Infant and young child feeding practices and associations between micronutrient adequacy, environmental enteric dysfunction and growth among 9-24 months old children in Nepal

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Summary

Background: Stunting in low- and middle-income countries has severe intergenerational consequences for health and human capital. Immediate causes of stunting are inadequate dietary intake due to poor feeding practices and disease. Environmental enteric dysfunction (EED), a subclinical infection of the intestine, influences associations between nutrient intake and linear growth, but the manner and extent of this influence is largely unknown.
Objective*:** This PhD thesis aims to assess tracking of WHO Infant and young child feeding (IYCF) practices, nutrient adequacy and associations between nutrient intake, EED and linear growth. ***Methods: Data from 231 children 9-24 months in the MAL-ED Nepal cohort was included. IYCF practices and nutrient adequacy was assessed by monthly 24-hour recalls. Anthropometry was measured monthly. Fecal markers for environmental enteric dysfunction (alpha-1-antitrypsin (AAT), myeloperoxidase (MPO) and neopterin (NEO)) were measured in stool samples collected quarterly, while lactulose:mannitol (L:M) ratio was assessed in urine collected at 15 months. Tracking of IYCF practices was assessed by Cohen's weighted kappa and Generalized estimating equations (GEE) models. Associations between fecal markers and linear growth were assessed by linear regression and GEE models, while associations between nutrient intake and fecal markers and L:M ratio were assessed by GEE models. ***Results:*** Consumption of iron- or vitamin A-rich foods was reported in 1/3 of recalls. Tracking was moderate for dietary diversity score (DDS) and meal frequency and low for intake of iron- and vitamin A-rich foods. Micronutrient adequacy was extremely low and main problem nutrients identified were iron, zinc, calcium, vitamin A and niacin. Associations between fecal markers and 3-month length velocity were overall weak with few significant findings, apart from for MPO and EE-score (based on all three fecal markers). Similarly, negative but weak associations were found between nutrient intake and fecal markers for EED and L:M ratio. Significant associations between intake of potassium, magnesium, phosphorous, folate, vitamin C and MPO and between zinc, calcium, potassium, magnesium, phosphorous and %lactulose, were found. ***Conclusion:*** The low tracking for key IYCF practices and low nutrient adequacy found implies that urgent interventions to improve complementary feeding practices are needed. The usefulness of fecal markers to assess associations between EED and growth and the effect of improved nutrient intakes on EED require further research.

Sammendrag

Bakgrunn: Veksthemming har alvorlige konsekvenser for helse og menneskelig kapital på tvers av generasjoner. Direkte årsaker til veksthemning er mangelfullt inntak av næringsstoffer og sykdom. Environmental enteric dysfunction (EED), en sub-klinisk infeksjon i tarmen, påvirker sammenhengen mellom inntak av næringsstoffer og lengdevekst, men i hvilken grad og på hvilken måte har vært lite undersøkt. ***Målsetning:*** Denne avhandlingen har som mål å undersøke stabilitet av WHO Infant and young child feeding (IYCF) - indikatorer samt sammenhenger mellom næringsstoffinntak, EED og lengdevekst. ***Metode:*** Analysene er basert på data fra 231 barn i alderen 9-24 måneder fra Bhaktapur, Nepal inkludert i MAL-ED studien. IYCF-indikatorer og tilstrekkelighet av næringsstoffer ble undersøkt ut fra månedlige 24-timers kostintervjuer. Vekt og høyde ble målt månedlig. Fekale markører for EED (alfa-1-antitrypsin (AAT), myeloperoxidase (MPO) og neopterin (NEO)) ble målt i feces prøver og samlet inn hver tredje måned, mens laktulose:mannitol (L:M) ratio ble målt i urin samlet inn ved 15 måneders alder. Stabilitet for IYCF-indikatorer ble undersøkt med Cohen's weighted kappa og Generalized estimating equations- (GEE) modeller. Sammenhenger mellom fekale markører og lengdevekst ble undersøkt med lineær regresjon og GEE-modeller og sammenhenger mellom inntak av næringsstoffer og fekale markører med GEE-modeller. ***Resultater:*** Inntak av mat rik på jern eller vitamin A ble registrert bare ved 1/3 av kostintervjuene. Stabiliteten var moderat for antall matgrupper i tilleggskosten og måltidsfrekvens og lav for inntak av mat rik på jern og vitamin A. Tilstrekkeligheten av næringsstoffer var svært lav, og i særdeleshet for jern, sink, kalsium, vitamin A og niacin. Sammenhenger mellom fekale markører og 3-måneders lengdevekst var svake med få signifikante funn, med unntak av MPO og en EE-score beregnet ut fra alle tre fekale markører. I hovedsak negative men svake sammenhenger ble også avdekket mellom inntak av næringsstoffer og fekale markører og L:M ratio. Vi fant signifikante sammenhenger mellom kalium, magnesium, fosfor, folat og vitamin C og MPO og mellom sink, kalsium, kalium, magnesium og fosfor og %laktulose. ***Konklusjon:*** Lav stabilitet for IYCF indikatorer og lav tilstrekkelighet av næringsstoffer understreker viktigheten av intervensjoner for å forbedre praksisen rundt tilleggskost. Bruken av fekale markører for å beskrive sammenhenger mellom EED og lengdevekst og effekten av økt inntak av næringsstoffer for å bedre EED bør undersøkes videre i fremtidige studier.

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Abbreviations

AAT	α-1 antitrypsin
AGP	Acid glycoprotein
ARI	Acute respiratory infection
ASF	Animal source food
BMI	Body mass index
BLUP	Best linear unbiased predictor
CRP	C-reactive protein
CV	Coefficient of variation
DDS	Dietary diversity score
DGLV	Dark green leafy vegetables
DHS	Demographic and health survey
DND	Desired nutrient density
EAR	Estimated average requirement
EE	Environmental enteropathy
EED	Environmental enteric dysfunction
EGP	Epiphyseal growth plate
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FCT	Food composition table
GEE	Generalized estimating equations
GH	Growth hormone
HAZ/LAZ	Height-for-age/length-for-age z-score
HDI	Human development index
HMO	Human milk oligosaccharide
HPLC	High-performance liquid chromatography
ICFI	Infant and child feeding index
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein

INFOODS	the FAO International Network of Food Data Systems
IOM	Institute of Medicine
IUGR	Intrauterine growth restriction
IYCF	Infant and young child feeding
Kcal	Kilocalorie
LC-MSMS	Liquid chromatography-tandem mass spectrometry
L:M	Lactulose:mannitol ratio
LMIC	Low- and middle-income country
LNS	Lipid based nutrient supplement
LPS	Lipopolysaccharide
LVZ	Length velocity z-score
MAD	Minimum adequate diet
MAL-ED	Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development
MDD	Minimum dietary diversity
MMF	Minimum meal frequency
MNDA	Mean nutrient density adequacy
MPA	Mean probability of adequacy
MPO	Myeloperoxidase
MSM	Multiple source method
MT	Microbial translocation
ND	Nutrient density
NDA	Nutrient density adequacy
NEO	Neopterin
NHRC	Nepal health research council
PA	Probability of adequacy
PCA	Principal component analysis
PUFA	Polyunsaturated fatty acid
Reg-1A	Regenerating protein - 1A
RNI	Recommended nutrient intake
SES	Socioeconomic status
SGA	Small-for-gestational age

SIBO	Small intestinal bacterial overgrowth
SPSS	Statistical Package for Social Sciences
SUN	Scaling Up Nutrition
TNF-α	Tumor necrosis factor alpha
WAMI	Water, assets, maternal education, income
WASH	Water, sanitation and hygiene
WHO	World Health Organization

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Paper 2

Morseth, MS, Torheim, LE, Chandyo, RK, Ulak, M, Shrestha, SK, Shrestha, B, Pripp, AH and Henjum, S: *Severely inadequate micronutrient intake among children 9.24 months in Nepal – The MAL-ED birth cohort study.* Matern Child Nutr.2018;14:e12552
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Paper 3

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Paper 4

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1.Introduction

1.1 Stunting

Stunting[i] is highly prevalent in low- and middle income countries (LMICs) and has severe consequences including increased risk of infections,[1] mortality[2,3] and loss of human capital.[1,4] The global prevalence of stunting decreased from 33% to 23% between 2000 and 2016.[5] Meanwhile, 37% of children in South Asia are stunted, and due to a large population size, the region bears about 40% of the global burden of stunting.[6] In Nepal, stunting has decreased from 57% in 2001 to 36% in 2016, with lower prevalence in urban than in rural settings.[7] The causes of stunting are complex and include infection and inadequate diet at the individual level, inadequate quality of care for children and women and food insecurity at the household level, poor accessibility to health services and clean water and sanitation at the community level and finally inadequate political and economic structures at the national level (Adapted from UNICEF framework, Fig.1).

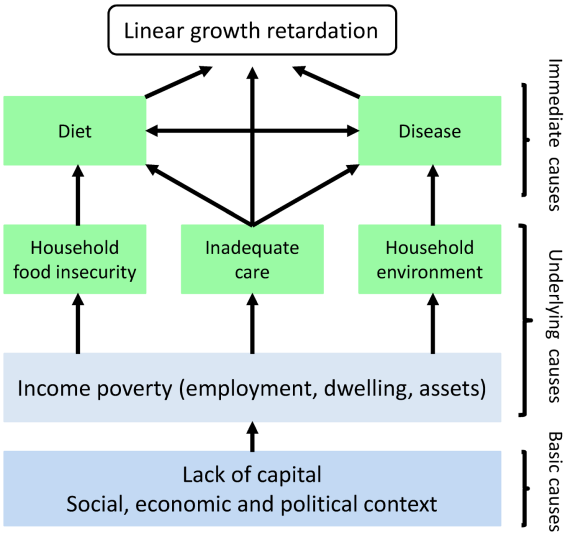

Figure 1 Modified version of the UNICEF conceptual framework for causes of malnutrition, adapted to the MAL-ED study[8]

A recent risk assessment analysis for 137 developing countries found that the leading risk factors for stunting were fetal growth restriction (birth weight <10th centile) followed by unimproved sanitation and diarrhea. It was estimated that 22% of stunting cases were

[i] Length-for-age (LAZ)/height-for age (HAZ) below 2 SD of the median in an age and sex matched reference population

attributable to environmental factors while 14% were attributable to child nutrition.[9] In addition, looking at trends from 1970 to 2012 for 116 countries, women's education, gender equality and finally quantity and quality of foods available at the country level have been instrumental in reducing stunting rates, while income growth and governance have played facilitating roles.[10] Finally, in Nepal short maternal stature,[11] low maternal education,[12,13] poor access to health services[13] and poverty[11,12] are strong determinants for stunting.

Almost all stunting occurs within the first 1000 days from conception to 2 years of age,[14,15] which constitutes a window of opportunity for growth promotion.[16] The recognition of pre-natal factors underlines the inter-generational aspects of growth,[17] and the need for early interventions. Maternal undernutrition increases the risk of stunting at 2 years age.[14] Based on data from 19 birth cohorts from LMICs, 20% of stunting is attributed to being born small-for-gestational-age (SGA).[18] Further, estimated stunting at 2 years attributed to fetal growth restriction and preterm birth in 2011 was 33% in all developing countries and 41% in South Asia.[9] Restricted pre- and postnatal growth are in turn important determinants of short adult height,[19] increasing the likelihood of the next generation also being stunted.[20] Balanced protein–energy supplementation in pregnancy seem to improve birth weight of children, with greater effects in undernourished women.[21] Meanwhile, micronutrient supplements and lipid based nutrient supplements (LNS) (providing both macro-and micronutrients) during pregnancy have shown mixed effects on birth weight and -length.[22,23] Similarly, studies supplementing LNS to mothers during pregnancy and lactation and their children during the complementary feeding period show heterogeneous results for stunting.[24,25]

1.2 Nature of growth

1.2.1 Growth patterns in early childhood

A child's growth results from a complex interaction between genetic and environmental factors.[26] The growth potential of an individual is genetically determined[27] and deviations from expected growth indicate unfavorable environments.[28] The 2006 World Health Organization (WHO) growth charts are based on anthropometric measurements of children in 6 sites in different regions of the world who were exclusively or predominantly breastfed for 4 months, introduced to complementary food by 6 months and who continued

breastfeeding until at least 12 months of age.[29] Based on a study comparing growth to WHO standards in 54 LMICs, length/height for age is close to the WHO standard at birth and falters dramatically until 24 months,[16] after which mean values tend to remain between 1.5 and 2 z-scores below the reference.[30] In the South East Asian region, the monthly decline in z-scores between 3 and 24 months age is 0.08.[16] The linear growth rate is highest during the first months of life with decelerating rates as the child ages,[31] and appears to stabilize between 18 and 24 months.[32] Faster growth requires more energy and nutrients.[33] Older children may thus be less responsive to insults on growth than rapidly growing infants.[32,34] Meanwhile, growth is saltatory with no growth occurring during 90-95% of the time from birth to 24 months.[35] Also, deviations from expected growth patterns are common. Weight faltering due to illness may precede linear growth faltering. Catch-up growth will occur if the illness is resolved and conditions for growth otherwise favorable.[36] Frequent illness, however, limits the periods with faster growth and stunting may result.[37,38] Linear catch-up growth can also occur in stunted children independent of illness.[39] To capture any deviations from expected growth pattern including the saltatory nature of growth, it is argued that growth to the extent possible should be assessed by longitudinal charts such as a velocity or increment reference[40,41] instead of cross-sectional measures (HAZ).

1.2.2 Physiology of growth

Linear growth takes place in the epiphyseal growth plates (EGP) of long bones.[42] In the growth plate, chondrocytes proliferate, hypertrophy and secrete cartilage extracellular matrix. New cartilage is subsequently remodeled into bone tissue, causing bones to grow longer.[43] Linear growth is a complex process regulated by the growth hormone (GH) - insulin-like growth factor-1 (IGF-1) axis, the thyroxine/triiodothyronine axis, androgens, estrogens, vitamin D, glucocorticoids and possibly leptin.[44] GH is secreted by the anterior pituitary gland in response to hypothalamic, pituitary and circulating factors. It affects growth by binding to receptors in the EGP,[42] and inducing production and release of IGF-1 by the liver.[45] IGF-1 has six binding proteins (IGFBPs), exhibiting different effects on body tissues, where IGFBP-3 is most abundant in human circulation.[46] IGF-1 initiates growth through differentiation and maturation of osteoblasts, and regulates release of GH from the pituitary through feedback mechanisms.[47] The GH/IGF-1 axis is responsive to dietary intake and infections. The endocrine system seems to allow for rapid growth only when the

organism is able to consume sufficient amounts of nutrients and signaling from key nutrients such as amino acids and zinc to induce production of IGF-1 is present.[44] At the same time inflammation and increased production of pro-inflammatory cytokines may cause GH resistance and a decrease in circulating IGF-1 and IGFBP-3 which in turn reduces endochondrial ossification and growth.[44,48] However, the EGP appears to conserve much growth capacity to allow for catch-up growth.[49] Concerns have been raised about associations between catch-up growth and increased risk of non-communicable diseases in adulthood.[4] In a large study based on 5 birth cohorts in Brazil, Guatemala, India, the Philippines and South Africa, faster linear growth at 0-2 years was associated with improvements in adult stature and school performance, but also an increased likelihood of overweight (mainly related to lean mass) and a slightly elevated blood pressure in young adulthood.[50]

1.3 Nutrition

1.3.1 Nutrient intake and growth

The energy and nutrient requirements that will allow moderately malnourished children to have catch-up growth, strengthened immune function and normalized mental, physical and metabolic development are high.[36] A system classifying nutrients as type 1 or type 2 nutrients depending on the body's response to their deficiency has been proposed by Golden. Type 1 nutrients (i.e Vitamin A, B-vitamins and iron) are needed for particular biochemical functions in the body. In case of deficiency clinical signs will develop and the child will be susceptible to stress and infection. Type II nutrients (i.e protein, potassium, sodium, magnesium, phosphorous and zinc) are building blocks of tissue and essential for child growth. Given the role of these nutrients in mitosis, cells with rapid turnover, such as intestinal and immune cells, are most vulnerable to insufficiency.[36]

It is generally believed that the protein density of complementary food in LMICs is adequate.[51] Meanwhile, an ecological study from 116 countries underlined the importance of protein quality when assessing risk of protein inadequacy, especially in poorer countries in Africa and Asia.[52] The quality of a protein depends on its ability to meet requirements for 9 essential amino acids,[53] and will depend on the food matrix in which the protein is consumed and the demands of the consumer which is influenced by age, health status and

energy balance.[54] It is presently unclear whether current recommendations for essential amino acids are sufficient in settings with a high burden of infectious disease and a substantial need for catch-up growth, but low levels of circulating essential fatty acids have been observed in stunted children.[55] Sulfur-containing amino acids should be used preferentially in stunted populations since sulfate is required for cartilage synthesis[36] which is essential for growth.[56]

Apart from protein, zinc is the only type II nutrient which has been thoroughly investigated in relation to growth. Modest long-term zinc deprivation results in detectable differences in growth and development,[57] while preventive zinc supplementation slightly improves linear growth.[58,59] Zinc deficiency induces anorexia with cyclical food intake and tissue catabolism and breakdown in murine models.[60] Similar responses to insufficiency as for zinc are likely for other type II nutrients for which there are no body stores.[61] In support of this, previous micronutrient supplementation studies where type II nutrients have not been provided have shown little or no effect on growth.[62,63] Malnourished children will likely suffer from multiple nutrient deficiencies,[36] underlining the need to improve whole diets through improved complementary feeding practices.

1.3.2 Complementary feeding

WHO recommends exclusive breastfeeding until the child is 6 months after which breast milk becomes insufficient, especially in iron and zinc, and complementary food should be provided.[64] High nutrient needs to support growth and development and small quantities of complementary foods consumed implies that nutrient density must be high.[33] Yet the opposite is often true in resource poor settings, where children are primarily fed bulky cereal-based porridges with low energy density[33] and low bioavailability of iron and zinc due to high levels of phytate.[65] Studies from diverse LMIC settings show that even in best case scenarios children are unable to meet their requirements, especially of iron, zinc and calcium, from family foods.[66,67]

Complementary feeding should be timely (starting at 6 months), adequate (providing the appropriate amount of nutrients in addition to breastmilk) and appropriate (diverse with appropriate texture and fed in appropriate quantities).[68] This is reflected in the WHO Infant and young child feeding (IYCF) indicators[69] which were constructed to assess and monitor child feeding practices within and between populations.[70] The indicators include

breastfeeding practices, timely introduction of solid, semi-solid or soft foods, minimum dietary diversity (MDD), minimum meal frequency (MMF) and minimum acceptable diet (MAD; MDD and MMF combined).[68] Out of these, timely introduction[71] and dietary diversity[71-73] have been associated with linear growth, while meal frequency in most studies is associated with weight.[71,74] For DDS and linear growth, associations are consistent across populations and in studies using different methodologies suggesting that they are robust.[71] Meanwhile, associations between complementary feeding practices and length increments seem less convincing, likely because tracking of good feeding practices and a cumulative positive effect is needed for a change in HAZ to occur.[75] Also, the indicators were not designed to be used separately,[70] and a complementary feeding index encompassing more than one aspect of IYCF has been proposed for studies assessing complementary feeding practices and growth.[76]

Specific food groups associated with improved linear growth are animal source foods,[15,77] and milk in particular.[78] Data from 39 Demographic and Health Surveys (DHS) showed that children who consumed no animal source foods (ASF) the previous day had a 1.44 higher odds of being stunted than children consuming all three types of ASF (egg, meat and dairy).[79] In support of this, since 1970, it is estimated that 18% of stunting reduction may be attributed to per capita dietary energy supply on a national level, while 15% is attributed to the share of energy consumed from non-staple foods.[10] The most recent estimates for South Asia showed that MDD and MAD was achieved by 33 and 21 percent, respectively. Grains were the main complementary food, with 1/3 of children 6-23 months being fed a vitamin-A rich fruit or vegetable and only 17% being fed animal source foods the previous day.[80] Acceptability, availability and affordability seem to limit improvements in dietary quality, especially consumption of animal source foods.[80,81]

1.3.3 Interventions to prevent stunting

Previous interventions to reduce stunting have shown modest effects. Multiple micronutrient supplementation shows only small benefits for linear growth[82] and results from studies supplementing LNS to children are inconclusive.[83,84] Educational interventions to improve complementary feeding may achieve behavioral change but have no or small effects on growth.[85,86] Further, studies on the effect of micronutrient fortification, increased availability of key nutrients or increased energy density of complementary foods on stunting

also show heterogenous results.[87] It is estimated that education interventions, if optimally designed and implemented, could reduce stunting by 0.6 z-scores while food-based interventions could reduce stunting by 0.5 z-scores,[87] which is moderate compared to the average global growth deficit.[30] Finally, the Lancet-series on maternal and child nutrition estimated that the impact of all existing interventions designed to improve nutrition and prevent related diseases in mothers and children, could reduce stunting at 3 years by merely 36%.[88] Hence, factors explaining the shortfall in observed associations between child feeding practices and nutrient intake and linear growth, have increasingly been the focus of scientific interest.[89]

1.4 Environmental enteric dysfunction (EED)

1.4.1 Intestinal inflammation and the role of the microbiota

Diarrhea has long been recognized as a main risk factor for child stunting,[14,90] with rotavirus, norovirus, cryptosporidum, shigella, campylobacter and E-coli among the most prevalent causative agents.[91,92] Meanwhile, studies from the early 1990s in the Gambia showed that children presented with abnormal intestinal architecture and function also in the absence of overt diarrhea.[93] The condition was histologically similar to the tropical enteropathy described since the 1960s in American military and Peace-corps personnel returning from work in Thailand.[94] The name was later changed to environmental enteropathy (EE), and more recently EED, recognizing the importance of environmental risk factors, particularly hygiene and sanitation[95] in the development of EED. The main cause of EED is likely repeated exposure to enteric pathogens through fecal contamination.[8,89,96] The key histological features are villous flattening, crypt hyperplasia and inflammation in the epithelium and lamina propria.[32,97] Intestinal inflammation interacts strongly with age,[98] and in the MAL-ED study appears to peak at about 9-12 months.[99,100] This likely in part reflects intestinal immunologic maturation, where some degree of self-limiting inflammatory response is protective against enteric pathogens.[98] Gut microbiota assembly and maturation (towards increased diversity) occur in the same age span as intestinal immunologic maturation[101] and microbiota containing low diversity is less resistant to enteropathogens.[102] Maturation of the microbiota and the intestinal immune system therefore likely interact in a reciprocal manner to promote healthy gut development.[103]

The intestinal mucosa is essential for nutrient absorption and acts as a barrier between the body and the environment.[28] The intestinal barrier function consists of a mechanical barrier formed by a single layer of epithelial cells joined by adherens and tight junctions, an antimicrobial barrier composed of defensins, immunoglobulins and mucins, an immunological barrier made up of immune cells in the sub-epithelial layer and finally an ecological barrier created by the gut microbiota which destroys pathogens.[95] Meanwhile, chronic T-cell mediated inflammation[104] seen in EED may pave the way for intestinal permeability with microbial translocation (MT), resulting in systemic inflammation.[105] EED is described as a reversible[94,106] condition which is probabilistically associated with poor development, but is neither a necessary nor a sufficient cause and may lead to no observable clinical outcomes.[107] This contributes to difficulties encountered when assessing EED.

