systemic inflammation.[31,45-47] Both conditions - malabsorption of nutrients and systemic immune response - are proposed pathways by which EED can inhibit growth in young children.

EED biomarkers

An intestinal biopsy (i.e. extracting a sample of the intestinal lining and examining it with a microscope) is required to assess the fore noted morphological changes, which characterize EED. However, given the costly and invasive nature of this procedure, there is a need for validated biomarkers to facilitate the diagnosis of EED in population-based studies in LMICs. To date, numerous biomarkers have been proposed across several bio-specimens, including serum, urine, and stool. However, a majority of these biomarkers measure only a single aspect of EED (ex. intestinal damage and repair, epithelial permeability and absorption, microbial translocation, intestinal inflammation, and systemic inflammation) and therefore fail to adequately diagnose EED across all of its functional domains.[48]

The L:M test

Currently, the most widely used proxy measure of EED is a dual sugar absorption test, most commonly the lactulose: mannitol (L:M) test. To conduct a dual sugar absorption test, a researcher administers a solution containing a standardized dose of one non-absorbed, disaccharide (i.e. lactulose) and one transcellularly-absorbed, monosaccharide (i.e. mannitol) with a timed urine collection, normally lasting 4-5 hours, to follow. The increased absorption of lactulose reflects impaired functioning of the tight junctions (i.e. intestinal permeability), and the decreased absorption of mannitol indicates villous atrophy and decreased absorptive surface area. The amounts of lactulose and mannitol are normally expressed as a ratio (lactulose: mannitol), with higher ratios indicating greater intestinal abnormality overall, reflective of EED.[47]

In a 2014 systematic review of 25 studies across geographic regions, Denno et al.[49] determined that the L:M test adequately captures both intestinal permeability and absorptive capacity (but not other EED domains, such as intestinal or systemic inflammation) and likely correlates with growth failure in young children. However, the L:M test has several disadvantages which make it difficult to perform in population-based studies in LMICs. Most notably, the test is time consuming and burdensome for both researchers and participants. Furthermore, the test continues to lack standardization across protocols, with differences reported regarding the dosage of sugars administered, the solution osmolality, the urine collection time, and the type of lab analysis (e.g. HPLC vs. LC-MSMS).[49]

Emerging biomarkers

Given the limitations of dual sugar absorption tests, numerous fecal and blood biomarkers have recently been proposed for the purposes of diagnosing EED. Building on the work of Denno et al.,[50] Harper et al.[48] characterized many of these proposed markers according to five functional EED domains, including 1) intestinal damage and repair, 2) epithelial permeability and absorption, 3) microbial translocation, 4) intestinal inflammation, and 5) systemic inflammation (see Table 1). Overall, these markers offer numerous advantages regarding the burden of sample collection and analysis relative to the L:M test. However, while several of these markers are becoming more common in the literature, none have been validated to date.

Because these biomarkers capture only a single aspect of EED, a panel of markers has also been proposed.[51] For example, several studies have calculated an "EED

composite score" comprised of three fecal markers a-1 antitrypsin (AAT), myeloperoxidase (MPO), and neopterin (NEO).[35,52,53] However, the few studies that have examined the correlation among a panel of biomarkers have found low agreement both internally and with the L:M test. Notably, Campbell et al.[54] measured serum and stool biomarkers (including myeloperoxidase AAT, MPO, NEO, endotoxin core antibodies (EndoCAb), glucagonlike peptide-2 (GLP-2), C-reactive protein (CRP), and a-1 acid glycoprotein (AGP)) in a sample of 539 18-month-old children in Bangladesh. From these, the authors were unable to find a subset of markers that closely approximated the L: M test.

Table 1: List of emerging EED biomarkers

Emerging EED Biomarkers			
Biomarker	Biospecimen	Description	EED Domain
Markers of Intestinal Damage and Repair			
Citrulline	Blood	A non-essential amino acid mainly produced by enterocytes *de novo*.	Intestinal surface area
Intestinal fatty acid binding protein (I-FABP)	Blood	An intracellular epithelial protein located primarily at the tips of small intestinal villi that is rapidly released into the circulation after injury to the epithelia.	Intestinal injury, especially recent
Regenerating (REG) proteins (e.g. REG-1B)	Stool	A family of proteins involved in tissue regeneration and cell proliferation.	Epithelial injury
Glucagon-like peptide 2 (GLP-2)	Blood	A gut trophic factor released by enteroendocrine L-cells of the ileum, which aids in mucosal regeneration.	Intestinal injury
Markers of Permeability and Absorption			

L:M test	Urine	Intestinal inflammation creates small pores between epithelial cells allowing for paracellular permeation of lactulose, while villous atrophy reduces epithelial surface area and mannitol absorption.	Permeability and absorptive capacity
Alpha-1-Antitrypsin (AAT)	Stool	A protein released during inflammation to protect cells against proteolytic enzymes released by neutrophils during infection.	Permeability, especially from the blood into the gut lumen.
Claudins (-2 and -15)/Zonulin	Blood	Proteins that modulate tight junctions, which form paracellular barriers between intestinal epithelial cells.	Claudins: absorptive capacity, zonulin: permeability
Markers of Microbial Translocation			
Lipopolysaccharide (LPS) and flagellin	Blood	Two outer components of bacterial structure.	Microbial translocation
Endotoxin core antibody (EndoCAb)/ Anti-LPS immunoglobulin G (IgG) and A (IgA)	Blood	Used to identify an immune response to systemic LPS.	Microbial translocation
Markers of Intestinal Inflammation			
Myeloperoxidase (MPO)	Stool	An enzyme stored inside neutrophils, involved in the process of killing bacteria.	Intestinal inflammation
Neopterin (NEO)	Stool	Produced by macrophages or dendritic cells upon stimulation by interferon-gamma, which is released during pro-inflammatory responses by Th1 lymphocytes.	Intestinal inflammation
Calprotectin	Stool	A calcium- and zinc-binding protein released by neutrophils as a result of cell stress or damage.	Intestinal injury/intestinal inflammation
Markers of Systemic Inflammation			

Cytokines (Interferon gamma (IFN-γ), tumor necrosis factor (TNF), and interleukins (e.g., IL-6, IL-10))	Blood	Signaling proteins that activate and drive differentiation of immune cells upon infection.	Systemic inflammation
Acute-phase proteins (Alpha-1-acid glycoprotein, C-reactive protein, and ferritin)	Blood	Proteins involved in the inhibition of microbial growth.	Systemic inflammation
Soluble CD14 (sCD14)	Blood	A circulating co-receptor for LPS secreted by monocytes and macrophages.	Systemic inflammation
Total Igs (IgG and IgM)	Blood	Antibodies released by antibody-secreting B-lymphocytes to fight infections.	Systemic inflammation
Kynurenine-tryptophan ratio (KTR)	Blood	Kynurenine is formed from the essential amino acid tryptophan by the enzyme indolamine 2,3-dioxygenase, which is upregulated by pro-inflammatory cytokines. Immune activation leads to formation of kynurenine and depletion of tryptophan.	Systemic inflammation

This table was developed from information cited in the article "Environmental enteric dysfunction pathways and child stunting: A systematic review" by Harper et al. (2018).

Etiology

Poor WASH conditions

It was first noted in the 1960s that children in LMICs are born with long villi that became flatter throughout infancy, suggesting that EED has an environmental rather than genetic etiology.[27,55] In 1993, an article by Solomons et al.[56] proposed that there is a connection between stunting in children in LMICs and the phenomenon of impaired growth of poultry and livestock reared under unsanitary conditions. Later, in a 2009

Lancet commentary, Jean Humphrey[33] expanded on this idea and formally hypothesized that EED is a key link between poor WASH conditions (i.e. poor household and community sanitation, lack of clean water, lack of hand washing facilities, and/or lack of sanitary food storage and preparation) and poor growth. Therefore, while the environmental etiology of EED has long been established, its proposed link to poor WASH conditions is fairly recent and remains speculative.

Although supporting evidence is relatively sparse, it is hypothesized that repeated exposure to enteropathogens early in life, as the result of living in poor WASH conditions, results in changes to the composition of the intestinal microbiota and eventually to the structure and function of the small intestine. This creates a "self-perpetuating cycle of pathology" (i.e. infection–malnutrition–infection).[57] Furthermore, it is hypothesized that many of these infections are subclinical, and that diarrhea only accounts for a small proportion of the development of EED throughout childhood.[10] For example, in the Gambia, Lunn et al.[30,33] observed that children had diarrhea only 7.3% of the time; however, elevated L:M ratios were observed in infants 76% of the time.

To date, a study by Lin et al.[34] is commonly cited as evidence in support of a link between poor WASH and stunting via EED. In the study of 119 children (≤ 48 months of age), the authors assessed the relationship between fecal environmental contamination and EED across rural Bangladesh. Household environmental cleanliness was defined by objective indicators of water quality and sanitary and hand-washing infrastructure. The authors found that children living in environmentally "clean" households had better intestinal health, characterized by lower L:M ratios (-0.32 SDs, 95% CI: -0.72, 0.08), and

higher HAZ (0.54 SDs, 95% CI: 0.06, 1.01) than children from "contaminated" households.

In addition to the Lin study, two articles by George et al.[53,58] provide further evidence of a link between poor WASH conditions and EED. The authors conducted a prospective cohort study of 216 children < 5 years of age, also in rural Bangladesh. EED in the study was measured using four fecal markers: alpha-1-antitrypsin, myeloperoxidase, and neopterin (all three combined to form an EED disease activity score) and calprotectin. The authors observed an association between geophagy (i.e. consumption of soil, dirt, or mud) and EED as well as between animal exposure and caregiver hygiene and EED. Children with caregiver-reported geophagy had significantly higher EED scores (0.72-point difference, 95% CI: 0.01, 1.42) and calprotectin concentrations (237.38 μg/g, 95% CI: 12.77, 462.00).[53] Furthermore, children with an animal corral in their sleeping room had significantly higher EED scores (1.0-point difference, 95% CI: 0.13, 1.88), and children of caregivers with visibly soiled hands had significantly higher fecal calprotectin concentrations (384.1 μg/g, 95% CI: 152.37, 615.83).[58]

Diet and nutritional causes

In addition to exposure to poor WASH conditions, other etiologies of EED have been proposed, including exposure to food contaminants, environmental toxins, and micronutrient deficiencies. Several studies have shown that children are more vulnerable to EED during certain periods, particularly when they move from exclusive breastfeeding to mixed feeding, ideally around 6 months of age, and when they cease breastfeeding,

ideally around 23 months of age.[41,59] It is hypothesized that these periods are associated with worsening EED status due to the fact that children are exposed to home-prepared complementary/weaning foods during this time, which may contain enteropathogens and enterotoxins.

Additionally, exposure to aflatoxins, or the toxic metabolites of fungi that frequently contaminate staple foods in LMICs, has been shown to impair intestinal integrity, although these data come primarily from animal studies. In one such study, Yunus et al.[60] found that the small intestines of chickens that were exposed to aflatoxin weighed less compared with intestine of unexposed chickens, suggesting a decrease in absorptive capacity. Furthermore, Applegate et al.[61] found that chickens exposed to aflatoxin had reduced crypt depth (but not villus length), which increased the villus: crypt ratio and also suggests a decrease in absorptive capacity due to aflatoxin exposure.

Finally, it has been suggested that certain micronutrient deficiencies, notably zinc and vitamin A, may also play a role in the development or exacerbation of EED. Zinc, which is recommended by WHO and UNICEF for the treatment of acute diarrhea, has also been shown to aid in the recovery of the intestinal mucosa following an episode.[62] In a study by Manary et al.[63] involving Malawian children aged 3-5 years, endogenous fecal zinc (EFZ) was positively correlated with the L:M test ($r = 0.62$, $p < 0.001$), and net zinc retention was negatively correlated with the L:M test ($r = -0.47$, $p = 0.02$), suggesting that perturbed zinc homeostasis is associated with EED. Furthermore, in a study by Roy et al.[64] involving Bangladeshi children aged 3-24 months, two-week zinc supplementation significantly reduced lactulose excretion in both children with acute and persistent diarrhea. Finally, in a study by Chen et al.[65] involving young children in Brazil, L:M

ratios were inversely correlated with serum retinol concentrations ($r = -0.55$, $p < 0.0005$). However, despite the possible links to specific micronutrients, supplementation trials have demonstrated mixed impacts with regard to EED among children in LMICs.[64-66] Notably, in a randomized, double-blind, placebo-controlled clinical trial by Wang et al.[67] involving Malawian children aged 12-35 months, the combined usage of albendazole, zinc, and a daily multiple micronutrient powder did not decrease EED or stunting.

Implications for growth

The hypothesis that EED may be linked to growth faltering in infants and young children was first examined by seminal research conducted in the early 1990s in the Gambia. Lunn et al.[30] reported that elevated L:M ratios were both highly prevalent in children aged 2-15 months, found in 76% of monthly assessments, and could explain up to 43% of observed stunting. While several subsequent studies have also suggested a link between EED and growth failure, the associations have been indisputably weaker in comparison. In a 2003 prospective study also from the Gambia, Campbell et al.[31] found that both intestinal permeability ($r = -0.41$, $p < 0.001$) and plasma concentrations of endotoxin and immunoglobulin (Ig) G-endotoxin core antibody ($r = -0.30$, $p < 0.02$; $r = -0.64$, $p < 0.0001$, respectively) were associated with impaired growth in children 8-64 weeks old.

More recently, two studies from Malawi have also shown there to be an association between EED and poor growth outcomes. In 2012, Weisz et al.[37] found better growth was associated with less urinary lactulose excretion in a sample of children aged 2-5 years old. Furthermore, in 2016, Ordiz et al.[36] found that the L:M test was inversely

correlated with linear growth over a 3-month period ($r = -0.32$, $p < 0.001$) and severe EED was associated with stunting ($p < 0.0001$) in a sample of 798 asymptomatic Malawian children aged 12-61 months. Outside of Africa, Lin et al.,[34] found that a 1-unit increase in the log L:M was associated with a 0.33 SD decrease in HAZ (95% CI = −0.62, −0.05) in a sample of 119 rural Bangladeshi children < 48 months old.

Finally, the "Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health" (MAL-ED) cohort study led by the Fogarty International Center of the National Institutes of Health and the Foundation for the National Institutes of Health aimed to assess enteropathogen infection and poor growth from birth-24 months in 8 sites in Bangladesh, Brazil, India, Nepal, Pakistan, Peru, Tanzania, and South Africa. Using samples from 537 children across the 8 study sites, Kosek et al.[35] created a disease activity score comprised of NEO, AAT, and MPO fecal markers. Children with the highest EED score grew 1.08 cm less than children with the lowest score over the 6-month period following the tests after controlling for the incidence of diarrheal disease.

Aflatoxin

Background and burden

Aflatoxins are a naturally-occurring secondary metabolite of *Aspergillus* molds, particularly *A. flavus* and *A. parasiticus*.[68] They are known to contaminate many staple crops (e.g. corn, cassava, wheat, rice, tree nuts, peanuts, chilies, and spices) particularly in environments where the temperatures 24-35°C and the moisture content exceeds 7% (or 10% with ventilation).[69] They are especially prevalent in the staple diets of many

LMICs, especially across Asia and Africa, where poor harvest and storage practices leave food supplies vulnerable to fungal contamination.[70,71]

Overall, it is estimated that 4.5 billion people, primarily in LMICs, are chronically exposed to largely uncontrolled amounts of the toxin in their diet.[69] Of these, ~0.5 billion are at significant risk of exposure.[72] In a 2017 study, Smith et al.[73] identified 12 epidemiologic studies from Africa, Asia, and the Middle East, including a total of more than 2,000 participants, which reported aflatoxin exposure in pregnant women and/or infant cord blood and found that prevalence of exposure ranged from 6%-100%, suggesting exposure is pervasive, if not ubiquitous, in certain populations. A 2014 pilot study from Southwest Uganda by Asiki et al.[74] analyzed sera from 100 adults and 96 children < 3 years of age and found detectable aflatoxin-albumin (AF-alb, a serum biomarker reflecting cumulative aflatoxin exposure over the prior 2-3 months)[75] in very adult and all but four children (levels ranged from 0 to 237.7 pg/mg). A 2015 follow-up study by Kang et al.[76] was conducted in the same population with 713 samples and found 90% were positive for AF-alb.

Implications for health

Aflatoxin exposure has been linked to a number of carcinogenic, teratogenic, and immunotoxic health effects in humans, most notably hepatocellular carcinoma (HC) or liver cancer,[77] which is the third leading cause of cancer deaths worldwide.[78] As a result, the International Agency for Research on Cancer (IARC) identifies aflatoxin as a Class 1 carcinogen, and many upper-middle- income countries (UMICs) have created regulations regarding allowable aflatoxin contamination in human foods, ranging from 4-30 ppb

aflatoxin.[79] However, aflatoxins are not as effectively controlled in the food system of most LMICs, which has resulted in a number of outbreaks of aflatoxicosis, or aflatoxin poisoning, most notably in India in 1975,[80] in Kenya in 1981,[81] and again in Kenya in 2004.[82]

In addition to these outbreaks, chronic aflatoxin exposure was linked to maternal anemia in a cross-sectional study of 755 pregnant women in Kumasi, Ghana by Shuaib et al [83] According to their findings, the odds of being anemic increased 21% (OR: 1.21, p = 0.01) with each quartile of AF-alb, reaching 85% increased odds in the "very high" compared with the "low" category (OR: 1.85, CI: 1.16–2.95). This study supports several vitro and in vivo animal studies that have shown relatively high aflatoxin exposures (range: 0.5–1 ppm) are cytotoxic and cause lysis of red blood cells.[84-86] Finally, in another study from Ghana by Jiang et al.,[87] aflatoxin exposure among study participants (n=64) was associated with impairments in immune function. Study participants with high AFB_1 levels had significantly lower percentages of CD3+ and CD19+ cells that showed the CD69+ activation marker (CD3+CD69+ and CD19+CD69+) compared to participants with low AFB_1 levels (p = 0.002 for both), and the percentages of CD8+ T cells that contained perforin or both perforin and granzyme A were also significantly lower in participants with high AFB_1.

Implications for growth

While the number of studies remains limited, aflatoxin exposure during the first 1,000 days increasingly been linked to poor growth outcomes. Studies have demonstrated that aflatoxins are capable of crossing the placental barrier;[88] however, to date only a few

studies have examined the association between maternal aflatoxin exposure during pregnancy and adverse birth outcomes. A prospective study of 201 women in the United Arab Emirates by Abdulrazzaq et al.[89] concluded that aflatoxin concentrations measured in cord blood were significantly negatively associated with birth weight ($p < 0.001$). Furthermore, a cross-sectional study of 785 pregnant women in Ghana by Shuaib et al.[90] showed that participants in the highest quartile of AFB_1 exposure were more than twice as likely to have a low birth weight baby compared to the lowest quartile (OR: 2.09, 95% CI, 1.19-3.68).

Additional studies have shown that aflatoxin exposure continues to affect growth in young children after birth. In a 2007 study by Turner et al.[91] involving 138 Gambian mother/infant pairs, average maternal AF-alb concentrations measured at 5 and 8 months gestation were significantly associated with lower weight-for-age (−0.249 Z-scores, $p = 0.012$) and lower height-for-age (−0.207 Z-scores, $p = 0.044$) in the first year. The authors concluded that a reduction of maternal AF-alb from 110 pg/mg to 10 pg/mg would lead to a 0.8 kg increase in weight and 2 cm increase in height within the first year of life. Furthermore, in a 2002 cross-sectional study by Gong et al.[92] involving 480 children aged 9 months-5 years from Benin and Togo, children with stunting or who were underweight had 30-40% higher mean AF-alb concentrations ($p = 0.001$ for HAZ, $p = 0.005$ for WAZ, and $p = 0.047$ for WHZ). Finally, in a 2004 longitudinal study also by Gong et al.[93] involving 200 children aged 16-37 months recruited from 4 villages in Benin (2 with high and 2 with low aflatoxin exposure) there was a strong negative correlation ($p < 0.0001$) between AF-alb and height increase over an 8-month follow-up.

In the study, the highest quartile of AF-alb was associated with a mean 1.7 cm reduction in growth compared with the lowest quartile.

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Chapter 3: Methodology

Aim #1 methodology

Study design

To assess the relationship among water quality, EED, and growth outcomes, we conducted a cross-sectional, observational study as a sub-study of the Uganda Birth Cohort Study (UBCS, see methods below) between July and August 2016 in 7 sub-counties (Bugangari, Buyanja, Bwizi, Kebisoni, Kibiito, Nyamweru, Ruhiija) in southwestern Uganda. In this study, EED was assessed using the L:M test when the children were 12-16 months of age.

The study was approved by the Tufts Health Sciences Institutional Review Board (IRB) in Boston, Massachusetts, USA; the IRB at Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA; the Makerere University Research Ethics Committee at the School of Public Health in Kampala, Uganda; and the Uganda National Council for Science and Technology in Kampala, Uganda.

The Uganda birth cohort study

With the exception of data from the L:M tests, data required for this study's analyses were extracted from the UBCS main dataset. The UBCS, conducted from 2014-2016 by the USAID Feed the Future Innovation Lab for Nutrition at Tufts University, was a longitudinal prospective study following > 5,000 recruited pregnant women in 16 sub-counties (12 districts) in Southwest and Northern Uganda. Participants were recruited into the study based on the following criteria: (1) participant had a confirmed pregnancy by a urine pregnancy test, (2) participant was between 15 and 49 years old, (3) participant

intended to reside in the study area through completion of follow-up, (4) participant intended to deliver in the study area, and (5) participant provided informed consent.

Throughout the duration of the UBCS, visits were conducted every 3 months from pregnancy until the infant turned either 6 or 9 months of age. Survey data were collected using tablets and Open Data Kit (ODK) software by a team of 35 enumerators and 16 supervisors. Data included in-depth information on agricultural production; demographics, socio-economic status, diets and nutritional/health status of women through pregnancy; birth outcomes; food security of the household; water, hygiene, and sanitation practices at the household level; and anthropometry in women and their infants. Blood samples were collected from both mothers (at prenatal visit #1, at birth of child, and when the child was 6 months) and their infant (at birth and at 6 months).

Height/length was measured in triplicate to the nearest 0.1 cm using a portable height board (ShorrBoard® infant/child/adult portable height-length measuring board; Weigh and Measure, LLC, Olney, MD, USA). Weight was measured in triplicate to the nearest 0.1 kg using an electronic scale (Seca, Hanover, MD, USA). MUAC was measured in triplicate on the left arm using a standard tri-colored, non-stretch measuring tape. Triplicate measurements were averaged to provide one measurement of length, weight, and MUAC per participant per visit. Hemoglobin was measured in infants at 6 months of age using a portable hemoglobinometer (HemoCue 301; HemoCue America, Brea, CA, USA).

Finally, drinking water quality in the UBCS was measured using a compartment bag test (CBT kit; Aquagenx, Chapel Hill, NC, USA) when the infant was 6 months of age. The CBT is a portable water quality test kit designed to detect and quantify

Escherichia coli (E. coli) bacteria. For the test, participants were asked to provide a glass of water from their primary drinking water storage container, and a 100 mL sample was mixed with an *E. coli* chromogenic growth medium. The sample was then poured into a plastic bag with 5 compartments of varying volumes, sealed, and incubated for a period of 48 hours. Risk categories were determined by noting which, if any, compartments changed from yellow to green/blue and matching that to a most probable number (MPN) table based on the World Health Organization (WHO) guidelines. Health risk categories are safe (<1 CFU/100 ml.), intermediate risk (1-10 CFU/100 ml.), high risk (>10-100 CFU/100 ml.), and very high risk/unsafe (>100 CFU/100 ml).[1]

Sample size and eligibility for sub-study

A sample size of 385 children for the sub-study was calculated with G*Power software for a multiple regression with the following parameters: medium effect size (f^2=0.15), 0.95 power, 0.05 type 1 error probability, and 7 predictors. Sample size was doubled to account for sub-county clustering and further increased by 25% to allow for potential challenges in conducting and analyzing the L:M test.

Eligible children were between 12 and 16 months of age, residing in one of the seven sub-counties, and had complete UBCS visits up to 9 months. Children were excluded from the study if they had had one or more episodes of diarrhea in the previous two weeks, were severely malnourished (MUAC < 11.5 cm), or had a serious illness at the time of the study.

L:M test

After an observed one hour fast, each child consumed 20 mL solution containing 5 grams of lactulose (Lactulose Solution; Mckesson, San Francisco, CA, USA) and 1 gram of mannitol powder (D-mannitol powder; Sigma-Aldrich, St. Louis, MO, USA) completely dissolved in sterile water. Children were carefully monitored to ensure that none of the solution was spilled, spit out, or vomited. If any of these events occurred, the test was rescheduled for a different day.

After children successfully consumed the L:M solution, urine was collected over a minimum of a 4-hour period using sterile adhesive urine collection bags (Thermo Fisher Scientific, Waltham, MA, USA), which were replaced after each urination episode. Collected urine was consolidated in a plastic container with thimerosal (Sigma-Aldrich, St. Louis, MO, USA) added to the container to prevent bacterial growth. Drinking water was provided and allowed *ad libitum* throughout the test, and breastfeeding and/or a small meal was allowed at the 3-hour mark. At the 4-hour mark, children were offered a juice drink to encourage a final urination episode, which marked the end of the test.

Total urine volume was measured to the nearest 1.0 mL using a graduated cylinder and the urine was aliquoted into plastic cryovials. The samples were transported on ice in plastic cooler boxes to the local health facility where they were stored at -20°C. Upon completion of the study, samples were stored at -80°C in Kampala before being transported on dry ice to the laboratory at Baylor College of Medicine for analysis (see below).

Statistical analysis

All statistical analyses for this study were carried out using STATA 15 software (Stata Corps, College Station, TX, USA). The primary outcomes of interest were the (ln) L:M ratio at 6 months and length-for-age Z-score (LAZ) at time of the L:M test. Secondary outcomes included percent lactulose excretion (%LE), percent mannitol excretion (%ME), and the lactulose mannitol excretion ratio (LMER) in addition to weight-for-age (WAZ) and weight-for-length (WLZ) Z-scores.

L:M ratios were calculated using the fractional excretion of each of the two sugars. Because of the right-skewed nature of its distribution, the L:M ratio was natural log transformed. %LE and %ME were calculated by first multiplying the concentration of excreted sugar (mg/mL) by the total urine volume and then dividing that amount by the initial dose of each sugar. The lactulose: mannitol excretion ratio (LMER) was calculated by taking the ratio of % LE to %ME.

Growth outcomes, including LAZ, WAZ, and WLZ, were calculated using the WHO Multicenter Growth Reference Study growth standards.[2] Dichotomous variables, stunting (LAZ < -2), underweight (WAZ < -2) and wasting (WLZ < -2), were also created. All extreme outliers (-6 > WAZ > 5, -6 > LAZ > 6, and -5 > WLZ > 5) were set to missing, per WHO recommendations. Anemia was defined as hemoglobin <11 g/dl.

Because no level of *E. coli* contamination is considered safe, a dichotomous (safe vs. unsafe) water variable was created, with safe water defined as no *E. coli* detected and unsafe water defined as any *E. coli* detected. Improved water sources were piped water, a public tap, a tube well/borehole, a protected well/spring, and rain water. Unimproved water sources were an unprotected well/spring, surface water, and other. An asset score

(0-4) was created based on household ownership of the following four items: telephone, bicycle, radio, and motorcycle.

Differences in means for L:M results between households with safe v. unsafe water and children who were stunted vs. not stunted were calculated using t-tests. Associations among water quality, L:M results, and growth outcomes were assessed using unadjusted and adjusted linear and logistic regression models. For all adjusted models, covariates from the 6-month time point were based on bivariate analyses and a p-value cut-point of 0.20. Associations were considered significant in the case of p-value < 0.05.

Analysis of lactulose and mannitol concentrations

Lactulose and mannitol concentrations were determined using a modification of the method of Catassi et al.[3] The urine was spun in a 1.5-mL microcentrifuge tube at 3000 rpm for 5 minutes at room temperature and then filtered through a 0.2-mm Micro prep disc membrane filter (Bio-Rad Laboratories, Richmond, CA). A 20-µL aliquot of the filtered material was injected onto an Aminex HPX 87C 300 7.8 mm cation-exchange column protected with a precolumn Bio-Rad Carbohydrate Deashing System (Bio-Rad Laboratories) and eluted with degassed pure water at a flow rate of 0.6 mL/min at 85°C. The column effluent was monitored with a differential refractometer (Millipore Corporation, Medford, MA) with the sensitivity setting of 128, scale factor at 25, and internal temperature at 50°C. The column was calibrated using lactulose and mannitol as standards. The interassay coefficient of variation (CV) of the method was $\leq 5\%$.

Aims #2 and #3 methodology

Study design

To examine the relationship between both maternal EED and aflatoxin exposure during pregnancy and subsequent infant birth outcomes, we performed a prospective cohort study between February and November 2017 in Mukono District, Central Region, Uganda. Mukono is a semi-urban district situated 20 kilometers east of the capital city, Kampala. The study was based at Mukono Health Center IV (MHC IV), a public outpatient health facility located in the center of Mukono Town.

The study was approved by the Tufts Health Sciences IRB in Boston, Massachusetts; the Mengo Hospital Research Ethics Committee in Kampala, Uganda; and the Uganda National Council for Science and Technology in Kampala, Uganda.

Sample size and eligibility

Based on studies of maternal idiopathic inflammatory bowel diseases (e.g., IBD) and adverse birth outcomes, the relative risk of maternal EED and subsequent preterm birth was assumed to be 2.0. Assuming 80% power, a significance level of 0.05, a frequency of preterm birth of 5%, and 15% loss to follow up, a sample size of 258 allowed for the detection of a relative risk of 2.0 within 50% of the true risk parameters.

Pregnant women were recruited during their first prenatal visit at MHC IV. Eligible women were 18-45 years of age, residing within 10 kilometers of Mukono Town, and carrying a singleton pregnancy. Women were excluded from the study if they were younger than 18 or older than 45 years, HIV positive (verified via routine rapid HIV test conducted at first prenatal visit), severely malnourished (defined as BMI <16.0

kg/m^2), severely anemic (defined as Hb <7 g/dl), or planning to move away from Mukono District prior to delivery.

Participation

Participation in the study involved 4 visits over a 4-6-month period: immediately following the first prenatal visit (MHC IV); conduct of the L:M test at the participant's residence within one week of the first visit; three weeks before the expected date of delivery (also at the participant's residence); and within 48 hours of delivery (either participant's residence, MHC IV, or other health facility).

Enrollment visit

An ultrasound scan was performed by a trained professional at MHC IV to both confirm a singleton pregnancy and determine participants' estimated date of delivery. Hemoglobin was measured using a portable hemoglobinometer (HemoCue Hb 301; HemoCue, Inc., Brea, CA, USA). A venous blood draw was performed by the phlebotomist at MHC IV (BD Vacutainer, Becton Dickinson, Durham, NC, USA). Systolic and diastolic blood pressure measurements were taken using a digital upper arm blood pressure monitor (Omron 10 Series, Omron Healthcare, Kyoto, Japan).

All anthropometry measurements were performed in triplicate and the mean was used for analyses. Weight was measured to the nearest 0.1 kg using a digital weight scale (Seca 874, Hanover, MD, USA). Height was measured to the nearest 0.1 cm using a portable, rigid height board (Infant/Child/Adult ShorrBoard, Shorr Production, Olney,

MD, USA). Mid-upper arm circumference (MUAC) was measured to the nearest 0.1 cm using a standard tri-colored, non-stretch adult MUAC tape.

Finally, a questionnaire was administered by the study nurse that included questions related to demographics, prior pregnancies, health status, diet, food security, and WASH practices.

L:M test visit

Within one week of the enrollment visit, a household visit was conducted to perform a L:M test. Following urination to void the bladder and an observed one hour fast, participants consumed a 50-mL solution containing 5 grams of lactulose (Lactulose Solution; Mckesson, San Francisco, CA, USA) and 2 grams of mannitol (D-mannitol powder; Sigma-Aldrich, St. Louis, MO, USA) completely dissolved in sterile water. Urine was collected for a period of 4 hours in a 2 L plastic collection bottle containing 0.05 mL of 50% thimerosal (Sigma-Aldrich, St. Louis, MO, USA) as a preservative. Water intake was permitted *ad libitum* one hour following ingestion of the solution, and women were encouraged to drink a minimum of 500 mL of water during the test to ensure sufficient urine output. A final urine sample was collected at the 4-hour time-point and total urine volume was measured to the nearest 1.0 mL using a graduated cylinder in the field. Samples were frozen at -20 °C at the MHC IV laboratory before being transferred to a -80 °C freezer in Kampala. Samples were analyzed at the laboratory at Baylor College of Medicine using the methods previously described (see above).

Follow-up visit

Three weeks prior to participants' estimated delivery date a second household visit was conducted, consisting of a follow-up survey, with questions related to pregnancy risk factors. In addition, weight and MUAC measurements were taken following the identical procedures used at the enrollment visit. Finally, participants were asked to provide a sample of water from their drinking water storage container for the purposes of a water quality test (see methods above).

Post-delivery visit

Newborn infant characteristics (live birth, date and time of delivery, sex, weight, and length) were collected within 48 hours of delivery. Weight was measured to the nearest 0.1 kg using a digital weigh scale (Seca 874, Hanover, MD, USA) and length was measured to the nearest 0.1 cm using a portable, rigid height board (ShorrBoard, Shorr Production, Olney, MD, USA). All anthropometry measurements were taken in triplicate and averaged. In the case of a stillbirth, only birth date, time, and infant sex were recorded.

Biomarkers aims #2 and #3

Analysis of anti-flagellin and anti-LPS Ig concentrations

Levels of flagellin- and LPS-specific IgA and IgG were measured via ELISA at Georgia State University. Microtiter plates were coated with purified *E. coli* flagellin (100 ng/well) or purified E. coli LPS (2 mg/well). Plasma samples diluted 1:200 were applied to wells coated with flagellin or LPS. After incubation and washing, the wells were

incubated either with anti-IgG coupled to horseradish peroxidase (GE, Catalog No. 375112) or, in the case of IgA-specific antibodies, with horseradish peroxidase–conjugated anti-IgA (KPL, Catalog No. 14-10-01). The specificity of the anti-human IgA and anti-human IgG was in accordance to the manufacturer's specifications, KPL and GE Healthcare Life Sciences, respectively.

Quantitation of total Igs was performed using the colorimetric peroxidase substrate tetramethylbenzidine, and optical density (OD) was read at 450 nm and 540 nm (the difference was taken to compensate for optical interference from the plate), with an ELISA plate reader. Data were reported as OD corrected by subtracting background (determined by readings in blank samples) and were normalized to each plate's control sample, which was prepared in bulk, aliquoted, frozen, and thawed daily as used. Standardization was performed using preparations of known concentrations of IgA and IgG. For quality control purposes, two duplicate plasma samples were measured in each batch.

Analysis of aflatoxin B1-lysine (AFB-lys) adduct levels

Serum samples were analyzed with an HPLC-fluorescence method, including measurement of albumin and total protein concentrations for each sample, digestion with protease to release amino acids, concentration and purification of the AF-lys adduct, and finally separation and quantitation by HPLC.[4,5] Specifically, thawed serum samples were inactivated for possible infectious agents via heating at 56 ℃ for 30 minutes, followed by measurement of albumin and total protein concentrations using modified procedures as previously described. A portion of each sample (approximately 150 μL) was digested by

pronase (pronase: total protein, 1:4, w: w) at 37°C for 3 h to release AFB-Lys. AFB-Lys in digests were further extracted and purified by passing through a Waters MAX SPE cartridge, which was pre-primed with methanol and equilibrated with water. The loaded cartridge was sequentially washed with water, 70% methanol, and 1% ammonium hydroxide in methanol at a flow rate of 1 ml/min. Purified AFB-Lys was eluted with 2% formic acid in methanol. The eluent was vacuum-dried with a Labconco Centrivap concentrator (Kansas City, MO) and reconstituted for HPLC-fluorescence detection.

The analysis of AFB-Lys adduct was conducted in an Agilent 1200 HPLC-fluorescence system (Santa Clara, CA). The mobile phases consisted of buffer A (20 mM $NH_4H_2PO_4$, pH 7.2) and buffer B (100% Methanol). The Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 x 250 mm) equipped with a guard column was used. Column temperature was maintained at 25°C during analysis, and a volume of 100 µL was injected at a flow rate of 1 mL/min. A gradient was generated to separate the AFB-Lys adduct within 25 min of injection. Adduct was detected by fluorescence at maximum excitation and emission wavelengths of 405 nm and 470 nm, respectively. Calibration curves of authentic standard were generated weekly, and the standard AFB-Lys was eluted at approximately 13.0 min. Quality assurance and quality control procedures were taken during analyses, which included simultaneous analysis of one authentic standard in every 10 samples and two quality control samples daily. The limit of detection was 0.2 pg/mg albumin. The average recovery rate was 90%. The AFB-Lys concentration was adjusted by albumin concentration for the report.

For quality control purposes, following completion of the laboratory analysis, sets of 3 samples were selected and pooled into 11 intra-day reproducibility samples, which

were analyzed twice on the same day by the same analyst, and 11 inter-day reproducibility samples, which were analyzed on different days by different analysts, to demonstrate laboratory precision and sampling reproducibility.

Study flow

Of the 300 pregnant women screened, 258 met the inclusion criteria and were enrolled in the study. Of these 258 participants, 247 had an L:M test conducted within one week of enrollment and 11 refused the test and dropped out of the study. Background characteristics, anti-flagellin and anti-LPS IgG concentrations, and AFB1 concentrations were analyzed for these 247 participants. Of these, 236 participants had a follow-up visit conducted prior to delivery and 11 were considered lost to follow up as they moved away from Mukono District. Birth outcome data were collected within 48 hours for 232 infants. Of these, ten infants were stillborn, and two infants died before anthropometry measurements could be taken. Thus, anthropometry measurements were collected and analyzed for 220 infants (see Figure 5.3).

Statistical analysis

All analyses were carried out using STATA 15 software (Stata Corps, College Station, TX, USA). Weight and length measurements were converted to Z-scores for weight-for-age (WAZ), length-for-age (LAZ), and weight-for-length (WLZ) using the World Health Organization standards. Outliers were defined as -6 > WAZ > +5, -5 > WLZ > +5, and -5 > LAZ > +5 and were excluded from analyses. Low birth weight was defined as weighing less than 2,500 grams at birth, and stunting at birth was defined as

LAZ <-2. Preterm birth was defined as birth before 37 weeks gestation, and still birth was defined as fetal death after 20 weeks gestation. In all cases, statistical significance was determined using a p-value < 0.05.

Statistical analysis: aim #2

Prior to analysis, distributions of biomarker values were assessed for outliers and normality. Because of their skewed distribution, L:M ratios were natural log (ln) transformed prior to all regression analyses. Pearson's correlation coefficients were calculated to evaluate agreement between log-transformed L:M ratio and log-transformed serum biomarkers. Associations between EED biomarkers (continuous, independent variables) and gestational age, length at birth, and LAZ at birth (continuous, dependent variables) were assessed using unadjusted and adjusted linear regression models. For all adjusted models, covariates were selected using bivariate analyses, with gestational age at birth and birth length as dependent variables, and a p-value < 0.25.

Statistical analysis: aim #3

Prior to analysis, distributions of biomarker values were assessed for outliers and normality. Because of their skewed distribution, AFB1 concentrations were natural log (ln) transformed prior to all regression analyses. Associations between means were assessed using t-tests, and bivariate correlations between variables were assessed using scatter plots and Pearson's correlation coefficients. Associations between ln maternal AFB1 concentrations and infant birth outcomes were assessed using unadjusted and adjusted linear regression. Bivariate analyses were conducted to test the association

between infant birth weight and potential covariates. Those with p-value <0.10 were included in the adjusted models. Among maternal anthropometry measurements, only maternal BMI was included to limit collinearity.

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Chapter 4: Unsafe drinking water is associated with environmental enteric dysfunction and poor growth outcomes in young children in rural southwestern Uganda

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Abstract

Environmental enteric dysfunction (EED), a subclinical disorder of the small intestine, and poor growth are associated with living in poor water, sanitation, and hygiene (WASH) conditions, but specific risk factors remain unclear. Nested within a birth cohort study, this study investigates relationships among water quality, EED, and growth in a sample of 385 children living in rural southwestern Uganda. Water quality was assessed using a portable water quality test when children were 6 months of age, and safe water was defined as lacking Escherichia coli contamination. EED was assessed using the lactulose: mannitol (L:M) test at 12-16 months. Anthropometry and covariate data were extracted from the cohort study, and associations were assessed using linear and logistic regression models. Under half of households (43.8%) had safe water, and safe vs. unsafe water did not correlate with improved vs. unimproved water source. Children from households with safe water had significantly lower mean ln L:M ratios (0.23-point difference, 95% CI: 0.09, 0.38) and significantly higher length-for-age (β: 0.34, 95% CI: 0.07, 0.61) and weight-for-age (β: 0.24, 95% CI: 0.10, 0.38) Z-scores at 12-16 months. Furthermore, children with higher length-for-age Z-scores at 6 and 9 months had significantly lower ln L:M ratios at 12-16 months (β: -0.06, 95% CI: -0.13, 0.00 and β: -0.05, 95% CI: -0.10, -0.001, respectively). To conclude, unsafe drinking water was strongly associated with EED and poor child growth. This suggests that programs seeking to improve nutrition should address poor WASH conditions simultaneously, particularly related to drinking water contamination.

Keywords**:** Environmental enteric dysfunction, L:M test, water quality, early growth, stunting

Introduction

An estimated 155 million children under 5 years of age are stunted, i.e. have a height-for-age Z-score (HAZ) of less than -2.[1] Stunting is associated with an array of health and economic consequences, including a greater risk of infections in childhood, diminished cognitive development, poorer educational outcomes, and lower economic productivity and earnings in adulthood.[2] However, despite the enormous global burden, mechanisms underlying stunting remain largely underexplored. That is, known interventions to resolve stunting implemented at 90% coverage would only avert 20% of the global burden, leaving most of the problem unaddressed.[3] One of the domains of potential concern for stunting is poor environmental conditions (water, sanitation, and hygiene or WASH) and associated intestinal health.

Some studies have demonstrated an association between poor WASH and poor growth outcomes[4,5,6,7] but the assumption that repeated symptomatic diarrheal infections are the main mechanism at work have not been supported. According to the 2008 Lancet Maternal and Child Nutrition Series, WASH interventions implemented at 99% coverage would reduce diarrhea incidence by 30%, which would reduce the prevalence of stunting by only 2.4% at 36 months of age.[8] Furthermore, in a pooled analysis of nine studies, only 25% of stunting at 24 months was attributable to a high burden of diarrhea (> or = 5 episodes before 24 months).[9]

That diarrheal infection is not more strongly linked to stunting as an outcome has promoted the hypothesis that the impact of poor WASH on nutrition operates through environmental enteric dysfunction (EED).[10] EED is a subclinical, inflammatory disorder of the small intestine characterized by altered gut morphology, reduced absorptive capacity, and impaired barrier function.[11,12] It is postulated that EED develops throughout

infancy as the result of chronic fecal-oral exposure to enteropathogens due to living in poor WASH conditions. However, to date, only a few studies have implicated WASH-related risk factors as being associated with EED, including unsafe child feces disposal,[13] mouthing of soil (geophagy),[14] and exposure to animals,[15] while several studies have implicated exposure to specific enteropathogens, including Giardia,[16] Shigella,[17] and rotavirus.[18,19] Furthermore, a study from Bangladesh found that children living in environmentally clean households had better intestinal health, characterized by lower L:M ratios (-0.32 SDs, 95% CI: -0.72, 0.08), and higher HAZ (0.54 SDs (95% CI: 0.06, 1.01) than children from contaminated households.[20] However, despite these findings, the exact risk factors for EED remain speculative, inconsistent across studies, and in need of further study.

While typically considered asymptomatic, EED is significant mainly due to its postulated association with poor growth outcomes, especially stunting, likely as the result of both malabsorption of nutrients and systemic immune activation.[21-26] The primary objective of this study was to investigate the relationship among water quality, environmental enteric dysfunction, and growth outcomes among children 12-16 months of age living in rural southwestern Uganda. We hypothesized that children from households with poor drinking water quality would have higher levels of EED, measured using a lactulose: mannitol (L:M) dual sugar absorption test, as well as poorer growth outcomes.

Materials and methods

Approvals

The study was approved by the Tufts Health Sciences Institutional Review Board in Boston, Massachusetts, USA; the Institutional Review Board at Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA; the Makerere University Research Ethics Committee at the School of Public Health in Kampala, Uganda; and the Uganda National Council for Science and Technology in Kampala, Uganda. Prior to enrollment in the study, written consent was obtained from the child's main caretaker.

Study design

This was a cross-sectional, observational study conducted as a sub-study to the Uganda Birth Cohort Study (UBCS) between July and August 2016 in seven sub-counties (Bugangari, Buyanja, Bwizi, Kebisoni, Kibiito, Nyamweru, Ruhiija) of rural southwestern Uganda. The UBCS was a prospective, observational study in 16 sub-counties across northern and southwestern Uganda that followed > 5,000 women through pregnancy, birth, and through 9 months of the infant's life.

Sample size and eligibility

The study reported here included a randomly selected sample of 385 children from the UBCS. Sample size was calculated with G*Power software for a multiple regression with the following parameters: medium effect size ($f2$=0.15),[27] 0.95 power, 0.05 type 1 error probability, and 7 predictors. Sample size was doubled to account for sub-county clustering and further increased by 25% to allow for potential challenges in conducting and analyzing the L:M test.

Eligible children were between the ages of 12 and 16 months, capturing a period of elevated L:M ratios observed in previous studies.[28,29] Furthermore, they were residing in one of the seven sub-counties and had complete UBCS visits up to 9 months. Children were excluded from the study if they had had one or more episodes of diarrhea in the previous two weeks, were severely malnourished (MUAC < 11.5 cm), or had a serious illness at the time of the study.

L:M test

EED in this study was measured using the lactulose: mannitol (L:M) dual sugar absorption test. While EED can only be diagnosed definitively through small intestinal biopsy,[30] the L:M test is the most commonly-used, non-invasive proxy marker.[31] In the test, mannitol recovery rates indicate absorptive capacity, lactulose recovery rates indicate permeability, and higher L:M ratios indicate greater intestinal abnormality, or EED.[32]

Standardized doses, consisting of a 20 mL solution containing 5 grams of lactulose (Lactulose Solution; Mckesson, San Francisco, CA, USA) and 1 gram of mannitol powder (D-mannitol powder; Sigma-Aldrich, St. Louis, MO, USA) completely dissolved in sterile water, were prepared in the Food Science laboratory at Makerere University in Kampala and transferred to refrigerators located in local health facilities. After the consent process and an observed one-hour fast, children were given a dose of solution using either a plastic cup or disposable dropper. Each child was carefully monitored to ensure that none of the solution was spilled, spit out, or vomited. If any of these events occurred, the test was rescheduled for a different day.

After children successfully consumed the L:M solution, urine was collected over a minimum of a 4-hour period using sterile adhesive urine collection bags (Thermo Fisher Scientific, Waltham, MA, USA), which were replaced after each urination episode. Collected urine was consolidated in a plastic container with thimerosal (Sigma-Aldrich, St. Louis, MO, USA) added to the container to prevent bacterial growth. Drinking water was provided and allowed ad libitum throughout the test, and breastfeeding and/or a small meal was allowed at the 3-hour mark. At the 4-hour mark, children were offered a juice drink to encourage a final urination episode, which marked the end of the test.