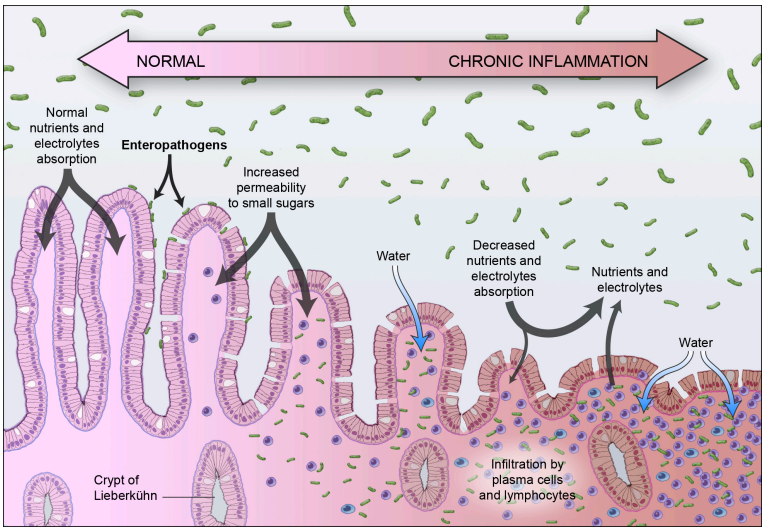

Figure 2 Schematic progression of EED (Illustration @ 2015 Haderer & Muller biomedical art, reprinted with permission)

1.4.2. Markers of EED

One main challenge in studies assessing EED is the lack of validated biomarkers.[32,108] Biopsies are used to diagnose diseases with similar pathological changes such as celiac disease.[109] However, biopsies are considered invasive in children without clinical illness,[108] unfeasible in endemic settings, and the sample collected may not be representative of the whole intestine.[107] A range of biomarkers measured in stool, urine or blood have therefore come into use to diagnose EED. These represent intestinal absorptive function (of various sugars, for instance lactulose and mannitol), intestinal barrier function (i.e alpha-1-antitrypsin (AAT)

and claudins), MT (i.e lipopolysaccharide (LPS), IgA and IgG anti-LPS and zonulin), intestinal inflammation (i.e myeloperoxidase (MPO), calprotectin, neopterin (NEO), lactoferrin and Reg1A), systemic inflammation (i.e acid glycoprotein (AGP), interleukins, TNFα, EndoCab and C-reactive Protein (CRP)) and finally metabolites/growth markers such as Tryptophan, Citrulline , IGFBP-3 and IGF-1.[107] The biomarkers have been found to correlate weakly with each other,[110,111] likely due to the diverse functions assessed and distinct physiological processes described. Further, the biomarkers show varying specificity for EED-induced growth faltering, with heterogeneous results among studies using the same biomarker.[112]

The lactulose:mannitol (L:M) ratio, measured in urine, has been most commonly used to assess EED in previous studies.[113] The test builds on the assumption that while mannitol is passively absorbed proportional to intestinal absorptive capacity, lactulose is a disaccharide which is not absorbed by the healthy intestine. Increased L:M ratio thus indicates reduced absorptive capacity and increased permeability.[105] EED is shown by numerous studies to be highly prevalent in LMICs,[32] but studies often lack reference values for diagnostic markers on which they base their findings.[113] Assignment of reference values is challenging because they may change with physiologic maturation. Also, a response to environmental exposures may initially reflect adaptive rather than pathologic processes.[113] Reference values for L:M ratio have usually been based on UK childhood values [114,115] or presumed norms for children in LMICs.[116,117] Most previous studies have applied 0.12 as reference.[113]

Compared to the L:M test, fecal markers are more readily collectible, and may be more feasible for surveillance of EED. MPO is a marker of neutrophil activity in the lamina propria[118] and has been correlated with disease activity and severity in inflammatory bowel disease.[119] MPO is a preferred biomarker in breastfed children since it is not elevated in breastmilk as are lactoferrin and calprotectin.[118] Neopterin is produced by macrophages and dendritic cells upon stimulation with inferferon-gamma (IFN-γ) produced by activated T-helper cells. It is thus a marker of TH1 stimulation[118] and has been linked to disease activity in celiac disease.[120] Finally, alpha-1-antitrypsin is a serum trypsin inhibitor which is excreted intact into stool.[121] It is thus a marker of intestinal permeability and protein losing enteropathies.[118] Due to large molecular polar surface area,[99] AAT is an indicator of relatively severe gut barrier disruption.[107] The level of fecal markers appear to be directly

associated with the number of pathogens in stool,[96,100] and most strongly with pathogens that are enteroinvasive or cause mucosal disruption.[122]

1.4.3 Dietary intake and EED

The role of nutrition in EED is increasingly being recognized.[107] EED is likely associated with energy deficiency and underweight. Mice fed a moderately energy- and protein deficient diet who are exposed to intestinal pathogens show traits similar to EED.[123] Further, weight gain in malnourished children is shown to improve EED.[124] Severe malnourishment is also likely associated with microbiota immaturity,[125] which might increase EED.[102] The intestinal mucosa turnover is dynamic, nutrient-dependent and rapid,[126] and malnourished children have rate-limiting stores for repairing mucosal damage.[38] The nutrients known to contribute to intestinal regeneration and improved barrier function are sulphur containing amino acids, [127] glutamine, vitamin A and zinc.[89,126] Meanwhile, studies investigating associations between glutamine[128] or vitamin A supplementation,[129,130] serum retinol[131,132] or zinc supplementation either alone,[133] in combination with vitamin A[134] or with micronutrients and antibiotics[135] and EED show mixed results.

Gut barrier repair and gut function may also be improved by a reduction in the inflammatory response. Short-chain fatty acids (SCFA) result from fermentation of non-starch polysaccharides in the colon.[126] It is likely that SCFA in addition to zinc[126] and poly-unsaturated fatty acids (PUFAs)[136] may reduce gastrointestinal inflammation. Although neither fibre nor PUFA provided as supplements improved L:M ratio or inflammation in intervention trials,[137,138] an increased protein and fibre intake from legumes as complementary food, might improve EED.[139,140] Cessation of breastfeeding and introduction of complementary foods, especially foods with high fibre and protein content, also likely increases microbiota diversity,[141] which might benefit the intestine. As for micronutrient intake and EED, studies from Africa have demonstrated that multiple micronutrient supplementation may improve L:M ratio in adults,[142] and transiently in children.[143] Finally, despite the diverse roles attributed to zinc in EED the effect of supplementation as prophylaxis is uncertain.[144] This may partly be due to the perturbed nutrient metabolism occurring in EED.[145]

1.5 Nutrient intake and nutritional status in EED

The relationship between dietary intake and infection is difficult to study since it is reciprocal in nature.[146,147] Further, the gut tissue consumes the nutrients it requires before passage of excess nutrients to the rest of the body.[130,148] The benefits achieved by improved nutrient intake on EED may thus be independent of nutritional status. Nutrient intake during inflammation is usually decreased. Reports of "poor appetite" by caregivers in LMICs,[149] and restriction of complementary foods during illness[150] is common. Appetite may be reduced both by pro-inflammatory cytokines and leptin[151] and low zinc status,[152] and may be continuous in children with EED[153]. Nutrient availability for growth in EED is further limited due to reduced intestinal surface area and loss of enzymatic activity causing malabsorption of nutrients[38,154] and, following MT, retention of circulating nutrients (i.e vitamin A, zinc and iron) in body tissues in order to starve pathogens.[153] Associations between nutrient intake and biomarkers for nutrient status[155] and nutrient status and growth[156] are thus likely distorted in children with inflammation. The systemic inflammation resulting from MT will increase basal metabolic rate and nutrient needs by the immune system.[105] At the same time, nutrient losses increase due to intestinal secretion.[57] The associations are thus complex, and further complicated by intestinal host-pathogen-microbiome interactions[95] and the effects of these interactions on intestinal nutrient availability,[101,157] where additional research is needed. Finally, evidence of whether nutrition interventions may be successful in children with repeated episodes of infection or persistent subclinical infection is scant.[153] Meanwhile, there seems to be agreement that successful interventions to improve complementary feeding practices[158] and reduce stunting[32,89] must encompass both immediate and underlying causes.

2. Objectives

The main objective was to assess tracking of infant and young child feeding practices, micronutrient adequacy and associations between micronutrient intake, environmental enteric dysfunction and linear growth among children aged 9-24 months in Bhaktapur, Nepal.

Specific objectives:

1. To assess infant and young child feeding practices and tracking of dietary diversity score (DDS), intake of iron- and vitamin A-rich foods and meal frequency and further to explore whether socioeconomic factors were associated with tracking patterns of these IYCF practices (paper 1).
2. To assess the micronutrient adequacy of complementary food and complementary food and breast milk combined and further to assess tracking of adequacy of micronutrients in complementary food (paper 2).
3. To measure associations between fecal markers for EED and length velocity z-scores (LVZ) and whether the associations are influenced by micronutrient adequacy (paper 3).
4. To measure associations between intake of specific nutrients and lactulose:mannitol ratio and fecal markers for EED (paper 4).

3. Materials and methods

3.1 Study area

Nepal is a land-locked country between China and India with a population of about 29 million and an annual population growth rate of 1.1%.[159] Nepal is dependent on outside donors for more than 50% for its annual budget. At the same time misuse of foreign aid appears to be on the rise.[160] Nepal is one of the world's least developed countries (ranked 144 on the 2016 Human development index) but has shown significant improvements in recent years. Fifteen percent of the population live below the poverty line[161] compared to 31% in 2010.[162] However, the country is facing an unprecedented spiral of income inequality.[160] The infant mortality rate is 29/1000 and the under-5 mortality rate 36/1000.[161] The illnesses contributing most to morbidity and mortality are acute respiratory infections (ARI) and severe diarrhea.[163] About half of mothers give birth by 20 years of age.[164] Similar to stunting, underweight (weight-for-age z-score < -2SD) and wasting (weight-for-height z-score <-2SD) has declined since the year 2000 from 42% to 27%, and 15% to 10% respectively.[7] This is likely in part attributable to an increased focus on maternal and child malnutrition with a national Multi-Sector Nutrition Plan launched in 2013.[165] A previous study showed that exclusive breastfeeding until 6 months was uncommon in Bhaktapur,[166] and breast milk substitutes was given to 15% of children within 30 days in our cohort.[167] The national micronutrient supplementation programme for children include biannual supplementation of vitamin A for children <5 and zinc supplementation in children with diarrhea. Finally, all wheat flour from roller mills should be fortified with iron, folate and vitamin A.[165]

Bhaktapur is a peri-urban society situated 15 km east of Nepal's capital city Kathmandu. The economy is primarily agriculture based. Socioeconomic status is above national averages.[163] Almost all households have access to improved water and sanitation,[168] although municipal drinking water supply is limited to a few hours per day.[163] The prevalence of iron deficiency anaemia in children 7-12 months is 26%.[169] Most mothers are literate, but only one-fourth have more than 10 years of schooling.[163] Rice is the staple food with typical side dishes of vegetable curry or lentil soup.[170] Meat, fish and dairy are not regularly consumed.[171] Eating patterns vary with the season and the availability of foods, with local green leafy vegetables consumed mainly in winter and spring.[163,171] Lentils and rice cooked together (jaulo) is the most common home-made complementary food, but roasted

grain/lentil flour cooked in water or milk is also commonly consumed. In addition, commercially produced snack food products (mainly sugary snacks) are consumed by a majority of children <2 years old in the Kathmandu valley.[172]

3.2 Subjects and study design

3.2.1 The MAL-ED study

The Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health (MAL-ED) Study, is a multicentre birth cohort led by the Fogarty International Center of the National Institutes of Health and Foundation for the National Institutes of Health (USA). The study aims to assess associations between enteropathogen infection, dietary intake, growth, vaccine response and cognitive development. Measures of gut integrity and inflammation, incidence and prevalence of enteric pathogens, diarrhea illness, anthropometry, breastfeeding and dietary intake, micronutrient status, cognitive function, socioeconomic status, immunization and vaccine response and finally other illnesses are obtained.

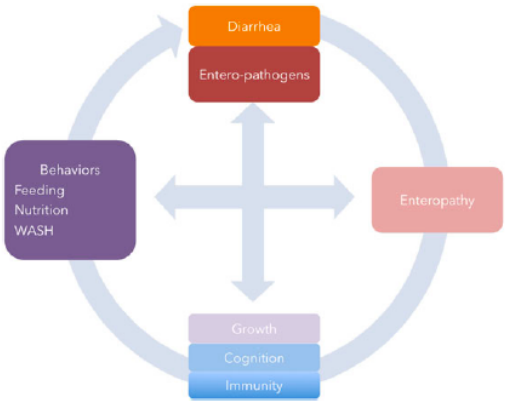

Figure 3 Interactive relationships between determinants and outcomes investigated in the MAL-ED study[173]

The 8 field sites are: Dhaka, Bhangladesh; Fortalesa, Brazil; Vellore, India; Loreto, Peru; Vellore, Pakistan; Venda, South Africa; Haydom, Tanzania and Bhaktapur, Nepal, all located in countries with high incidence of diarrhea and undernutrition. In each site children were enrolled within 17 days from birth and followed longitudinally to 24 months age. Based on census data, each site defined a catchment area where it was estimated that the target number of >200 children would be born within the 2 years enrolment period.[173]

3.2.2 Inclusion at the MAL-ED Bhaktapur site

In Bhaktapur, the MAL-ED census was carried out in March 2010. About 16 000 households were identified in the municipality and surrounding neighbourhoods and 487 pregnant women were identified in the study catchment area with an estimated 35 births per month. During the enrolment period of the study (June 2010 to February 2012), 668 deliveries were recorded and 97% occurred at the hospital. Households with recent deliveries were randomized on a weekly basis using simple random technique in Microsoft Excel (Microsoft Corporation, Redmond, Washington). Randomized households were visited and briefed about the MAL-ED study. All consenting participants were screened for enrollment.[163] The following inclusion criteria were applied:

1. Healthy infants enrolled within 17 days from birth.
2. No plans to move out of the catchment area for at least 6 months following enrolment.
3. Willingness of caregiver to be visited in the home twice weekly.

Children were excluded if:

1. The family had plans to move out of the catchment area for >30 consecutive days during the first 6 months of follow-up.
2. The mother was <16 years of age.
3. A sibling was already enrolled in the MAL-ED study.
4. The child was not a singleton.
5. The infant had indications of serious disease such as hospitalization for something other than normal birth, severe or chronic condition (i.e neonatal disorder; renal, liver, lung and/or heart disease; congenital condition) or enteropathies diagnosed by a medical doctor.
6. The guardian did not provide informed consent.
7. Weight at birth or enrolment was <1500 g[173].

Out of 275 children screened, 240 were included and 227 completed all study activities. The main reason for withdrawal was moving outside the study area.

Due to a change in methodology for dietary data collection after 9 months,[174] and need for consistency in the data, we use data from children 9-24 months only. Data was divided into time slots of different length depending on the study objectives in each paper. In paper 1 and 2 we used 4-months (9-12, 13-16, 17-20 and 21-24) time slots, while in paper

3 and 4 we used 3-months (9-12, 12-15, 15-18, 18-21 and 21-24) time slots. Data collection for this age interval took place between February 2011 and February 2014. The study received ethical approval from Nepal Health Research Council (NHRC) and the Walter Reed Institute of Research (Silver Springs, Maryland).

3.3 Data collection methods

Table 1 MAL-ED variables included in thesis and time of measurement

Variables	Age (months)															
	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Nutrition 24h dietary recall	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Gut integrity (L:M)							x									
Gut inflammation (AAT, MPO, NEO)				x			x			x			x			x
Anthropometry	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Socioeconomic status				x												

3.3.1 Infant and young child feeding practices

Child feeding practices were assessed by monthly 24h recalls. The fieldworkers went through a 3-day training with local or regional experts in recall technique and periodic 1-day refresher training sessions. Parents/caregivers were asked about the child's food intake from morning the previous day to the morning of the day of the interview. A structured form (Appendix 1) was used asking the caregivers about all food and drink offered to the child, the time and place of consumption, whether the food was raw or cooked, the amount served and the amount left over. A multiple pass method was applied. Separate forms were used to collect details on recipes (Appendix 2). Picture books, common household utensils and play dough were used to estimate amounts.[174] The raw data was double-entered into a pre-structured database at Siddhi hospital, Bhaktapur and then sent to the data coordinating centre at the US National Institutes of Health (Bethseba, Maryland) where nutritional experts processed the data and calculated nutrient intakes. DDS was calculated based on the 7 food groups grains; roots and tubers; legumes and nuts; dairy products; flesh foods; eggs; vitamin A rich fruits and vegetables and other fruits and vegetables.[69] MMD was defined as a DDS of ≥ 4 based on the previous days consumption. MMF was 3 times or more of solids,

semi-solids or soft foods per day for breastfed children and 4 times or more for non-breastfed children. Meals included both meals and snacks (apart from minimal amounts). MAD was defined as those who had MDD and MMF. Milk and milk products were excluded when calculating MAD for non-breastfed children, and only those receiving two or more milk feeds the previous day were counted as having received the milk food group.[69] The proportion of children having consumed an iron-rich food (meat or organ meat) or a vitamin A-rich food (yellow fruits and vegetables or dark green leafy vegetables) during the previous day was calculated (Table 1, paper 1).

3.3.2 Micronutrient intake and adequacy

Micronutrient intake was estimated based on the monthly 24h recalls. To enhance the validity of intake data and enable calculations of within-subject variation, secondary recalls were performed within 2-7 days of the original recall once during the age interval 9-24 months for each child.[174] To estimate nutrient intake, the FAO International Network of Food Data Systems (INFOODS) database for ASIA[175] was used, with supplementary nutrient values from other food composition tables (paper 2).

3.3.2.1 Probability of adequacy

The Institute of Medicine (IOM) probability approach[176] was used to calculate probability of adequacy (PA) based on intake from both complementary foods and breast milk after 12 months age. Breast milk intake was not measured, but estimated in the following way: the energy received from complementary foods was subtracted from the FAO total energy requirements with moderate physical activity level (kcal/kg/day) for each child.[177] The FAO energy requirement (kcal/kg) was multiplied by each child's body weight which was measured monthly. To calculate the assumed intake of breast milk (L), the calculated energy need from breast milk was further divided by the energy density of breast milk in developing countries (0.63 kcal/g) proposed by the WHO[178]. The assumed amount of breast milk was multiplied by the amount of nutrients in mature breast milk. For participants who consumed more than their nutrient requirement from complementary food, estimated nutrient intake from breast milk was set to 0. Values from LMICs were applied for nutrients where breast milk content depends on maternal status (further described in paper 2).[179,180] Finally, the

estimated amount of nutrients from breast milk was added to the amount consumed in complementary food for total intake for each child.

PA was calculated for 10 micronutrients: thiamine, riboflavin, niacin, vitamin B_6, folate, vitamin C, vitamin A, calcium, iron and zinc. Estimated average requirements (EAR) were based on back-calculation from FAO/WHO (2002) Reference nutrient intakes, defined as EAR + $2SD_{EAR.}$[181] EARs were calculated using variability coefficients from IOM.[182-184] Requirements for zinc were based on the phytate:zinc ratio, with low absorption if mean phytate:zinc ratio for the time slot was >15 and medium absorption otherwise.[185] Adequacy of iron intake was assessed based on table I-5 in the IOM report on iron requirements[184] by comparing participant's intakes with a matrix for iron requirements consistent with 5% absorption (based on phytate:iron ratio)[65] (Supplement table 1, paper 2). The PA (%) was based on mean usual intakes from 4 recalls within each time slot (13-16, 17-20 and 21-24 months, respectively) or five in time slots with secondary recalls. Skewed values were transformed using a Box-Cox transformation. After calculating within- and between person variance, the best linear unbiased predictor (BLUP) for usual intake was calculated for each nutrient for each child. BLUPs were then used to estimate PA for each micronutrient. The mean probability of adequacy (MPA) represents the PA across all 10 micronutrients.