Total urine volume was measured to the nearest 1.0 mL using a graduated cylinder and the urine was aliquoted into plastic cryovials. The samples were transported on ice in plastic cooler boxes to the local health facility where they were stored at -20°C. Upon completion of the study, samples were stored at -80°C in Kampala before being transported on dry ice to the laboratory at Baylor College of Medicine for analysis. Concentrations of lactulose and mannitol were analyzed using high-performance liquid chromatography (HPLC) using previously described methods.[33,34]

Anthropometry and covariates

With the exception of the L:M data, data required for analysis were extracted from the UBCS main dataset. Data were collected by trained research assistants in 3-month intervals from pregnancy until the infant turned 9 months of age using electronic tablets. Covariates were obtained from the 6-month time-point and anthropometry measurements were obtained at birth, 6 months, and 9 months. Information included household characteristics; water, sanitation, and hygiene; diet; health; food security;

gender and decision-making; agricultural production; and anthropometry, including length, weight, mid-upper arm circumference (MUAC), and head circumference.

Length was measured in triplicate to the nearest 0.1 cm using a portable height board (ShorrBoard® infant/child/adult portable height-length measuring board; Weigh and Measure, LLC, Olney, MD, USA). Weight was measured in triplicate to the nearest and 0.1 kg using an electronic scale (Seca, Hanover, MD, USA). MUAC was measured in triplicate on the left arm using a standard tri-colored, non-stretch measuring tape. Triplicate measurements were averaged to provide one measurement of length, weight, and MUAC per participant per visit. Hemoglobin was measured at 6 months of age using a portable hemoglobinometer (HemoCue 301; HemoCue America, Brea, CA, USA).

Water quality

Water quality was assessed in the UBCS using a compartment bag test (CBT kit; Aquagenx, Chapel Hill, NC, USA) at the 6-month time-point. The CBT is a portable water quality test kit designed to detect and quantify Escherichia coli (E. coli) bacteria. For the test, participants were asked to provide a glass of water from their primary drinking water storage container and a 100 mL sample was mixed with an E. coli chromogenic growth medium. The sample was then poured into a plastic bag with 5 compartments of varying volumes, sealed, and incubated for a period of 48 hours. Risk categories were determined by noting which, if any, compartments changed from yellow to green/blue and matching that to a most probable number (MPN) table based on the World Health Organization (WHO) guidelines. Health risk categories are safe (< 1

CFU/100 ml.), intermediate risk (1-10 CFU/100 ml.), high risk (> 10-100 CFU/100 ml.), and very high risk/unsafe (> 100 CFU/100 ml.).[35]

Statistical methods

All analyses were carried out using STATA 15 software (Stata Corps, College Station, TX, USA). The primary outcomes of interest were the (ln) L:M ratio at 6 months and length-for-age Z-score (LAZ) at time of the L:M test. Secondary outcomes included percent lactulose excretion (%LE), percent mannitol excretion (%ME), and the lactulose mannitol excretion ratio (LMER) in addition to weight-for-age (WAZ) and weight-for-length (WLZ) Z-scores.

L:M ratios were calculated using the fractional excretion of each of the two sugars. Because of the right-skewed nature of its distribution, the L:M ratio was natural log transformed. %LE and %ME were calculated by first multiplying the concentration of sugar (mg/mL) by the total urine volume and then dividing that amount by the initial dose of each sugar. The lactulose: mannitol excretion ratio (LMER) was calculated by taking the ratio of % LE to %ME.

Growth outcomes, including LAZ, WAZ, and WLZ, were calculated using the WHO Multicenter Growth Reference Study growth standards.[36] Dichotomous variables, stunting (LAZ < -2), underweight (WAZ < -2) and wasting (WLZ < -2), were also created. All extreme outliers (-6 > WAZ > 5, -6 > LAZ > 6, and -5 > WLZ > 5) were set to missing, per WHO recommendations. Anemia was defined as hemoglobin <11 g/dl. A dichotomous (safe vs. unsafe) water variable was created, with safe water defined as no E. coli detected in the CBT and unsafe water defined as any E. coli detected. Improved

water sources were piped water, a public tap, a tube well/borehole, a protected well/spring, and rain water. Unimproved water sources were an unprotected well/spring, surface water, and other. An asset score (0-4) was created based on household ownership of the following four items: telephone, bicycle, radio, and motorcycle.

Differences in means for L:M results between households with safe v. unsafe water and children who were stunted vs. not stunted were calculated using t-tests. Associations among water quality, L:M results, and growth outcomes were assessed using unadjusted and adjusted linear and logistic regression models. For all adjusted models, covariates from the 6-month time point were based on bivariate analyses and a p-value cut-point of 0.20. Associations were considered significant in the case of p-value < 0.05.

Results

Study population

Background characteristics of the 385 participating children and their households are presented in Table 1. Half of children were female and mean age at enrollment into the sub-study was ~15 months. As expected, mean LAZ, WLZ, and WAZ declined over time from birth until the L:M test. At the time of the L:M test, 35.5% of the participants were stunted, 8.9% were underweight, and 2.1% were wasted. On average, households had ~6 members, and the majority of dwellings had an earth floor, an unimproved pit latrine, and no electricity.

Water source vs. water risk

Table 2 shows the percentage of households with safe vs. unsafe water, disaggregated by main water source. Among sampled households, 43.8% had safe water and 56. 2% had unsafe water. Overall, we observed no correlation between having an improved water source and having safe drinking water. Among the 210 households with an improved water source, 44.3% had safe water and 55.7% had unsafe water. Similarly, among the 167 households with an unimproved water source, 43.3% had safe water and 56.7% had unsafe water (chi-square p-value = 0.82). Furthermore, we observed no association between water source and either EED risk or stunting risk.

L:M results

Table 3 shows the results of the L:M test, disaggregated by safe and unsafe water. The arithmetic mean ± SD L:M ratio for participants was 0.34 ± 0.27. The mean L:M ratio and ln L:M ratio were significantly lower in children from households with safe vs. unsafe water (0.08-point difference, 95% CI: 0.02-0.14 and 0.23-point difference, 95% CI: 0.09-0.38, respectively) as were the %LE (0.09-point difference, 95% CI: 0.03-0.15) and LMER (0.02-point difference, 95% CI: 0.005-0.03). No significant difference in %ME between households with safe vs. unsafe water was observed.

Figure 1 shows the median ln L:M ratio for children from households in each of the four WHO health risk categories for water contamination. With increasing risk there was a progressive increase in median ln L:M ratios (safe = -1.50, intermediate risk = -1.33, high risk = -1.18, and unsafe = -1.10, chi-square p-value = 0.004).

Finally, in addition to water quality, ln L:M ratios in children were significantly associated with household water quantity, measured in number of 5-liter jerrycans of water utilized per household per day. On average, in an unadjusted linear regression model, each additional jerrycan of water was associated with a 0.08-point decrease in ln L:M ratio (95% CI: -0.13, -0.02) (Supplemental Table 1).

Association between water quality and growth outcomes/iron status

Table 4 shows the association between water quality and growth outcomes/iron status using unadjusted and adjusted regression models. In addition to better intestinal health, children from households with safe water had significantly better overall LAZ and WAZ, but not WLZ. In an adjusted linear regression model, safe water was significantly associated with better LAZ at birth (β: 0.57, 95% CI: 0.10, 1.04) and at the time of the L:M test (β: 0.34, 95% CI: 0.07, 0.61). Furthermore, safe water was significantly associated with better WAZ at 6 months (β: 0.29, 95% CI: 0.09, 0.48), 9 months (β: 0.28, 95% CI: 0.04, 0.52), and at the time of the L:M test (β: 0.24, 95% CI: 0.10, 0.38). In an adjusted logistic regression model, children from households with unsafe water were 1.68 times more likely to be stunted at birth (95% CI: 1.22, 2.32), 2.09 times more likely to be stunted at 6 months (95% CI: 1.30, 3.37), and 1.56 times more likely to be stunted at the time of the L:M test (95% CI: 1.05, 2.33).

Finally, safe water was significantly associated with better iron status assessed at 6 months. On average, safe water was associated with a 0.37-point (95% CI: 0.05, 0.68) increase in hemoglobin level in an adjusted linear regression model. Furthermore, in an unadjusted logistic regression model, children from households with unsafe water were

1.68 (95% CI: 1.08, 2.60) times more likely to be anemic compared to children from households with safe water.

Association between intestinal health and past LAZ

Higher L:M ratios were observed in children who were stunted vs. not stunted at birth (-0.10-point difference, 95% CI: -0.16, -0.04), 6 months (-0.10-point difference, 95% CI: -0.16, -0.03), and 9 months (-0.07-point difference, 95% CI: -0.13, -0.004). Table 5 shows the association between past LAZ and L:M results in unadjusted and adjusted linear regression models. In the adjusted linear regression model, higher LAZ at 6 months was significantly associated with a lower L:M ratio (β: -0.02, 95% CI: -0.05, -0.00) and ln L:M ratio (β: -0.06, 95% CI: -0.13, 0.00). Furthermore, higher LAZ at 9 months was significantly associated with a lower ln L:M ratio (-0.05, 95% CI: -0.10, -0.00).

Discussion

In this study of 385 children living in rural southwestern Uganda, we found that those from households with safe drinking water, assessed using a CBT at the 6-month time-point, had significantly lower mean L:M ratios, ln L:M ratios, %LE, and LMERs at 12-16 months. In addition, they had significantly higher LAZ and WAZ at 12-16 months. Contrastingly, children from households with unsafe water were 1.56 times more likely to be stunted at this time. Finally, we found children who were stunted at birth, 6 months, and 9 months had significantly higher mean L:M ratios compared to children who were not stunted. Overall these results add to the growing body of literature connecting poor WASH conditions, EED, and poor growth outcomes. Specifically, our results indicate

that contaminated household drinking water may be an important contributing factor to the high burden of both EED and stunting in southwestern Uganda as well as in other LMICs with poor WASH conditions.

To our knowledge, this is one of the first studies to use an objective measure of drinking water quality to link both EED and poor child growth with a specific water-borne enteric pathogen, in this case E. coli. Conventionally, water quality is assessed using a sole indicator of "improved drinking water source" vs. "unimproved drinking water source.[34] However, had this definition of water quality been employed, we would have observed no association between water quality and either EED risk or stunting risk in this study. Our finding that safe vs. unsafe water did not correlate with improved vs. unimproved water source is consistent with findings from rural Peru where the authors used the same CBT and determined that improved water sources were not associated with decreased contamination risk.[37] Together, these findings demonstrate a need for more objective WASH indicators that adequately assess risk of exposure to pathogens.

Furthermore, our study adds to the growing body of literature that supports a link between poor WASH conditions and the development of EED in young children. In a study by Lin et al. of 119 rural Bangladeshi children ≤ 48 months of age, the authors assessed the relationship between fecal environmental contamination and EED. They found that children living in environmentally "clean" households, defined using objective indicators of water quality and sanitary and hand-washing infrastructure, had better intestinal health, characterized by lower L:M ratios (-0.32 SDs, 95% CI: -0.72, 0.08), compared to children from "contaminated" households.[20] Additionally, in a prospective cohort study of 216 children < 5 years of age also in rural Bangladesh, George et al.

observed an association between geophagy (i.e. consumption of soil, dirt, or mud) and EED as well as between animal exposure and caregiver hygiene and EED, measured using four fecal markers: alpha-1-antitrypsin, myeloperoxidase, and neopterin (all three combined to form an EED disease activity score) and calprotectin . Children with caregiver-reported geophagy had significantly higher EED scores (0.72-point difference, 95% CI: 0.01, 1.42) and calprotectin concentrations (237.38 μg/g, 95% CI: 12.77, 462.00).[38] Furthermore, children with an animal corral in their sleeping room had significantly higher EED scores (1.0-point difference, 95% CI: 0.13, 1.88), and children of caregivers with visibly soiled hands had significantly higher fecal calprotectin concentrations (384.1 μg/g, 95% CI: 152.37, 615.83).[39]

This study has several limitations, and it also points to several areas in need of further research. First, both the L:M test and CBT water quality test have inherent disadvantages. The L:M test, while still the most commonly used measure of EED in the field, suffers from significant variability in methods related to aspects such as fasting time, dosing amount, urine collection time, and laboratory analysis. Furthermore, there is debate regarding both the L:M test's ability to adequately assess EED as well as its correlation with poor growth.[40] The CBT water quality test, while a convenient, inexpensive method of measuring water quality in the field, provides a statistical "most likely mean" measure of CFUs per 100 ml. and approximate risk categories rather than a precise measure of contamination.

While many data elements in this study were collected prospectively, other elements were collected at a subsequent time point, limiting capacity to establish causality. Both the L:M test and the CBT water quality test were conducted at only one

point in time, which fails to capture potential variability in these indicators over time. Furthermore, anthropometry data were not collected beyond the point of the L:M test, and therefore we can only conclude that L:M results were associated with past growth rather than future growth. To provide additional causal support of our hypothesis, a randomized controlled trial nested within a prospective birth cohort study that offers improvements in household water quality would be a valuable next step.

The authors acknowledge that measuring E. coli alone is an imperfect proxy for water contamination; however, this likely means that the role of water quality in the etiology EED and poor growth outcomes is, in fact, greater than we observed. In addition to E. coli, it would be useful to look at the association between other pathogens, including Cryptosporidum, Shigella, Salmonella, and viruses, and their relationship with EED. Furthermore, in addition to EED and iron status, it would be useful to examine the role of EED in other micronutrient deficiencies, including addition to zinc, vitamin A, folate, and vitamin B12. Finally, given the significant association between household water quality and stunting at birth, it is worth exploring whether maternal EED is linked to certain negative birth outcome, such as reduced in-utero growth.

In conclusion, amid mounting evidence, efforts to tackle stunting in LMICs must begin to recognize the role of poor water quality and subsequent poor intestinal health. From a programmatic perspective, WASH interventions should focus on preventing EED by reducing children's fecal-oral exposure to enteropathogens. This should include an emphasis on improving water quality in settings where water contamination is prevalent and likely a predominate underlying cause of EED

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Tables and figures

Table 2: Characteristics of 385 rural Ugandan children and their households

Characteristic	**Mean ± SD or n (%)**
Child characteristics	
Female	195 (50.7)
Age, months	14.8 ± 1.1
Anthropometry at birth	
Length-for-age z-score	-0.93 ± 1.54
Weight-for-length z-score	0.57 ± 1.54
Weight-for-age z-score	-0.17 ± 0.96
Anthropometry at 6 months of age	
Length-for-age z-score	-0.98 ± 1.51
Weight-for-length z-score	0.63 ± 1.41
Weight-for-age z-score	-0.25 ± 1.19
Anthropometry at 9 months of age	
Length-for-age z-score	-1.21 ± 1.46
Weight-for-length-z score	0.42 ± 1.35
Weight-for-age z-score	-0.41 ± 1.21
Anthropometry at time of L:M test	
Length-for-age z-score	-1.55 ± 1.14
Weight-for-length z-score	0.24 ± 1.10
Weight-for-age z-score	-0.58 ± 1.04
Hemoglobin at 6 months of age, g/dL	11.2 ±1.2
Household characteristics	
Individuals in household	5.7 ± 2.4
Female household head	16 (4.2)
Caregiver education years	5.9 ±3.0
Asset score	1.8 ± 0.9
Earth floor	334 (86.8)
Electricity, grid or solar	61 (15.8)
Unimproved pit latrine	368 (95.6)
Water quantity per household, jerrycans per day	2.4 ± 1.3
Boil water	274 (71.2)
Water quality (n=377)	
Safe	165 (43.8)
Intermediate risk	51 (13.5)
High risk	46 (12.2)
Very high risk	115 (30.5)

Table 3: Comparison of water quality by main water source

Main water source	**Total**	**Safe[1] n (%)**	**Unsafe n (%)**
Piped	8	4 (50.0)	4 (50.0)
Public tap	45	26 (57.8)	19 (42.2)
Tubewell/borehole	57	17 (29.8)	40 (70.2)
Protected well/spring	85	35 (41.2)	50 (58.8)
Unprotected well/spring	110	54 (49.1)	56 (50.9)
Rain water	15	11 (73.3)	4 (26.7)
Surface water	54	17 (31.5)	37 (68.5)
Other	3	1 (33.3)	2 (66.7)
Total	377	165 (43.8)	212 (56.2)

[1]Safe water defined as the lack of presence of E. coli contamination according to the results of a compartment bag test. Unsafe water defined as any E. coli contamination detected.

According to the WHO, improved drinking water sources are considered to be piped water, public taps, tubewells/boreholes, protected wells/springs, and rainwater. Unimproved sources are considered unprotected wells/springs and surface water. No correlation between improved vs. unimproved water source and safe vs. unsafe water observed (chi-square p-value =0.82).

Table 4: Comparison of L:M results from households with safe vs. unsafe water

	All (n=385)[2]	**Safe water (n=165)**	**Unsafe water (n=212)**	**Difference (95% CI)[3]**	**p-value**
L:M ratio, arithmetic mean	0.34 ± 0.27	0.30 ± 0.24	0.38 ± 0.29	0.08 (0.02, 0.14)	0.0050
Ln L:M ratio	-1.31 ± 0.70	-1.44 ±0.69	-1.21 ± 0.70	0.23 (0.09, 0.38)	0.0014
Urinary lactulose, % dose excreted	0.32 ± 0.28	0.27 ± 0.22	0.36 ± 0.31	0.09 (0.03, 0.15)	0.0016
Urinary mannitol, % dose excreted	5.32 ± 3.48	5.14 ± 3.59	5.40 ± 3.42	0.26 (-0.46, 0.98)	0.4705
LMER	0.07 ± 0.05	0.06 ± 0.05	0.08 ± 0.06	0.02 (0.005, 0.03)	0.0043

[1]Safe water defined as the lack of presence of E. coli contamination according to the results of a compartment bag test. Unsafe water defined as any E. coli contamination detected.

[2]Mean ± SD for all such values

[3]Differences are children from households with unsafe water minus children from households with safe water

Abbreviations: L:M, lactulose: mannitol ratio; LMER, lactulose: mannitol excretion ratio

Table 5: Association between water quality and growth outcomes/iron status

Outcome	Unadjusted linear/logistic regression model	Adjusted linear/logistic regression model controlled for sub-county clustering
Water quality and growth outcomes (LAZ, WAZ, WLZ)[2]		
LAZ at birth	0.65 (0.34, 0.96)*	0.57 (0.10, 1.04)*
WAZ at birth	0.18 (-0.01, 0.38)	0.15 (-0.12, 0.42)
WLZ at birth	-0.38 (-0.72, -0.03)*	-0.38 (-1.02, 0.27)
LAZ at 6 months	0.40 (0.10, 0.71)*	0.31 (-0.29, 0.90)
WAZ at 6 months	0.35 (0.11, 0.59)*	0.29 (0.09, 0.48)*
WLZ at 6 months	-0.02 (-0.31, 0.27)	0.003 (-0.55, 0.56)
LAZ at 9 months	0.25 (-0.05, 0.55)	0.21 (-0.31, 0.72)
WAZ at 9 months	0.35 (0.11, 0.60)*	0.28 (0.04, 0.52)*
WLZ at 9 months	0.18 (-0.10, 0.46)	0.16 (-0.27, 0.59)
LAZ at L:M test	0.39 (0.16, 0.62)*	0.34 (0.07, 0.61)*
WAZ at L:M test	0.29 (0.08, 0.51)*	0.24 (0.10, 0.38)*
WLZ at L:M test	0.08 (-0.14, 0.31)	0.06 (-0.26, 0.37)
Water quality and growth outcomes (stunted and underweight)[2]		
Stunted at birth	1.88 (1.14, 3.11)*	1.68 (1.22, 2.32)*
Underweight at birth	0.98 (0.26, 3.72)	0.78 (0.28, 2.18)
Stunted at 6 months	2.31 (1.37, 3.88)*	2.09 (1.30, 3.37)*
Underweight at 6 months	1.70 (0.71, 4.04)	1.52 (0.66, 3.51)
Stunted at 9 months	1.66 (1.03, 2.67)*	1.57 (0.97, 2.54)
Underweight at 9 months	2.36 (1.03, 5.42)*	2.05 (1.12, 3.76)*
Stunted at L:M test	1.67 (1.08, 2.58)*	1.56 (1.05, 2.33)*
Underweight at L:M test	1.29 (0.63, 2.67)	1.15 (0.62, 2.13)
Water quality and iron status[3]		
Hemoglobin, g/dL at 6 months	0.41 (0.15, 0.66)*	0.37 (0.05, 0.68)*
Anemia[4] at 6 months	1.68 (1.08, 2.60)*	1.59 (0.89, 2.84)

[1]Safe water defined as the lack of presence of E. coli contamination according to the results of a compartment bag test. Unsafe water defined as any E. coli contamination detected.

[2]Adjusted regression model was adjusted for sex of child, sex of household head, mother's height caregiver education level, family size, and asset score

[3]Adjusted regression model was adjusted for sex of child, sex of household head, caregiver education level, and asset score

[4]Hemoglobin <11 g/dl considered anemic

Abbreviations: L:M, lactulose: mannitol ratio; LMER; lactulose: mannitol excretion ratio; LAZ, length-for-age z-score; WAZ, weight-for-age z-score; WLZ, weight-for-length z-score; g/dl, grams per deciliter

* P-value < 0.05

Table 6: Association between prior LAZ and L:M results

	Unadjusted linear regression model			**Adjusted linear regression model controlled for sub-county clustering[1]**		
Outcome	**LAZ at birth**	**LAZ at 6 months**	**LAZ at 9 months**	**LAZ at birth**	**LAZ at 6 months**	**LAZ at 9 months**
L:M ratio	-0.02 (-0.04, -0.003)*	-0.03 (-0.05, -0.009)*	-0.02 (-0.04, 0.002)	-0.01 (-0.03, 0.006)	-0.02 (-0.05, -0.001)*	-0.02 (-0.03, -0.005)
Ln L:M ratio	-0.06 (-0.11, -0.02)*	-0.07 (-0.11, -0.02)*	-0.05 (-0.10, 0.002)	-0.05 (-0.11, 0.003)	-0.06 (-0.13, 0.000)*	-0.05 (-0.10, -0.001)*
Urinary lactulose, % dose excreted	-0.009 (-0.03, 0.009)	-0.02 (-0.04, -0.001)*	-0.02 (-0.04, -0.003)*	0.002 (-0.02, 0.03)	-0.01 (-0.04, 0.02)	-0.02 (-0.04, 0004)
Urinary mannitol, % dose excreted	0.17 (-0.06, 0.40)	0.03 (-0.21, 0.27)	-0.01 (-0.26, 0.24)	0.27 (0.01, 0.52)*	0.13 (-0.21, 0.47)	0.05 (-0.24, 0.35)
LMER	-0.004 (-0.008, -0.001)*	-0.006 (-0.01, -0.002)*	-0.004 (-0.008, 0.000)*	-0.003 (-007, 0.001)	-0.005 (-0.01, 0.001)	-0.003 (-0.008, 0.002)

[1]Adjusted regression model was adjusted for sex of child, age, sex of household head, caregiver education level, asset score, safe water, and birth weight

Abbreviations: L:M, lactulose: mannitol ratio; LMER; lactulose: mannitol excretion ratio; LAZ, length-for-age z-score

* P-value < 0.0

Table 7: Association between water quantity and L:M results

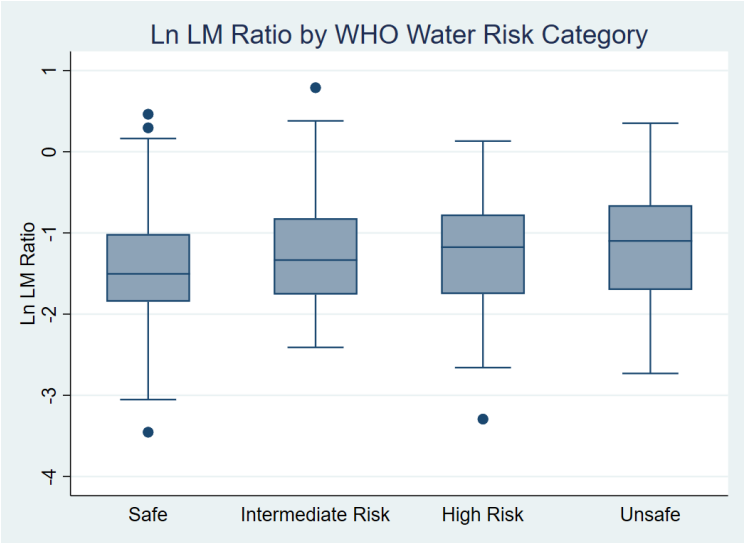
Outcome	**Unadjusted linear regression model**	**Adjusted linear regression model controlled for sub-county clustering**
L:M ratio	-0.03 (-0.05, -0.005)*	-0.02 (-0.07, 0.03)
Ln L:M ratio	-0.08 (-0.13, -0.02)*	-0.06 (-0.19, 0.06)
Urinary lactulose, % dose excreted	-0.01 (-0.03, 0.01)	-0.008 (-0.04, 0.03)
Urinary mannitol, % dose excreted	0.23 (-0.04, 0.51)	0.27 (-0.07, 0.62)
LMER	-0.005 (-0.01, -0.0009)*	-0.004 (-0.01, 0.005)

[1]Adjusted regression model was adjusted for sex of child, age, sex of household head, caregiver education level, asset score, safe water, and birth weight

* P-value < 0.05

Figure 1: L:M ratio by drinking water quality

Figure shows box plot of ln L:M ratio by WHO health risk category for E. coli contamination in drinking water. Health risk categories are safe (< 1 CFU/100 ml.), intermediate risk (1-10 CFU/100 ml.), high risk (> 10-100 CFU/100 ml.), and very high risk/unsafe (> 100 CFU/100 ml.). Each box shows median value and interquartile range.



Chapter 5: Systemic inflammation markers of maternal environmental enteric dysfunction are associated with shorter gestation and reduced length in newborn infants in Uganda

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Abstract

Background: Adverse birth outcomes, including preterm birth and stunting at birth, have long-term health implications. The relationship between adverse birth outcomes and chronic, asymptomatic gastrointestinal inflammation (environmental enteric dysfunction, or EED) is poorly understood.

Objective: To examine the relationship between maternal EED and adverse birth outcomes in a sample of pregnant Ugandan women and their newborn infants.

Design: We conducted a prospective cohort study in Mukono, Uganda. A total of 258 pregnant women were enrolled at their first prenatal visit. We measured EED biomarkers, namely lactulose: mannitol (L:M) ratios and serum concentrations of antibodies to the bacterial components flagellin and lipopolysaccharide (LPS). Data on covariates were obtained from two surveys. Associations were assessed using unadjusted and adjusted linear regression models.

Results: Birth outcome data were recorded for 232 infants within 48 hours of delivery. Mean ± SD gestational age was 39.4 ± 2.9 weeks and 10% were born preterm (<37 weeks gestation). Mean ± SD length and length-for-age Z-score (LAZ) at birth were 48.7 ± 2.0 cm and -0.44 ± 1.07, respectively. Mean ± SD L:M ratio was 0.08 ± 0.12 and was not associated with any birth outcome. In adjusted models, higher concentrations of anti-flagellin IgG and anti-LPS IgG were significantly associated with shorter length of gestation [(β: -1.35, 95% CI: -2.64, -0.07) and (β: -0.89 [95% CI: -1.52, -0.25)] and with lower length at birth [(β: -0.97, 95% CI: -1.79, -0.14) and (β: -0.45, 95% CI: -0.87, -0.02)] and lower LAZ at birth [(β -0.52, 95% CI: -0.95, -0.08) and (β: -0.25, 95% CI: -0.48, -0.03)].

Conclusion: In this Ugandan sample**,** markers of maternal EED, specifically anti-flagellin and anti-LPS IgG, are associated with shorter gestation and reduced length at birth. This association may be mediated via systemic inflammatory response rather than nutrient malabsorption, but further research is needed.

Keywords: Environmental enteric dysfunction, inflammation, intestinal permeability, intestinal biomarkers, dual sugar absorption test, L:M test, anti-flagellin antibodies, anti-LPS antibodies, birth outcomes, Uganda

Introduction

Stunting, defined as a height-for-age Z-score (HAZ) of less than -2, remains a pervasive form of undernutrition globally, affecting roughly 155 million children under five years of age.[1] Stunted children are not only short for their age but are at an increased risk of developing the "stunting syndrome,"[2] characterized by numerous health and economic consequences including increased morbidity and mortality,[3,4] impaired cognitive development,[5,6] and lower economic productivity later in life.[7] An estimated 20% of stunting has in-utero origins driven largely by intrauterine growth restriction, premature births, or both.[8]

Environmental enteric dysfunction (EED) is a subclinical inflammatory disorder of the small intestine characterized by altered gut morphology, reduced absorptive capacity, and impaired barrier function.[9,10] The condition is thought to result from chronic fecal-oral exposure to enteropathogens as the result of living in a contaminated environment with poor water, sanitation, and hygiene (WASH) conditions.[11,12] EED is an alarming global health concern, especially given its high prevalence and demonstrated association with poor growth outcomes in young children living in LMICs.[13,14,15]

EED is clinically diagnosed via small bowel biopsy; however, the invasive nature of this approach is ill-suited for population-based research studies. Numerous biomarkers for EED have been proposed which measure different domains of EED, including intestinal permeability, absorption, and inflammation.[16] One common marker is the lactulose: mannitol (L:M) dual sugar absorption test. While the L:M test is a widely-used proxy for EED, it has several limitations. Most notably, in addition to being time-consuming and expensive to implement, the test lacks formal evaluation studies, and its correlation with EED symptoms and growth outcomes is debated.[17]

Drawbacks of the L:M test have underpinned a search for new EED biomarkers. Anti-flagellin and anti-lipopolysaccharide (anti-LPS) Igs have been recently proposed supported by their elevated presence in a range of diseases associated with and/or promoted by inflammation such as short bowel syndrome (SBS),[18] Crohn's disease (CD),[19,20] and irritable bowel syndrome (IBS).[21] Since large bacterial-derived molecules like flagellin LPS do not readily cross the epithelium, elevated concentrations of anti-flagellin and anti-LPS antibodies are thought to signify a state of increased intestinal permeability, microbial translocation, and systemic immune activation. To date, elevations in these serum markers have been linked to increases in underweight in a sample of young Tanzanian children[22] and to stunting in a sample of young children in Northeast Brazil.[23]

While we are unaware of any study that has examined the role of maternal EED as a risk factor for adverse birth outcomes in LMICs, women with idiopathic inflammatory bowel disease (IBD) have pregnancies marked by higher rates of preterm birth, small for gestational age infants, and other complications.[24-27] The primary objective of the study was to examine the association between maternal EED and birth outcomes. We hypothesized that maternal EED would be associated with shorter gestational age at birth and reduced birth length in infants.

Subjects and methods

Study design

We performed a prospective cohort study between February and November 2017 in Mukono District, Central Region, Uganda. Mukono is a semi-urban district situated 20

kilometers east of the capital city, Kampala. The study was based at Mukono Health Center IV (MHC IV), a public outpatient health facility located in the center of Mukono Town.

Pregnant women were recruited during their first prenatal visit at MHC IV. Eligible women were 18-45 years of age, residing within 10 kilometers of Mukono Town, and carrying a singleton pregnancy. Women were excluded from the study if they were younger than 18 or older than 45 years, HIV positive (verified via routine rapid HIV test conducted at first prenatal visit), severely malnourished (defined as BMI <16.0 kg/m2), severely anemic (defined as Hb <7 g/dl), or planning to move away from Mukono District prior to delivery.

The study was approved by the Tufts Health Sciences Institutional Review Board in Boston, Massachusetts; the Mengo Hospital Research Ethics Committee in Kampala, Uganda; and the Uganda National Council for Science and Technology in Kampala, Uganda. Prior to enrollment, written consent in either Luganda or English was obtained from each participant.

Participation

Participation in the study involved 4 visits over a 4-6-month period: immediately following the first prenatal visit (MHC IV); conduct of the L:M test at the participant's residence within one week of the first visit; three weeks before the expected date of delivery (also at the participant's residence); and within 48 hours of delivery (either participant's residence, MHC IV, or other health facility).

Enrollment visit

An ultrasound scan was performed by a trained professional at MHC IV to both confirm a singleton pregnancy and determine participants' estimated date of delivery. Hemoglobin was measured using a portable hemoglobinometer (HemoCue Hb 301; HemoCue, Inc., Brea, CA, USA). A venous blood draw was performed by the phlebotomist at MHC IV (BD Vacutainer, Becton Dickinson, Durham, NC, USA). Systolic and diastolic blood pressure measurements were taken using a digital upper arm blood pressure monitor (Omron 10 Series, Omron Healthcare, Kyoto, Japan).

All anthropometry measurements were performed in triplicate and the mean was used for analysis. Weight was measured to the nearest 0.1 kg using a digital weight scale (Seca 874, Hanover, MD, USA). Height was measured to the nearest 0.1 cm using a portable, rigid height board (Infant/Child/Adult ShorrBoard, Shorr Production, Olney, MD, USA). Mid-upper arm circumference (MUAC) was measured to the nearest 0.1 cm using a standard tri-colored, non-stretch adult MUAC tape.

Finally, a questionnaire was administered by the study nurse that included questions related to demographics, prior pregnancies, health status, diet, food security, and WASH practices.

L:M test visit

Within one week of the enrollment visit, a household visit was conducted to perform a lactulose: mannitol (L:M) dual sugar absorption test. Following urination to void the bladder and an observed one hour fast, participants consumed a 50-mL solution

containing 5 grams of lactulose (Lactulose Solution; Mckesson, San Francisco, CA, USA) and 2 grams of mannitol (D-mannitol powder; Sigma-Aldrich, St. Louis, MO, USA) completely dissolved in sterile water. Urine was collected for a period of 4 hours in a 2 L plastic collection bottle containing 0.05 mL of 50% thimerosal (Sigma-Aldrich, St. Louis, MO, USA) as a preservative. Water intake was permitted ad libitum one hour following ingestion of the solution, and women were encouraged to drink a minimum of 500 mL of water during the test to ensure sufficient urine output. A final urine sample was collected at the 4-hour time-point and total urine volume was measured to the nearest 1.0 mL using a graduated cylinder in the field. Samples were frozen at -20 °C at the MHC IV laboratory before being transferred to a -80 °C freezer in Kampala.

Follow-up visit

Three weeks prior to participants' estimated delivery date a second household visit was conducted, consisting of a follow-up survey, with questions related to pregnancy risk factors. In addition, weight and MUAC measurements were taken following the identical procedures used at the enrollment visit. Finally, participants were asked to provide a sample of water from their drinking water storage container for the purposes of a water quality test.

Post-delivery visit

Infant characteristics (live birth, date and time of delivery, sex, weight, and length) were collected within 48 hours of delivery. Weight was measured to the nearest 0.1 kg using a digital weigh scale (Seca 874, Hanover, MD, USA) and length was

measured to the nearest 0.1 cm using a portable, rigid height board (ShorrBoard, Shorr Production, Olney, MD, USA). All anthropometry measurements were taken in triplicate and averaged. In the case of a stillbirth, only birth date, time, and infant sex were recorded.

Laboratory analyses

Urine samples were analyzed for concentrations of lactulose and mannitol using previously described high-performance liquid chromatography (HPLC) methods at the Shulman Laboratory at Baylor College of Medicine.[28] The L:M ratio was calculated by dividing the urinary lactulose concentration by the urinary mannitol concentration. Lactulose (%LE) and mannitol (%ME) excretion ratios were calculated from the measured amount of each in urine (concentration × total urine volume) relative to the initial dose of each sugar. Finally, lactulose: mannitol excretion ratios (LMERs) were calculated from the division of the %LE by the %ME.

Blood samples were centrifuged at MHC IV for five minutes at speed of 4,000 rpm, and serum was aliquoted into 2.0 mL clear plastic cryovials. Anti-flagellin- and anti-LPS Ig concentrations (IgA and IgG) were measured at the Gewirtz Laboratory at Georgia State University using previously described ELISA methods.[18]

Water quality was assessed in the field using a compartment bag test (CBT, Aquagenx, Chapel Hill, NC, USA). One hundred ml. of drinking water from each household was mixed with an E. coli chromogenic growth medium and incubated for a period of 48 hours inside a sealed plastic bag containing 5 compartments of varying volumes. Risk categories were determined by noting which, if any, compartments

changed from yellow to green/blue during the incubation period and matching that to a most probable number (MPN) table based on the World Health Organization (WHO) guidelines.[29] As there is no safe level of E. coli contamination, a dichotomous (safe vs. unsafe) water variable was created for the purpose of this analysis corresponding to no E. coli detected vs. any E. coli detected.

Statistical analyses

Based on studies of maternal idiopathic inflammatory bowel diseases (e.g., IBD) and adverse birth outcomes, the relative risk of maternal EED and subsequent preterm birth was assumed to be 2.0.[30,31] Assuming 80% power, 0.05 significance, a frequency of preterm birth of 5%, and 15% loss to follow up, a sample size of 258 allowed for the detection of a relative risk of 2.0 within 50% of the true risk parameters.

All analyses were carried out using STATA 15 software (Stata Corps, College Station, TX, USA). Weight and length measurements were converted to Z-scores for weight-for-age (WAZ), length-for-age (LAZ), and weight-for-length (WLZ) using the World Health Organization standards.[32] Outliers were defined as -6 > WAZ > +5, -5 > WLZ > +5, and -5 > LAZ > +5 and were excluded from analyses. Low birth weight was defined as weighing less than 2,500 grams at birth, and stunting at birth was defined as LAZ <-2. Preterm birth was defined as birth before 37 weeks gestation, and still birth was defined as fetal death after 20 weeks gestation.

Prior to analysis, distributions of biomarker values were assessed for outliers and normality. Because of their skewed distribution, L:M ratios were natural log (ln) transformed. Pearson correlation coefficients were calculated to evaluate agreement

between log-transformed L:M ratio and log-transformed serum biomarkers. Associations between EED biomarkers (continuous, independent variables) and gestational age, length at birth, and LAZ at birth (continuous, dependent variables) were assessed using unadjusted and adjusted linear regression models. For all adjusted models, covariates were selected using bivariate analyses, with gestational age at birth and birth length as dependent variables, and a p-value < 0.25. In all cases, statistical significance was determined using a p-value < 0.05.

Results

A flow diagram for the prospective cohort study is presented in Figure 1. Of the 300 pregnant women screened, 258 met the inclusion criteria and were enrolled in the study. Of these 258 participants, 247 had an L:M test conducted within one week of enrollment and 11 refused the test and dropped out of the study. Background characteristics and anti-flagellin and anti-LPS IgG concentrations were analyzed for these 247 participants. Of these, 236 participants had a follow-up visit conducted prior to delivery and 11 were considered lost to follow up as they moved away from Mukono District. Birth outcome data were collected within 48 hours for 232 infants. Of these, ten infants were stillborn, and two infants died before anthropometry measurements could be taken. Therefore, anthropometry measurements were collected and analyzed for 220 infants.

The background characteristics and L:M test results for 247 participants are presented in Table 1. On average, participants were ~24 years old and ~18 weeks pregnant at the time of enrollment. Background characteristics and EED biomarkers were not significantly different between women who dropped out of the study or were lost to

follow up and those that remained in the study. Table 2 presents the pregnancy characteristics and birth outcome data for 232 infants. Half of the infants were female, and mean ± SD gestational age at birth was 39.4 ± 2.9 weeks. The vast majority of women took iron supplementation and intermittent preventative therapy (IPT) during their pregnancy, but amounts of each varied widely. Exposure to risk factors such as tobacco and alcohol were minimal.

As shown in Figure 2, median maternal anti-flagellin and anti-LPS Ig concentrations obtained at ~18 weeks gestation were higher in women who subsequently delivered preterm infants compared to women who delivered term infants across all serum EED. These differences were significant for IgG (but not IgA) antibodies to flagellin and LPS. Furthermore, higher maternal anti-flagellin- and anti-LPS Ig concentrations were associated with shorter infant gestational age at birth. Once again, correlations were significant for IgG (but not IgA) antibodies to flagellin and LPS (Figure 3).

Table 3 shows the association between maternal EED biomarkers and infant gestational age at birth. In both unadjusted and adjusted analyses, increased concentrations of anti-flagellin IgG and anti-LPS IgG were significantly associated with lower gestational age at birth [(adjusted β: -1.35, 95% CI: -2.64, -0.07) and (adjusted β: -0.89 [95% CI: -1.52, -0.25)]. In contrast, no association was observed between ln L:M ratio and gestational age.

Table 4 shows the association between maternal EED biomarkers and infant length and LAZ at birth using unadjusted and adjusted linear regression models. In adjusted analyses, increased concentrations of anti-flagellin IgG and anti-LPS IgG were

significantly associated with lower infant length at birth [(β: -0.97, 95% CI: -1.79, -0.14) and (β: -0.45, 95% CI: -0.87, -0.02), respectively] and LAZ at birth [(β -0.52, 95% CI: -0.95, -0.08) and (β: -0.25, 95% CI: -0.48, -0.03), respectively] for infants born at term. Once again, no association was observed between ln L:M ratio and either infant length at birth or LAZ at birth.

Pearson correlation coefficients between continuous natural log-transformed maternal L:M ratios and continuous natural log-transformed anti-flagellin and anti-LPS Ig concentrations are presented in Table 5. Overall, there were modest negative correlations ranging from -0.08 to -0.19 between ln L:M ratios and ln anti-flagellin and anti-LPS Ig concentrations. Significant negative associations were observed between ln L:M ratio and ln anti-flagellin IgG and ln anti-LPS IgG. As expected, since both measure exposure to bacterial components, pairs of ln anti-flagellin and ln anti-LPS Ig concentrations had significant positive associations ranging from 0.21 between ln anti-flagellin IgA and ln anti-flagellin IgG to 0.72 between ln anti-flagellin IgA and ln anti-LPS IgA.

Discussion

In this prospective cohort study of 258 pregnant women in Mukono, Uganda, we tested the hypothesis that maternal EED biomarkers, measured at ~18 weeks of gestation using both the L:M test as well as serum concentrations of anti-flagellin and anti-LPS Igs, are associated with adverse birth outcomes, specifically shorter gestational age and reduced length at birth. We found significant differences in anti-flagellin and anti-LPS IgG concentrations in mothers that delivered preterm compared with those who delivered term infants. Furthermore, we found that higher maternal anti-flagellin and anti-LPS IgG,

but not IgA, concentrations were significantly associated with shorter gestation and lower length and LAZ at birth in adjusted linear regression models. We did not find this relationship between LM scores and any birth outcome. To our knowledge, this is the first study to examine the association between maternal EED biomarkers and adverse birth outcomes in newborn infants.

In addition, we found anti-flagellin and anti-LPS Igs to be poorly correlated with the L:M test. This finding is consistent with results from previous studies, which found low correlation among a panel of EED biomarkers. In a sample of 539 18-month-old children in rural Bangladesh, Campbell et al. found that serum and stool biomarkers (including myeloperoxidase (MPO), a-1 antitrypsin (AAT), neopterin (NEO), endotoxin core antibodies (EndoCAb), glucagonlike peptide-2 (GLP-2), C-reactive protein (CRP), and a-1 acid glycoprotein (AGP)) demonstrated low agreement with the L:M test.[33] Similarly, in a sample of 375 6-26-month-old children in Northeast Brazil, Guerrant et al. also found low correlation among 18 proposed EED biomarkers, including between anti-flagellin/LPS Igs and the L:M test.[23]

One reasonable explanation for this disparity is that the L:M test measures intestinal permeability and absorptive capacity rather than intestinal or systemic inflammation.[34] On the other hand, anti-flagellin and anti-LPS IgG concentrations more generally reflect increased systemic exposure to these microbial products, which could reflect increased permeability to macromolecules, alterations in microbiota composition, and/or increased translocation of bacteria. Because we found no associations between maternal L:M ratio and adverse birth outcomes, we hypothesize that the primary

mechanism by which EED maybe associated with adverse birth outcomes is via increased systemic inflammatory response rather than via nutrient malabsorption.

While not established in cases of EED, studies in patients with similar intestinal disorders demonstrate that gut barrier dysfunction results in systemic exposure to flagellin and LPS, which ultimately stimulates an immune response. In one such study, Ziegler et al. compared serum from parenteral nutrition (PN)-dependent patients with SBS (n=23) to non-SBS control subjects (n=48 healthy adults and n=37 adults requiring PN during critical illness) and found flagellin, LPS, or both in 61% of SBS patients versus 0% in control subjects. Furthermore, patients with SBS had significantly elevated anti-flagellin Igs (IgA, IgG, and IgM) compared to control subjects ($p<0.001$).[18] Similarly, Dlugosz et al. compared serum from patients with three different subtypes of IBS (total n=88), and to healthy control subjects (n=106) and found significantly elevated concentrations of LPS in patients with diarrhea-predominant IBS ($p=0.0155$) compared to control subjects as well as significantly elevated concentrations of antibodies to flagellin in all patients with IBS compared to control subjects ($p=0.0018$).[21]

While this is the first study to examine the association between maternal anti-flagellin and anti-LPS Igs and birth outcomes, several studies have previously demonstrated an association between adverse birth outcomes and maternal inflammation biomarkers, particularly pro-inflammatory cytokines[35,36] and CRP.[37,38] In a prospective cohort study of HIV-positive (n=44) and HIV-negative (n=70) Tanzanian mothers and their infants, Wilkinson et al. found that systemic inflammation, measured by a 9-plex panel of maternal plasma cytokines, was associated with poorer birth anthropometry. In the study, greater maternal plasma tumor necrosis factor (TNF)-α concentrations were

associated with earlier delivery (-1.7 weeks, p = 0.039) and lower birthweights (-287 g, p = 0.020), and greater umbilical cord TNF-α (-1.43 cm, p = 0.036) and interleukin (IL)-12p70 (-2.4 cm, p = 0.008) were associated with shorter birth length.[39] Furthermore, in a longitudinal study of Filipino mothers and their infants (n=327), Kuzawa et al. found that systemic inflammation, measured by maternal CRP during pregnancy, was associated with reduced infant body weight (-0.047 ± 0.017 kg/log-mg/l), length (-0.259 ± 0.092 cm/log-mg/l) and sum of skinfolds (-0.520 ± 0.190 mm/log-mg/l) (all $p<0.05$).[40]

Overall, our study design had several strengths. Date of delivery was estimated more exactly through the use of an ultrasound scan rather than calculated from an estimate of the first day of the last menstrual period. Furthermore, loss to follow up in the study was relatively low, and birth outcome measurements, including birth length, were collected within 48 hours of delivery. There were, however, several limitations. This study was designed as a pilot or proof of concept study and our sample size of 258 enrolled women was relatively small. We were underpowered to determine associations between EED biomarkers and less common adverse birth outcomes such as spontaneous abortion and stillbirth. Furthermore, we obtained measures of only two proposed EED biomarkers at only one point in time, ~18 weeks gestation. Overall, we recommend larger, more robust studies which examine a more extensive panel of biomarkers at different gestational time points to better determine the effects of EED on adverse birth outcomes.

In conclusion, maternal anti-flagellin and anti-LPS IgG concentrations were significantly associated with shorter gestational age and lower length and LAZ at birth in a sample of pregnant Ugandan women and their newborn infants. These results provide

preliminary evidence that maternal EED, especially the component of systemic inflammation, may be related to adverse birth outcomes. From this, we surmise that reducing the presence of maternal EED, through reducing exposure to enteropathogens prior to and during pregnancy, will result in improved birth outcomes. However, larger and more robust studies are needed to provide further evidence and generalizable results.