3.3.2.2 Nutrient density adequacy

Nutrient density (ND) (amount of nutrient per 100 kcal of complementary food) and nutrient density adequacy (NDA) was calculated based on methodology by Dewey et al.[186] for the same 10 micronutrients and for all four time slots. Context specific desired nutrient densities (DND) were calculated in the following way:

[RNI of nutrient χ – (concentration of nutrient χ in breastmilk x median breast milk intake in time slot)/median energy intake from complementary food in time slot]*100.

using the same requirements, amounts of micronutrients in breast milk and absorption rates for iron and zinc as was used when calculating PA. For children who were not breastfed, FAO/WHO nutrient requirements were divided by the median energy intake in the non-breastfed group. Individual NDs were calculated as mean of four measurements within each time slot and NDAs were calculated as the percentage of the DND. NDAs for separate

nutrients were further calculated as mean NDA of four recalls within each time slot and mean nutrient density adequacy (MNDA) was the mean of individual NDAs for all ten micronutrients each capped at 100%.

3.3.3 Multiple source method

In paper 4, the Multiple Source Method[187] was used to calculate individuals' usual intake of nutrients. The probability of consumption of a food on a given day and the usual amount of food intake on days of consumption is estimated for each individual. The usual food intake on all days is then calculated by multiplying the probability of consumption with the usual amount of a food on days of consumption.[188] Three recalls in each time slot or four recalls in time slots with secondary recalls were used.

3.3.4 Anthropometry

Anthropometric measurements were performed monthly. A standard length board (ShorrBoard; Weigh and Measure, LLC, Olney, Maryland) was used to measure length, and an infant scale (Seca, Chino, California) was used to measure weight. In case of unlikely measurements, raw values were plotted on growth curves on site, and new measurements were done immediately if deviations from previous values were substantial. Each month, ten percent of measurements were duplicated within 24 hours by a supervisor for quality control. The inter-observer technical error of measurement for these repeated measurements was 0.343 for height and 0.070 for weight. Anthropometric data was double entered into a database. The site data supervisor checked for discrepancies and missing data and new measurements were performed, generally within 48 hours, if needed. Following an external quality control at the Data Coordinating Center (Bethseba, Maryland) implausible increments in subsequent measurements (>1.5 kg for weight and >3.5 cm for length) were returned for review by the study site.

3.3.5 Fecal markers for intestinal inflammation

Routine stool samples collected quarterly were used to assess MPO, AAT and NEO in paper 3 and 4. Samples were stored for processing at -70°C without fixative[106] and further analyzed by ELISA test at Walter Reed/AFRIMS Research Unit, Katmandu, Nepal. Initial dilutions were 1:500 ng/mL for MPO (ALPCO, Salem, NH) and AAT (BioVendor, Chandler, NC) and 1:1000

nmol/L for NEO (GenWay Biotech, San Diego, CA). Any test showing out of range values, was run again at a 2-fold higher or lower (as appropriate) concentration.[106] Stool samples collected <7 days after an episode of diarrhea or at the same time as the lactulose:mannitol test were excluded to avoid overly diluting the biomarker concentrations. The definition of diarrhea applied in MAL-ED is consistent with WHO guidelines.[189]

The environmental enteropathy (EE) score was calculated based on methodology by Kosek et al (2013) using weighting factors from a principal component analysis of the natural log of the three biomarkers and percentile scores for AAT, MPO and NEO.
The score ranges from 0-10 and was calculated the following way:
EE-score = (2 x AAT category) + (2 x MPO category) + (1 x NEO category), where categories were defined as 0 (<= 25th percentile), 1, (25-75th percentile), or 2 (>= 75th percentile).[118]

3.3.6 Lactulose:mannitol ratio

Children fasted for 2 hours prior to and 30 minutes after the L:M test and were recommended to void before administration of the sugar dose. Lactulose was administered at 250 mg/mL and mannitol at 50mg/mL at a dose of 2 mL/kg to a maximum administered dose of 20 mL at a concentration of 1002 mOsm/L. The urine collection period was 5 hours. To avoid bacterial growth, aliquots were stored at -70°C on ice until testing. Concentrations of lactulose and mannitol were measured by high-performance liquid chromatography (HPLC) and pulsed amperometric detection.[190]

3.3.7 Socioeconomic status

Data from a questionnaire on socio-economic status administered at 12 months is used in this dissertation. The WAMI index (range 0-1) is a measure of socioeconomic status developed for MAL-ED and is composed of the following variables: access to improved **W**ater and sanitation, number of **A**ssets, **M**aternal education and household **I**ncome. The eight assets used were separate room for a kitchen, household bank account, mattress, refrigerator, TV, people per room (mean), table and chair or bench.[168]

3.4 Data management and statistics

Statistical Package for Social Science (SPSS) version 23.0 and 24.0 and STATA version 14.0 were used to analyse data. Continuous data were presented as mean and standard deviation

(SD) if normally distributed and as median and inter-quartile range (IQR) if not normally distributed. The significance level was 0.05.

3.4.1 IYCF practices (paper 1)

Differences in mean and median values for complementary feeding practices across time slots were analysed using One-Way repeated measures ANOVA or Friedmann's test. Stability coefficients (tracking) for all 4 time slots were calculated using Generalized Estimating Equations (GEE) models. The models were first unadjusted, then adjusted for WAMI, and finally for WAMI, maternal age, parity and child's gender, but only unadjusted models and models adjusted for WAMI are presented. Correlation coefficient of <0.30 were viewed as low, 0.30-0.60 as moderate and >0.60 as moderately high.[191] Tracking of tertile membership was done using Weighted Cohen's kappa, and stability is presented as the percentage of participants remaining in their tertile. The Weighted kappa procedure is not available in SPSS, so data from Crosstabs analysis were entered into a syntax from the IBM website.[192] A weighted kappa value <0.02 was viewed as slight agreement, 0.21-0.40 as fair agreement and 0.41-0.60 as moderate agreement.[193] Finally, multinominal logistic regression was performed to assess factors associated with the odds of maintaining stable low vs. stable high consumption patterns from the first to the last time slot. Independent variables were WAMI (above median (0.703) as reference), maternal age (>25 as reference) and parity (single child as reference). The effect of season on the outcome variables was tested, but left out since associations were not significant.

3.4.2 Nutrient adequacy (paper 2)

GEE models with unstructured correlation structure were used to calculate stability coefficients for NDA across all time slots. Models were adjusted for WAMI. The same classification of correlations was used as in paper 1.

3.4.3 EED and linear growth (paper 3)

Growth velocity z-score was calculated based on WHO standards.[194] Associations between EE-score, AAT, MPO and NEO (independent variables) and length velocity (LVZ) (dependent variable) were measured using multiple linear regression models. The fecal marker

concentrations were log-transformed to obtain normality and a base 2-transformation was used to ease interpretation of results. Models were constructed for 3- and 6-month growth periods starting at the time of fecal marker sampling. Models were adjusted for baseline (at the beginning of the time slot) HAZ, child's gender, WAMI, diarrhea (proportion of days in time slot) and, for the last time slot, breastfeeding status. Models were further adjusted for MNDA. GEE models with first order autoregressive (AR-1) covariance matrix and similar adjustments were used to assess associations for the entire follow-up period. Finally, associations between quartile groups of fecal markers and 3-month LVZ were assessed using multiple linear regression models adjusted for baseline HAZ and proportion of days with diarrhea in time slot.

3.4.4 Nutrient intake and EED (paper 4)

Both L:M ratio and variables for fecal markers were log-transformed. Models for nutrient intake and L:M ratio and fecal markers were adjusted for energy intake from complementary food, WAMI, gender, season and age (only for fecal markers), while models with MNDA were not adjusted for energy intake. Associations between nutrient intake and L:M ratio were assessed using multiple linear regression analysis while all other analysis was performed using GEE with autoregressive (AR-1) covariance structure. Season was coded according to the date when the fecal sample was taken as pre-monsoon (March-May), monsoon (June-August), post-monsoon (September-November) and winter (December-January).

4.0 Results

4.1 IYCF practices (paper 1)

Dietary diversity increased through follow-up. The proportion of children meeting MDD was far lower than the proportion meeting MMF (65% vs. 98% in the last time slot). The proportion achieving MAD was 39% in the first and 64% and 25% in the last time slot for breastfed and non-breastfed children, respectively. About 1/3 of recalls showed consumption of iron- and vitamin A-rich foods. DDS, intake of iron- and vitamin A-rich foods and meal frequency all increased with age and differed significantly between time slots ($p < 0.01$).

The unadjusted stability coefficients calculated by GEE models were moderate for DDS (0.48) and meal frequency (0.53), and low for intake of iron- (0.20) and vitamin A-rich foods (0.26). Tracking of tertile membership of DDS and meal frequency was moderate and stable in the first three, but increased slightly in the fourth time slot (to 0.48 and 0.56, respectively). For intake or iron- and vitamin A-rich foods, coefficients were mainly fair and decreased throughout follow-up from 0.27 at 13-16 months age to 0.22 for iron- and 0.19 for vitamin A-rich foods at 21-24 months age. A low WAMI index significantly increased the odds of tracking low DDS (OR=3.31, CI 1.44, 7.60) and meal frequency (OR=3.46, CI 1.54, 7.76) and likely increased the odds of maintaining a low intake of iron-rich foods (OR=2.68, CI 1.01, 7.17) between the first and the last time slot. Having three or more children compared with one child increased the odds of stable low intake of iron-rich foods (OR=7.29, CI 1.62, 32.8) and low meal frequency (OR=6.31, CI 1.89, 21.1).

In conclusion, infant and young child feeding practices improved slightly with age. Tracking of poor feeding practices was associated with socioeconomic status. Low tracking for intake of iron- and vitamin A-rich foods implies that interventions targeting these IYCF practices must address underlying causes for irregular intake in order to have sustainable effects.

4.2 Nutrient adequacy (paper 2)

The estimated median (IQR) percent of energy intake from breast milk out of total energy intake decreased from 65% (48, 79) to 35% (15, 55) through time slots.

PA for B vitamins (except riboflavin), vitamin A, calcium, iron and zinc (low absorption group) ranged from 0% to 8% for all time slots. PA for riboflavin and zinc (medium absorption group) increased markedly from the first to the last time slot, while PA for vitamin C decreased by half in the last time slot. Median (IQR) MPA increased from 11% (10, 15) at 13-16 months to 21% (10, 35) at 21-24 months.

The lowest median NDAs of complementary food at baseline (9-12 months) were found for iron (4.3%), zinc (17%), vitamin A (22%) and calcium and niacin (31%). Apart from a slight drop in NDA for calcium and niacin between the first and the second time slot, NDA of all nutrients showed increasing trends through follow-up and was 17% for iron and about 40% for zinc and vitamin A in the last time slot. NDA for vitamin C was above 100% for the first three time slots. Finally, median MNDA decreased from 42% to 39% in the second time slot, and then increased to 52% in the last time slot.

Stability coefficients calculated by GEE models adjusted for WAMI were low (< 0.30) for thiamin, niacin, vitamin B_6, C, A, iron and MNDA. For the remaining nutrients, coefficients were moderate.

In conclusion, both PA and NDA increased with age, with the exception of vitamin C. Iron, zinc, vitamin A, calcium and niacin were identified as main problem nutrients by both methods. The extremely low PA found for most nutrients raise grave concern about micronutrient adequacy among Nepalese children < 2 years of age. Urgent interventions are needed.

4.3 EED and linear growth (paper 3)

Mean length was 50 (SD 2.1) cm and 12% of children were stunted at birth. Concentrations of fecal markers decreased gradually through time slots, with the largest decrease found for MPO. For all three fecal markers, more than half of children had concentrations above reference values for healthy populations in all time slots, and the highest concentrations compared to reference values were measured for NEO. Mean LVZ was lowest at 15-18 months (-0.72, SD 1.12).

Associations between fecal markers and LVZ for 3-month growth periods were overall weak with few significant findings. When MNDA was not adjusted for, EE-score (-0.03, CI -0.05, 0) and MPO (-0.03, CI -0.06, 0) were significantly associated with LVZ for the whole follow-up period, while adjusting for MNDA slightly weakened the associations. For 6-

month growth periods, only MPO was significantly associated with lower LVZ for the whole period 9-24 months. Similarly, only quartile groups for MPO were significantly associated with LVZ and only at 12-15 months. Very little variation (< 5%) was explained in the models.

In conclusion, we found weak associations between fecal markers for EED and LVZ. The only fecal marker significantly associated with growth in our population was MPO. Micronutrient adequacy slightly modified these associations.

4.4 Nutrient intake and EED (paper 4)

The median (IQR) L:M ratio was 0.07 (0.05, 0.12) and 26% had values above the reference (0.12). AAT was similar to the reference value in the last time slot.

Associations between individual nutrients and MNDA and L:M ratio and fecal markers were negative but weak. We found significant associations between intake of potassium (-0.33, C.I -0.61, -0.05), magnesium (-2.81, C.I -5.36, -0.26), phosphorous (-0.58, C.I -1.14, -0.02), folate (-2.08, C.I -3.90, -0.25), vitamin C (-0.01, C.I -0.001, 0) and MNDA (-0.01, C.I -0.01, 0) and log MPO. Weak but significant negative associations were also found between intake of zinc, calcium, potassium, magnesium, phosphorous and lactulose, while for nutrient intake and mannitol or L:M ratio no significant associations were found.

In conclusion, mainly negative but weak associations were found between intake of specific nutrients, MNDA and markers of intestinal inflammation. Significant negative associations were found between intake of various nutrients and MPO and %lactulose.

5.0 Discussion

5.1 Methodological considerations

5.1.1 Subjects and study design

The MAL-ED study is a multicenter prospective cohort study aiming to assess interrelationships between diet, environmental exposures, EED and child growth.[8] A cohort study is the most suitable design when assessing child growth and development since the temporal sequence allows for distinguishing causes from effects, and several outcomes can be examined in a single study. A methodological challenge in cohort studies is confounding.[195] The main advantage in this study was the close follow-up with monthly measurements of dietary intake and anthropometry and biweekly visits to assess child morbidity. This improved the validity of dietary intake data by enabling calculations of usual intake and the validity of fecal markers for intestinal inflammation which should only be included from non-diarrheal stool. Finally, frequent growth assessment allowed for measures of attained growth and growth velocity to be used. Selection and loss to follow-up is a major potential source of bias in cohort studies.[195] Attrition rate was low in the MAL-ED Bhaktapur cohort. Out of 240 participants screened, 227 completed all study activities. The main reason for attrition was moving out of the study area, and there is little reason to believe that children who withdrew differed significantly from those who remained in the cohort.

The age span selected for analysis in this PhD was 9-24 months. This was based on our objective to assess dietary intake during the complementary feeding period, and to investigate growth during the time where most growth faltering occurs.[20] The age span 6-9 months was, however, omitted since only after 9 months the energy intake from complementary food was considered sufficient to estimate adequacy of intake from non-breastmilk sources.[174] Meanwhile, the choice of age span prevented the inclusion of other variables known to influence the associations between diet, EED and growth such as breast feeding initiation and timely introduction of complementary food.[196]

5.1.2 Dietary intake

IYCF practices

Twenty-four-hour recall is the preferred method for collecting dietary data in children over time, and may be used for calculating IYCF indicators.[174]

The IYCF indicators have shown mixed associations with anthropometry in various settings, and in many instances seem to lack sensitivity or specificity for analyzing causal pathways to growth faltering.[197] Out of the indicators, only MDD has been validated against a gold standard.[70] DDS seems to reflect micronutrient density in the complementary feeding period, and is more strongly correlated with adequacy in children than in adults.[198] MMF is intended as a proxy measure of energy intake from complementary food and relies on the assumption that energy density is adequate,[199] but this is often not the case.[51] Also, the validity of the indicator depends on the minimum amounts of food needed in order to count as a meal.[70] In MAL-ED all meals and virtually all snacks were counted,[174] and in Nepal, children are usually fed small amounts of rice and dipping sauces by hand. In such settings, the usefulness of MMF in calculating MAD has been questioned.[200] The lack of portion size requirements might also have skewed the DDS indicator, since DDS seems to better reflect nutrient adequacy when minimum portion size requirements are applied.[201,202] In addition to MDD, intake of iron-rich foods and MAD have been suggested as meaningful indicators in this age group.[70] Meanwhile, MDD and MAD were deemed less useful than DDS in tracking analysis based on rank measures since they are binominal outcomes. It would also be problematic to determine based on 4 recalls within a time slot whether the child was fed complementary food consistent with MDD or MAD.

Probability of adequacy

Probability of adequacy, comparing usual intakes to EAR, may give approximately unbiased estimates of prevalence of inadequacy.[203] Another advantage compared to the cut-point method, is that PA may be applied in populations where the prevalence of inadequacy is high, such as in our sample.[204] The method relies on repeated measurements of dietary intake for assessment of between- and within subject variability.[205] The longitudinal design with regular 24h recalls performed thus greatly improved the quality of dietary intake data in MAL-ED.[174] Episodically consumed foods are challenging when calculating PA, and the IOM

approach that we used does not account for this.[205] Meanwhile, since PA was calculated for micronutrients and not for food groups, non-consumption was very unlikely. Further, the number of recalls within each time slot in our study (4-5) seems adequate to assess micronutrient intake in our age group, where intra-individual variability is usually small.[206,207]

Dietary intake measurements are prone to bias. Misreporting might occur, but over-reporting seems to be more common than underreporting in young children.[208] The numerous recalls performed might have improved validity of data, since training in portion size estimation seems to improve performance.[209] Detailed collection of recipes was an advantage since dipping sauces in Nepal likely are important nutrient sources, but the content will vary depending on availability of ingredients.[174] Finally, food composition databases may be a source of error when estimating nutrient intake,[210] and the quality of FCTs worldwide is uneven.[174] In MAL-ED, existing site-specific databases were primarily used. These tables contain nutrient values for commonly consumed local foods and the validity of data for nutrient intake was thus likely improved.

The PA in this study was based on the combined intake from complementary food and breast milk. The method used to calculate amounts of breast milk may constitute a validity threat in our study. Meanwhile, test weighing of children to estimate total breast milk intake[211] in this setting where breastfeeding is very frequent (paper 2, table 1) is probably unfeasible. Further, with presumably small amounts of breast milk consumed in each feed, the sensibility of test weighing may be questionable.[212] Other studies have used similar approaches to calculate amounts of complementary food or breast milk[213-215], but have based their assessment on average breast milk intake (L) as reported by WHO.[178] This is likely not ideal in settings where breast milk intake is high and energy intake from complementary foods varies substantially between participants (from about 50 to >2000 kcal, data not shown), such as in our study. Hence, the ability to impute breast milk intake from monthly anthropometric measurements was a great advantage in this study. A previous study from Bangladesh showed that energy intakes in infants 9-12 months was closely tied to body weight.[213] Also, our estimated proportions of energy intake from breast milk and complementary food (paper 2) greatly resemble estimates for high breast milk consumers reported in the Technical update report for complimentary feeding by Dewey and Brown.[215] Setting the nutrient intake from breast milk to 0 for participants who consumed more than their energy requirement from complementary food, might have

caused lower PA. However, this only applied to a few participants, so the impact was likely limited. Also, the energy density of breast milk may depend on maternal BMI,[216] but BMI among breastfeeding women in Bhaktapur has previously been shown to be normal range,[170] and imputing WHO estimates for energy content in breast milk[178] was likely appropriate.

Finally, the concentration of micronutrients in breast milk is a cause of great uncertainty. Previous studies usually rely on IOM estimates for Western women, but studies determining these estimates have generally been small and heterogeneous with regards to use of supplements or fortified foods and time postpartum.[217] In addition, methods used for analysing B-vitamins in breast milk have rarely been validated for the complex human milk matrix.[218] The validity and sensitivity of these methods is essential to understanding nutrient intake from breast milk and further nutrient needs from complementary foods. For nutrients where maternal status is known to influence breast milk content, we used concentrations found in LMICs. We based this decision on the low PA found for these same nutrients among lactating women in Bhaktapur.[170] PA for vitamin A was only 11%, while recent research showed that the prevalence of marginal- and vitamin A deficiency among lactating women in Bhaktapur was only 13% and 3%, respectively.[219] This discrepancy might imply that PA for vitamin A and MPA in our study was underestimated. Consequently, if we had been able to measure micronutrient content in breast milk from a subsample of mothers of included children, this would have greatly improved the validity of our findings.

Besides intake distributions, the PA approach also makes use of requirement distributions to assess risk of inadequacy.[203] Meanwhile, information about nutrient requirements of infants and children is relatively limited.[217] Apart from for iron and zinc, estimated average requirements are not available for children under 12 months,[215] which limited the age span for analysis of PA in our study. Further, requirements for iron, zinc and calcium are based on a factorial method[217] and might be overestimated.[51] Although differences in requirements by various organizations are usually moderate, for some nutrients (i.e vitamin C, calcium and zinc), the RNI used may alter conclusions about which nutrients constitute problem nutrients in a given setting.[215] We chose to use FAO/WHO[181] over IOM requirements,[176] mainly because they provide requirements for low and middle absorption or iron and zinc, and thus seem more appropriate to use in LMICs. The choice to use requirements consistent with 5% absorption for iron was well funded based on a very

high phytate:iron ratio (paper 2). Meanwhile, applying the cut-off for mean phytate:zinc ratio may have divided participants with quite similar absorption rates into different groups. Given the large differences in PA caused by absorption group (paper 2), this categorization may have unduly influenced the results.

Nutrient density adequacy

Required nutrient densities in complementary food are substantially affected by the estimated contribution of nutrients from breast milk.[217] The decision to calculate context specific DND instead of applying the DND calculated by Dewey et al.[186] was based on the high proportion of energy intake from breast milk (paper 2), and the assumed low level of several micronutrients in breast milk in this population. Although PA is based on EAR while NDA is calculated using RNI, applying the same amounts of breast milk and nutrient content in breast milk increased the comparability of the two methods for adequacy used. Meanwhile, using context specific NDA makes our results less comparable to other studies where DND based on Dewey et al.[202,215] are applied.

Multiple source method

MSM was developed to overcome the challenges encountered by other statistical models estimating usual intakes when assessing rarely eaten foods,[187] and has proven useful even when a sizeable proportion of the population are non-consumers.[188] The use of MSM was chosen over PA in paper 4 since it enabled us to calculate usual intake for ASF and PUFA. Changing methodology from PA which depends on EAR for calculation, also allowed for the youngest age group to be included.