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Tables and figures

Table 8: Baseline characteristics of 247 pregnant women and their households

Characteristic	**Mean ± SD or n (%)**
Age, years	23.9 ± 4.2
Gestational age	17.9 ± 3.4
Weight, kg	60.8 ± 9.7
Height, cm	158.4 ± 6.0
BMI, kg/m^2	24.2 ± 3.6
MUAC, cm	27.0 ± 3.3
Hemoglobin, g/dl	11. 9 ± 1.4
Anemic (Hb < 12)	118 (47.8)
Systolic blood pressure, mmHg	109.5 ± 11.3
Diastolic blood pressure, mmHg	72.3 ± 8.3
Pulse Pressure, mmHg	37.2 ± 9.3
First pregnancy	92 (37.2)
Prior fetal loss[1]	26 (16.7)
Household head	9 (3.6)
Household members	3.4 ± 2.0
Marital status	
Married/cohabitating, monogamous	215 (87.0)
Married/cohabitating, polygamous	20 (8.1)
Single	12 (4.9)
Employed	108 (43.7)
Education, years	9.9 ± 2.9
HFIAS	
Food secure	66 (26.7)
Mildly food insecure	69 (27.9)
Moderately food insecure	94 (38.1)
Severely food insecure	18 (7.3)
Lactulose: mannitol (L:M) test	
Urine volume, mL	243.5 ± 228.2
L:M ratio	0.08 ± 0.12
Urinary lactulose, % dose excreted	0.50 ± 0.74
Urinary mannitol, % dose excreted	16.87 ± 7.12
LMER	0.03 ± 0.05

[1]Among those reporting a prior pregnancy (n=156)

Abbreviations: MUAC, mid-upper arm circumference; Hb, Hemoglobin; HFIAS, household food insecurity access scale; L:M, lactulose: mannitol ratio; LMER, lactulose: mannitol excretion ratio

Table 9: Pregnancy and birth outcome characteristics for 232 women and their newborn infants

Characteristic	**Mean ± SD or n (%)**
Pregnancy characteristics	
Antenatal visits	3.4 ± 0.8
Weight change, g/week	290.1 ± 186.1
Iron supplementation, >=60 days	67 (28.9)
IPT, courses	2.2± 1.0
Deworming medication, y/n	143 (61.6)
Second-hand tobacco exposure	20 (8.6)
Safe water	98 (42.2)
Infant characteristics at birth	
Female, sex	119 (51.3)
Still birth[1]	10 (4.3)
Neonatal death	2 (0.9)
Preterm[2]	23 (9.9)
Gestational age, weeks	39.4 ± 2.9
Birth weight, kg[3]	3.3 ± 0.4
Birth length, cm	48.7 ± 2.0
Birth weight < 2500 g	8 (3.6)
Weight-for-length Z-score	0.47 ± 1.54
Weight-for-age Z-score	-0.13 ± 0.95
Length-for-age Z-score	-0.44 ± 1.07

[1]Defined as fetal death after 20 weeks gestation

[2]Defined as born before 37 weeks gestation

[3]Anthropometry data (weight, length, WLZ, WAZ, LAZ) collected only on live-born infants (n=220)

Abbreviations: IPT, intermittent preventive treatment/therapy

Table 10: Biomarkers of maternal EED as predictors of infant gestational age at birth

	Unadjusted	**Adjusted[1]**
Ln L:M	-0.03 (-0.37, 0.31) p=0.878	0.02 (-0.33, 0.37) p=0.904
Anti-flagellin IgA	-0.14 (-0.69, 0.42) p=0.626	-0.27 (-0.86, 0.33) p=0.376
Anti-LPS IgA	-0.33 (-0.99, 0.34) p=0.333	-0.36 (-1.06, 0.34) p=0.312
Anti-flagellin IgG	**-1.45** **(-2.68, -0.21)** **p=0.022***	**-1.35** **(-2.64, -0.07)** **p=0.039**
Anti-LPS IgG	**-0.84** **(-1.44, -0.23)** **p=0.007***	**-0.89** **(-1.52, -0.25)** **p=0.006**

Cell values are beta coefficients, 95% confidence intervals, and p-values

[1]Adjusted models control for mother's age, height, mid-upper arm circumference (MUAC), pulse pressure (SBP-DBP), years of education, safe water, first pregnancy (y/n), and household food insecurity access scale (HFIAS) score

Abbreviations: L:M, lactulose: mannitol ratio; LPS, lipopolysaccharide

* $p<0.05$

Table 11: Biomarkers of maternal EED as predictors of infant length and LAZ at birth[1]

	Length, cm		**LAZ**	
	Unadjusted	Adjusted[2]	Unadjusted	Adjusted
Ln L:M	0.04 (-0.21, 0.30) p=0.746	-0.004 (-0.24, 0.23) p=0.973	0.03 (-0.11, 0.16) p=0.712	-0.003 (-0.12, 0.13) p=0.963
Anti-flagellin IgA	0.04 (-0.39, 0.46) p=0.867	-0.05 (-0.45, 0.36) p=0.821	-0.002 (-0.23, 0.22) p=0.987	-0.05 (-0.27, 0.16) p=0.618
Anti-LPS IgA	-0.27 (-0.78, 0.24) p=0.301	-0.30 (-0.77, 0.17) p=0.208	-0.17 (-0.43, 0.10) p=0.228	-0.19 (-0.43, 0.06) p=0.135
Anti-flagellin IgG	-0.75 (-1.67, 0.18) p=0.112	**-0.97** **(-1.79, -0.14)** **p=0.022***	-0.40 (-0.88, 0.09) p=0.112	**-0.52** **(-0.95, -0.08)** **p=0.020***
Anti-LPS IgG	-0.22 (-0.69, 0.25) p=0.357	**-0.45** **(-0.87, -0.02)** **p=0.041***	-0.13 (-0.38, 0.12) p=0.305	**-0.25** **(-0.48, -0.03)** **p=0.029***

Cell values are beta coefficients, 95% confidence intervals, and p-values

[1] Regression analyses done for infants born at term (>37 weeks)

[2]Adjusted models control for mother's age, height, MUAC, pulse pressure (SBP-DBP), years of education, safe water, first pregnancy (y/n), household food insecurity access scale (HFIAS) score, weight change, and birth weight

Abbreviations: L:M, lactulose: mannitol ratio; LPS, lipopolysaccharide

*p<0.05

Table 12: Correlation among maternal L:M ratios and serum EED biomarkers[1]

Biomarker	**L:M**	**Anti-flagellin IgA**	**Anti-LPS IgA**	**Anti-flagellin IgG**	**Anti-LPS IgG**
L:M	1.000				
Anti-flagellin IgA	-0.09 (0.16)	1.000			
Anti-LPS IgA	-0.14 (0.03)*	0.72 (0.00)*	1.000		
Anti-flagellin IgG	-0.08 (0.21)	0.21 (0.0008)*	0.22 (0.004)*	1.000	
Anti-LPS IgG	-0.19 (0.002)*	0.32 (0.00)*	0.56 (0.00)*	0.24 (0.002)*	1.000

[1]Cell values are Pearson correlation coefficients between log-transformed biomarkers and (p-values)

Abbreviations: L:M, lactulose: mannitol ratio; LPS, lipopolysaccharide

*$p<0.05$

Figure 2: Flow diagram for prospective birth cohort study

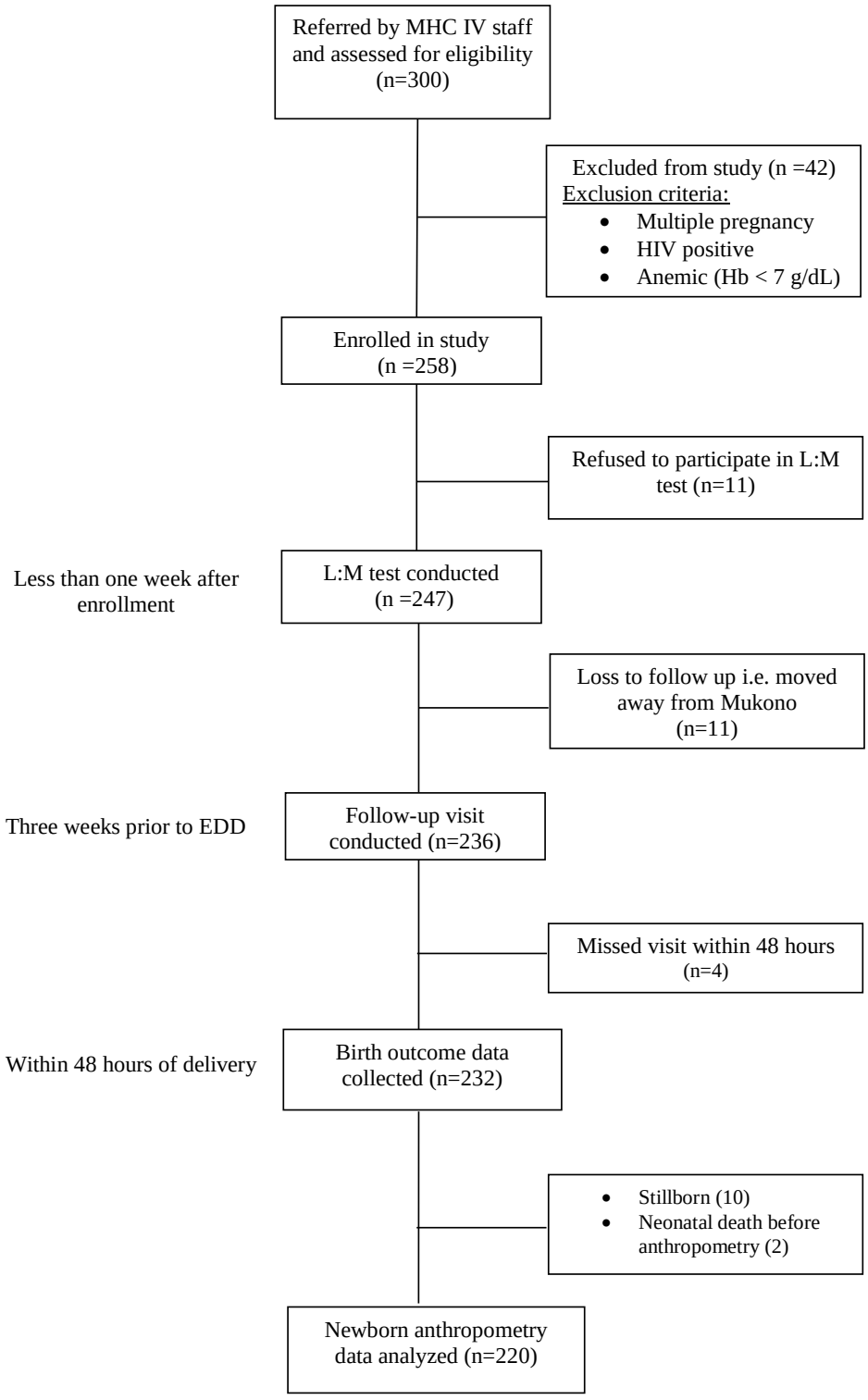


Figure 3: Serum EED biomarkers for mothers who gave birth term vs. preterm

Figure is box plot of serum EED biomarker levels for mothers who gave birth term vs. preterm (<37 weeks gestation). Plot shows median, minimum, maximum, and inter-quartile range for given concentrations. Differences are significant ($p < 0.05$) for anti-flagellin and anti-LPS IgG.

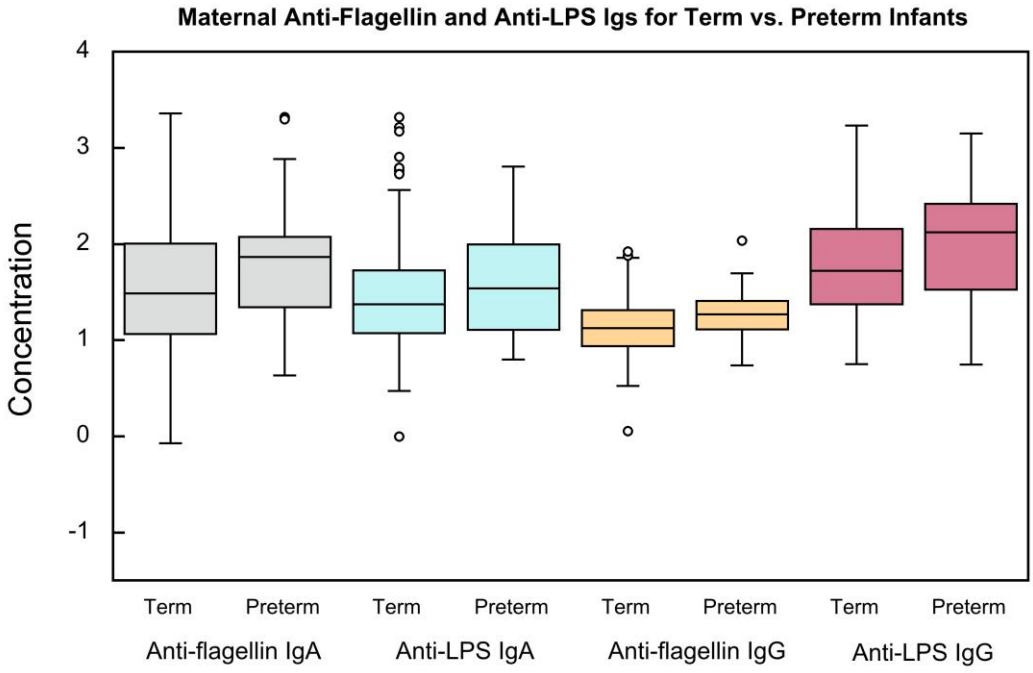
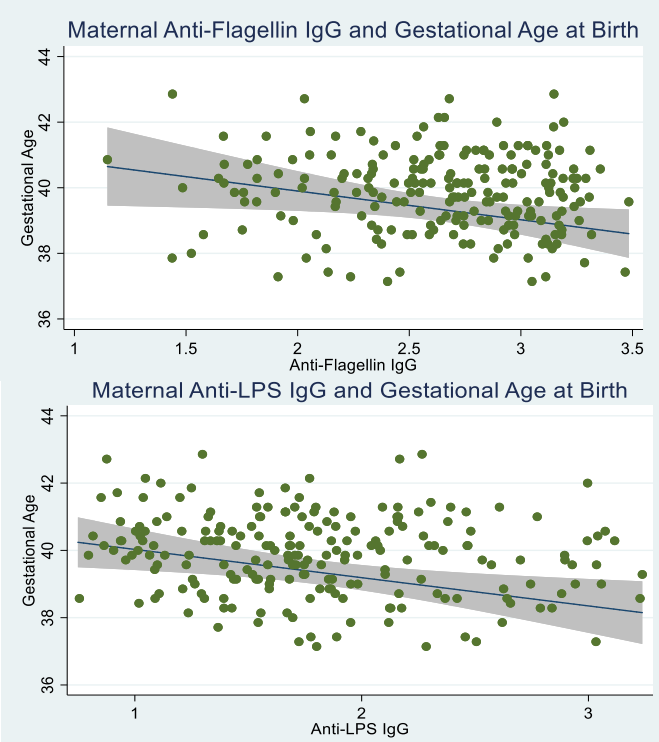


Figure 4: Maternal serum EED biomarkers and infant gestational age at birth

Figure is maternal anti-flagellin and anti-LPS IgG and their correlation with infant gestational age at birth. Graphs show best-fit trend line (red line) with 95% confidence interval (gray area) for infants born at term (>37 weeks gestation). Correlations are significant ($p<0.05$).



Chapter 6: Association between maternal aflatoxin exposure during pregnancy and adverse birth outcomes in Uganda

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Abstract

Aflatoxins are toxic metabolites of *Aspergillus* molds and are widespread in the food supply, particularly in low- and middle-income countries (LMICs). Both in utero and infant exposure to aflatoxin B_1 (AFB_1) have been linked to poor child growth and development. The primary objective of this prospective cohort study was to investigate the association between maternal aflatoxin exposure during pregnancy and adverse birth outcomes in Mukono District, Uganda. Maternal aflatoxin exposure was assessed in 247 participants by measuring the serum concentration of AFB_1-lysine (AFB-Lys) adduct at 17.9 ± 3.4 (mean ± SD) weeks gestation using high performance liquid chromatography (HPLC). Anthropometry and birth outcome variables (sex, gestational age, weight, length, and head circumference) for 220 live infants were obtained within 48 hours of delivery. Associations between maternal aflatoxin exposure and birth outcomes were assessed using multivariate linear regression models adjusted for confounding factors. Median maternal AFB-Lys level was 5.71 pg/mg albumin (range: 0.71-95.60 pg/mg albumin, IQR: 5.98 pg/mg albumin). In adjusted linear regression models, elevations in natural log transformed maternal AFB-Lys levels were associated with lower weight (adjusted β: -0.07; 95% CI: -0.13, -0.002; p=0.045), lower weight-for-age Z-score (WAZ) (adjusted β: -0.16; 95% CI: -0.30, -0.03; p=0.021), smaller head circumference (adjusted β: -0.27; 95% CI: -0.51, -0.03; p=0.029), and lower head circumference-for-age Z-score (HCZ) (adjusted β: -0.23; 95% CI: -0.41, -0.04; p=0.016) in infants at birth. Data from our study suggests an association between maternal aflatoxin exposure during pregnancy and adverse birth outcomes.

Keywords: Aflatoxin, aflatoxin B_1-lysine adduct, pregnancy, maternal exposure, birth weight, head circumference

Introduction

Worldwide, an estimated 15.5% of infants are born low birth weight (LBW), i.e. birth weight < 2500 g, with the vast majority (> 95%) of cases occurring in low- and middle- income countries (LMICs) (Wardlaw, 2004). Overall, birth weight is an indicator of maternal health and nutrition status as well as infants' short-and long-term wellbeing. Infants born low birth weight are at an increased risk of a number of short- and long-term consequences, including neonatal mortality and morbidity, impaired immune function, childhood stunting, reduced cognitive development, and chronic diseases later in life (Wardlaw, 2004).

Aflatoxins are naturally-occurring, toxic secondary metabolites of Aspergillus molds, particularly A. flavus and A. parasiticus. They are widely prevalent in staple foods, such as maize, sorghum, and groundnuts, particularly in LMICs where poor harvest and storage practices leave food supplies vulnerable to contamination (Hell, Cardwell, Setamou, & Poehling, 2000; Kachapulula, Akello, Bandyopadhyay, & Cotty, 2017). Overall, it is estimated that 4.5 billion people, mainly in LMICs, are at risk of chronic exposure to aflatoxins (Williams et al., 2004), which have been linked to a number of carcinogenic, teratogenic, and immunotoxic health effects, most notably liver cancer (Liu & Wu, 2010).

Aflatoxin B1 (AFB1), the most prevalent and toxic type of aflatoxin (Hussein & Brasel, 2001), has also been linked to poor growth and development (Y. Gong et al., 2004; Y. Y. Gong et al., 2002; Shirima et al., 2015) and immune function impairment (Turner, Moore, Hall, Prentice, & Wild, 2003) in young children. Furthermore, AFB1 can cross the placental barrier during pregnancy (Denning, Allen, Wilkinson, & Morgan, 1990; Partanen et al., 2010), putting the fetus at risk of exposure. In a limited number of

studies, maternal aflatoxin exposure during pregnancy has been linked to adverse birth outcomes, (Abdulrazzaq, Osman, & Ibrahim, 2002; De Vries, Maxwell, & Hendrickse, 1989; Shuaib et al., 2010) particularly low birth weight, as well as continued poor growth during the first 1,000 days (Groopman et al., 2014; Turner et al., 2007).

Previous studies have shown that aflatoxin exposure is widespread in both the food supply (Kaaya & Kyamuhangire, 2006; Kitya, Bbosa, & Mulogo, 2010) and population (Asiki et al., 2014) in Uganda. The primary objective of this study was to investigate the association between maternal exposure to aflatoxin during pregnancy and adverse birth outcomes, particularly lower weight and length, smaller head circumference, and shorter gestational age at birth in Mukono District, Uganda. In this study, maternal aflatoxin exposure was measured at ~18 weeks gestation using the serum concentration of the AFB1-Lysine (AFB-Lys) adduct, which is an established biomarker of dietary aflatoxin exposure over the previous 2-3 months (Wild et al., 1992). We hypothesized that higher levels of AFB-Lys in pregnant women would be associated with adverse birth outcomes, particularly lower birth weight.

Key messages

- AFB-Lys levels were detected in 100% of maternal serum samples (n=247), indicating widespread exposure to AFB1 in the diets of the sample population.
- Maternal AFB-Lys levels were positively correlated with age of gestation at the time of the blood draw whereas maternal albumin concentrations were negatively correlated with age of gestation at the time of the blood draw.

• Elevated maternal AFB-Lys levels were significantly associated with lower birth weight, lower WAZ, smaller head circumference, and lower HCZ in infants at birth.

• Initiatives to reduce aflatoxin exposure, especially targeted at women of reproductive age, may result in improved birth outcomes in LMICs.

Methods

Study site and population

This was a prospective cohort study conducted in Mukono District, Uganda from February-November 2017. Two hundred fifty-eight pregnant women were initially enrolled at Mukono Health Center IV (MCH IV) during their first prenatal visit. Women qualified for the study if they were between 18 and 45 years old, resided within 10 kilometers of MHC IV, carried a singleton pregnancy, and planned to remain in Mukono District throughout their pregnancy. Women were excluded if they were < 18 years old or > 45 years old, HIV-positive (verified via routine rapid HIV test conducted at first prenatal visit), severely malnourished (defined as BMI <16.0 kg/m2), severely anemic (defined as Hb < 7 g/dL), or planned to move away from Mukono District prior to delivery.

This study was approved by the Tufts Health Sciences Institutional Review Board in Boston, Massachusetts; the Mengo Hospital Research Ethics Committee (REC) in Kampala, Uganda; and the Uganda National Council for Science and Technology (UNCST) in Kampala, Uganda. Written consent was obtained from all participants prior to enrollment. The study was registered at ClinicalTrials.gov, ID # NCT03429257.

Study parameters and measurement

Following enrollment, height (0.1 cm precision; Infant/Child/Adult ShorrBoard, Shorr Production, Olney, MD, USA), weight (0.1 kg precision; Seca 874, Hanover, MD, USA), mid-upper arm circumference (MUAC) (0.1 cm precision; tri-colored, non-stretch adult MUAC tape), blood pressure (Omron 10 Series, Omron Healthcare, Kyoto, Japan), and hemoglobin (HemoCue Hb 301; HemoCue, Inc., Brea, CA, USA) measurements were taken by the study nurse. All anthropometry measurements, including height, weight, and MUAC, were taken in triplicate and averaged. Date of delivery was estimated from an obstetric ultrasound examination performed at enrollment (i.e. between 9-27 weeks gestation) by a trained technician. A venous blood draw (BD Vacutainer, Becton Dickinson, Durham, NC, USA) was performed by the phlebotomist at MHC IV, and blood samples were centrifuged at MHC IV for 5 minutes at speed of 4,000 rpm to separate serum. Finally, a questionnaire was administered by the study nurse that included questions related to demographics, prior pregnancies, health status, diet, food security, and water, hygiene, and sanitation (WASH) practices. Diet was assessed using a diet diversity questionnaire that asked participants to respond yes/no to having eaten > 50 specific foods in the previous 24 hours.

Infant anthropometry data, including length (0.1 cm precision; Infant/Child/Adult ShorrBoard, Shorr Production, Olney, MD, USA), weight (0.1 kg precision; Seca 874, Hanover, MD, USA), head circumference (0.1 cm precision; flexible measuring tape), were assessed within 48 hours of delivery. All anthropometry measurements were taken in triplicate and averaged. Head circumference was measured as the largest possible occipital-frontal circumference.

Chemicals

Aflatoxin B1 (> 98% purity), albumin determination reagent bromocreosol purple, and normal human serum, were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). Pronase (25kU, Nuclease-free) was purchased from Calbiochem (La Jolla, CA). Protein assay dye reagent concentrate and protein standards were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). Mixed mode solid phase extraction (SPE) cartridges were purchased from the Waters Corp. (Milford, MA). Authentic AFB-Lys was synthesized as previously described (Sabbioni, Skipper, Büchi, & Tannenbaum, 1987). All other chemicals and solvents used were of highest grade commercially available.