5.1.3 Tracking

Tracking analysis has many potential pitfalls. First, there are no universally accepted cut-offs to classify good or poor tracking, since the magnitude of the coefficients may depend on the length of follow up and measurement error in the variable being tracked.[220] Higher tracking coefficients have been found in younger age groups and with shorter follow-up periods[221,222] such as in our study. At the same time, considerable changes in food intake are expected to occur during our follow-up period. Variables associated with lifestyle (such as dietary intake)

are more prone to measurement error and usually show larger variation than biological outcomes (i.e cholesterol), causing lower tracking coefficients.[223]

Tracking calculated for percentile groups might be considered arbitrary, and interpretation of results could potentially have been easier if objective cut-offs such as MDD or MFF had been used. Meanwhile, there was little variation in DDS and meal frequency. Measurements for DDS further centred around the cut-off for MDD, and objective cut-offs would likely have made little difference to the interpretation of our findings. In this regard, the usefulness of applying the weighted kappa, which assigns less weight to categories further apart[192] may also be questioned, since the difference between tertiles is so small. Further, tracking in extreme groups is usually stronger than tracking in middle groups,[224] and in studies with many non-consumers, the stability coefficient may be artificially inflated.[225] This likely applied to the kappa statistic for intake of iron- and vitamin A-rich foods in our study.

Finally, the tracking phenomenon assessed by GEE models may in many instances be underestimated because the model does not consider the within-person variability which might influence the initial measurement on which the remaining measurements are regressed. This variability can be reduced by averaging values from more than one measurement and use this average as the initial measurement,[223] which was done in the present study.

5.1.4 Anthropometry

Linear growth was expressed as length velocity z-scores. Length velocity is a more sensitive indicator than attained length in the detection of growth faltering[40] and reflects the longitudinal and multifactorial aspects of growth better than single measurements of HAZ.[41] Longitudinal growth measures further have theoretical advantages as they reflect current growth trend while HAZ is a cumulative measure of altered growth rate.[27,226] All anthropometric measurements in MAL-ED were performed within the maximum tolerable difference from target age (5 days for 6-12 months and 7 days for 12-24 months).[194] Charts for LVZ should only be used for full-term children with a weight and length appropriate for gestational age who are not malnourished,[227] and thus might be unsuitable for many children born in LMICs. The mean birth weight in our cohort (3000 g) (paper 3) suggests that the charts are suitable to use in our population. Further, LVZ show higher instability in

subsequent short time-periods,[228] and it has been argued that longer growth periods produce more clinically relevant results than shorter age spans.[118] Three-month growth periods as used here represent a balance between risk of measurement error and clinical relevance.

Finally, an important disadvantage of using LVZ in our study was the inability to directly compare our results (paper 3) to previous studies on fecal markers and linear growth where for the most part change in HAZ[118,229] have been used. In contrast to length velocity, change in HAZ does not take into account the different probability at different ages to track on a certain z-score. Meanwhile, deviations in z-score (centile crossing) is more common at younger ages, and the two methods are expected to give comparable results in the age span investigated in our study.

5.1.5 Markers for EED

Fecal markers

One advantage of the fecal biomarkers used in this study is that they can easily be evaluated using kits with internal standards and demonstrated high reproducibility in MAL-ED. Ongoing quality control was performed in all MAL-ED laboratories during the study period.[100] The concentration of fecal markers is highly dependent on water content in the samples.[100] Biweekly diarrhea surveillance which allowed for exclusion of watery samples thus greatly improved the quality of data for fecal markers. The assessment of stool consistency in non-diarrheal stool, however, might have been a source of bias. A main challenge with the fecal markers used is that they correlate with other GI diseases and thus are not specific to EED.[32] For instance, MPO reflect neutrophil or macrophage activation caused by any inflammation and NEO is a non-specific marker of activated cell-mediated immunity involving release of INF-γ.[105] Apart from enteric infection, the level of fecal markers is also influenced by breastfeeding (MPO and AAT)[100] and age,[98,100] which complicates their use as biomarkers in a cohort study among breastfed children. Although the main aspects of EED (inflammation, permeability and absorption) are assessed in paper 4, a composite index of markers should likely have been considered.[100] The EE-score could have been included, but the number of regression models presented in the paper was already extensive.

L:M ratio

Although L:M ratio is the most commonly used biomarker for EED, it has not been validated for this purpose.[113] Differences in the dosage of sugars given, the solution osmolarity and the urine collection time, are known to affect L:M ratio.[230] In previous studies sugar dose loadings varied and only half the studies reported having dosed according to body weight.[113] L:M ratio also depends on the type of analytic platform used. HPLC was used in MAL-ED, but this platform overestimates the disaccharide concentrations in the lower range to a greater extent than liquid chromatography-tandem mass spectrometry (LC-MSMS) which has the best accuracy.[231] L:M z-scores have recently been calculated to allow for comparison to other age- and sex specific populations.[99] However, since we only assessed one cohort site, z-scores were less relevant in our study and would limit the opportunity to compare results to previous studies. Interestingly, in the Bhaktapur cohort, both mannitol and lactulose were elevated to a similar degree. In such instances, the L:M ratio is likely not a good reflection of the severity of EED, since while the L:M ratio appears normal, increased %lactulose indicates that an aspect of EED (permeability) is in fact present. For these reasons, it has been suggested that recovery of each probe should be reported separately[99]. In paper 4, this was done only in the text, since the number of regression models presented was so vast. It is further argued that among the two probes, %lactulose should be chosen as biomarker since there is uncertainty about the interpretation of mannitol absorption; the same para-cellular pathway by which lactulose enters the systemic circulation is one by which mannitol can be absorbed as well.[100,232] Significant associations found between nutrient intake and lactulose, but not mannitol or L:M ratio should likely be viewed in light of this.

5.1.7 Statistics

Variables to be adjusted for in paper 3 were selected based on theory and previous publications. Adjustment for maternal height was initially considered but left out based on theoretical considerations since our outcome was length velocity and not attained height. Meanwhile, some studies imply that maternal height is also likely associated with length velocity,[233,234] but maybe more so in younger infants.[235] In hindsight, maternal height should probably have been adjusted for, but would likely have made few changes to regression coefficients in our analyses.

Associations between fecal markers and length velocity were assessed by GEE which are partial likelihood models. One advantage compared to full likelihood models (mixed-effects) is that they are computationally easier and generalize easily to different distributional forms.[236] Further advantages of GEE are the production of reasonably accurate standard errors and confidence intervals,[237] the possibility to include both time dependent and time independent variables and robustness under a wrong choice of correlation structure.[220] The disadvantages are the inability to estimate person-specific effects[237] that could be useful for understanding individual variability in the longitudinal response process, and more restrictive assumptions regarding missing data.[236] It is further argued that random-effects regression modelling should preferably be used for studying associations between biomarkers and growth,[41] likely because it allows researchers to simultaneously predict individual growth curves and investigate effects of exposure variables.[238] Meanwhile, we did not see the need for investigating associations at the individual level.

The vast number of regression models constructed in paper 4 increases the risk of reporting significant associations by chance. However, adjustment for multiple testing is not required in exploratory analysis [239] A purposeful selection of covariates[240] was attempted, but was unfeasible due to strong correlations between nutrients found in the same foods (i.e Iron and zinc in meat and calcium and riboflavin in milk). We chose to report results from all models to avoid emphasizing only significant results.

Finally, we only present data from the MAL-ED Bhaktapur site, and the sample size is relatively limited. A sample size determines whether the study has statistical power to detect meaningful effects and provide reliable answers to research questions.[241] The adequacy of a sample size depends on the strength of the association investigated, with stronger associations requiring fewer participants, and the variability of the variables measured, with high levels of variability requiring more participants.[242] In our study, the strengths of the associations are weak and both measures of fecal markers and dietary intake show large variability. Consequently, our study may have been underpowered to detect all meaningful effects. However, it seems unlikely that large effects have been overlooked.

5.2 Discussion of results

5.2.1 IYCF practices

The lower proportion of participants meeting MDD than MMF is comparable to regional[200] and global[243] estimates. Our results clearly indicate that MAD is mainly an effect of DDS and not MMF. Meanwhile, it is doubtful whether MAD represents adequate feeding practices in our sample since the validity of DDS as a measure of nutrient adequacy[201] and MMF as a measure of energy adequacy[199] is dependent on portion size. A similarity in determinants for MDD and MAD has previously been reported.[200] In our study, socioeconomic status was a determinant for stability of poor feeding behavior both for DDS and MMF. Low consumption of iron- and vitamin A-rich foods likely contributed to low or moderate DDS, and all three IYCF practices increased gradually with age. A previous study from Nepal saw a jump in food groups consumed between 12 and 17 months,[244] consistent with cultural taboos reported for eggs and flesh foods which are primarily fed after 12 months due to teething,[200] and the perception of mothers that these foods are hard to digest.[169]

Over recent years, there has been modest improvements in the percentage of children fed flesh foods nationally.[245] At the same time, there has been improvements in the linear growth of Nepalese children, with greater declines in stunting among wealthier than among poorer quintiles.[12] Increments in height depend, among other things, on stability of good feeding practices.[36,75] A possible explanation contributing to the differences in linear growth among wealth quintiles based on our logistic regression analysis is that intake of animal source foods and dietary diversity has increased as a consequence of economic development, but mostly in the wealthier groups. However, the notion that intake of animal source foods is determined by economic factors is at odds with results from our tracking analysis. Tracking for intake of iron-rich foods was low, and if intake of these foods was mainly dependent on family income, higher tracking coefficients would probably be seen. The unstable intake of iron-rich foods therefore seems to reflect the mainly vegetarian Nepalese diet. Increased income and diet diversification in LMICs usually results in increased consumption of prestigious, non-staple foods.[246] It could be that consumption of other foods which improve growth, such as milk products,[78] have increased in the wealthier quintiles even if consumption of meat is rare.

Finally, tracking for intake of vitamin A-rich foods might have been more tied to season than we were able to capture. We tested this by assessing the difference in DDS between seasons, but the months included in each season might not adequately coincide with the months where availability of DGLV is highest. Stronger stability of an infant and young child feeding index was found at 18-24 months than at 12-18 months in Senegal,[75] which is supported by larger tracking coefficients with age for DDS and MMF in our study. Meanwhile, lower tracking with increasing age for iron- and vitamin A-rich foods was unexpected. One possible explanation could be inflated tracking coefficients in the first two time slots due to many non-consumers. Otherwise, the small differences between groups might cause different tracking coefficients from one time slot to the next purely by chance.

5.2.2 Nutrient adequacy

The problem nutrients discovered by both PA and NDA (iron, zinc, calcium, vitamin A and niacin) have repeatedly been singled out as problem nutrients among children in LMICs.[66,67,247] This is mainly due to low intake of animal source foods and vitamin A-rich fruits and vegetables[15] and poor absorbability of iron and zinc from staple foods.[65] Nutrient adequacy in older children and women is correlated with energy intake[198] and micronutrient density adequacy was higher among non-breastfed than breastfed 6-23 months old children in Madagascar.[202] The improved nutrient adequacy with age found in our study is thus likely in part caused by increased total energy intake and cessation of breastfeeding for some children in the last time slot.

PA for most micronutrients was extremely low, but in the last time slot approached PA among lactating women in Bhaktapur.[170] To our knowledge, no previous studies have assessed PA from a combined intake from complementary food and breast milk, so we have no studies with similar methodology to compare with. However, the difference in PA between vitamin C (high content in breast milk) and iron and the drop for adequacy of vitamin C in the final time slot underlines the immense importance of nutrient content in breast milk in our setting. Our finding that high breast milk intake is beneficial for adequacy of vitamin C is further supported by previous studies. [213,247]

A study from Bangladesh with a similar age group and a similar proportion of energy intake from breast milk to our study showed that breast milk was the main source of all

nutrients apart from iron and vitamin B_6. The diet was, however, deficient in many nutrients[213] similar to our findings. Whether adequacy will be improved by a higher proportion of energy from complementary food ultimately depends on the quality of complementary food, and will vary in different settings.[215] It has been calculated that with average LMIC breast milk intake, 95% of iron, 40% of zinc and 10-30% of vitamin A must be provided by complementary foods,[178] which for iron seems impossible in our setting. In the study from Bangladesh, a 100 kcal replacement of breast milk with complementary food slightly increased the adequacy of iron, zinc and calcium,[213] while in Guatemala the adequacy of iron and zinc was unchanged when calculated from both breast milk and complementary food compared to from complementary food alone.[247] In the end, since iron and zinc content in breast milk is unaffected by maternal status in our age group,[248] the potential for improving adequacy of main problem nutrients is through improving the quality of complementary food and then increasing the amounts of complementary food fed. IYCF interventions emphasizing DDS and particularly animal source foods which contains more easily absorbable vitamin A, riboflavin, calcium, iron and zinc[15] are of utmost importance. This was firmly re-established in this thesis.

In our study MNDA, reflecting the quality of complementary food was low to moderate. NDA is based on RNIs which likely overestimate desired nutrient densities[176] and thus may have underestimated NDA on a group level. The low nutrient densities and MNDA found in our study compared to similar age groups in other settings[202,247] is likely partly explained by our choice to apply context specific NDs. For nutrients where the content in breast milk is low, high breast milk consumption increases desired ND from complementary food resulting in lower NDA than if nutrient densities based on average breast milk consumption had been applied. Absorption rates are also different in our study compared to those described in the report by Dewey et al.[186] NDA for iron and zinc when calculated with desired NDs by Dewey et al. in our cohort was ~35% and 55% at 12-23 months (data not shown), compared to 10-17% and 24-43% across time slots with context specific NDA. Although this must be considered a statistical artefact, it again underlines the great methodological importance of the proportion of complementary food vs. breast milk and absorption rates applied when assessing adequacy in breastfed children.

There is uncertainty about bioavailability of minerals during the complementary feeding period.[51] Bioavailability from breast milk compared to complementary food is

favourable,[249] although it too may be lowered by a high phytate:zinc ratio.[65] Of interest to our findings is that while milk iron concentration seems to decrease with weaning, prolonged breastfeeding increases zinc in breast milk independent of maternal status.[248] These differences will not be detected by either method for assessing adequacy, but might influence the amounts of problem nutrients which must be supplied by complementary food throughout the weaning period to improve nutrient adequacy.

If requirements for iron, zinc and calcium are overestimated, as has previously been suggested,[51] this could influence our assessment of adequacy by both methods. How the effect of absorption rates of complementary foods is related to level of breastfeeding was also demonstrated by our findings. While PA for iron and zinc (low absorption group) was close to 0 throughout follow-up, PA for zinc (medium absorption group) improved greatly in the final time slot with cessation of breastfeeding for some children and a larger proportion of energy from CF for all. Rice contributes about 50% to zinc intake among healthy women of reproductive age in Bhaktapur,[250] and despite high phytate content, likely also contributes to the improved adequacy of zinc found among older children with moderate absorption in our study.

Finally, tracking coefficients for iron and vitamin A were low, which corresponds to random consumption and low coefficients for intake of iron- and vitamin A-rich foods found in paper 1.

5.2.3 EED and linear growth

The weak associations found in our study between fecal markers, EE-score and linear growth[118,229] and significant associations between MPO[39,110,118,229] and AAT[39,98] and linear growth are consistent with previous studies. Meanwhile, studies showing significant associations between all three fecal markers and 6-month growth,[118] no associations between EE-score and 3-month growth[229] and no association between any of the fecal markers and delta HAZ in the second year[122] contrasts our findings. A recent systematic review found that among 5 domains in EED (intestinal damage and repair, permeability, translocation, inflammation and systemic inflammation), intestinal inflammation for which MPO is a marker, was most consistently associated with linear growth.[112] MPO had the highest partial R^2 explaining 2.4% of variability in LAZ 3 months later (vs. 0.2 for NEO and 0.7

for AAT) in the MAL-ED Peru cohort, and performed consistently across different lag lengths for growth.[98] MPO is associated with enteroaggressive pathogens[122] prevalent in all MAL-ED sites[92] and likely targets growth hormone through effects on IGF-1.[48,110]

Decreasing concentrations of MPO and AAT after 9 months age is similar to findings from other MAL-ED sites[98,100] and is likely caused by intestinal immunological maturation[98] and improved microbiota maturity with increasing age[101]. In our study, higher values for fecal markers seemed to have a neutral or weakly positive effect on growth up to 12 months. Although such weak associations should be interpreted with great caution, this fits the notion that some elements of EED may be adaptive, rather than pathologic[112] where some self-limiting inflammation is positive up to a certain age, after which EED becomes deleterious for growth[98].

In all MAL-ED sites, recent illness was associated with an increase in NEO but a small decrease in MPO in the second year of life.[100] NEO was the fecal marker most elevated compared to reference values in our study. Meanwhile, it appears not to be associated with pathogen burden[110] and has limited or no direct effect on linear growth after 9 months,[110,122] which was also found in our study. We assessed the associations of each biomarker with LVZ separately, which might have been a limitation considering how the fecal markers interact. For instance, in the MAL-ED Brazil cohort, high MPO combined with high NEO was associated with the poorest growth, while NEO in the absence of MPO was associated with improved growth.[39] Also, AAT appears to be associated with growth only in the absence of MPO, which might imply that permeability of the intestine is a more distal determinant than inflammation on the pathway between pathogen exposure and undernutrition.[98] The stronger negative associations between MPO and growth at 12-15 months and AAT and growth at 18-21 months might support a temporal shift in relevance of pathways (from inflammation to permeability) to explain linear growth faltering. On the other hand, a recent systematic review found that associations between intestinal inflammation (MPO) and biomarkers for permeability (including AAT) are not well established.[112]

Large inter- and intra-individual variation for AAT, MPO and NEO has been found in all MAL-ED sites,[98,122] and is a main limitation in applying these markers for diagnosing EED. The variation reflects the multi-dimensional aspects of intestinal health and growth with great differences in resistance to pathogens, normal microbiota and acquired immunity between individuals and in the same individual across time.[107] Several different gut

community configurations are associated with moderate or severe malnutrition.[156] These configurations may be differently affected not only by pathogen burden,[100] but also changes in diet or antibiotic treatment.[196] In this complex picture, low adjusted R^2 for single biomarkers, comparable to our findings, are expected.[41,98] Meanwhile, explained variance may also depend on how associations are modelled. We assumed a linear relationship between fecal markers and growth. This is a strong assumption and we may have overlooked significant non-linear associations. For instance, in the MAL-ED Peru cohort, about 90% of observed associations between fecal markers and LAZ was explained by including polynomial terms for age and subject-specific random effects.[98] Our choice of statistical model (GEE) does not include subject-specific effects (random intercepts and slopes for each child), which increased the variance in fecal markers explained in all MAL-ED sites.[100] The baseline characteristics we adjusted for (i.e baseline HAZ, SES, gender) might have captured some of the between-subject variation, but very small differences in regression coefficients between unadjusted and adjusted models suggests that the variables were unable to explain much variability.

Several factors which have not been accounted for might have biased our results. First, both MPO and AAT are significantly elevated by recent breast milk intake,[100] so high breastfeeding frequency likely overestimated the severity of EED in our population. Secondly, since all households have access to improved water and sanitation, WASH was not adjusted for. Meanwhile, almost half of all families shared their toilet facilities with up to 10 other households,[251] which likely increased their risk of fecal contamination. Thus, the variable for WASH could potentially have been more nuanced and included in our models. Finally, the length of the growth period applied (3 months) might have influenced our results through influencing the stability of LVZ assessments.[228] The strongest association between MPO and LAZ appears to be over a 2-month time lag,[98] but quarterly fecal sampling in our study determined the length of the growth period used.

MNDA slightly modified associations between MPO and length velocity, which might be caused by beneficial effects of several micronutrients on MPO found in paper 4. Meanwhile, it seems that micronutrient adequacy overall contributes little to improving linear growth when mediated through improvements in EED, supported by other studies.[135,143,252] Both associations between fecal markers and LVZ and effects of MNDA on these associations are too small to be of clinical significance. Finally, MNDA only assesses

adequacy of complementary food, which might be less suitable than adequacy of total micronutrient intake in our group of high breast milk consumers. Meanwhile, adjusting for MNDA rather than PA allowed us to also include the youngest age group in the analysis.

5.2.4 Nutrient intake and EED

The median L:M ratio was in the lower range for reference values used in previous studies.[112] It thus appeared that our participants were not severely inflicted by EED. However, since both lactulose and mannitol were high, it seems that mannitol might not have reflected absorptive surface the way it was intended.[232] A potentially unclear association between the severity of EED and L:M ratio in our study greatly complicates interpretation of results. Meanwhile, we chose to use L:M ratio to enable comparison to previous studies. In hindsight, associations between nutrient intake and %Lactulose should likely have been presented in table 3 (paper 4), but instead of L:M ratio to avoid increasing the number of regression models further. Significant associations between nutrient intake and %Lactulose will thus be discussed in this thesis.

The weak associations found between nutrient intake and fecal markers could be due to a number of reasons. First, the relationship between dietary intake and EED is bidirectional. On the one hand, poor nutrient status may increase the likelihood of[131] or perpetuate adverse outcomes of EED,[253] while EED in turn leads to poor absorption and poor nutrient status.[153,253] Whether nutrient intake is reflected by nutrient status also depends on absorption rates. MSM does not account for this, which may be a source of bias. Especially for iron and zinc, the regression models should likely have been adjusted for average phytate:zinc ratio. Second, changes in gut microbiota may influence bioavailability and metabolism of nutrients in infants[156,254] where age-associated changes in genes involved in vitamin biosynthesis and metabolism during the first 3 years have been described.[101] This could bias associations between nutrient intake and severity of EED across different ages. Finally, intestinal cells have the ability to use nutrients directly from the lumen,[130] which complicates any inferences about adequacy of nutrient intake, improvements in EED and nutrient status.

The amount of complementary food on which we based our calculations for usual intakes was low, especially in the first time slots, and our results are likely biased by high

breast milk consumption. Breast milk contains epidermal growth factors and IGF-1 which aids in maintaining gut health.[255,256] The numerous immunologic functions of human milk are also well recognized.[256] For instance, human milk oligosaccharides (HMOs) alter gut microbiota and inhibit adhesion of pathogenic bacteria[257], while Lactoferrin has bactericidal properties.[258] The limited intake of complementary food on which we base our models is further problematic since a large proportion of nutrients potentially influencing EED comes from breastmilk and is unaccounted for. Inferences about the associations studied could likely have been made with more confidence if older non-breastfed children had been investigated. Finally, we assessed breastfeeding at the end of each time slot while many mothers stopped breastfeeding during the last two time slots. Since cessation of breastfeeding has been associated with changes in L:M status[231,259] and improved microbiota maturity[141] in previous studies this might have biased our results.

Given the vast number of regression models, it seems inadvisable to emphasize significant findings without plausible biological explanations. Significant associations between potassium, magnesium, phosphate, vitamin C, folate, MNDA and MPO are hard to explain each separately, but consumption of fruits and vegetables which are good sources of potassium, folate and vitamin C, seem to protect from inflammatory bowel disease,[260] which is also partly characterized by neutrophil activation and increased levels of MPO[119]. Also, associations between depletion of magnesium, potassium and zinc and decreased plasma and tissue IGF-1 levels have been shown in animal models. Decreased levels of IGF-1 could affect gut growth.[261] Further, potassium, magnesium, phosphate, calcium and zinc are all type II nutrients which are needed for mitosis and are particularly important in rapidly dividing tissue such as intestinal and immune cells.[36] Significant associations for type II nutrients and %Lactulose may be caused by an increased need in EED for intestinal “repair” nutrients to limit permeability. Few studies have previously assessed these associations, but zinc intake or status has been associated with reduced L:M ratio or %Lactulose in various settings.[34,134,262] Potential beneficial effects of specific nutrients on markers of intestinal inflammation need to be corroborated by further studies.

Other aspects could have confounded our results. Small intestine bacterial overgrowth (SIBO) is prevalent in LMICs and leads to impaired micronutrient absorption, increased GI permeability and disrupted mucosal immunity. In Bangladeshi children, SIBO did not increase L:M ratio which was interpreted as SIBO and EED being separate but

associated conditions.[263] With similar pathology to EED, SIBO may have confounded our results. Further, to fully assess the relationships under study, a deeper understanding of the associations between nutrient intake, microbiota maturity and intestinal inflammation would be preferable. Given the influence of recent breast milk intake on concentrations of MPO and AAT[100] and increased lactulose excretion,[231] timing for cessation of breastfeeding could have been included. On this note, interaction terms between age and breastfeeding were tested, but were not significant. The main factor influencing fecal markers in univariable models was age.

6.0 Conclusion

The present thesis concludes with the following:

- The proportion of children meeting minimum meal frequency was far greater than the proportion meeting minimum dietary diversity among children 9-24 months in Bhaktapur, Nepal. The stability of complementary feeding practices was moderate for dietary diversity and meal frequency and low for intake or iron- and vitamin A-rich foods. Low socioeconomic status (WAMI) significantly increased the odds of being in the low tertile at 9-12 months and 21-24 months for DDS, meal frequency and intake of iron-rich foods.
- Nutrient adequacy increased with age. Iron, zinc, vitamin A, calcium and niacin were identified as main problem nutrients both when adequacy of combined intake from complementary food and breast milk (PA) and complementary food alone (NDA) was assessed. Stability coefficients were low for thiamin, niacin, vitamin B_6, C, A, iron and MNDA, and moderate for the remaining nutrients.
- Associations between fecal markers for EED and length velocity were generally weak with few significant findings. Significant associations between EE-score and MPO and 3-month LVZ and MPO and 6-month LVZ were found for the entire follow-up period. Micronutrient adequacy of complementary food slightly modified these associations.
- Mostly negative but weak associations were found between nutrient intake, MNDA and markers of intestinal inflammation. Type II nutrients, including zinc and calcium, were associated with reduced permeability assessed by %Lactulose, while antioxidants (i.e folate and vitamin C) were associated with reduced MPO. Weak associations are likely due to the complexity of the associations investigated and the low proportion of total nutrient intake assessed in our study.

In summary, complementary feeding practices, especially dietary diversity and intake of animal source foods, need to be improved in order to increase intake of bioavailable iron, zinc, calcium and vitamin A. Some of the nutrients with the lowest adequacy in our study may potentially improve EED through reduced intestinal permeability, which in turn might benefit child growth, but further studies are needed.

7.0 Future perspectives

7.1 Improving complementary feeding practices and EED

Although Nepal was an early riser in the Scaling Up Nutrition (SUN) movement[12] and has a national multi-sectoral nutrition strategy in place,[165] a gap has been observed in the policy and programming environment around complementary feeding practices.[264] The low prevalence and low stability of adequate complementary feeding practices and inadequacy of micronutrient intake in our study underlines a need for interventions.

Intensive interventions to improve complementary feeding practices encompassing interpersonal counselling, mass media and community mobilization have proven effective in a South Asian setting.[85] Interventions should identify cultural barriers and enablers and seek to learn from intermediary behaviour changes.[265] Also, improving the level of maternal education[12] and empowerment[266] and teaching mothers responsive feeding practices[267] might increase their effectiveness. Improved feeding practices have been associated with number of antenatal and postnatal visits in Nepal.[268] At the same time, there is shortage of capital and human resources at the community level,[165] which should be addressed. Further, frontline health workers should continuously receive standard training packages about IYCF,[264] including improved complementary feeding practices during disease.[150] Finally, equity-based approaches to improve child health and nutrition have proven most cost-effective.[269], underlining the importance of addressing barriers to adequate IYCF practices in food-insecure families.[85]

The low adequacy of iron, zinc and calcium underlines the importance of interventions focusing on improved intake of animal source foods.[79] However, it is very unlikely that requirements, especially for iron, may be met without some form of supplementation.[33] Meanwhile, additional iron has been associated with increased intestinal inflammation,[270,271] so advantages of iron supplementation must be carefully weighed against risks in this setting. The success of interventions to improve complementary feeding practices in children with frequent clinical and subclinical infections has been questioned.[33] As a starting point, such interventions must provide nutrients, including type II nutrients[36] and vitamin A[126] to improve EED, but must also seek to eliminate infections.[33] However, the multiple causal pathways for EED underlines the need for multifaceted interventions targeting both immediate and underlying causes.[89] The roles and responsibilities of different

stakeholders working towards improved IYCF practices in Nepal must be clarified.[264] Importantly, intervention aiming to improve IYCF or reduce EED should go through rigorous evaluations.[33]

7.2 Need for research

This thesis has highlighted a number of areas where further research is needed. First, a systematic review of optimal amounts of supplemental micronutrients to raise concentrations in breast milk is needed.[179] Larger studies assessing micronutrient content in breast milk of women in LMICs would be useful. Meanwhile, it is advisable that future studies aiming to assess adequacy of total micronutrient intake in breastfed children measure micronutrient composition in milk from mothers of the children included in the study.

Second, how nutrient depletion relates to EED requires further research.[272] Bioavailability studies and metabolic phenotyping[273] in children with EED would be welcome. Further, there is a great need to improve our understanding of how components in breast milk,[274] cessation of breastfeeding,[141] components of complementary foods,[275] and micro- and macronutrient deficiencies[156] influence microbiota maturity. Some of these issues would likely be better highlighted in children slightly older than the age group included in our study. Further research on associations between microbiota maturity and EED are needed. Overall, it is still unclear what composition of microbiota is beneficial and which constitutes dysbiosis,[107] and how determinants are linked to microbiota composition, which likely differs in various settings.

Finally, there is an acute need for validated non-invasive biomarkers for EED and further research to establish reference values in children in LMICs is required. Future use of the L:M ratio should probably be discouraged due to challenges in interpreting %mannitol. As for fecal markers, MPO should likely be the preferred biomarker in settings similar to ours. If all three markers are to be applied in combination, further studies investigating determinants of their joint variability[111] are needed. Optical probes assessing tissue activity in situ[276] and stable isotope techniques[277] with the potential to assess multiple dimensions of intestinal functioning have recently been proposed as novel techniques in the assessment of EED. These techniques are seen as part of the solution to the practical and ethical

difficulties of validation against biopsy and histology, and will likely contribute to improved quality of studies on EED and growth in the future.

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Paper 1

Tracking of infant and young child feeding practices among 9- to 24-month-old children in Nepal: the MAL-ED Birth Cohort Study

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Tracking of infant and young child feeding practices among 9- to 24-month-old children in Nepal: the MAL-ED Birth Cohort Study

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Abstract

Objective: The present study aimed to assess infant and young child feeding (IYCF) practices and the tracking of dietary diversity score (DDS), intakes of Fe- and vitamin A-rich foods and meal frequency in a peri-urban area in Nepal. Furthermore, to explore whether sociodemographic factors were associated with tracking patterns of these IYCF practices.

Design: Longitudinal study. Monthly food intake was measured by 24 h recall. Four time slots were used (9–12, 13–16, 17–20 and 21–24 months). Tracking of IYCF practices was investigated using generalized estimating equations (GEE) models and Cohen's weighted kappa. Multinominal logistic regression was used to identify determinants for tracking of the IYCF practices.

Setting: Bhaktapur municipality, Nepal.

Subjects: Children (*n* 229) aged 9–24 months, randomly selected.

Results: Prevalence of minimum meal frequency was higher than for minimum dietary diversity at all time slots. Tracking based on absolute measures (GEE models) was moderate for DDS (0·48) and meal frequency (0·53), and low for intakes of Fe- (0·23) and vitamin A-rich (0·35) foods. Tracking based on rank measured was moderate for DDS and meal frequency, and fair for Fe- and vitamin A-rich foods. Low socio-economic status significantly increased the odds (OR; 95 % CI) of tracking of low *v.* high DDS (3·31; 1·44, 7·60) and meal frequency (3·46; 1·54, 7·76).

Conclusions: Low tracking for intakes of Fe- and vitamin A-rich foods implies that interventions to improve these IYCF practices must address underlying causes for irregular intake to have sustainable effects.

Keywords
MAL-ED
Nepal
Complementary feeding
Infant and young child practices
Tracking
Dietary diversity

Maternal and child undernutrition was estimated to account for 45 % of all child deaths globally in 2011[1]. Among the underlying nutrition-related causes of death in children under 5 years of age are stunting (low height-for-age), wasting (low weight-for-height), deficiencies of vitamin A and Zn, and suboptimum breast-feeding[2,3]. While the global prevalence of child malnutrition has decreased in the past decades, it continues to be high in South Asia and Africa[1]. Adequate complementary feeding practices have been associated with improved child growth[4–6] and reduced under-5 mortality[7]. The target age for complementary feeding is generally accepted to be 6 to 24 months[8]. During this critical time period for child growth and development, breast milk is no longer sufficient to cover the child's growing nutritional needs and should be supplemented by foods that fill the gaps for energy and nutrients[9], including animal-source foods and fresh vegetables and fruits[4,10].

The WHO has developed eight core infant and young child feeding (IYCF) indicators, designed to reflect adequate intakes of energy and key nutrients. These include breast-feeding practices, timely introduction of solid, semi-solid or soft foods, minimum dietary diversity (MDD), minimum meal frequency (MMF) and intake of Fe-rich foods[11]. In several studies, compliance with IYCF indicators has been linked to improved nutritional status in children[5,6,12], while other studies have found inconsistent or non-existent relationships between separate IYCF indicators and child growth[13]. These studies have in general been cross-sectional, and associations between compliance over time and anthropometric outcomes have rarely been investigated. In one study by Moursi *et al.*,

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an infant and child feeding index composed of current breast-feeding and bottle-feeding, dietary diversity, food group frequency and feeding frequency in the past 24 h showed relative stability over time (from 6 to 17 months) and was associated with increased length-for-age but not weight-for-age[14].

Tracking is defined as the stability of a health behaviour over time or as the tendency of an individual to maintain his/her rank or position within a group over time[15]. Tracking studies are useful to determine at what age health behaviours stabilize. Health interventions implemented before this age are generally believed to be more effective[16]. Interventions may also become more targeted since high-risk groups for continued poor health behaviour and future health problems may be discovered. To our knowledge, there is little information in the scientific literature on the tracking of food patterns in infants and children from low- and middle-income countries. One study from Australia, where food intakes were recorded at 9 and 18 months, showed a high degree of tracking for energy-dense, nutrient-poor foods. Intakes of healthier foods such as fruits, vegetables, eggs and fish were also relatively stable from infancy to toddlerhood[17]. Another study from the UK on infants (6 and 12 months old) found that dietary patterns, the first characterized by fruits, vegetables and home-made foods and the second characterized by bread, savoury snacks, biscuits and chips, were correlated between ages and associated with maternal and family characteristics, most importantly maternal diet[18].

The present paper focuses on children included in the study titled 'Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development' (MAL-ED) in Nepal. The seven other cohort sites are in Bangladesh, Brazil, India, Pakistan, Peru, South Africa and Tanzania. The aim of MAL-ED is to improve understanding of the interrelationships between gut microbial ecology, enteropathogen infection, diet, nutritional status, gut physiology, growth, immune function and cognitive development[19]. The first objective of the paper was to assess IYCF practices in a cohort of children aged 9 to 24 months in Bhaktapur, Nepal. The second objective was to assess tracking of dietary diversity score (DDS), intakes of Fe- and vitamin A-rich foods and meal frequency, and the sociodemographic factors associated with tracking of these complementary feeding practices.

Methods

Design and participants

The MAL-ED study is based on birth cohorts followed longitudinally in each of the eight study sites. The data in the present paper pertain to children aged 9 to 24 months included in the MAL-ED study in Bhaktapur, a peri-urban agriculture-based community located 15 km east of Kathmandu, the capital city of Nepal. Data from 6 to 9 months were omitted due to a change in the methodology for dietary data collection at 9 months[20] and a need for consistency in the data. In total, 240 infants were enrolled within 17 d from birth and 229 had completed nutritional data at 24 months. Initial enrolment started in June 2010, while data collection for this age group took place between February 2011 and November 2012. The MAL-ED study in Nepal received ethical approval from Nepal Health Research Council and the Walter Reed Institute of Research (Silver Springs, MD, USA). Signed informed consent was collected from the mother or caregiver of each participating child. Further details on study design and methods are reported elsewhere[19].

24 h recall

The food intake of the children was collected by monthly 24 h recalls, where the mother or caregiver answered questions about all meals, foods and amounts consumed on the day prior to the interview. Local fieldworkers went through an initial three-day training with follow-up exercises and periodic one-day refresher training sessions led by experts in dietary recall technique. A structured form was used to write down all foods and drinks offered to the child, when and where it was consumed, whether it was raw or cooked, the amount served and the amount left over. Play dough, common household utensils and pictures of portion sizes were used to estimate amounts of food. Another form was used to collect details on the recipes[20].

Infant and young child feeding indicators

Three of the eight core IYCF indicators[11,21] were assessed in addition to adapted indicators for intakes of Fe- and vitamin A-rich foods (Table 1). DDS was calculated based on seven food groups as follows: grains, roots and tubers; legumes and nuts; dairy products; flesh foods; eggs; vitamin A-rich fruits and vegetables; and other fruits and vegetables[21]. DDS was calculated by summing up the number of food groups consumed by each child in the last 24 h. MDD was defined as a DDS of ≥4. MMF was defined as solids, semi-solids or soft foods ≥3 times/d for breast-fed children and ≥4 times/d for non-breast-fed children. Meals included both meals and snacks (apart from minimal amounts). Minimum acceptable diet (MAD) was defined as those who had at least MDD and MMF for the relevant time slot. Milk is considered a required element in the diet of non-breast-fed children[11]. To avoid counting the milk group twice, milk and milk products were excluded when calculating DDS for the MAD indicator for non-breast-fed children. Further, MAD in this group was defined as those with DDS ≥ 4/6 who had received ≥2 portions of dairy and/or infant formula and ≥4 portions of dairy and/or infant formula and solid/semi-solid foods[11]. Results are reported separately for breast-fed and non-breast-fed children in the 21–24 months time slot.

Table 1 WHO infant and young child feeding indicators adapted for the present study

Indicator	Description*
Minimum dietary diversity (MDD)	Proportion of children 9–24 months of age who receive foods from four or more food groups during the previous day
Minimum meal frequency (MMF)	Proportion of breast-fed and non-breast-fed children 9–24 months of age who receive solid, semi-solid or soft foods (also including milk feeds for non-breast-fed children) the minimum number of times during the previous day
Minimum acceptable diet (MAD)	Proportion of children 9–24 months of age who had at least the minimum dietary diversity and minimum meal frequency (apart from breast milk) during the previous day
Consumption of Fe-rich foods†	Proportion of children 9–24 months who received an Fe-rich food (meat or organ meat) during the previous day
Consumption of vitamin A-rich foods†	Proportion of children 9–24 months who received a vitamin A-rich food (yellow fruits and vegetables or dark green leafy vegetables) during the previous day

*Original indicators cover the age group 6–23 months.
†Adapted from WHO *Indicators for Assessing Infant and Young Child Feeding Practices*[11] for the purpose of the study.

Socio-economic status

A questionnaire on socio-economic status was administered at 12, 18 and 24 months. The WAMI (Water, Assets, Mother's education and Income) index, a measure of socio-economic status developed for MAL-ED, was composed of the following variables: access to improved water and sanitation; number of assets; maternal education; and household income. The eight assets used were: separate room for a kitchen; household bank account; mattress; refrigerator; television; people per room (mean); table; and chair or bench. The WAMI index based on data from all eight MAL-ED sites showed a significantly stronger association with stunting than maternal education or more complete measures of wealth alone[22].

Statistical analysis

Data analysis was performed using the statistical software packages IBM SPSS Statistics version 23·0 and STATA version 14·0. A probability level of 0·05 was used. Continuous data were presented as mean and SD[23] if normally distributed, and as median (minimum, maximum) if not normally distributed. After reviewing trends for age in the data it was decided to use four time slots, each with four months of measurements (9–12, 13–16, 17–20 and 21–24 months, respectively). Characteristics of adequate complementary feeding are based on all observations within time slots. This prevents exclusion of participants with missing data. Seventeen participants lacked one measurement, one participant in the 9–12 months time slot lacked two measurements, while for the last two time slots one participant lacked three measurements.

Differences between mean and median values across time slots were analysed using one-way repeated-measures ANOVA or Friedman's test. Stability coefficients for all four time slots for DDS, intakes of Fe- and vitamin A-rich foods and meal frequency were calculated using generalized estimating equations (GEE) models. A GEE model has the advantage of providing one stability coefficient taking into account that measurements within one individual are correlated and may be adjusted for both time-dependent and time-independent covariates. The regression of the value of the outcome variable at time 1 is performed *v.* the longitudinal development of the outcome variable from time 2 to time *m* (number of measurements) while adjusting for covariates, yielding a single regression coefficient[24]. First, unadjusted models and models adjusting for WAMI were calculated. Then, models adjusted for WAMI, maternal age, parity and child's gender were performed. Since these adjustments made no further changes to the estimates, only results from the unadjusted models and those adjusted for WAMI are presented. Correlation coefficients <0·30 were classified as low, 0·30 to 0·60 as moderate, and >0·60 as moderately high[25].

Tracking of tertile membership from one time slot to the next and from the first to the last time slot was done using Cohen's weighted kappa (κ_w), which takes into account the squared concordance of position among groups[26]. Stability is presented as the percentage of participants remaining in their tertile, while the increase and decrease categories represent change in tertile membership since the previous time slot or from the first to the last time slot. Since the weighted kappa procedure is not available in IBM SPSS Statistics, data from Crosstabs analysis and syntax from the IBM website[27] were used. According to Landis and Koch[28], a κ_w of 0·01–0·20 represents slight agreement, 0·21–0·40 fair agreement, 0·41–0·60 moderate agreement, 0·61–0·80 substantial agreement, and 0·81–1·00 almost perfect agreement.

Multinomial logistic regression was then conducted to assess factors associated with the odds of maintaining stable low (being in the lower tertile for the dietary behaviour at 9–12 months and at 21–24 months) *v.* stable high (being in the upper tertile for the dietary behaviour at 9–12 months and at 21–24 months) consumption patterns. The independent variables were WAMI index (divided into high (above the median, 0·703) and low (below median)), maternal age and parity. WAMI above the median value, maternal age above 25 years and single child were treated as reference for the independent variables. The analysis was performed comparing the first and last time slot only.

To account for possible changes in outcome variables due to seasonality, the date of each observation was recoded into pre-monsoon (March–May), monsoon (June–August), post-monsoon (September–November) and winter (December–February). One-way ANOVA or the Kruskal–Wallis test was performed, but no significant

differences between seasons were found for either of the outcome variables. Consequently, seasonality was left out from the multinominal regression analyses.

Results

The mean age of the mothers was 27·4 (SD 3·7) years, their mean number of years of education was 8·2 (SD 4·0), their mean parity was 1·7 (SD 0·8) children and the predominant caste was Newari (89·2 %). The mean WAMI index (range 0–1)[22] was 0·71 (SD 0·13), where all households had access to improved water and sanitation. Out of 240 infants included at baseline, 130 (54·2 %) were male (Table 2).

Nearly all children (>97 %) were breast-fed up to 20 months and the children were breast-fed frequently (about 10 times/d). The mean DDS increased gradually through the four time slots, and the prevalence of MDD increased from 40 % in the 9–12 months time slot to 65 % in the 21–24 months time slot. All children had received food from the grains/cereal category (data not shown) and the most common staple food was rice. The prevalence of MMF increased from 88 % in the 9–12 months time slot to 98 % in the final time slot. The prevalence of MAD increased from 39 % in the 9–12 months time slot to 64 % among breast-fed children aged 21–24 months, while the corresponding prevalence for non-breast-fed children aged 21–24 months was 26 %. Although increasing slightly with age, only one-third of recalls showed consumption of Fe- and vitamin A-rich foods in all four time slots (Table 3).