Analysis of Aflatoxin B1-lysine (AFB-Lys) adduct levels

Mid-gestation maternal aflatoxin exposure was assessed using the serum AFB-Lys adduct biomarker. Serum samples were transported on dry ice to the Wang laboratory at the University of Georgia, Athens, USA and analyzed with a high-performance liquid chromatography (HPLC)-fluorescence method. This included measurement of albumin and total protein concentrations for each sample, digestion with protease to release amino acids, concentration and purification of the AFB-Lys adduct, and finally separation and quantification by HPLC (G Qian, Tang, Liu, & Wang, 2010; Guoqing Qian et al., 2013).

Specifically, thawed serum samples were inactivated for possible infectious agents via heating at 56℃ for 30 minutes, followed by measurement of albumin and total protein concentrations using modified procedures as previously described. A portion of

each sample (approximately 150 μL) was digested by pronase (pronase: total protein, 1:4, w: w) at 37°C for 3 hours to release AFB-Lys. AFB-Lys in digests were further extracted and purified by passing through a Waters MAX SPE cartridge, which was preprimed with methanol and equilibrated with water. The loaded cartridge was sequentially washed with water, 70% methanol, and 1% ammonium hydroxide in methanol at a flow rate of 1 ml/min. Purified AFB-Lys was eluted with 2% formic acid in methanol. The eluent was vacuum-dried with a Labconco Centrivap concentrator (Kansas City, MO) and reconstituted for HPLC-fluorescence detection.

The analysis of AFB-Lys adduct was conducted in an Agilent 1200 HPLC-fluorescence system (Santa Clara, CA). The mobile phases consisted of buffer A (20 mM NH4H2PO4, pH 7.2) and buffer B (100% Methanol). The Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 x 250 mm) equipped with a guard column was used. Column temperature was maintained at 25°C during analysis, and a volume of 100 μL was injected at a flow rate of 1 mL/min. A gradient was generated to separate the AFB-Lys adduct within 25 minutes of injection. Adduct was detected by fluorescence at maximum excitation and emission wavelengths of 405 nm and 470 nm, respectively. Calibration curves of authentic standard were generated weekly, and the standard AFB-Lys was eluted at approximately 13.0 minutes. The limit of detection was 0.2 pg/mg albumin. The average recovery rate was 90%. The AFB-Lys concentration was adjusted by albumin concentration.

Quality assurance and quality control procedures were maintained during analyses, which included simultaneous analysis of one authentic standard in every 10 samples and two quality control samples daily. Furthermore, following completion of the

laboratory analysis, sets of three samples were selected and pooled into 11 intra-day reproducibility samples, which were analyzed twice on the same day by the same analyst, and 11 inter-day reproducibility samples, which were analyzed on different days by different analysts, to demonstrate laboratory precision and sampling reproducibility.

Statistical analysis

All statistical analyses were performed using STATA 15 software (Stata Corps, College Station, TX, USA). Variables were first assessed for outliers and normality. Because of their skewed distribution, AFB-Lys levels were natural log-transformed prior to all regression analyses.

Weight, length, and head circumference measurements were converted to Z-scores for weight-for-age (WAZ), length-for-age (LAZ), weight-for-length (WLZ), and head-circumference-for age (HCZ) using the World Health Organization standards (WHO, 2006). Outliers were defined as -6 > WAZ > +5, -5 > WLZ > +5, -5 > LAZ > +5, and -5 > HCZ > +5 and excluded from analysis. Low birth weight was defined as weighing < 2,500 grams at birth.

Associations between means were assessed using t-tests, and bivariate correlations between variables were assessed using scatter plots and Pearson's correlation coefficients. Associations between ln maternal AFB-Lys levels and infant birth outcomes were assessed using unadjusted and adjusted linear regression models. Bivariate analyses were conducted to test the association between infant birth weight and potential covariates. Those with p-value < 0.10 were included in the adjusted models. Among

maternal anthropometry measurements, only maternal BMI was included to limit collinearity. For all analyses, $p < 0.05$ was considered statistically significant.

Results

Of the 258 participants enrolled, 11 dropped out of the study, and therefore background characteristics and AFB-Lys levels were analyzed for 247 participants. Birth outcome data were collected within 48 hours for 232 infants. Of these, 10 infants were stillborn, and two infants died before anthropometry measurements could be taken. Therefore, anthropometry measurements were collected and analyzed for 220 infants. At the time of enrollment, participants were (mean ± SD) 23.9 ± 4.2 years of age and 17.9 ± 3.4 weeks gestation. Select characteristics of participants and their association with infant birth weight are presented in Table 1.

All maternal serum samples had detectable albumin (g/dl) and AFB-Lys (pg/mg albumin) levels. The arithmetic mean ± SD albumin level was 3.96 ± 0.45 g/dl, and the geometric mean albumin level was 3.93 g/dl (range: 2.84-6.08 g/dl). The arithmetic mean ± SD AFB-Lys level was 8.55 ± 11.09 pg/mg albumin, and the geometric mean AFB-Lys level was 5.77 pg/mg albumin (range: 0.71-95.60 pg/mg albumin)

Among the 220 live-born infants with birth outcome data, 115 (52%) were female. Mean ± SD gestational age at birth was 39.7 ± 2.1 weeks. Mean ± SD weight and length at birth were 3.3 ± 0.4 kg and 48.7 ± 2.0 cm, respectively. Mean ± SD WHZ, WAZ, and LAZ scores were 0.47 ± 1.54, -0.13 ± 0.95, and -0.44 ± 1.07, respectively. Mean ± SD HCZ was 0.88 ± 1.19. Eight (3.6%) were born low birth weight, and 15 (6.8%) were born stunted at birth.

Maternal characteristics and albumin/ AFB-Lys concentrations

Ln AFB-Lys levels were significantly negatively associated with maternal MUAC measurements at enrollment (β: -0.51; 95% CI: -1.0, -0.01; p = 0.044). No association was observed between ln AFB-Lys levels and levels of hemoglobin (p = 0.975). However, there was a significant association between albumin and hemoglobin levels (β: 0.46; 95% CI: 0.06, 0.86; p = 0.023).

Furthermore, mean ± SD levels of ln AFB-Lys were significantly higher in participants who reported eating cassava in the previous 24 hours compared to those that did not (1.95 ± 0.93 vs. 1.69 ± 0.80 pg/mg albumin; diff: 0.27; 95% CI: 0.03, 0.51; p=0.0254). No association was observed between ln AFB-Lys levels and other foods or food groups.

Albumin levels were significantly negatively associated with gestational age of the fetus at the time the blood sample was collected (β: -1.25; 95% CI: -2.21, -0.29; p = 0.011). On the other hand, ln AFB-Lys levels were significantly positively associated with gestational age of the fetus at the time of the blood sample was collected (β: 0.70; 95% CI: 0.19, 1.21; p = 0.007). Mean ± SD ln AFB-Lys levels for gestation = <18 weeks and >18 weeks were 1.62 ± 0.81 and 1.89 ± 0.85 (diff=-0.26; 95% CI: -0.47, -0.06; p = 0.0134). Correlations between albumin/ ln AFB-Lys levels and gestational age of the fetus at the time the blood sample was collected were also assessed using scatter plots and correlation analyses (Figure 1).

AFB1 exposure and birth outcomes

Table 2 shows the association between maternal AFB-Lys levels and birth characteristics. In adjusted linear regression models, controlling for mothers' age, body mass index (BMI), pulse pressure, first pregnancy (y/n), years of education, and gestational age at birth, ln AFB-Lys concentrations were significantly associated with lower birth weight (adjusted β: -0.07; 95% CI: -0.13, -0.002; p = 0.045) and lower WAZ at birth (adjusted β: -0.16; 95% CI: -0.30, -0.03; p = 0.021). In a one-tailed t-test, the 8 infants born LBW had significantly higher mean ± SD ln AFB-Lys levels compared to infants born not LBW (2.27 ±1.10 vs. 1.75 ± 0.85; diff=0.52; 95% CI: -0.09-1.13; p = 0.0466).

Additionally, in adjusted linear regression models with the same controls, ln AFB-Lys levels were significantly associated with smaller head circumference at birth (adjusted β: -0.27; 95% CI: -0.51, -0.03; p = 0.028) and lower HCZ (adjusted β: -0.23; 95% CI: -0.41, -0.04; p = 0.016). Correlations between AFB-Lys concentrations and infant birth weight and head circumference were also assessed using scatter plots and correlation analyses (Figures 2, 3).

No significant associations were observed between ln AFB-Lys levels and infant length, WHZ, LAZ, or gestational age at birth or between albumin levels and any of the measured birth characteristics.

Discussion

In this prospective cohort study conducted in Mukono, Uganda, we examined the relationship between maternal aflatoxin exposure during pregnancy (i.e. AFB-Lys levels measured at enrollment, or between 9 and 27 weeks gestation) and adverse birth outcomes. Our results showed that exposure to dietary aflatoxins during pregnancy is

widespread in the population, with 100% of samples having detectable AFB-Lys levels ranging from 0.71 to 95.60 pg/mg albumin. Furthermore, we observed that levels of albumin were significantly negatively associated with gestational age of the fetus at the time of the blood draw and ln AFB-Lys levels were significantly positively associated with gestational age of the fetus at the time of the blood draw. Finally, we found that elevated ln AFB-Lys levels were significantly associated with lower weight, lower LAZ, smaller head circumference, and lower HCZ in infants at birth.

Our findings that higher AFB-Lys levels are positively associated with gestational age at the time of the blood draw are consistent with the findings from Castelino et al. which found a significant difference between aflatoxin-albumin (AF-alb) levels between early (<16 weeks) and later (> 16 weeks) stages of pregnancy in the dry season in the Gambia (geometric mean: 34.5 vs. 41.8 pg/mg, $p < 0.05$) (Castelino et al., 2014). Furthermore, they are also consistent with findings from animal models that show that pregnancy enhances the toxicological impact of AFB1 exposure. In one recent study, pregnant C57BL/6J mice given a single dose of AFB1 accumulated 2-fold higher AFB1-N7-guanine DNA adducts in the liver compared to nonpregnant controls (Sriwattanapong et al., 2017).

AFB1 is metabolically activated to the toxic AFB1-8, 9-epoxide via various cytochrome P450 enzyme families (CYP1A2, CYP3A4, CYP3A5) (Guengerich et al., 1998). This aflatoxin-epoxide is capable of binding to DNA, proteins, and other macromolecules, resulting in adduct formation as well as mutagenic and carcinogenic responses. In the case of some of these enzymes, pregnancy may increase their activity, causing more AFB1 to be metabolized and converted to aflatoxin-epoxides (Tracy,

Venkataramanan, Glover, & Caritis, 2005). Furthermore, the early presence of CYP3A7 in the fetal liver indicates that the fetus may be able to convert maternal transplacental AFB1 to AFB1-8, 9-epoxides as well (Doi, Patterson, & Gallagher, 2002; Hashimoto et al., 1995). Overall, these results indicate that pregnancy may be a window of high risk to aflatoxin exposure for pregnant women and their fetuses.

While previous findings have demonstrated that aflatoxins are capable of crossing the placental barrier (Denning et al., 1990; Partanen et al., 2010) and that infant exposure is associated with poor growth outcomes (Y. Gong et al., 2004; Y. Y. Gong et al., 2002; Turner et al., 2003), only a few studies have examined the association between maternal aflatoxin exposure and adverse birth outcomes. In a prospective study of 201 women in the United Arab Emirates, aflatoxin levels measured in cord blood were significantly negatively associated with birth weight ($p < 0.001$) (Abdulrazzaq et al., 2002). Furthermore, in a cross-sectional study of 785 pregnant women in Ghana, participants in the highest quartile of AFB1 exposure were more than twice as likely to have a low birth weight baby (OR: 2.09, 95% CI, 1.19-3.68) (Shuaib et al., 2010). However, findings were inconsistent across studies, with a study by Maxwell et al. reporting no association between in-utero aflatoxin exposure measured in cord blood samples and infant birth weight in a sample of 625 Nigerian infants (Maxwell, Familusi, Sodeinde, Chan, & Hendrickse, 1994). It is worth noting, however, that only 14.6% of serum samples in this study detected the presence of aflatoxin.

Previous studies have established that AFB1 can cross the blood brain barrier (A. Oyelami, M. Maxwell, A. Adelusola, A. Aladekoma, & O. Oyelese, 1995; Qureshi et al., 2015). As far as we are aware, this is the first human study to examine the association

between maternal aflatoxin exposure during pregnancy and infant head circumference and HCZ at birth. We found a significant negative association, which is particularly noteworthy given the association between infant head circumference and brain size (Cooke, Lucas, Yudkin, & Pryse-Davies, 1977; H Bartholomeusz, Courchesne, & Karns, 2002) as well as cognitive ability later in life (Gale, O'Callaghan, Bredow, & Martyn, 2006; Veena et al., 2010).

Very few studies have looked at the association between in utero aflatoxin exposure and infant length, head circumference, or gestational age at birth. The study by Shuaib et al. found no association between preterm birth (< 37 weeks gestation) and AF-alb biomarkers (Shuaib et al., 2010), which was consistent with our findings of no association. However, there is a clear need for additional human studies that examine the relationship between maternal aflatoxin exposure and a diverse set of birth outcomes besides only infant birth weight.

In conclusion, mid-gestation exposure to aflatoxin in pregnant women was significantly associated with lower birth weight, WAZ, head circumference, and HCZ in infants at birth in Uganda. Our findings suggest that interventions to reduce dietary exposure to aflatoxin may have positive effects on birth outcomes in LMICs.

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Tables and figures

Table 13: Characteristics of study mothers and their association with infant birth weight[1]

	Mean ± SD or n (%)	**β - coefficient**	**SE**	**95% CI**	**p-value**
Age, years	23.9 ± 4.2	0.02	0.007	0.004-0.03	0.010*
Weight, kg	60.8 ± 9.7	0.01	0.003	(0.008, 0.02)	0.000*
Height,	158.4 ± 6.0	0.01	0.005	(0.004, 0.02)	0.005*
MUAC	27.0 ± 3.3	0.03	0.009	(0.01, 0.05)	0.001*
BMI, kg/m^2	24.2 ± 3.6	0.03	0.008	0.01-0.04	0.000*
Pulse pressure, mmHg	37.2 ± 9.3	0.006	0.003	-0.0002-0.01	0.061
First pregnancy, y/n	92 (37.2)	-0.12	0.06	-0.23-0.002	0.053
Education, years	9.93 ± 2.87	0.02	0.01	0.0006-0.04	0.044*
Gestational age at birth, weeks	39.7 ± 2.1	0.04	0.01	0.007-0.07	0.016*

[1] β-coefficients, SEs, 95% CIs, and p-values derived from bivariate regression analyses with infant birth weight as a continuous dependent variable

Abbreviations: CI, confidence interval; kg, kilogram; m, meter; mmHg; millimeters of mercury; MUAC, mid-upper arm circumference; SD, standard deviation; SE, standard error

*$p < 0.05$

Table 14: Association between maternal aflatoxin exposure and birth characteristics[1]

	Unadjusted Model	**Adjusted Model[1]**
Weight	-0.06 (-0.13, 0.002) p=0.059	**-0.07 (-0.13, -0.002) p=0.045***
Length	-0.09 (-0.42, 0.23) p=0.566	-0.12 (-0.44, 0.21) p=0.476
Weight-for-age Z-score	**-0.16 (-0.31, -0.01) p= 0.032***	**-0.16 (-0.30, -0.03) p=0.021***
Weight-for-height Z-score	-0.13 (-0.39, 0.12) p=0.301	-0.14 (-0.40, 0.11) p=0.274
Length-for-age Z-score	-0.07 (-0.24, 0.10) p=0.444	-0.08 (-0.25, 0.09) p=0.357
Head circumference	-0.23 (-0.47, 0.003) p=0.053	**-0.27 (-0.51, -0.03) p=0.028***
Head circumference-for-age Z-score	**-0.19 (-0.37, -0.007) p=0.042***	**-0.23 (-0.41, -0.04) p=0.016**
Gestational age at birth	-0.10 (-0.43, 0.23) p=0.561	-0.08 (-0.42, 0.26) p=0.642

Cells present β coefficient, 95% confidence interval, and p-value

[1]Adjusted linear regression model controls for mother's age, body mass index (BMI), pulse pressure, first pregnancy (y/n), and years of education in all models. Gestational age was controlled for in all models except for when it was an outcome.

Figure 5: Maternal AFB-Lys/albumin levels and gestational age of the fetus

Figure shows the correlation between maternal ln AFB-Lys levels and albumin levels and gestational age of the fetus (weeks) at the time of the blood draw. AFB-Lys was positively correlated with gestational age at the time of the blood draw (r=0.1709; p=0.0079) and albumin was negatively correlated with gestational age at the time of the blood draw (r=-0.1622; p=0.0107).

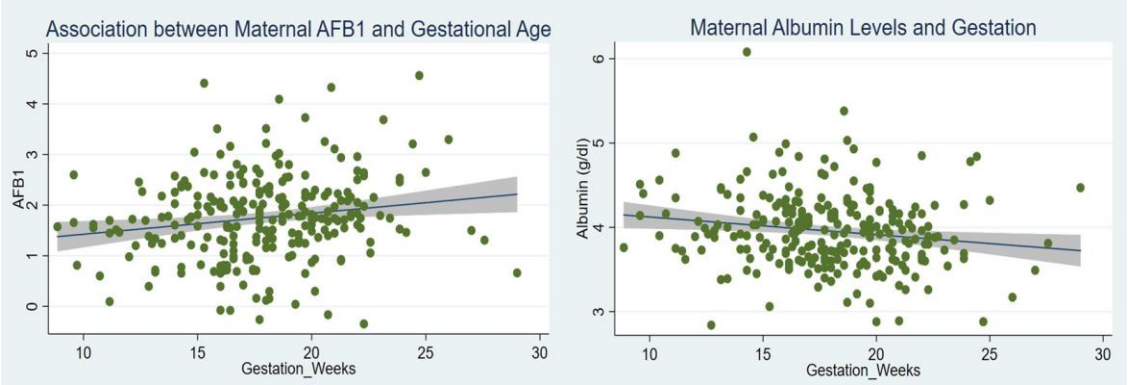


Figure 6: Maternal AFB-Lys levels and infant birth weight

Figure shows the correlation between maternal ln AFB-Lys levels and infant birth weight (r=-0.1280; p=0.0586)

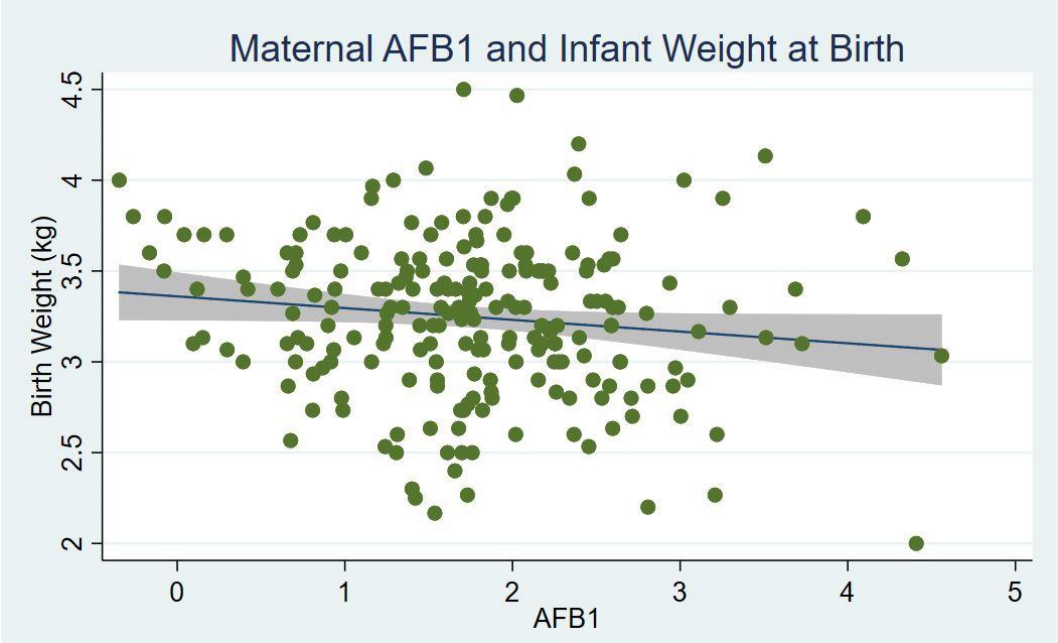
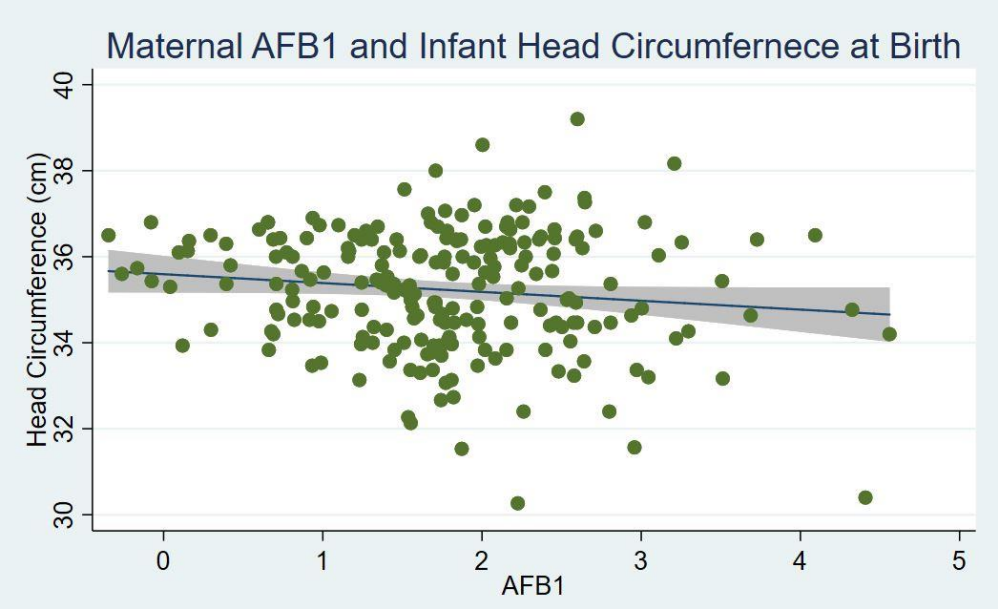


Figure 7: Maternal AFB-Lys levels and infant head circumference at birth

Figure shows the correlation between maternal ln AFB-Lys levels and infant head circumference at birth (r=-0.1309; p=0.0526)



Chapter 7: Conclusions

Summary of findings

The first aim of this dissertation was to examine the association among poor WASH conditions, specifically poor household drinking water quality, EED, and growth outcomes in children aged 12-16 months living in rural southwestern Uganda. We found that children from households with safe drinking water, defined as a lack of *E. coli* contamination assessed using a CBT at the 6-month time-point, had better intestinal health (i.e. significantly lower mean L:M ratios, ln L:M ratios, %LE, and LMERs) at 12-16 months. Furthermore, they had better growth outcomes, specifically significantly higher LAZ and WAZ, at 12-16 months. On the other hand, children from households with unsafe water were > 2.0 times as likely to be stunted at 6 months and > 1.5 times as likely to be stunted at 12-16 months using adjusted linear regression models. Finally, we found that higher LAZ scores at 6 and 9 months were significantly associated with lower mean L:M ratios using adjusted linear regression models. Overall, our findings suggest that poor drinking water quality, measured using a CBT, may be an important underlying cause of EED, and poor growth outcomes in children in LMICs like Uganda may be mediated through EED.