Table 2 Selected characteristics of mother–child pairs (*n* 231), Bhaktapur, Nepal, February 2011–November 2012

Characteristic*	
Mother's age (years), mean	27·4
SD	3·7
Family's caste	
Newar (%)	89·2
Chettri (%)	5·2
Others (%)	5·7
Parity, mean	1·7
SD	0·8
One child (%)	46·3
Two children (%)	41·6
Three or more children (%)	12·1
Number of assets†, median	6
Minimum, maximum	1, 8
Mother's education (years), mean	8·2
SD	4·0
Household income ($US)‡, median	151·2
Minimum, maximum	23, 720
WAMI§, mean	0·71
SD	0·13
Child's gender, male (%)	54·2

*Measured at 12 months age.
†Out of a total of eight assets as suggested by Psaki *et al.*[22].
‡Exchange rates from Oanda.com.
§WAMI (Water, Assets, Mother's education and Income) index as a measure of socio-economic status proposed by Psaki *et al.*[22].

Repeated-measures ANOVA with a Greenhouse–Geisser correction showed that mean DDS (F (2·87, 655·32) = 57 429, $P < 0·001$) and meal frequency (F (2·83, 645·68) = 189·8, $P < 0·001$) differed significantly between time points. Friedman's test showed a significant difference in rank between time slots for portions of Fe- ($P < 0·001$) and vitamin A-rich foods ($P = 0·006$; Table 4). The stability coefficients calculated by unadjusted GEE models for DDS (0·48) and meal frequency (0·53) were moderate, while for portions of Fe-rich (0·20) and vitamin A-rich foods (0·26), correlations were low (Table 5). Adjusting for WAMI made minor changes to the coefficients for DDS (0·44) and meal frequency (0·50), while the coefficients for Fe- and vitamin A-rich foods remained the same. Additional adjustment for maternal age, parity and child's gender made no further difference to the estimates (data not shown).

Tracking of tertile membership of DDS and meal frequency was moderate and stable from 9–12 months to 17–20 months, but increased slightly at 21–24 months (Cohen's $\kappa_w = 0·48$ and 0·56, respectively; Table 6). For intakes of Fe- and vitamin A-rich foods, tracking coefficients were for the most part fair and decreased throughout follow-up from 0·27 and 0·27 for the 13–16 months time slot to 0·22 and 0·19 for the 21–24 months time slot, respectively. Tracking between the first and last time slot was fair to moderate for DDS (0·40) and meal frequency (0·41), and fair for portions of Fe- (0·23) and vitamin A-rich (0·35) foods.

A low WAMI index significantly increased the odds of tracking of low DDS (OR = 3·31; 95 % CI 1·44, 7·60) and meal frequency (OR = 3·46; 95 % CI 1·54, 7·76) between the first and last time slot compared with tracking of stable high DDS and meal frequency (Table 7). There was also a borderline significant association between low WAMI and maintaining stable low intake of Fe-rich foods (OR = 2·68; 95 % CI 1·01, 7·17). The odds of stable low intake of Fe-rich foods was significantly higher for mothers with three or more children (OR = 7·29; 95 % CI 1·62, 32·8) compared with mothers with only one child. Finally, the odds of tracking of low meal frequency was significantly higher for mothers with three or more children (OR = 6·31; 95 % CI 1·89, 21·1) compared with mothers with only one child.

Discussion

The prevalence of MDD, MMF and MAD increased gradually through follow-up and was higher for MMF than for MDD for all time slots. Prevalence of MDD and MMF was higher than respective national (30 and 77 %)[29] and global (<33 and 50 %)[30] estimates, but comparable to findings in a recent study on children aged 6–23 months in the Kathmandu valley[31]. Since dietary diversity increases with age[32], the higher prevalence of MDD in our study may be caused by exclusion of children below 9 months old.

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Table 3 Selected characteristics of infant and young child feeding practices, by age, among 9- to 24-month-old children (*n* 924)*, Bhaktapur, Nepal , February 2011–November 2012

	Child's age (months)							
	9–12 (*n* 909)		13–16 (*n* 921)		17–20 (*n* 912)		21–24 (*n* 910)	
Characteristic	Mean or %	SD	Mean or %	SD	Mean or %	SD	Mean or %	SD
Breast-feeding (%)	99·9	–	99·9	–	97·3	–	78·1	–
Number of breast-feeds/d†	11·1	2·8	10·8	2·8	9·8	2·8	8·3	3·1
DDS	3·2†	1·1	3·5†	1·2	3·7†	1·2	3·9/4·1‖	1·2/1·2
MDD (≥4 food groups; %)	39·8	–	49·2	–	54·8	–	65·4	–
Meal frequency	4·3	1·6	4·9	1·9	5·5	2·0	6·2	2·0
MMF‡ (%)	87·8	–	92·7	–	95·0	–	98·2	–
MAD§ (%)	38·8†	–	48·6†	–	53·9†	–	64·1/25·9‖	–
Consumption of Fe-rich foods								
Meat, fish, poultry and liver/organ meats (%)	27·1	–	29·2	–	33·2	–	35·4	–
Consumption of vitamin A-rich foods								
Yellow fruits and vegetables & DGLV (%)	28·5	–	32·7	–	31·7	–	34·8	–

DDS, dietary diversity score; MDD, minimum dietary diversity; MMF, minimum meal frequency; MAD, minimum acceptable diet; DGLV, dark green leafy vegetables.
*Number of observations in a time slot, secondary recalls excluded.
†Only children breast-fed the previous day included in the analysis.
‡ ≥ 3 times/d for breast-fed and ≥4 times/d for non-breast-fed children.
§For breast-fed children: MDD and MMF; for non-breast-fed children: DDS ≥ 4/6, ≥2 portions of dairy and/or infant formula and ≥4 portions of dairy and/or infant formula and solid- or semi-solid foods.
‖Breast-fed/non-breast-fed.

Table 4 Dietary diversity, portions of iron-rich and vitamin A-rich foods and meal frequency* by age, among 9- to 24-month-old children, Bhaktapur, Nepal, February 2011–November 2012

	Child's age (months)								
	9–12 (*n* 231)		13–16 (*n* 231)		17–20 (*n* 230)		21–24 (*n* 229)		
	Mean or median	SD or min, max	Mean or median	SD or min, max	Mean or median	SD or min, max	Mean or median	SD or min, max	*P*†
DDS, mean and SD	3·3	0·8	3·6	0·9	3·8	0·9	4·1	0·9	<0·001
Portions of Fe-rich foods, median and min, max	0·25	0, 2·0	0·25	0, 2·0	0·5	0, 2·3	0·5	0, 2·0	<0·001
Portions of vitamin-A-rich foods, median and min, max	0·25	0, 2·3	0·25	0, 1·8	0·25	0, 2·0	0·5	0, 2 0	0·006
Meal frequency, mean and SD	4·3	1·2	4·9	1·4	5·5	1·5	6·2	1·5	<0·001

DDS, dietary diversity score.
*Average for each time slot calculated.
†*P* value for difference between time points calculated by one-way repeated-measures ANOVA or Friedman's test.

Table 5 Tracking of complementary feeding behaviours among 9- to 24-month-old children (*n* 229), Bhaktapur, Nepal, February 2011–November 2012

	Unadjusted model		Adjusted for WAMI	
	Coefficient*	95 % CI	Coefficient*	95 % CI
DDS	0·48	0·40, 0·56	0·44	0·35, 0·52
Portions of Fe-rich foods	0·20	0·12, 0·28	0·20	0·11, 0·29
Portions of vitamin-A-rich foods	0·26	0·18, 0·34	0·26	0·18, 0·35
Meal frequency	0·53	0·45, 0·61	0·50	0·42, 0·58

WAMI, Water, Assets, Mother's education and Income index; DDS, dietary diversity score.
*Stability coefficients calculated by general estimating equations (GEE) analysis.

Meanwhile, Bhaktapur is a peri-urban society with higher socio-economic status than the national average[33], which probably has a positive influence on child feeding practices. The high prevalence of MMF may be caused by the study design where snacks were recorded as meals. At the same time, previous research has revealed that compliance with adequate meal frequency is higher than for dietary diversity both in multi-country studies[5,13] and Nepal[5]. Finally, prevalence of and risk factors for MAD have in previous research been closely linked to MDD,

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Table 6 Tracking of complementary feeding practices between time slots among 9- to 24-month-old children, Bhaktapur, Nepal, February 2011–November 2012

	Child's age (months)																			
	9–12 (*n* 231)	13–16 (*n* 231)					17–20 (*n* 230)					21–24 (*n* 229)					21–24 *v.* 9–12 (*n* 229)				
	%*	%*	% D†	% S‡	% I§	κ_w‖	%*	% D†	% S‡	% I§	κ_w‖	%*	% D†	% S‡	% I§	κ_w‖	%*	% D†	% S‡	% I§	κ_w‖
Complementary feeding behaviour																					
DDS																					
Low tertile	29·0	27·7	46·9	53·1	NA		28·3	47·7	52·3	NA		32·8	45·3	54·7	NA		32·8	49·3	50·7	NA	
Middle tertile	33·3	35·9	33·7	39·8	26·5		37·4	33·7	38·4	27·9		29·3	29·9	44·8	25·4		29·3	43·3	31·3	25·4	
High tertile	37·7	36·4	NA	59·5	40·5	0·417	34·3	NA	54·4	45·6	0·407	38·0	NA	33·3	66·7	0·475	38·0	NA	54·0	46·0	0·404
Fe-rich foods																					
Low tertile	33·8	29·9	52·2	47·8	NA		26·5	49·2	50·8	NA		19·2	59·1	40·9	NA		19·2	47·7	52·3	NA	
Middle tertile	27·3	46·3	38·3	28	33·6		38·3	22·7	53·4	23·9		44·5	34·3	40·2	25·5		44·5	40·2	26·5	33·3	
High tertile	39·0	23·8	NA	58·2	41·8	0·271	35·2	NA	33·3	66·7	0·255	36·2	NA	45·8	54·2	0·217	36·2	NA	49·4	50·6	0·233
Vitamin A-rich foods																					
Low tertile	39·8	29·9	39·1	60·9	NA		29·0	53·7	46·3	NA		28·8	62·1	37·9	NA		28·8	40·9	59·1	NA	
Middle tertile	24·2	39·4	40·7	27·5	31·9		42·0	28·9	48·5	22·7		35·8	24·4	45·1	30·5		35·8	31·7	26·8	41·5	
High tertile	35·9	30·7	NA	47·9	52·1	0·273	28·6	NA	45·5	54·4	0·234	35·4	NA	39·5	60·5	0·194	35·4	NA	55·6	44·4	0·346
Meal frequency																					
Low tertile	38·5	29	28·4	71·6	NA		30·4	42·9	57·1	NA		31·9	41·1	58·9	NA		31·9	41·1	58·9	NA	
Middle tertile	24·2	36·4	34·5	34·5	31·0		32·6	29·3	48·0	22·7		35·4	32·1	43·2	24·7		35·4	32·1	28·4	39·5	
High tertile	37·2	34·6	NA	66·3	33·8	0·510	37·0	NA	61·2	38·8	0·501	32·8	NA	70·7	29·3	0·556	32·8	NA	62·7	27·3	0·405

DDS, dietary diversity score; NA, not applicable.
*Proportion of mother–child pairs with feeding behaviour at the time slot.
†Proportion of mother–child pairs with a decrease in feeding behaviour from the previous time slot.
‡Proportion of mother–child pairs with stable feeding behaviour since the previous time slot.
§Proportion of mother–child pairs with an increase in feeding behaviour from the previous time slot.
‖Tracking coefficient of Cohen's weighted kappa.

Table 7 Associations of tracking of low tertile of dietary diversity, intakes of iron- and vitamin A-rich foods and meal frequency with sociodemographic characteristics, by multinominal logistic regression, among 9- to 24-month-old children (*n* 229), Bhaktapur, Nepal, February 2011–November 2012

		Tracking stable low tertile of DDS, intake of Fe- and vitamin A-rich foods and meal frequency*											
		DDS			Fe-rich foods			Vitamin A-rich foods			Meal frequency		
Characteristic	*n*	OR†	95 % CI	*P*	OR†	95 % CI	*P*	OR†	95 % CI	*P*	OR†	95 % CI	*P*
WAMI													
Low‡	115	3·31	1·44, 7·60	0·001	2·68	1·01, 7·17	0·05	1·18	0·54, 2·62	0·68	3·46	1·54, 7·76	0·003
High	114	1·00	–		1·00	–		1·00	–		1·00	–	
Maternal age													
Low (19·0–24·9 years)	49	1·31	0·50, 3·44	0·58	1·65	0·54, 5·05	0·38	1·68	0·68, 4·14	0·26	0·71	0·28, 1·83	0·48
High (≥25·0 years)	180	1·00	–		1·00	–		1·00	–		1·00	–	
Parity													
Three or more children	28	3·05	0·86, 10·8	0·08	7·29	1·62, 32·8	0·010	1·09	0·31, 3·80	0·90	6·31	1·89, 21·1	0·003
Two children	96	1·68	0·70, 4·02	0·24	1·62	0·55, 4·78	0·38	1·00	0·43, 2·34	1·00	1·25	0·53, 2·98	0·61
One child	105	1·00	–		1·00	–		1·00	–		1·00	–	

DDS, dietary diversity score; WAMI, Water, Assets, Mother's education and Income index.
*Low tertile both at 9–12 and 21–24 months time slots.
†High tertile at both 9–12 and 21–24 months time slots used as the reference category.
‡WAMI below median value (7·03) at 12 months.

especially in populations with high meal frequency[34], as reflected in our data. The validity of meal frequency in explaining MAD may thus be questioned.

Our data showed that nearly all children (>97 %) were breast-fed up to 20 months and that breast-feeding frequency was high (about 10 times/d). Mothers in our study thus seem to comply with recommendations for on-demand breast-feeding up to 2 years age[8]. In our data, dietary diversity increased somewhat with age as breast-feeding frequency decreased, which is consistent with other studies[32,35]. Further, non-breast-fed children in our last time slot had slightly higher DDS than breast-fed children, a finding supported by others[36]. On the one hand, this seems to suggest that frequent breast-feeding in this age group may lower micronutrient adequacy. On the other hand, in most low- and middle-income countries, complementary foods are based on staples and contain only small amounts of key nutrients such as vitamin B_6, Fe and Zn[37]. Further research on the relationship between the quality of complementary foods and the amount of breast milk *v.* complementary foods that should be consumed by children in different settings to maximize nutrient intake is thus warranted.

We found moderate tracking for DDS and meal frequency using GEE models. In addition, tracking based on rank measures was moderate and stable for these variables up to 20 months and increased slightly at 21–24 months. We have not been able to find other studies investigating tracking of DDS during early childhood, but moderate correlations or tracking of dietary intake in children has been observed in Western populations[16]. Moderate tracking for DDS is expected since dietary practices are expected to change, likely at an uneven pace for different children, through these age groups. At the same time, a higher tracking coefficient for DDS than for separate food groups is likely since DDS reflects complete family food- and maternal dietary patterns, which have relatively stable determinants. Dietary intake in Bhaktapur is homogeneous[38], which may influence tracking positively. Finally, tracking coefficients for meal frequency are most likely influenced by daily routines. Moderate tracking for DDS and meal frequency implies that early interventions to improve these complementary feeding behaviours might have sustainable effects.

The low correlation coefficients and fair tracking coefficients (0·21–0·40) found for Fe- and vitamin A-rich foods may reflect that these foods are consumed only occasionally. Only about 30 % of observations showed consumption of Fe- or vitamin A-rich foods the previous day, which is comparable to other studies[5,39]. Low tracking of healthy dietary behaviours in this population compared with the study on Australian children[17] probably signifies that cultural and environmental factors influence intake in Nepal more than in Western settings. For instance, meat consumption has been shown to depend on religion and caste[40]. In addition, a previous study has shown that Nepali mothers may find animal-source foods unsuitable for young infants since they are perceived as difficult to digest[41]. Finally, vitamin A-rich green leafy vegetables are widely consumed in Bhaktapur, but mainly in winter and spring[33]. Fair tracking coefficients may therefore imply that time slots have been comprised of measurements made in different seasons. The low or fair tracking coefficients for intakes of Fe- and vitamin A-rich foods imply that early interventions to improve these complementary feeding behaviours might have limited sustained effect.

The odds of tracking of low (low tertile at both first and last time slot) compared with high DDS, intakes of Fe-rich foods and meal frequency were significantly higher in the low WAMI group. This is consistent with other findings[34], where children from poor households and children with

illiterate mothers were found to have poor complementary feeding practices. Low family income and education level are relatively stable determinants for mothers in our sample, increasing the odds of tracking low on complementary feeding practices. The role of maternal education for improved complementary feeding practices has been highlighted in previous studies from low- and middle-income countries[5,39]. While some researchers claim that this effect is conditioned by resource availability at household level[42,43], others point to the significant differences in nutritional status observed among children from the same households[44]. The higher odds of tracking of low intake of Fe-rich foods and meal frequency for mothers with three or more children may simply reflect the cost of feeding a large family compared with a smaller one.

Our data portray a nutrition situation typical of many low- and middle-income countries where cereals and grains are used as staple foods while nutrient-dense foods such as meat, fruits and vegetables are consumed sparingly[10,45]. The Nepal Demographic and Health Survey 2011 data showed a deterioration of complementary feeding practices among Nepali mothers since 2006[46]. At the same time, consumption of sweet snacks is a problem in Nepal[31] and may lead to preference for sweet taste and displacement of nutrient-rich foods[47]. Our findings imply that complementary feeding practices of 9- to 24-month-old children in Bhaktapur need to be improved. Education of mothers either alone or in combination with food supplements has in previous meta-analyses been linked to improved complementary feeding and child growth[48,49], and should be strengthened in this population. Mothers with low education level should be prioritized since they are a high-risk group for poor complementary feeding practices. Such education should stress the importance of acquiring nutrient-dense complementary foods to the extent possible and feeding children these foods[50] with adequate frequency[8] from the time when complementary feeding commences.

A major strength of the current study is the longitudinal design providing detailed data on the development of complementary feeding practices across a crucial period in children's lives. Four measurements within each time slot may increase the precision of estimates for complementary feeding performance. However, intake of Fe- and vitamin A-rich foods, which is more random than DDS and meal frequency, probably requires more measurements to be adequately assessed. Retention was almost complete throughout the study. Inclusion of children directly after birth is another strength, since according to the 2011 Nepal Demographic and Health Survey, only 29 % of children below 2 years of age possess birth certificates[29] and precise estimates of children's ages may be difficult.

The current study also had some limitations. Misreporting cannot be ruled out and previous research implies that over-reporting is more common than under-reporting in studies on young children[17]. Further, dietary diversity is attractive for its simplicity, adaptability to different settings[4,51] and its ability to reflect dietary adequacy[52]. The method is particularly beneficial in food cultures with a common food bowl such as Nepal, where precise estimation of intake is difficult[53], and is the preferred data collection method for young children over time[20]. However, previous articles show heterogeneity in the number of food groups used, the length of reference periods, which foods are grouped together and the application of intake limits, which makes direct comparisons of results difficult. In the current study, we chose to use seven food groups and a cut-off of ≥4 for dietary adequacy as recommended by FAO/WHO, since this is inherent in the MAL-ED protocol and enables cross-site comparisons. In addition, no minimum intake limit was used. This may result in a high DDS, which to a lesser degree reflects nutrient adequacy than if portion size requirements were included[51].

Tracking analysis has some potential pitfalls[24]. First, if the reproducibility of the outcome variable is poor, the tracking coefficient will also be low. The age group investigated in the current study may show low reproducibility because feeding practices are expected to change somewhat with food preferences and less breast-feeding. For instance, children are expected to fully adjust to family foods only after 12 months age[8]. This was a contributing factor in our choice to divide the population into tertiles instead of using fixed cut-offs for intake. At the same time, tertiles may be somewhat misleading, since high tertile membership does not necessarily reflect meeting recommendations. Closely spaced measurements in our study may influence tracking positively, but the time period is one where substantial changes in food intake are expected to occur. Finally, minor shifts at the borders of groups will influence the tracking coefficient, although such shifts may not be clinically relevant[24]. This was shown in our study where extremely low median intakes of Fe- and vitamin A-rich foods, and small differences in intake between tertiles, were found (Table 4).

Finally, the population of Bhaktapur represents a semi-urban population residing close to the capital Kathmandu, which may limit the generalizability to other populations in Nepal and South Asia.

In summary, tracking for DDS and meal frequency was moderate, while tracking for intakes of Fe- and vitamin-A rich foods was low among infants and toddlers in this peri-urban area in Nepal. The odds of tracking of low compared with high DDS, intake of Fe-rich foods and meal frequency was higher among mothers with low socio-economic status. Our data highlight the importance of educating mothers about feeding children nutrient-rich complementary foods, particularly those rich in Fe and vitamin A, on a regular basis from the time complementary feeding commences. Irregular intake and low tracking for intakes of Fe- and vitamin A-rich foods imply that a broader approach targeting reasons for not feeding these foods to children regularly should be applied.