The second aim of this dissertation was to examine the association between maternal EED, measured using L:M ratios and serum concentrations of antibodies to the bacterial components flagellin and LPS, and adverse birth outcomes. We found significantly higher anti-flagellin and anti-LPS IgG concentrations in mothers that delivered preterm compared with those who delivered term infants. Furthermore, using adjusted linear regression models, we found that higher maternal anti-flagellin and anti-

LPS IgG, but not IgA, concentrations were significantly associated with shorter gestation and lower infant length and LAZ at birth. We did not observe an association between L:M scores and any birth outcome. These findings suggest that maternal EED, especially the systemic inflammation component of the condition, may be a contributor to adverse birth outcomes, particularly stunting at birth and preterm birth

Finally, the third aim of this dissertation was to examine the association between maternal aflatoxin exposure during pregnancy (AFB_1-Lys concentrations) and adverse birth outcomes. We found that elevated (ln) AFB_1-Lys concentrations were significantly associated with lower weight, lower LAZ, smaller head circumference, and lower HCZ in infants at birth using adjusted linear regression models. These findings suggest that maternal exposure to aflatoxin during pregnancy may also contribute to adverse birth outcomes, particularly LBW and small head circumference.

Implications of findings

If current trends continue, the WHA target to reduce stunting by 40% by 2025 will almost certainly go unrealized. While notable progress has been made, particularly in Asia, the focus of nutritional interventions remains narrowly focused on addressing macro- and micronutrient intake, while other assaults to growth-including chronic exposure to enteropathogens and aflatoxins-remain de-prioritized. In part, as a consequence of this, nutritional interventions have failed to achieve their anticipated impacts, and stunting remains pervasive in young children across LMICs.[1]

While a more holistic approach to nutrition is undoubtedly warranted, significant challenges to implementation exist. Currently, at least 1.8 billion people worldwide are estimated to drink fecally-contaminated water, and only 68% have access to “improved”

sanitation facilities.[2] At the same time, 4.5 billion people in the world, primarily in LMICs, are chronically exposed to aflatoxins in their diet.[3] Given the scope of the problem, approaches to tackle improvements in both WASH conditions and agricultural practices must be adequately funded, locally implemented, and grounded in evidence that supports their ability to deliver significant health and nutritional gains.

Directions for future EED research

Currently, there is sufficient evidence to suggest that repeated exposure to enteropathogens due to living in poor WASH conditions is associated with a continuum of changes in the small intestine for the majority of those in LMICs. Furthermore, there is mounting evidence to suggest that this condition, termed environmental enteric dysfunction or EED, is associated with suboptimal growth and potentially also adverse birth outcomes. However, as it stands today, EED lacks an agreed upon case definition and diagnostic criteria. Recently, evidence from Campbell et al. has led to the conclusion that the L:M ratio is not a suitable gold standard measure of EED, and that a better diagnostic test is essential to allow for further progress in this field of study.[4] Furthermore, according to Denno et al., "given its [EED's] obscure etiology, largely sub-clinical nature, and no clear role for a single biomarker to identify a case, it is currently impossible to diagnose a specific child with EED."[5] Therefore, filling in these gaps in our understanding of EED should be an immediate priority in order to facilitate more effective clinical trials.

In the meantime, numerous clinical trials involving EED are ongoing across LMICs, with a variety of stated objectives. Several large multi-country observational studies are currently being conducted, including the MAL-ED[6,7] study and the SHINE

trial,[8,9] which will help improve our understanding of the both the etiology and nutritional consequences of EED and allow for a comparison across different settings. While the SHINE trial in particular has highlighted the need to study EED in the context of aflatoxin exposure, this is an area in need of further study. Furthermore, research is ongoing to both validate non-invasive biomarkers of EED with small intestinal biopsy research (e.g. the Bangladesh Environmental Enteric Dysfunction (BEED) study),[10] as well as to assess the impact of EED through the use of less invasive procedures, particularly the proposed use of 'optical biopsy' techniques.[11]

A range of trials have assessed or are currently assessing the use of therapeutics, both nutritional (e.g. vitamin A, zinc, glutamine, multiple micronutrients) and medical (e.g. antibiotic, anti-inflammatory, probiotics), on EED and growth with mixed results reported.[12] In addition, two prospective randomized controlled clinical trials were conducted to determine whether common beans or cowpeas improve growth, ameliorate EED, and alter the intestinal microbiome in rural Malawian children.[13] According to results published by Agapova et al.,[14] there was no significant effects on LAZ, change in LAZ, or WLZ due to either intervention legume, compared to the control, but %L was reduced with common bean consumption (effect estimate was -0.07 percentage points of lactulose, $p = 0.0007$). Furthermore, in a sub-study nested within a cluster-randomized controlled trial of complementary food supplements (CFSs) in Bangladesh, Campbell et al.[15] found that CFSs had no impact on L:M ratios or on PCA-generated inflammation and permeability scores. Finally, a prospective randomized placebo-controlled parallel-group randomized controlled trial is currently ongoing to determine if a daily supplement

of lactoferrin and lysozyme, two important proteins found in breast milk, can decrease the burden of EED and stunting in rural Malawian children.[16]

Across all EED trials, the emphasis continues to be on child growth as an outcome, while less known about the impact of EED on (micro)nutrient absorption, immune response to oral vaccines, and cognitive development, which are all areas in need or further research. Furthermore, as far as we are aware, our study is the first to examine the association between maternal EED during pregnancy and its association with adverse birth outcomes. Considering our findings, larger, more robust studies, across different, LMICs and which include a range of EED biomarkers are warranted.

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Appendix 1: Informed consent form for the study "Assessment of environmental causes and nutritional effects of environmental enteric dysfunction (EED) among infants born to mothers in the Nutrition Innovation Lab Birth cohort study"

Effectiveness of integrated livelihood and nutrition interventions to improve maternal and child nutrition and health in rural Uganda: A birth cohort study **Sub-Study titled assessment of environmental causes and nutritional effects of environmental enteric dysfunction (EED) among infants born to mothers in the Nutrition Innovation Lab Birth cohort study**
Principal Investigators: Bernard Bashaasha PhD (Makerere University), Joyce Kikafunda PhD (Makerere University), Christopher Duggan MD MPH (Harvard School of Public Health), Wafaie Fawzi MD DrPH (Harvard School of Public Health), Jeffrey Griffiths MD MPH&TM (Tufts University), Shibani Ghosh PhD (Tufts University)
Description of Subject Population: Children ages 12 months to 15 months enrolled in the NILA Birth Cohort Study
Version 1.4**,** July 15, 2016

INTRODUCTION

Although undernutrition is a complicated problem with many causes, past research has shown that poor intestinal health may be one cause. In order to have a better understanding of this cause, we believe it is important to study intestinal health in children in the Uganda birth cohort study.

In the field, we will use a non-invasive test that looks at the absorption of two different sugars. This test will tell us about the ability of the intestine to absorb nutrients, which the child needs to grow properly.

HOW TO PARTICIPATE

Before you decide if you want your child to participate, we want to explain the purpose of the research, what you will be asked to do, the possible risks and benefits of participation, how we will protect your child's information, and who to contact if you have any questions or concerns about the project.

We will review this consent with you and answer any questions you may have about study participation. If you decide to have your child take part in the study, you will be asked to sign this consent form or make your mark in front of someone. You will be given a copy of this consent form for you to keep for the future.

Please note that:

- Your child's participation in this research project is entirely voluntary.

- You may decide your child not to take part or to withdraw your child from the study at any time.
- Your decision for your child not to participate or to withdraw will not affect your or the child's relationship with this project or any of the collaborating institutions, including Uganda Community Connecter Project (CC), now or in the future.

PEOPLE WILL TAKE PART IN THIS RESEARCH STUDY

We aim to recruit 400 infants at the age of 12 months to 15 months across 7 sub counties in Southwest Ugandan Birth Cohort study sites who are participating in the birth cohort study. Children who are eligible to participate in the study are those that have complete NILA cohort data, have had a water quality test conducted at their household, are between 12 and 15 months, and have proper consent from their caretaker. Children who have diarrhea two weeks prior to the study or vomiting and/or skin infections in the area of the groin on the day of the study will not be able to participate or may be rescheduled for a different time.

PURPOSE OF THE PROJECT

Urine specimens collected in the study will be used to address two primary aims: 1) to examine the underlying environmental contributors to intestinal health in children under the age of two, and 2) to examine the association of intestinal health and nutritional outcomes, including stunting and wasting, in children under the age of two.

PROCEDURES

The test involves a non-invasive lactulose to mannitol (L:M) test on urine specimens. For the urine specimen collection, mothers will be requested to fast their children for one hour before the test begins. An oral solution consisting of two sugars will be given to the children using a disposable dropper. After drinking the solution, fieldworkers will collect urine in pediatric urine collection bags which stick over the groin over the course of up to 5 hours. The children will not be able to eat or drink for two hours after drinking the solution. At that time they can drink some water, but cannot breast feed or take a meal until after the third hour. After the test, the caretakers and children will be given a drink. During the time of the LM test, caretakers will be given a questionnaire regarding livestock. Questions will be regarding ownership, where the animals reside in the daytime and nighttime, and whether or not the animals interact with the child.

RISKS and/or DISCOMFORTS

Overall, the risks involved in the L:M test are minimal, although diarrhea has been reported. Children who experience diarrhea during the test may receive support from the referral system to nearby health facilities that is already in place for the birth cohort study.

In addition, there is the risk that the child will cough up the solution while drinking it. However, this risk is no greater than if the child were drinking any other type of liquid.

POTENTIAL BENEFITS

There are no direct benefits to you or your household by participating in this study.

NEW FINDINGS

You will be told of any new information learned during the study that might cause you to change your mind about staying in the study and participating in interviews. If the study is cancelled or stopped for any reason by the sponsors, the funding agency, or the ethical committee, you will also automatically cease to be a member of the study without your consent.

COSTS TO YOU

Other than your time, there is no direct cost to you for participating in this study.

PAYMENT

You will be given a small gift for your time, which includes a bar of soap and one kilogram of sugar. Refreshments will be served after the study procedure has been conducted.

CONFIDENTIALITY

We will take strict precautions to safeguard you and your child's personal information throughout the study. Identifying data will be coded and only the researchers will have access to the code. All information that is entered in a computer or electronic device will be protected with a password. Your child will not be personally identified in any reports made public about this study.

STORAGE OF SAMPLES

Urine samples will be temporarily stored before being transferred to the laboratory for testing. After this, samples will be disposed of appropriately.

PERSONS TO CONTACT FOR PROBLEMS OR QUESTIONS

If you have questions about this study, you should contact Prof. Bernard Bashaasha, Principal Investigator, Makerere University, Kampala (Tel: 0772627249, 414542277, or 414543880). If you wish to speak to someone not directly involved with this research study about your rights as a research subject, you should contact Dr. Suzanne Kiwanuka, Chair of the Higher Degrees, Research and Ethics Committee at Makerere University School of Public Health. She can be reached at P.O. Box 7062, Kampala, or by calling 0718060387 or 0312291397 for any of the following reasons: your questions, concerns, or complaints are not being answered by the research team; you cannot reach the research team; you want to talk to someone besides the research team; you have questions about your rights as a research participant; you want to get information or provide input about this research. This study has been reviewed and approved by the Tufts Medical Center/Tufts University Health Sciences IRB. Tufts IRB can be reached by calling 617-636-7512.

YOUR PRIVACY RIGHTS

- You have the right not to sign this form permitting us to use and share your child's information for research. If you do not sign this form, your child cannot take part in this research study. This is because we need the information of everyone who takes part.
- You have the right to withdraw your permission for us to use your child's information for this research study. If you would like to withdraw your permission, you must notify a member of the study staff.
- If you withdraw your permission, we will not be able to take back any information that has already been used. This includes information used to carry out the research study or to be sure the research is safe and of high quality.
- If you withdraw your permission, you cannot continue to take part in this research study.

SIGNATURES

If you have read the informed consent, or had it read and explained to you, and you understand the information and voluntarily agree to join this study, please sign your name or make your mark below.

PARTICIPANT'S STATEMENT

I have read the information in this consent form including risks and possible benefits, or it has been read to me. All my questions about the research study have been answered to my satisfaction

I consent to participate in the study. I authorize the use and disclosure of my child's information to the parties authorized and my child to be involved in this research.

SIGNATURE

I, _____, have read the contents in this form. My questions have been answered. I agree for my child to participate in this study.

_____ _____

Signature of Index Mother/Index child's care giver Date

_____ _____

Signature of Study Representative Date

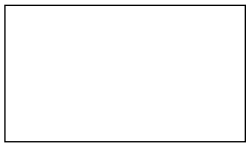
If participant is illiterate:

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

*Print name of witness*_____ *AND Thumbprint of participant*

Signature of witness _____

Date _____



Appendix 2: Protocol for preparing the thimerosal/L:M solution and conducting urine collection

Preparing the Thimerosal Solution

Items needed:

- 1 50 mL graduated cylinder
- 1 scale that measures to 100th decimal digit
- Water
- Thimerosal powder
- Weigh boats
- Spoon
- 5mL falcon tubes for storing thimerosal solution

Procedure (makes enough for about 750 samples):

1. Weigh 7.5g of thimerosal
2. Measure 15mL of water in graduated cylinder
3. Add thimerosal to water and swirl until powder dissolves (takes a while)
4. Aliquot solution into 5mL falcon tubes

Making the lactulose: mannitol solution

Items needed:

- 1 1000 mL graduated cylinders
- Distilled water
- 1 scale that measures to 100th decimal digit
- D-Mannitol powder
- Lactulose (10g/15mL solution)
- 1 spoon
- Weigh boats

Procedure (this is for 50 children, so repeat as needed):

1. Measure 375 mL of lactulose (equal to 250 grams of lactulose)
2. Weigh out 50 grams of mannitol with weigh boat and scale.
3. Pour 50 grams of mannitol into the graduated cylinder with the lactulose.
4. Pour water up to the 1000 mL line. Gently tilt cylinder to mix mannitol, lactulose, and water and ensure correct volume.
5. Dissolve lactulose and mannitol using stir bar and stir plate
6. Transfer 20 ml to glass viles (28 ml.; autoclavable)
7. Autoclave, screw on tops, and refrigerate

Urine Collection

Items needed:

- Urine Bags (about 5/child)
- Cotton Wipes
- Urine Collection Containers with Lids
- Medical Tape

Procedure:

1. Wipe the child with cotton.
2. The perineum should be clean and dry before applying urine bag; make sure the sticky part is attached to the perineum to ensure no urine loss.
3. At 2-3 hours out from the time the first child got a urine bag, start giving water. If children are thirsty before this mark, it's okay to have them drink.
4. Collect all urine in urine collection containers and add thimerosal as a preservative.
5. After a child is finished, use the disposable pipet to aliquot two 2 ml volumes of urine into each cryovials.
6. Use cryovials markers and labels to write child's ID Number and either 1 or 2
7. Put cryovials on ice and transport to medical facility.
8. Store at -80; transport to Kampala for shipment to US

Appendix 3: Checklist for enumerators conducting the L:M test in the field

Date	Household ID Number	Time Start	Time Finish	Urination Episodes (#)	Urine Volume (ml.)	Enumerator Signature

① The child **has NOT had diarrhea** (3 loose stools/day) in the last 2 weeks (confirm with caretaker)

② The **consent form** is explained to the caretaker and signed (one copy left with family, one placed in binder**)**

③ The child **fasts for one hour** while the solution warms and the **livestock survey** is given

④ The child **drinks ALL 20 ml. of solution** from cup or disposable dropper

⑤ **One drop of thimerosal** (antiseptic) is placed in the urine collection container

⑥ ALL urine is collected using a **new urine bag each time** and **ALL the urine** is put in the urine collection container

⑦ The child urinates **once AFTER the 4-hour mark** and this is the last sample (test is over)

⑧ **Two cryovials are filled with 1.5 ml of urine each**, lids are screwed on tightly, and **labeled with the correct household IDs** (both A and B)

⑨ The **two samples are put in a cool box**, transferred to the health center, and **stored in the cryovial box in freezer**

⑩ Used equipment is placed in **biohazard bag** and properly discarded by health facility

Appendix 4: Informed consent form for the study "Examining the presence of Environmental Enteric Dysfunction (EED) in pregnant women and its association with birth outcomes in Mukono, Uganda"

"Examining the presence of Environmental Enteric Dysfunction (EED) in pregnant women and its association with birth outcomes in Mukono, Uganda."
Principal Investigator: Shibani Ghosh PhD (PI, Tufts University)
Description of Subject Population: Pregnant women 18-45 utilizing Mukono Health Center IV for prenatal care and delivery.
Version 4.0**,** February 2017

Introduction

You are being asked to volunteer for a research study because you are pregnant woman using Mukono Health center IV for prenatal care and delivery. The Principal Investigator in charge of this study is Shibani Ghosh, PhD from Tufts University in Boston, MA, USA. Before you decide if you want to participate, we want to explain the purpose of the research, what you will be asked to do, the possible risks and benefits of participation, how we will protect your information, and who to contact if you have any questions or concerns about the project.

Taking part in this research study is entirely your choice. You can decide to refuse to participate in this study. If you decide to participate in this study, you can then choose to stop taking part in the study at any time for any reason. If you refuse to participate in the study or stop being in this study, it will not affect your care or treatment outside this study, payment for your health care, or your health care benefits.

Please read all the following information carefully. Ask the study representative to explain any words, terms, or sections that are unclear to you. Ask any questions that you have about this study. Do not sign this consent form unless you understand the information in it and have had your questions answered to your satisfaction.

If you decide to take part in this research study, you will be asked to sign this form, or make a mark in front of someone. You will be given a copy of the signed form. You should keep your copy for your records. It has information, including important names and telephone numbers, to which you may wish to refer in the future.

If you are eligible to participate and decide to be in the study, the Principal Investigator may still choose to stop your participation in this study if she thinks it is in your best medical interest. You may also be withdrawn from the study if you move away from the study site. If you withdraw or are withdrawn from the study, any data collected from you before your withdrawal will still be used for the study.

As a participant in this study, your identity, medical records, and data relating to this study will be kept confidential, except as required by law.

Purpose of the study
The purpose of the study is to look at the association between poor intestinal health in pregnant women and birth outcomes, such as low birth weight and low birth length. The study is a collaboration between Tufts University (USA) and Uganda Christian University (Uganda). Funding for the study is provided by the United States Agency for International Development (USAID).

Length of your participation
Your participation in the study will last approximately 6 months and will involve four different visits (an initial prenatal visit, a home visit, a final prenatal visit, and delivery) over the course of your pregnancy. The study is expected to take up to approximately nine hours of your time in total.

Participants in the research study
This study is being conducted in Mukono at Mukono Health Center IV. In total, we aim to recruit 258 women for participation in the study. You are eligible to participate in this study if you are 18-45 years old, will continue utilizing Mukono Health Center IV for prenatal care, are not severely anemic, are HIV negative, are carrying a single child, and provide proper consent.

Study Procedure
Initial Prenatal Visit (2 hours):
This session will take place today if you choose to take part in this study.
1) A needle prick on your finger to test for anemia (lack of healthy red blood)
2) A blood draw of 10 ml., or about two teaspoons
3) An ultrasound. An ultrasound is a safe and painless test that uses sound waves to show an image of your fetus. It can be used to estimate gestational age.
4) We will collect information on your height, weight, mid-upper arm circumference (MUAC), and blood pressure.

Household Visit (4 hours and 45 minutes):
This visit will be followed up by a visit by an enumerator who will administer a questionnaire in addition to a lactulose: mannitol (sugars) test. This visit will take place in your home within one to five days of the initial prenatal visit.

1) Questionnaire: The questionnaire will include questions regarding demographic data, socio-economic status, antenatal care, health status before and during pregnancy, former pregnancies, diet quality/quantity, food insecurity, and other factors that could potentially be associated with negative birth outcomes.

2) Lactulose: mannitol test: this is a test which is a test of gut health. It is a non-invasive test on urine. For the urine collection, you will be requested to fast (not eat or drink anything except water) for one hour before the test begins. An oral solution consisting of the two sugars (5 grams of lactulose and 2 grams of mannitol) will be given. After drinking the solution, all your urine over the course of four hours will be collected using urine collection containers. You will not be able to eat or drink for two hours after drinking the solution, except for water. You cannot take a meal until after the third hour. You can do normal activities, such as cooking and housework, during the lactulose: mannitol test.

Final Prenatal Visit (2 hours):
1) Questionnaire: The questions will be the same as the first questionnaire.
2) A blood draw of 10 ml. or about two teaspoons.
3) We will collect information on your height, weight, mid-upper arm circumference (MUAC), and blood pressure.

Postnatal Visit/Delivery (15 minutes):
Upon delivery, your child's height, weight, and head circumference will be measured and recorded at the hospital on a tablet computer by the nurse. If the birth occurs outside of the hospital, you will call the study coordinator or a member of the research team and these measurements will be taken within 72 hours of delivery. If a final prenatal visit is not possible, for example in the case of premature delivery, the second questionnaire, blood draw, and anthropometric measurements will take place as soon as possible after delivery.

Possible risks or side effects of taking part in this study
Overall, the risks involved with the study are very minimal. You may feel some discomfort or get redness, pain, bruising or infection when we draw blood from you. We would like to reassure you that our study team members are well trained and will collect your blood with great care, and will try to make you feel as comfortable as possible. Recommended universal infection control precautions will be followed to minimize risk of infections.

The ultrasound is an extremely safe procedure for you and your fetus. You may, however, experience some discomfort or pressure from the ultrasound machine on your abdomen.

The risks involved in the lactulose: mannitol test are minimal, although mild cases of diarrhea have been reported in rare circumstances. In addition, you may also experience feelings of hunger, lightheadedness, and/or low energy as a result of the fasting period. With regard to the questionnaire, you may feel uncomfortable answering some of the interview questions that ask about sensitive topics such as your household's food security or income. Our study interviewers and staff are specifically trained to administer these

interviews and recognize that some individuals may feel uncomfortable discussing certain topics. You can refuse to answer any question at any time.

In the event that you experience physical harm or injury as a result of participation in the study, you will receive payment for your treatment.

Possible benefits to you for taking part in the study
We will inform you of the results of our blood, ultrasound, and other assessments described above. If we find that you or your newborn are in need of medical attention (for example, if you are severely underweight), we will advise you and refer you to the appropriate health services.

Otherwise, there are no direct benefits to you for participating in this study. However, your participation in this study may enable the researchers to better understand the causes of negative birth outcomes in Uganda.

Alternatives
The alternative to participating in the research study is not to participate. If you choose not to participate, you will still receive the same standard of care for your pregnancy and delivery.

Costs to you
Other than your time, there is no direct cost to you for participating in this study.

Payment
You will be given 10,000 Ugandan Shillings at each of the four visits as compensation for your time. The four visits where you will receive the 10,000 UGS compensation are at the initial prenatal visit, at the home visit, at the last pre-natal visit, and after delivery. This means that, in total, you will receive 40,000 UGS for complete participation in the study.

Confidentiality of study records and medical records
Information collected for this study is confidential. We will take strict precautions to safeguard you and your personal information throughout the study. Identifying data will be coded and only the researchers will have access to the code. All information that is entered in a computer or electronic device will be protected with a password. All information collected on paper will be stored in locked file cabinets. You will not be personally identified in any reports made public about this study.

The Uganda National Council for Science and Technology (UNCST) will receive copies of the study records. The UNCST and the Mengo Hospital Research Ethics Committee (REC) may see parts of your medical records related to this study.

Storage of samples
All samples collected will be temporarily stored before being transferred to the laboratory for testing. After this, samples will be disposed of appropriately.

Your privacy rights

- You have the right not to sign this form permitting us to use and share your information for research. If you do not sign this form, you cannot take part in this research study. This is because we need the information of everyone who takes part.
- You have the right to withdraw your permission for us to use your information for this research study. If you would like to withdraw your permission, you must notify a member of the study staff.
- If you withdraw your permission, we will not be able to take back any information that has already been used.

New findings
You will be told of any new information learned during the study that might cause you to change your mind about staying in the study and participating in interviews. If the study is cancelled or stopped for any reason by the sponsors, the funding agency, or the ethical committee, you will also automatically cease to be a member of the study without your consent.