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Paper 2

Severely inadequate micronutrient intake among children 9.24 months in Nepal – The MAL-ED birth cohort study

II

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ORIGINAL ARTICLE

Severely inadequate micronutrient intake among children 9–24 months in Nepal—The MAL-ED birth cohort study

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Abstract

Prevalence of micronutrient deficiencies is high among infants and children in low- and middle income countries, but knowledge about nutrient adequacy across the complementary feeding period is limited. We investigated probability of adequacy (PA) of breast milk and complementary food combined and nutrient density adequacy (NDA) of complementary food and tracking of NDA over time among 229 children from 9–24 months of age in Bhaktapur, Nepal. Monthly, 24 h dietary recalls (16 in total) were performed and subgrouped into four 4-month time periods. Ten micronutrients (thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin C, vitamin A, calcium, iron, and zinc) were assessed. Nutrient density was defined as the amount of a nutrient in a child's complementary food per 100 kcal, whereas NDA was the nutrient density as percentage of the context specific desired nutrient density. Tracking of NDA was investigated using generalized estimating equations models. PA for B vitamins (except riboflavin), vitamin A, calcium, iron, and zinc (low absorption group) was very low (0% to 8%) at all time slots. Median (IQR) mean PA (of all 10 micronutrients) increased from 11% (9, 15) in the second to 21% (10, 35) in the last time slot. Median value for mean nutrient density adequacy of all micronutrients varied between 42% and 52%. Finally, tracking of NDA was low (correlation <0.30) or moderate (0.30–0.60) indicating poor association between the first and subsequent measurements of NDA. These findings raise grave concerns about micronutrient adequacy among young children in Nepal. Urgent interventions are needed.

KEYWORDS

birth cohort, complementary feeding, infant and child nutrition, low income countries, micronutrients, Nepal

1 | INTRODUCTION

Micronutrient deficiencies are associated with infectious disease, cognitive impairment, and growth retardation in infants and children (Black et al., 2013). According to World Health Organization (WHO), infants should be exclusively breastfed until 6 months after which breast milk alone is no longer sufficient and complementary foods should be introduced (Pan American Health Organization/WHO, 2003). Nutrient requirements per kilogram bodyweight are high due to intensive growth and development in infancy (Dewey, 2013). In addition, children in low- and middle income countries (LMICs) have high susceptibility to intestinal infections and parasitic infestations, which decrease nutrient absorption and appetite (Dewey & Mayers, 2011; Ochoa, Salazar-Lindo, & Cleary, 2004; The MAL-ED Network Investigators, 2014). Because of limited gastric capacity and increased nutrients requirements of infants (Lutter & Rivera, 2003), complementary foods should be diverse with frequent consumption of nutrient dense foods such as meat, milk, eggs, and fruits and vegetables (WHO, 2001). At the same time, diets in LMICs are often cereal-based and monotonous (Dewey, 2013) where particularly iron, zinc, and calcium have been singled out as problem nutrients for children (Abeshu, Lelisa, & Geleta, 2016; Ferguson, Chege, Kimiywe, Wiesmann, & Hotz, 2015). These nutrients are found in foods consumed sparingly due to economic constraints (Neumann, Murphy, Gewa, Grillenberger, & Bwibo, 2007; Solomons & Vossenaar, 2013), and the high phytate and fibre content of cereal-based complementary foods may further inhibit mineral absorption (Gibson, Bailey, Gibbs, & Ferguson, 2010; Lonnerdal, 2000).

In Nepal, the diet is primarily based on rice, lentils, and seasonal vegetables with irregular consumption of meat, dairy, and fish (Ulak et al., 2016). The most commonly consumed home-made complementary foods in the Kathmandu valley is jaulo (lentils and rice mashed together) and lito (roasted grain/lentil flour cooked with water or milk), whereas few (<15% of children 6–23 months) consume industrially produced infant cereals (Pries et al., 2016). The diet is usually monotonous, and prevalence of micronutrient deficiencies among children ranges from 6% to 59% depending on the nutrient (Bhandari & Banjara, 2015; Ulak et al., 2016). The government runs a vitamin A supplementation program where children 6–59 months are supplemented twice a year and provides zinc supplementation to children with diarrhoea (Bhandari & Banjara, 2015). The national prevalence of stunting (41%) and wasting (29%) among children under 5 years of age is high (Ministry of Health and Population (MOHP) [Nepal]/ New ERA/ ICF International Inc, 2012), whereas the prevalence in Bhaktapur District is considerably lower at 19% and 7%, respectively (Shiwakoti, Devkota, & Paudel, 2017).

In a previous paper, we found low to moderate (3–4 out of 7) dietary diversity score (DDS) in our sample (Morseth et al., 2017). Tracking, measuring the stability of behaviour over time (Kelder, Perry, Klepp, & Lytle, 1994), was moderate for DDS but low for intake of iron- and vitamin A-rich food (Malina, 2001; Morseth et al., 2017). Although DDS is strongly associated with nutrient adequacy (Moursi et al., 2008; Working Group on Infant and Young Child Feeding Indicators, 2006), probability of adequacy (PA) based on usual intake is preferable to evaluate the probability of meeting nutrient requirements (Dodd et al., 2006). Few previous studies assess micronutrient adequacy in the same cohort of children across the complementary feeding period, and no previous studies, to our knowledge, evaluate PA longitudinally among children below the age of two. The objectives of this study were to assess PA calculated from estimated breast milk intake and complementary foods and nutrient density adequacy (NDA) of complementary foods in up to four time slots and to assess tracking of NDA among children 9–24 months in Bhaktapur, Nepal.

Key messages

- For most micronutrients, probability of adequacy was extremely low and increased slightly with age, whereas nutrient density adequacy varied greatly between nutrients and increased with age in this group of children aged 9–24 months.
- Both probability of adequacy and nutrient density adequacy identified iron, zinc, vitamin A, calcium, and niacin as main problem micronutrients.
- Health authorities should support families through education about the importance of feeding their children high quality complementary foods in adequate amounts and with adequate frequency.

2 | METHODS

2.1 | Study design and study population

Participants were children 9 to 24 months living in Bhaktapur, a peri-urban agriculture-based community situated 15 km east of Kathmandu, the capital city of Nepal. All children were enroled within 17 days of birth. Children with very low birth weight (<1500 g) were excluded. Enrolment started in June 2010, and data collection took place between February 2011 and November 2012. Out of 240 enroled infants, 229 had complete nutritional data at 24 months. Four time slots (9–12, 13–16, 17–20 and 21–24 months) were used. The MAL-ED Nepal cohort received ethical approval from the Nepal Health Research Council and Walter Reed Institute of Research (Silver Springs, Maryland), and all participants signed informed consent forms prior to participation. Further details on design and methodology are reported elsewhere (The MAL-ED Network Investigators, 2014).

2.2 | Dietary data collection

Monthly, 24-hr recalls (16 in total) were performed to collect data on foods and amounts consumed the previous day. Local fieldworkers were trained by experts in dietary recall technique through initial 3-day training sessions with periodic 1-day refresher sessions (Caulfield et al., 2014). Household utensils, portion size booklets, and play dough were used to estimate amounts. Details on recipes were also collected. To enable assessment of within-subject variation and increase the precision of estimated intakes, secondary recalls within 1 week of the original recall were conducted for each child once during the 16 months follow-up period (Caulfield et al., 2014).

2.3 | Dietary data analysis

To estimate nutrient intake, the Food and Agriculture Organization of the United Nations (FAO) International Network of Food Data Systems database for Asia (FAO, 2014) was used. When needed, supplementary nutrient values from other food composition tables, for instance, US Food composition table (United States Department of Agriculture), NUTTAB (Food Standards Australia New Zealand, 2010), and CoFIDS (Public Health England), were used.

PA for nutrient intake was calculated using the Institute of Medicine (IOM) probability approach (IOM, 2000a) based on the total nutrient intake from complementary foods and from breast milk. Breast milk intake was not measured but estimated based on the assumption that there is an inverse relationship between energy intake from complementary foods and energy intake from breast milk (Kimmons et al., 2005). First, the energy received from complementary foods was subtracted from the total energy requirements with moderate physical activity level (kilocalorie per kilogram per day) estimated by FAO (2004) for each child. The FAO energy requirement (kilocalorie per kilogram) for the appropriate age was multiplied by each child's body weight, which was measured monthly. The assumed energy needed from breast milk was then divided by the energy density of breast milk in developing countries (0.63 kcal/g) as described by WHO (1998) in order to estimate the assumed amount of breast milk consumed (converted from grams to litres). For each

nutrient, this amount was multiplied by the amount of nutrient in mature breast milk. For participants who consumed more than their energy requirement from complementary food, estimated nutrient intake from breast milk was set to zero. Because the breast milk content of thiamin, riboflavin, vitamin B_6, and vitamin A depends on maternal status (WHO, 1998), and PA of these nutrients among breastfeeding women in Bhaktapur is low (Henjum et al., 2015), nutrient values found among women in LMICs were used. These were 0.16 mg/L for thiamin, 0.22 mg/L for riboflavin, 0.10 mg/L for vitamin B_6 (Allen, 2012), and 227 μg/L for vitamin A (Rice et al., 1999). For the remaining nutrients, WHO's values were used (WHO, 1998). Finally, the estimated amount of nutrients consumed from breast milk was added to the amount consumed from complementary food for total intake for each child.

Due to uncertainty concerning requirements for children <12 months (Dewey & Brown, 2003; Moursi et al., 2008), PA was calculated only for the 13 to 24 month age groups. Also, because the content of vitamin B_{12} in breast milk decreases with child's age (Hampel & Allen, 2016) and few previous studies to our knowledge have assessed the content of vitamin B_{12} in breast milk fed to children >12 months, we decided to exclude vitamin B_{12} from our analysis. As a result, PA was calculated for 10 micronutrients: thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin C, vitamin A, calcium, iron, and zinc. Estimated average requirements (EARs) were based on a back calculation from FAO/WHO (2002) reference nutrient intakes (RNIs). RNI is defined as EAR + 2 SD_{EAR} (FAO/WHO, 2002). EARs were calculated using the variability coefficients from IOM, namely, 15% for niacin, 20% for vitamin A, 25% for zinc, and 10% for the remaining nutrients (IOM, 2000c, 2000b, 2001). For participants with a mean phytate:zinc ratio > 15 for the time slot, requirements based on low absorption were used (WHO/FAO/International Atomic Energy Agency, 2002), otherwise requirements were based on medium absorption. Because iron requirements are skewed (IOM, 2000a), adequacy was estimated based on Table I-5 in the IOM report on iron requirements (IOM, 2001). The 5th percentile for phytate:iron ratio in our population was three, whereas the recommended ratio is <1 (Gibson et al., 2010). Probability of adequate iron intakes was therefore assessed by comparing participant's intakes with a matrix for iron requirements consistent with very low absorption (5%; Table S1).

The probability (%) of adequate nutrient intake was assessed based on mean usual intakes calculated from four recalls in each time slot or five recalls for time slots with secondary recalls. Skewed nutrient intakes were transformed using a Box–Cox transformation. After calculating within- and between person variance, the best linear unbiased predictor (BLUP) for usual intake was calculated for each nutrient for each child. A BLUP minimizes the prediction error variance and shrinks the individual means of a variable towards the group-level means. BLUPs were then used to estimate PA for each micronutrient. Mean probability of adequacy (MPA) was calculated using PA across all micronutrients for each participating child. Due to highly skewed data, median values for MPA are presented.

Nutrient density (ND) and NDA of complementary foods were calculated based on methodology by the Working Group on Infant and Young Child Feeding Indicators (Working Group on Infant and Young Child Feeding Indicators, 2006), for the same 10 micronutrients. The nutrient densities of complementary food were defined as the amount of nutrient consumed per 100 kcal of complementary food. For each time slot and for each nutrient, desired nutrient densities were calculated the following way:

$$[\text{RNI of nutrient } \chi - (\text{concentration of nutrient } \chi \text{ in breastmilk} \times \text{median breast milk intake in time slot}) / \text{median energy intake from complementary food in time slot}] * 100.$$

Requirements, amounts of micronutrients in breast milk, and absorption rates for iron and zinc were similar to those used when calculating PA. For recalls where the child had not been breastfed FAO/WHOs (2002) requirements were divided by median energy intake in the nonbreastfed group. Individual NDs were calculated as mean of four measurements within each time slot. NDAs were calculated for each nutrient for each observation as the ND as percentage of the desired nutrient density. Further, individual NDAs were calculated as mean NDA of four recalls within each time slot. Finally, mean nutrient density adequacy (MNDA) was calculated as the mean of individual NDAs for all 10 micronutrients each capped at 100%. Due to skewed data, median values are reported.

2.4 | Socioeconomic status

Socioeconomic status was measured at 12, 18, and 24 months by the WAMI index (Psaki et al., 2014). The WAMI index, a measure of socioeconomic status developed for MAL-ED, ranges from 0 to 1 and consists of the following variables: **W**ater and sanitation, **A**ssets, **M**aternal education, and **I**ncome. The eight assets included are separate room for a kitchen, household bank account, mattress, refrigerator, TV, people per room (mean), table, and chair or bench (Psaki et al., 2014). In our sample, all households had access to improved water and sanitation.

2.5 | Statistical methods

Statistical Package for Social Science version 23.0 and STATA version 14.0 were used to analyse data. The significance level was 0.05. Continuous data were presented as mean and standard deviation if normally distributed, and as medians if not normally distributed.

Generalized estimating equations (GEE) models with unstructured correlation structure were used to calculate stability (tracking) coefficients for NDA across all four age intervals. In a GEE model, the value of the outcome variable at Time 1 is regressed on the longitudinal development of the outcome variable from Time 2 to Time m (number of measurements). This provides one stability coefficient taking into account that measurements within one individual are correlated and may be adjusted for both time dependent and time independent covariates (Twisk, 2003). Models were adjusted for WAMI measured at 12 months. Stability correlation coefficients of <0.30 were classified as low, 0.30 to 0.60 as moderate, and >0.60 as moderately high (Malina, 2001).

3 | RESULTS

The mothers were on average 27 (SD 4) years old, and the average number of years of education was 8 (SD 4). The average WAMI-index in our sample was 0.7, representing middle to high socioeconomic status in a Nepali context. Fifty-four percent of participants were male.

Data on breastfeeding and complementary feeding are presented in Table 1. Breastfeeding was frequent (about 10 times per day), and almost all children (> 97%) were breastfed up to 21 months. The estimated median (IQR) percent of energy intake from breast milk out of total energy intake decreased from 65% (48, 79) in the first to 35% (15, 55) in the last time slot.

Micronutrient usual intakes and PA are presented in Table 2. Mean usual intake was below the EAR for all nutrients, apart from vitamin C and riboflavin, across all time slots. PA for B vitamins (except riboflavin), vitamin A, calcium, iron, and zinc (low absorption group) was very low, ranging from 0% to 8% for all time slots. There was a marked increase in PA for riboflavin from 4% to 87% and zinc (medium absorption group) from 12% to 47% through time slots, whereas PA for vitamin C decreased from 100% to 54% in the last time slot. For the remaining nutrients, there were small or marginal improvements in PA. Median (IQR) MPA increased from 11% (10, 15) to 14% (10, 24), and finally, 21% (10, 35) through the three time slots compared. Corresponding numbers when excluding vitamin C were 1.6%, 7%, and 17%, respectively (data not shown).

ND and NDA of complementary food are presented in Table 3. The lowest median NDAs at baseline were 4.3% for iron, 17% for zinc, 22% for vitamin A, and 31% for calcium and niacin. Median NDA for iron, zinc, and vitamin A increased gradually through time slots to 17%, 43%, and 39% in the last time slot, respectively. NDA for calcium and niacin dropped slightly between the first and the second time slot then increased to 52% and 43% in the last time slot, respectively. The same pattern was seen for thiamin and folate. The highest median NDA was found for vitamin C, with values above 100% for the first three time slots. The only significant difference ($p < .05$) in ND between recalls with and without breastfeeding was found for calcium (data not shown). Median MNDA decreased from 42% in the first to 39% in the second time slot then increased to 52% in the last time slot.

Tracking of NDA is presented in Table 4. The stability coefficients calculated by GEE models adjusted for WAMI were low for thiamin (0.27), niacin (0.22), vitamin B_6 (0.12), vitamin C (0.21), vitamin A (0.25), and iron (0.28). For the remaining nutrients, stability coefficients were moderate, with the highest value found for calcium (0.47). The stability coefficient for MNDA was 0.27.

4 | DISCUSSION

In this longitudinal study of micronutrient adequacy among infants and young children in Nepal, we found that PA was extremely low for most micronutrients, apart from vitamin C and riboflavin, whereas NDA varied greatly between nutrients and was lowest for iron, vitamin A, and zinc. Tracking of NDA was low or moderate.

The low median PA observed for most micronutrients reflects poor quality of complementary food and inadequate nutrient content in breast milk to cover the nutrient gap. Although the proportion of energy intake from complementary foods compared to breast milk should increase with age, the optimal balance will depend on the quality of complementary foods available and will differ in various settings (Dewey & Brown, 2003). In one study on 6–12 months old children in Bangladesh, the intake of 100 additional kcal of complementary foods led to a small increase in adequacy for iron, calcium, zinc, and riboflavin, and a small decrease for vitamin C (Kimmons et al., 2005). The same trends are reflected in our data. The median of MPA in our study was lower than MPA in 24–71 month old nonbreastfeeding Filipino children (Kennedy, Pedro, Seghieri, Nantel, & Brouwer, 2007). Also, PA for all nutrients, apart from vitamin C and calcium, and MPA were higher in a study on children 24–48 months in Bangladesh compared to our findings (Arsenault et al., 2013). No previous studies, to our knowledge, assess PA in the same age group as our study.

The drop in NDA between the first and second time slot for many nutrients is likely caused by increased requirements (FAO/WHO, 2002) and low energy intake from complementary food in the second time slot, causing high desired nutrient densities. The high NDA for vitamin C may be caused by a combination of fruit intake and fruit juice intake and low desired NDs due to high vitamin C content in breast milk. ND was overall lower in our study (first time slot) compared to ND for infants 10–12 months (Campos, Hernandez, Soto-Mendez, Vossenaar, & Solomons, 2010) and lower for all nutrients apart from riboflavin (second time slot) than among infants 12–15 months in Guatemala (Dewey & Brown, 2003). Further, ND in our study (first time slot) was higher for all nutrients apart from thiamin, niacin, vitamin A, and iron than ND among infants 9–11 months in Bangladesh (Dewey & Brown, 2003). Finally, mean micronutrient density adequacy was higher (66–67 vs. 42–52%) among children 9–23 months in Madagascar than in our study (Moursi et al., 2008). Meanwhile, none of these previous studies used context specific NDA. Our desired NDs were generally higher than those proposed by Dewey and Brown (2003). Direct comparisons should thus be made with caution.

The problem nutrients identified by both PA and NDA were primarily iron, vitamin A, zinc, calcium, and niacin, comparable to other studies (Campos et al., 2010; Dewey & Brown, 2003; Fahmida & Santika, 2016; Vossenaar, Hernandez, Campos, & Solomons, 2013). For iron and zinc in particular, small amounts are found in breast milk (WHO, 1998), and requirements are high, especially for children <12 months. In addition, bioavailability is a significant problem (Gibson et al., 2010; Lonnerdal, 2000), which was clearly demonstrated by differences in PA between groups with different absorption rates for zinc in our data. During complementary feeding, meat is an invaluable source of readily absorbed iron and zinc (Hambidge et al., 2011). An intervention study showed that 86% of 9 months old infants met EAR for zinc when meat intake was 60 g/day (Krebs et al., 2012). Such high intakes imply that meeting requirements through consumption of family foods alone seems near impossible (Dewey, 2013; Krebs et al., 2012; Solomons & Vossenaar, 2013), especially in the youngest age groups. Meat intake in our population is probably limited by cost (Dewey, 2013) and religious and cultural factors (Siegel et al., 2006). In addition, animal source foods are not commonly served to the youngest children in Nepal,

TABLE 1 Breastfeeding and energy intake from complementary food, children 9–24 months, Bhaktapur, Nepal (n = 909)

	9–12 months	13–16 months	17–20 months	21–24 months	p-value
Children being breastfed, %	99.9	99.9	97.3	78.8	
Breastfeeding frequency (times/day), mean (SD)	11 (3)	11 (3)	10 (3)[a]	8 (3)[a]	<.001[b]
Total energy needs (kcal), mean (SD)[c]	662 (81)	742 (90)	794 (93)	844 (95)	<.001[d]
Energy intake from food (kcal), median (IQR)	227 (137, 342)	316 (208, 451)	424 (274, 580)/871 (694, 1201)[e]	542 (383, 719)/ 873 (651, 1161)[e]	<.001[b]
Estimated amount of breast milk consumed (L), median (IQR)[f]	0.68 (0.50, 0.84)	0.67 (0.44, 0.86)	0.60 (0.33, 0.80)	0.47 (0.20, 0.75)	<.001[b]
Estimated percent energy intake from breast milk out of total energy intake, median (IQR)[a]	65 (48, 79)	57 (39, 73)	48 (28, 65)	35 (15, 55)	<.001[b]

Note. Calculations based on total number of observations within each time slot.

Parts of this table has been presented previously.

[a]Only children breastfed the previous day included.

[b]Friedman's test.

[c]Estimated based on FAO (2004) energy requirements and body weight measured monthly.

[d]Repeated measures ANOVA.

[e]Breastfed/non-breastfed children.

[f]Calculated only if assumed amount of breast milk >0. Energy density of breast milk based on WHO (1998).

due to a common perception that these foods are hard to digest (Chandyo et al., 2016).

Tracking of NDA was low or moderate, which indicates that nutrient density of complementary food was not very stable within our participants over time (Kelder et al., 1994). Little is known about tracking of dietary intake for infants and young children (Robinson et al., 2007), but moderate tracking for dietary behaviours has been observed among children and from childhood to adolescence in Western populations (Madruga, Araujo, Bertoldi, & Neutzling, 2012). Tracking of micronutrient intake between 9 and 18 months among Australian children was slightly higher for vitamin A and niacin but lower for minerals, B-vitamins, and vitamin C than in our sample (Lioret, McNaughton, Spence, Crawford, & Campbell, 2013). On the one hand, tracking of nutrient density in our age group might be low due to changes in types of foods that are introduced with increasing age. On the other hand, closely spaced observations increases the likelihood of higher tracking coefficients (Twisk, 2003). The lowest tracking was seen for vitamin B_6 and vitamin A. For vitamin A, this may in part be due to seasonal variations in intake of green leafy vegetables (Shrestha et al., 2014).