Names of contact for questions about the study
This research study has been reviewed and approved by the IRB of Tufts University Health Sciences and the IRB of Mengo Hospital in Uganda. If you have question about your rights as a research study subject, call the Tufts Medical Center and Tufts University Health Sciences Institutional Review Board (IRB) at +1-617-636-7512 or the Mengo Hospital Research Ethics Committee (REC) at 0772 084737. The IRB/REC is a group of doctors, nurses, and non-medical people who review human research studies for safety and protection of people who take part in the studies. Federal law requires the IRB/REC to review and approve any research study involving humans. This must be done before the study can begin. The study is also reviewed on a regular basis while it is in progress. If you have any questions about taking part in this study, or if you think you may have been injured because of the study, call Dr. Shibani Ghosh at +1-617-259-7752 or Jacqueline Lauer, the study coordinator, at 0779 584778. If you have any questions about your rights as a research subject, you can call the Chairman Institutional Review Board at 0772 505189.

Participant consent page
I certify that I have read or have had read to me the above document describing the benefits, risks and procedures for the study titled ““Examining the presence of Environmental Enteric Dysfunction (EED) in pregnant women and its association with birth outcomes in Mukono, Uganda.”, or that it has been read and explained to me, and

that I understand it. I have been given an opportunity to have any questions about the study answered to my satisfaction. I agree to participate voluntarily.

_____	_____
Date	Signature or mark of participant
	_____
	Name of participant (print)

Appendix 5: Survey tool for enrollment interview

Section 1: Interview Information

1.1	Date of Interview		HDATEINT
1.2	District		HDIST
1.3	Sub-County		HSCOUNT
1.4	Parish		HPARISH
1.5	Village		HVILA
1.6	Participant's ID		HHID
1.7	Participant's Initials		HPINIT
1.8	Interviewer Name		HINTNAME

Section 2: Participant Information

2.1	How old were you at your last birthday?	_____completed years	HAGE
2.2	What is your marital status?	1=married, monogamous; 2=married, polygamous; 3=cohabiting; 4=single; 5=widowed; 6=divorced; 7=separated	HMARTS
2.3	What is your primary occupation?	1=housewife 2=farmer 3=merchant 4=daily laborer 5=other _____	MOCCUPAT
2.4	Are you also the household head?	1=yes 0=no	HHEAD
2.5	How many people live in the household (normally live there and share meals)?	_____people	HMEMBERS
2.6	Is this household located in an urban or rural environment? (Enumerator can answer)	1=urban 2=rural 3=peri-urban	MURBAN
2.7	How many years of education have you completed?	_____completed years	HEDUC

2.8	What is the highest level of school you attended?	1=none 2=primary 3=secondary 4=higher	HLEVEL
2.9	Are you literate?	1=yes 0=no	HLITERATE

Section 3: Current Pregnancy

3.1	During this pregnancy, how many antenatal visits have you attended thus far?	_____visits	MANTVISNO
3.2	During this pregnancy, have you taken iron tablets or syrup?	1=yes 0=no	MANCIRON
3.3	If yes, for how many days have you taken the iron tablets/syrup so far?	_____days	MIRONDAY
3.4	During this pregnancy, have you taken any malaria prophylaxis?	1=yes 0=no	MPROPH
3.5	During this pregnancy, have you taken any drug for intestinal worms?	1=yes 0=no	MWORMS

Section 4: Former Pregnancies

4.1	How many pregnancies have you had, including this one?	_____pregnancies	MNOPREG
4.2	What was your age the first time you became pregnant?	_____years old	MFPREGAGE
4.3	How many live born children have you ever had?	_____live born children	MALIVEB
4.4	Have you ever lost a fetus during pregnancy (spontaneous abortion)?	1=yes 0=no	MFETUSDIE
4.5	If yes, how many?	_____fetuses	MNOFEDIE
4.6	Have you ever lost a child at birth (stillbirth)?	1=yes 0=no	MCHILDDIE
4.7	If yes, how many?	_____stillbirths	MNOCHDIE
4.8	How old is your youngest child that you gave birth to? (Answer in months if under two years old. If the child	_____years or _____months	MAGEYOUN

	has passed away, put how old he/she would be)		
4.9	Was this child born low birth weight?	1=yes 0=no	MLBW
4.10	Are you currently breastfeeding another child?	1=yes 0=no	MCRBREAS

Section 5: Health Risks and Health Status

5.1	During this pregnancy, have you contracted malaria (diagnosed or presumed)?	1=yes 0=no	LMALARIA
5.2	If yes, how many times?	_____times	LMALTIMES
5.3	During your pregnancy, have you smoked cigarettes/tobacco?	1=yes 0=no	LSMOKE
5.4	If yes, how many times do you smoke in a week, on average, while pregnant?	_____times	LSMOKENO
5.5	Are you exposed to second hand smoking in your household?	1=yes 0=no	LSMOKESEC
5.6	If yes, how many times are you exposed to second hand smoke in a week, on average, while pregnant?	_____times	LSMKSECNO
5.7	During your pregnancy, have you drank alcohol?	1=yes 0=no	LDRINK
5.8	If yes, how many alcoholic drinks do you take in a week, on average, while pregnant?	_____drinks	LDRINKNO
	Have you ever been diagnosed with the following conditions?		
5.9	Tuberculosis?	1=yes 0=no	LTBER
5.10	If yes, do you still suffer from tuberculosis today?	1=yes 0=no	LTBERNOW
5.11	Diabetes?	1=yes 0=no	LDIAB
5.12	If yes, do you still suffer from diabetes today?	1=yes 0=no	LDIABNOW
5.13	Heart disease?	1=yes 0=no	LHEART

5.14	If yes, do you still suffer from heart disease today?	1=yes 0=no	LHDNOW
5.15	Renal disease (kidney disease)	1=yes 0=no	LRENAL
5.16	If yes, do you still suffer from renal disease today?	1=yes 0=no	LRENALNOW
5.17	Sexually transmitted infection (chlamydia, gonorrhea, syphilis, herpes, etc.)?	1=yes 0=no	LSTI
5.18	If yes, do you still suffer from a sexually transmitted infection today?	1=yes 0=no	LSTINOW
5.19	Any other chronic medical conditions you currently suffer from?	1=yes _____ 0=no	LOTHER

Section 6: Diet Information-MDD-W

I am going to ask you to describe everything that you ate or drank yesterday during the day or night, whether you ate it at home or anywhere else. Please include all foods and drinks, any snacks or small meals, as well as any main meals. Remember to include all foods you may have eaten while preparing meals or preparing food for others. Please also include food you ate even if it was eaten elsewhere, away from your home.

	Food Group	Consumed	
6.1	Grains/cereals (millet, maize, sorghum, rice, wheat, pasta, bread)	1=yes 0=no	DGRAINS
6.2	White roots, tubers, plantains (white potato, white yams, cassava, beet root, plantains)	1=yes 0=no	DWROOTS
6.3	Vitamin A rich roots and tubers (pumpkin, squash, carrot, sweet potato, sweet red pepper)	1=yes 0=no	DVAROOTS
6.4	Dark green leafy vegetables (spinach, greens)	1=yes 0=no	DDGLV
6.5	Other vegetables (tomato, onion, eggplant, okra, green pepper)	1=yes 0=no	DOTHERVEG
6.6	Vitamin-A rich fruits (papaya, mango, passion fruit)	1=yes 0=no	DVAFRUITS
6.7	Other fruits (oranges, limes, lemons, pineapple, jack	1=yes 0=no	DOTHERFR

	fruit, watermelon, guava, avocado)		
6.8	Flesh foods and organ meat (beef, goat, mutton, pork, chicken, rabbit, rat, liver, other organs)	1=yes 0=no	DMEAT
6.9	Eggs	1=yes 0=no	DEGGS
6.10	Fish and sea foods (dry fish, fresh fish)	1=yes 0=no	DFISH
6.11	Pulses (beans, peas, lentils)	1=yes 0=no	DPULSES
6.12	Nuts and seeds (groundnuts, sunflower seeds, simsim)	1=yes 0=no	DNUTS
6.13	Milk and milk products (milk, yoghurt, cheese)	1=yes 0=no	DDAIRY
6.14	Oils and fats (vegetable cooking oils, solid fats)	1=yes 0=no	DFATS
6.15	Sweets (sugar, honey, candies, cakes)	1=yes 0=no	DSWEETS
6.16	Spices and condiments (chili, salt, pepper, chicken/beef cubes, herbs, ketchup)	1=yes 0=no	DSPICES
6.17	Sugar-sweetened beverages (tea with sugar, coffee with sugar, soda, fruit drinks)	1=yes 0=no	DBEVERAGE

Section 7: Household Food Insecurity Access Scale

I am going to ask you questions about your household's food supply over the past four weeks. Food supply includes staples, sauces, and any other foods in your diet and the diets of all members of your household.

7.1	In the past four weeks, did you worry that your household would not have enough food?	1=yes 0=no	SWORRY
7.2	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)	SWORRFQ
7.3	In the past four weeks, were you or any household member not able to eat the	1=yes 0=no	SKIND

	kinds of foods you preferred because of a lack of resources?		
7.4	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)	SKINDFQ
7.5	In the past four weeks, did you or any household member have to eat a limited variety of foods due to a lack of resources?	1=yes 0=no	SLIMIT
7.6	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)	SLIMITFQ
7.7	In the past four weeks, did you or any household member have to eat some foods that you really did not want to eat because of a lack of resources to obtain other types of food?	1=yes 0=no	SDISLIKE
7.8	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)	SDISLIKEFQ
7.9	In the past four weeks, did you or any household member have to eat a smaller meal than you felt you needed because there was not enough food?	1=yes 0=no	SSMALL
7.10	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)	SSMALLFQ

7.11	In the past four weeks, did you or any household member have to eat fewer meals in a day because there was not enough food?	1=yes 0=no	SFEW
7.12	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)	SFEWFQ
7.13	In the past four weeks, was there ever no food to eat of any kind in your household because of lack of resources to get food?	1=yes 0=no	SNFOOD
7.14	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)	SNFOODFQ
7.15	In the past four weeks, did you or any household member go to sleep at night hungry because there was not enough food?	1=yes 0=no	SSLEEP
7.16	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)	SSLEEPFQ
7.17	In the past four weeks, did you or any household member go a whole day and night without eating anything because there was not enough food?	1=yes 0=no	SNODAY
7.18	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)	SNODAYFQ

Section 8: Wealth Index

	Does your household own/have the following assets?		
8.1	Radio	1=yes 0=no	ARADIO
8.2	Television	1=yes 0=no	ATELEV
8.3	Telephone	1=yes 0=no	ATELEPH
8.4	Refrigerator	1=yes 0=no	AREFRIG
8.5	Bicycle	1=yes 0=no	ABIKE
8.6	Boda/motorcycle	1=yes 0=no	ABODA
8.7	Automobile	1=yes 0=no	ACAR
8.8	Running water	1=yes 0=no	ARWATER
8.9	Stone/cement/tile floor	1=yes 0=no	AHARDFL
8.10	Grid electricity	1=yes 0=no	AGRELEC
8.11	Domestic helper (unrelated to household head)	1=yes 0=no	ADHELP

Section 9: Water and Sanitation

9.1	What type of toilet do you primarily use?	1=None/bush/garden 2=Unimproved pit latrine 3=Improved pit latrine 4=Flush toilet 5=Community owned latrine	WTOILET
9.2	What is your household's most commonly used source of water?	1=Piped water 2=Public tap 3=Tube well or borehole 4=Protected well or spring 5=Unprotected well or spring 6=Truck/cart/bottled 7=Rainwater 8=Surface water	WSOURCE
9.3	What is the distance from your household to this source of water (one-way)?	_____ km (if the source of water is in the compound, then put 0)	WDISTANC

9.4	How much time does it take to bring water (one roundtrip, including waiting time, by usual means)?	_____ minutes	WTIME
9.5	What, if anything, do you usually do to your household water before drinking it?	1=Nothing 2=Boiling 3=Use chemicals 4=Filter 5=Other	WTREAT
	Under what circumstances do you wash your hands?		
9.6	Not at all	1=yes 0=no	WNIL
9.7	When they have visible dirt	1=yes 0=no	WDIRT
9.8	After bathroom use	1=yes 0=no	WBATH
9.9	After cleaning child following defecation	1=yes 0=no	WCLCHILD
9.10	Before preparing food	1=yes 0=no	WFOOD
9.11	Before serving a meal	1=yes 0=no	WSERVE
9.12	Before eating	1=yes 0=no	WEAT
9.13	Before feeding a child	1=yes 0=no	WFEEDCH

Appendix 6: Survey tool for third visit, conducted three weeks prior to participants' estimated date of delivery

Section 1: Interview Information

1.1	Date of Interview	
1.2	Participant's ID Number	
1.3	Participant's Name	
1.4	Interview Time Point	
1.5	Interviewer Name	

Section 2: Current Pregnancy

2.1	How many times did you seek antenatal care during this pregnancy?	_____ times
2.2	During this pregnancy, were you given or did you buy any iron tablets/syrup ("blood medicine")?	☐ 1=Yes ☐ 0=No
2.3	If yes, during the whole pregnancy, for how many days did you take the iron tablets/syrup?	_____ days
2.4	During this pregnancy, were you given or did you buy any multivitamins?	☐ 1=Yes ☐ 0=No
2.5	If yes, during the whole pregnancy, for how many days did you take the multivitamins?	_____ days
2.6	During this pregnancy, did you receive prophylaxis for malaria (IPT)?	☐ 1=Yes ☐ 0=No
2.7	If yes, how many times did you take the malaria (IPT) tablets?	_____ times
2.8	During this pregnancy, did you take any drug for intestinal worms?	☐ 1=Yes ☐ 0=No
2.9	If yes, how many times did you take drugs for intestinal worms?	_____ times

Section 3: Health Risks and Health Status

3.1	During this pregnancy, have you <u>smoked</u> cigarettes/tobacco?	☐ 1=Yes ☐ 0=No
3.2	If yes, how many times did you <u>smoke</u> cigarettes/tobacco during this pregnancy?	_____times per week /or/ _____ times per month

3.3	During this pregnancy, were you exposed to second hand smoking in your household or workplace?	☐ 1=Yes ☐ 0=No
3.4	If yes, how many times were you exposed to second hand smoking (tobacco)?	_____times per week /or/ _____ times per month
3.5	During this pregnancy, have you chewed/sniffed tobacco?	☐ 1=Yes ☐ 0=No
3.6	If yes, how many times did you chew/sniff tobacco during this pregnancy?	_____times per week /or/ _____ times per month
3.7	During this pregnancy, have you drank alcohol (excluding small sips for holy communion and/or nausea)?	☐ 1=Yes ☐ 0=No
3.8	If yes, how many times did you drink alcohol?	_____times per week /or/ _____ times per month

Section 4: Diet Information-MDD-W

	Did you eat the following foods in the last 24 hours?	**Participant consumed?**
	Cereals	
4.1	Millet (meal, porridge)	☐ 1=Yes ☐ 0=No
4.2	Maize (meal, porridge)	☐ 1=Yes ☐ 0=No
4.3	Sorghum (meal, porridge)	☐ 1=Yes ☐ 0=No
4.4	Rice	☐ 1=Yes ☐ 0=No
4.5	Wheat (bread, chapatti, buns, cakes, mandazi)	☐ 1=Yes ☐ 0=No
4.6	Other cereals	☐ 1=Yes ☐ 0=No
	Roots/ Tubers/Plantain	
4.7	Cassava	☐ 1=Yes ☐ 0=No
4.8	Sweet Potatoes - orange	☐ 1=Yes ☐ 0=No
4.9	Sweet Potatoes – other colors	☐ 1=Yes ☐ 0=No
4.10	Irish Potatoes	☐ 1=Yes ☐ 0=No
4.11	Yams	☐ 1=Yes ☐ 0=No
4.12	Matooke, Plantain	☐ 1=Yes ☐ 0=No
4.13	Other roots, tubers, or plantains	☐ 1=Yes ☐ 0=No
	Legumes	
4.14	Beans	☐ 1=Yes ☐ 0=No

	Did you eat the following foods in the last 24 hours?	**Participant consumed?**
4.15	Peas (field, cow, pigeon, lapane)	☐ 1=Yes ☐ 0=No
4.16	Ground nuts or Bambara nuts	☐ 1=Yes ☐ 0=No
4.17	Soybeans	☐ 1=Yes ☐ 0=No
4.18	Other legumes	☐ 1=Yes ☐ 0=No
	Oil Seeds	
4.19	Sunflower	☐ 1=Yes ☐ 0=No
4.20	Simsim (sesame)	☐ 1=Yes ☐ 0=No
4.21	Other oil seeds	☐ 1=Yes ☐ 0=No
	Vegetables	
4.22	Dark green leafy vegs (e.g., dodo, bugga, nakati)	☐ 1=Yes ☐ 0=No
4.23	White/light green leafy vegs (e.g., cabbage)	☐ 1=Yes ☐ 0=No
4.24	Vitamin A vegs (e.g., carrots, pumpkin)	☐ 1=Yes ☐ 0=No
4.25	Tomato	☐ 1=Yes ☐ 0=No
4.26	Okra	☐ 1=Yes ☐ 0=No
4.27	Other vegetables	☐ 1=Yes ☐ 0=No
	Fruits (including juices)	
4.28	Citrus (e.g., oranges, limes)	☐ 1=Yes ☐ 0=No
4.29	Vitamin A fruits (papaya, mangoes)	☐ 1=Yes ☐ 0=No
4.30	Passion fruit, pineapples, jack fruit	☐ 1=Yes ☐ 0=No
4.31	Avocado	☐ 1=Yes ☐ 0=No
4.32	Other fruits or fruit juices	☐ 1=Yes ☐ 0=No
	Meats	
4.33	Beef, goat, mutton	☐ 1=Yes ☐ 0=No
4.34	Pork	☐ 1=Yes ☐ 0=No
4.35	Liver	☐ 1=Yes ☐ 0=No
4.36	Other organs, blood, &offals	☐ 1=Yes ☐ 0=No
4.37	Poultry	☐ 1=Yes ☐ 0=No
4.38	Eggs	☐ 1=Yes ☐ 0=No
4.39	Dried fish	☐ 1=Yes ☐ 0=No
4.40	Fish (all other types)	☐ 1=Yes ☐ 0=No
4.41	Other meats and non-dairy animal products	☐ 1=Yes ☐ 0=No

	Did you eat the following foods in the last 24 hours?	**Participant consumed?**
	Dairy Products	
4.42	Milk	☐ 1=Yes ☐ 0=No
4.43	Yoghurt, Bongo	☐ 1=Yes ☐ 0=No
4.44	Cheese	☐ 1=Yes ☐ 0=No
4.45	Other dairy	☐ 1=Yes ☐ 0=No
	Fats/Oils	
4.46	Veg cooking oils (e.g. Mukwano, Sunflower)	☐ 1=Yes ☐ 0=No
4.47	Vegetable solid fats (e.g.Kimbo, Blue Band)	☐ 1=Yes ☐ 0=No
4.48	Animal fats (ghee, butter)	☐ 1=Yes ☐ 0=No
	Others	
4.49	Sugar/Sugar cane/honey	☐ 1=Yes ☐ 0=No
4.50	Coffee, tea	☐ 1=Yes ☐ 0=No
4.51	Condiments	☐ 1=Yes ☐ 0=No
4.52	Soda, Safi, packed sugary drinks	☐ 1=Yes ☐ 0=No
5.53	Alcohol or beer (any type)	☐ 1=Yes ☐ 0=No

Section 5: Household Food Insecurity Access Scale

5.1	In the past four weeks, did you worry that your household would not have enough food?	☐ 1=Yes ☐ 0=No
5.2	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)
5.3	In the past four weeks, were you or any household member not able to eat the kinds of foods you preferred because of a lack of resources?	☐ 1=Yes ☐ 0=No
5.4	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks)

		3=Often (more than 10 times in the past four weeks)
5.5	In the past four weeks, did you or any household member have to eat a limited variety of foods due to a lack of resources?	☐ 1=Yes ☐ 0=No
5.6	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)
5.7	In the past four weeks, did you or any household member have to eat some foods that you really did not want to eat because of a lack of resources to obtain other types of food?	☐ 1=Yes ☐ 0=No
5.8	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)
5.9	In the past four weeks, did you or any household member have to eat a smaller meal than you felt you needed because there was not enough food?	☐ 1=Yes ☐ 0=No
5.10	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)
5.11	In the past four weeks, did you or any household member have to eat fewer meals in a day because there was not enough food?	☐ 1=Yes ☐ 0=No
5.12	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)

5.13	In the past four weeks, was there ever no food to eat of any kind in your household because of lack of resources to get food?	☐ 1=Yes ☐ 0=No
5.14	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)
5.15	In the past four weeks, did you or any household member go to sleep at night hungry because there was not enough food?	☐ 1=Yes ☐ 0=No
5.16	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)
5.17	In the past four weeks, did you or any household member go a whole day and night without eating anything because there was not enough food?	☐ 1=Yes ☐ 0=No
5.18	If yes, how often did this happen?	1=Rarely (once or twice in the past four weeks) 2=Sometimes (3 to 10 times in the past four weeks) 3=Often (more than 10 times in the past four weeks)

Section 6: Wealth Index

	Does your household own/have the following assets?	
6.1	Radio	☐ 1=Yes ☐ 0=No
6.2	Television	☐ 1=Yes ☐ 0=No
6.3	Telephone	☐ 1=Yes ☐ 0=No
6.4	Computer/Laptop	☐ 1=Yes ☐ 0=No
6.5	Refrigerator	☐ 1=Yes ☐ 0=No

6.6	Bicycle	☐ 1=Yes ☐ 0=No
6.7	Boda/motorcycle	☐ 1=Yes ☐ 0=No
6.8	Automobile	☐ 1=Yes ☐ 0=No
6.9	Land	☐ 1=Yes ☐ 0=No
6.10	Domestic helper (unrelated to household head)	☐ 1=Yes ☐ 0=No

Section 7: Water and Sanitation

7.1	What is your most commonly used source of water?	1=piped water 2=public tap 3=borehole 4=protected well or spring 5=unprotected well or spring 6=rainwater 7=surface water 8=bottled water 9=other (specify_____)
7.2	What type of toilet do you use most of the time?	1. none/bush/garden 2. unimproved pit latrine 3. improved pit latrine (VIP) 4. flush toilet 5. other (specify _____)
7.3	Is your toilet community owned or shared by multiple families?	☐ 1=Yes ☐ 0=No
7.4	Does your household have running water (piped)?	☐ 1=Yes ☐ 0=No
7.5	Do you do anything to your water before drinking it?	1=do nothing 2= boil 3=use traditional herbs 4=use chemicals 5=filter/sieve 6=other (specify_____)
7.6	Where do you store your household drinking water?	1=traditional pot with cover 2=traditional pot without cover

		3=plastic jerry can with cover 4=plastic jerry can without cover 5=other (specify_____)
7.7	How much water (in 20L jerry cans) does your household usually use in one day?	_____ 20L jerry cans or equivalent
7.8	How do you most commonly store prepared food?	1. uncovered container/plate 2. covered container or plate 3. on fire or hot ash 4. other (specify_____)
7.9	When do you wash your hands?	
7.9.1	Not at all	☐ 1=Yes ☐ 0=No
7.9.2	When they have visible dirt	☐ 1=Yes ☐ 0=No
7.9.3	After toilet use/defecation/urination	☐ 1=Yes ☐ 0=No
7.9.4	After cleaning child following defecation	☐ 1=Yes ☐ 0=No
7.9.5	Before preparing the food	☐ 1=Yes ☐ 0=No
7.9.6	Before serving a meal	☐ 1=Yes ☐ 0=No
7.9.7	Before eating	☐ 1=Yes ☐ 0=No
7.9.8	Before feeding a child	☐ 1=Yes ☐ 0=No

Section 8: Basic Anthropometry

8.1	MUAC 1		☐☐.☐ cm	
8.1.1	MUAC 2		☐☐.☐ cm	
8.1.2	MUAC 3		☐☐.☐ cm	
8.2	Weight (1, 2, 3)	☐☐.☐ kg	☐☐.☐ kg	☐☐.☐ kg