In general, our results paint a very bleak picture regarding micronutrient adequacy among young children in Nepal. Bhaktapur is a peri-urban society with higher socioeconomic status than national averages (Shrestha et al., 2014), and it is likely that nutrient adequacy is higher here than in other regions. There is thus an urgent need for measures to improve the situation. A study on complementary feeding practices among Nepali mothers showed that only 16% fed their children appropriate complementary foods in adequate amounts and with adequate frequency (Chapagain, 2013). Advice about feeding the most nutrient dense foods in the household (Dewey & Mayers, 2011) in adequate amounts and with adequate frequency to the infants should thus be given before and during the period of complementary feeding. In addition, fortification (Arsenault et al., 2010) or dephytinization (Gibson et al., 2010) of cereals either at home or industrially may also be viable options to increasing the amounts of absorbed iron and zinc.

The major strength of this study is the longitudinal design and the large number of dietary recalls performed, providing estimates for usual intakes that account for between- and within subject variance (Dodd et al., 2006). The number of dietary measurements (4–5 in each time slot) seems adequate to describe nutrient intake in this age group, where intraindividual variation is usually small (Lanigan, Wells, Lawson, Cole, & Lucas, 2004; Piernas, Miles, Deming, Reidy, & Popkin, 2016). Another strength of the study is the inclusion of children directly after birth, because few children <2 years possess birth certificates (Ministry of Health and Population (MOHP) [Nepal]/ New ERA/ ICF International Inc, 2012), and reporting of exact age may be challenging. Finally, retention throughout the study was nearly complete at 95%.

The major weakness of our study was lack of data on the amount of breast milk consumed and the amount of nutrients in breast milk. Our method for calculating PA and NDA, using the amount of nutrients from breast milk based on total energy needs, is admittedly not ideal. However, given the substantial variation in energy intake from complementary foods and frequent breast feeding observed, this method was deemed superior to using average amounts of breast milk intake for the whole group as proposed by WHO (1998). Also, others

TABLE 2 Micronutrient usual intake and probability of adequacy, children 12–24 months, Bhaktapur, Nepal (*n* = 229)

		13–16 months			17–20 months			21–24 months		
	Requirement[a] EAR (SD)	Usual intake according to BLUP[b] Mean (SD)	Median	PA[b] % Median (IQR)	Usual intake according to BLUP Mean (SD)	Median	PA % Median (IQR)	Usual intake according to BLUP Mean (SD)	Median	PA % Median (IQR)
Nutrient										
Thiamin (mg/d)	0.4 (0.04)	0.23 (0.06)	0.22	0 (0, 0.1)	0.26 (0.07)	0.25	0 (0, 1.4)	0.3 (0.09)	0.29	0.6 (0, 20)
Riboflavin (mg/d)	0.4 (0.04)	0.34 (0.1)	0.31	3.8 (0, 62)	0.39 (0.13)	0.36	31 (4.7, 96)	0.47 (0.19)	0.44	87 (4.1, 100)
Niacin (mg/d)	4.6 (0.69)	2.2 (0.4)	2.1	0 (0, 0.1)	2.5 (0.5)	2.4	0 (0, 0.8)	2.9 (0.7)	2.7	0.4 (0.1, 3.1)
Vitamin B_6	0.4 (0.04)	0.24 (0.08)	0.22	0 (0, 0.1)	0.29 (0.1)	0.28	0.5 (0, 23)	0.36 (0.14)	0.32	8.4 (0.1, 76.9)
Folate (µg/d)	133 (13.3)	91.9 (18.1)	90.2	0 (0, 0.5)	95.9 (19.7)	94.5	0 (0, 2.1)	101.3 (23)	98.6	0.2 (0.1, 6.1)
Vitamin C (mg/d)	25 (2.5)	31.7 (8.4)	31.5	99 (38, 100)	31.6 (9.1)	31.5	100 (31.4, 100)	28.1 (9.7)	28.4	54 .3 (1.4, 100)
Vitamin A (µg/d)	286[c] (57.1)	52.8 (42.5)	43.5	0 (0, 0)	77 (52.5)	65.6	0 (0, 0.8)	112.1 (75.2)	101.5	0.4 (0, 16)
Calcium (mg/d)	417 (41.2)	291 (69)	286	0.1 (0, 2.5)	305 (81)	292	0.1 (0, 4.4)	337 (101)	329	0.9 (0.1, 30.8)
Iron (mg/d)	NA[d]			0 (0, 0)			0 (0, 0)			0 (0, 0.1)
Zinc $(mg/d)_{low}$[1]	5.6[e] (1.4)	1.8 (0.4)	1.7	0.8 (0.4, 0.9)	2 (0.4)	2	0.4 (0.3, 0.8)	2.3 (0.6)	2.1	0.5 (0.2, 1)
Zinc $(mg/d)_{medium}$[2]	2.7[e] (0.7)	1.9 (0.4)	1.9	12 (7, 24)	2.2 (0.5)	2.2	27 (13, 44)	2.6 (0.6)	2.5	47 (29, 68)
MPA, median (IQR)				11 (10, 15)			14 (10, 24)			21 (10, 35)

Note. EAR = estimated average requirement; PA = probability of adequacy; BLUP = best linear unbiased predictor.

[a]Based on RNIs from FAO/WHO Vitamin and Mineral Requirements (2002). EAR back calculated with a variability coefficient of 15% for niacin, 20% for vitamin A, 25% for zinc, and 10% for the remaining nutrients (IOM). PA for iron based on Table I-5 in IOM (2001) iron requirements report.

[b]Based on four recalls (or five if secondary recall in time slot).

[c]From British Nutrition Foundation (2014), UK Nutrition requirements.

[d]Iron requirements are not normally distributed, PA calculated by comparing usual intake to Table I-5 from IOM (2001) iron requirements report converted to 5% bioavailability (Table S1).

[e]EAR and SD based on low (phy:zn ratio > 15) and median zinc absorption respectively.

[1]*n* = 129 (13–16), 117 (17–20) and 103 (21–24 months) respectively.

[2]*n* = 101 (13–16), 112 (17–20) and 126 (21–24 months) respectively.

TABLE 3 Median nutrient density and nutrient density adequacy of complementary food intake, children 9 to 24 months, Bhaktapur, Nepal (n = 229)

	9–12 months			13–16 months			17–20 months[a]			21–24 months			
										Desired ND			
	Desired ND[b]	ND[c]	NDA median (IQR)	Desired ND	ND	NDA median (IQR)	Desired ND	ND	NDA median (IQR)	BF	NonBF[e]	ND[d]	NDA median (IQR)
Nutrient													
Thiamin (mg/100 kcal)	0.08	0.04	49 (35, 61)	0.1	0.04	41 (31, 50)	0.1	0.04	46 (34, 57)	0.08	0.06	0.04	52 (40, 67)
Riboflavin (mg/100 kcal)	0.1	0.06	58 (39, 85)	0.1	0.06	60 (39, 82)	0.09	0.06	67 (52, 89)	0.07	0.06	0.06	94 (64, 127)
Niacin (mg/100 kcal)	1.3	0.40	31 (25, 37)	1.6	0.4	26 (21, 33)	1.2	0.43	36 (30, 45)	0.1	0.7	0.37	43 (36, 54)
Vitamin B_6 (mg/100 kcal)	0.1	0.06	56 (43, 72)	0.1	0.06	56 (44, 70)	0.1	0.06	61 (46, 77)	0.08	0.06	0.05	72 (58, 98)
Folate (µg/100 kcal)	9.8	10.6	111 (81, 159)	33	11.4	36 (28, 47)	26	11	44 (35, 57)	22	18	10.3	51 (40, 66)
Vitamin C (mg/100 kcal)	1.2	1.3	119 (79, 199)	1.1	1.6	196 (108, 345)	1.4	1.9	156 (86, 251)	2.1	3.4	1.2	79 (49, 128)
Vitamin A (µg/100 kcal)	87	19	22 (13, 33)	79	20.5	26 (16, 36)	62	19.9	33 (23, 45)	55	46	19.4	39 (29, 55)
Calcium (mg/100 kcal)	93	29.3	31 (17, 48)	100	28.7	30 (17, 45)	78	30.3	39 (25, 54)	68	57	30.4/36.4[f]	52 (35, 69)
Iron (mg/100 kcal)[g]	8.3	0.36	4.3 (3.2, 5.7)	3.7	0.36	10 (8, 13)	2.8	0.37	13 (11, 16)	2.2	1.4	0.33	17 (14, 21)
Zinc (mg/100 kcal)	3.3/1.5[h]	0.36	17 (12, 23)	2.4/1	0.34	24 (17, 32)	1.8/0.8	0.33	32 (23, 42)	1.5/0.7	1/0.5	0.33	43 (31, 53)
MNDA[i], median (IQR)			42 (35, 49)			39 (33, 45)			44 (38, 51)				52 (43, 58)

Note. ND = nutrient density; NDA = nutrient density adequacy; BF = breastfed; Non-BF = nonbreastfed; MNDA = mean nutrient density adequacy.

[a]Calculated only for breastfed children.

[b]Based on RNIs from FAO/WHO Vitamin and Mineral Requirements (2002), median breast milk intake and median contribution of energy (kilocalorie) from complementary foods for time slot.

[c]Median values reported.

[d]Based on all observations within time slot.

[e]Based on RNIs from FAO/WHO and median amount of energy (kilocalorie) from recalls where child was breastfed.

[f]Breastfed/nonbreastfed.

[g]Requirements corresponding to 5% absorption rates used in calculation.

[h]Requirements based on low (phy:zn ratio > 15) and medium absorption used in calculation.

[i]Average NDA for all 10 micronutrients, each capped at 100%.

TABLE 4 Tracking of nutrient density adequacy of complementary food intake, children from 9 to 24 months, Bhaktapur, Nepal (n = 229)

Nutrient	Coefficient[a] (CI)
Thiamin (mg/100 kcal)	0.27 (0.18, 0.35)
Riboflavin (mg/100 kcal)	0.38 (0.28, 0.48)
Niacin (mg/100 kcal)	0.22 (0.15, 0.29)
Vitamin B_6 (mg/100 kcal)	0.12 (0.04, 0.20)
Folate (μg/100 kcal)	0.37 (0.25, 0.49)
Vitamin C (mg/100 kcal)	0.22 (0.07, 0.36)
Vitamin A (μg/100 kcal)	0.25 (0.16, 0.34)
Calcium (mg/100 kcal)	0.47 (0.37, 0.57)
Iron (mg/100 kcal)	0.28 (0.19, 0.36)
Zinc (mg/100 kcal)	0.33 (0.28, 0.42)
MNDA[b]	0.27 (0.15, 0.38)

Note. Generalized estimating equations analysis, adjusted for WAMI.

[a]Tracking coefficients calculated by general estimating equations.

[b]Average nutrient density adequacy for all 10 micronutrients, each capped at 100%.

have estimated energy intake from complementary food (Dewey & Brown, 2003; Kimmons et al., 2005) and breast milk (Mallard et al., 2016) based on total energy requirements similar to our approach. A previous study showed that infants aged 9–12 months in Bangladesh consumed slightly more (105%) energy than their requirements per body weight but slightly less (94%) energy than their requirements per ideal body weight for length (Kimmons et al., 2005). Although it seems unrealistic to assume that all our participants cover their energy needs, monthly anthropometric measurements in our view improve the validity of our imputation method for breast milk intake because the estimated energy need of a growth faltering child was reduced the subsequent month. Finally, our estimated intakes from breast milk and complementary foods resemble estimates for children with high breast milk consumption reported by Dewey and Brown (2003).

To our knowledge, no data exist on the content of the 10 micronutrients assessed in breast milk of Nepali women. For nutrients known to vary with maternal status (Allen, 2012), we assumed a breast milk content comparable to that of women from other LMICs (i.e., India and Bangladesh; Allen, 2012; Rice et al., 1999). Based on low probability of micronutrient adequacy among breastfeeding women in Bhaktapur (Henjum et al., 2015), it is probable that our estimates are more valid than if WHO values based on breast milk from western women (WHO, 1998) had been used. Finally, over-reporting of intake has been found to be more common than under-reporting in this age group (Lioret et al., 2013). This could have led to lower estimated energy and nutrient intakes from breast milk when calculating PA and lower desired nutrient densities resulting in slightly higher NDA.

The severe micronutrient inadequacy demonstrated, especially for iron, vitamin A and zinc among Nepali children 9–24 months in our study reflect low micronutrient content of complementary food and inadequate content in breast milk to cover the nutrient gap. In light of the potentially severe consequences for health, growth, and cognitive development, health authorities should urgently consider measures to improve micronutrient intake in this age group. Educating mothers about the importance of feeding children nutrient dense complementary foods is an important first step. However, because achieving micronutrient adequacy consuming local foods or/and breast milk seems improbable, micronutrient supplementation of breastfeeding women and dephytinization or fortification of foods commonly consumed by breastfeeding women and children must also be considered.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

CONTRIBUTIONS

MSM performed the statistical analysis and drafted the manuscript. LET and SH assisted with statistical analysis and contributed to revising the manuscript for intellectual content. RKC, MU, and SKS supervised and contributed to data aquisition. BS was in charge of data management. AHP conducted GEE analysis. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Supplemental table 1

PA[a]	13-24 mo
0	< 3.96[b]
0.04	3.96 - 4.49
0.08	4.50 - 5.57
0.15	5.58 - 7.08
0.25	7.09 - 8.38
0.35	8.39 - 9.60
0.45	9.61 - 10.86
0.55	10.87 - 12.23
0.65	12.24 - 13.78
0.75	13.79 - 15.79
0.85	15.80 - 18.93
0.92	18.94 - 21.84
0.96	21.85 - 24.52
1	>24.52

Table converted from table 1-5 in IOM iron requirements report (IOM, 2001)

[a] Probability of adequacy

[b] Iron requirements (mg/d) with 5% absorption rate

Paper 3

Environmental enteropathy, micronutrient adequacy and length velocity in Nepalese children – the MAL-ED birth cohort study

Status: Accepted in Journal of Paediatric Gastroenterology Nutrition

III

Adequacy of micronutrient intake among children 9-24 months in Nepal – The MAL-ED birth cohort study

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CONFLICT OF INTEREST

None

Environmental enteropathy, micronutrient adequacy and length velocity in Nepalese children – the MAL-ED birth cohort study

Abstract

Objectives: Environmental enteropathy (EE) is likely associated with growth retardation in children, but the association between EE and length velocity z-score (LVZ) has not been investigated. The objective of the study was to assess associations between fecal markers for intestinal inflammation and LVZ and whether these associations were influenced by micronutrient adequacy among 9-24 months old children in Bhaktapur, Nepal.

Methods: Data was divided into 5 time slots (9-12, 12-15, 15-18, 18-21 and 21-24 months). Anthropometric measurement and dietary assessment (by 24h recall) were performed monthly. Mean nutrient density adequacy (MNDA) was calculated based on nutrient density adequacy (NDA) of ten micronutrients (thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin C, vitamin A, calcium, iron and zinc). Anti-1-antitrypsin (AAT), myeloperoxidase (MPO) and neopterin (NEO) were measured in stool samples collected at the beginning of each time slot. An environmental enteropathy (EE) score was calculated based on all three fecal markers. Associations between AAT, MPO, NEO and EE-score and LVZ were assessed by multiple linear regression analyses and Generalized estimating equations (GEE) models.

Results: Associations between fecal markers and EE-score and LVZ were generally weak. EE-score and MPO for 3-month- and MPO for 6-month growth periods were significantly associated with LVZ from 9-24 months. These associations were slightly modified by MNDA.

Conclusions: EE-score and MPO were significantly associated with LVZ in 9-24 months old Nepali children. Further studies to establish the usefulness of AAT, MPO and NEO in assessing EE and growth retardation are warranted.

Key words: anti-1-antitrypsin, myeloperoxidase, neopterin, nutrient density adequacy, GEE model

What is known:

- Environmental enteropathy (EE) combined with poor micronutrient intake likely contributes to the global burden of growth faltering in children.
- The fecal markers alpha-1-antitrypsin (AAT), myeloperoxidase (MPO) and neopterin (NEO) have been linked to decreases in height-for-age, but associations with length velocity z-score (LVZ) have not been assessed.

What is new:

- Associations between fecal markers and LVZ were weak
- Environmental enteropathy (EE) score (based on all three fecal markers) and MPO were significantly associated with LVZ from 9-24 months
- These associations were slightly modified by nutrient density adequacy

Introduction

Child undernutrition is a widespread global health problem, with the majority of affected children living in Sub-Saharan Africa and South Asia (1). While the global prevalence of stunting decreased from 33 to 23% between 2000 and 2016, the prevalence of wasting was 8% in 2016 (2). The causes of growth faltering are complex and include intrauterine growth retardation (IUGR) (3), diarrhea (4) and inadequate infant and young child feeding (IYCF) practices (5), while a recent meta-analysis showed heterogeneous results for associations

between environmental enteropathy (EE) and impaired growth (6). The consequences of growth faltering for affected children are cognitive deficits and reduced human capital (7) and increased risk of infections (8) and mortality (9).

Dietary interventions among children in LMICs generally fail to achieve normal linear growth (10). This may be partly caused by weaning environments with high exposure to environmental pathogens (11), resulting in chronic inflammation of the intestine and malabsorption of nutrients, described as environmental enteropathy (EE), or more recently, environmental enteral dysfunction (EED) (12). Histologically, EE presents as villous blunting, crypt hyperplasia, inflammation in the epithelium and lamina propria as well as damaged tight junction integrity and microerosions, increasing the risk of systemic inflammation (12). Biopsies of healthy children are considered invasive and efforts are made to validating simple, non-invasive biomarkers associated with EE and subsequent growth faltering (11, 13). Among the biomarkers investigated for this purpose are fecal alpha-1-antitrypsin (AAT) (marker for protein losing enteropathy), myeloperoxidase (MPO) (marker of neutrophil activity) and neopterin (NEO) (marker of TH1 immune activation) (14).

While Bhaktapur has higher socioeconomic status than national averages (15), micronutrient adequacy in our sample was extremely low (16) and multiple micronutrient deficiency has been shown to histologically mimic environmental enteropathy in animal models (17). Previous studies have demonstrated associations between MPO and short-term (3 months) (18) and AAT, MPO and NEO and long-term (6 months) (14) changes in height (ΔHAZ). While ΔHAZ uses the same scale (z-scores) independent of age, length velocity assesses expected growth incorporating different growth velocities across infancy and childhood, and may thus be a more suitable method to assess determinants of growth faltering (19). The aim

of this study was to assess the association between intestinal inflammation and length velocity z-scores (LVZ) and whether these associations were influenced by micronutrient adequacy among 9 to 24 months old children in Bhaktapur, Nepal.

Methods

Design and subjects

This study was part of the Etiology, Risk Factors, and Interactions of Enteric Infections and Consequences for Child Health and Development study (MAL-ED). Data was collected in Bhaktapur, a peri-urban community situated 15 km east of Kathmandu, the capital of Nepal. The number of children to be included each week was based on a pre-study census providing information on the expected birth rate and the target sample size (> 200 children). Participants were enrolled within 17 days from birth and followed up until 24 months. Children who were singletons, born to mothers > 16 years of age, weighed >1500 grams at birth and were without indications of serious disease were eligible. Out of 275 children screened, 240 were included. Data collection took place between February 2011 and February 2014. In this paper, we used data of children aged 9-24 months only. Data was divided into five time slots (9-12, 12-15, 15-18, 18-21 and 21-24 months). The study received ethical approval from Nepal Health Research Council (NHRC) and the Walter Reed Institute of Research (Silver Springs, Maryland) and all parents signed informed consent forms. Further information on design and methodology is reported elsewhere (20).

Anthropometry

Anthropometric measurements were performed monthly. A standard length board (ShorrBoard; Weigh and Measure, LLC, Olney, Maryland) was used to measure length, and an infant scale (Seca, Chino, California) was used to measure weight. If concern was raised

about measurements, raw values were plotted on growth curves on site. If deviations from previous values were substantial, measurements were redone immediately. To monitor the quality of the data, 10% of measurements each month were duplicated within 24 hours by a supervisor. Anthropometric data was double entered into a database. The site data supervisor checked for discrepancies and missing data. If needed, new measurements were performed, generally within 48 hours. Following an external quality control implausible increments in subsequent measurements (>1.5 kg for weight and >3.5 cm for length) were returned for review by the study site.

Intestinal inflammation markers

Routine stool samples were collected monthly for children < 12 months, then quarterly up to 36 months age (20). Stool samples collected at 9, 12, 15, 18 and 21 months were used in the analyses. The samples were stored for processing at -70°C without fixative (21). The concentrations AAT, MPO and NEO were measured by ELISA tests at Walter Reed/AFRIMS Research Unit, Katmandu, Nepal, using initial dilutions of 1:500 ng/mL for MPO (ALPCO, Salem, NH) and AAT (BioVendor, Candler, NC) and 1:1000 nmol/L for NEO (GenWay Biotech, San Diego, CA). Tests showing out of range values were run again at a 2-fold higher or lower (as appropriate) concentration (21). In order to avoid overly diluting the biomarker concentrations, we excluded stool samples collected < 7 days after a diarrheal episode or at the same time as the urine sample for the lactulose:mannitol test of intestinal permeability inherent in MAL-ED protocol (13). Due to highly skewed distributions (with few very high values), the variables were $\log_2$ transformed to obtain normality and to ease the interpretation of results.

The environmental enteropathy (EE) score was calculated from weighting factors from a

126 principal component analysis of the natural log of the three biomarkers and percentile scores
127 for AAT, MPO and NEO, based on methodology by Kosek et al (14). The EE-score (range 0-
128 10) was calculated as follows:
129 EE-score = (2 x AAT category) + (2 x MPO category) + (1 x NEO category),
130 where categories were defined as 0 (< = 25th percentile), 1, (25–75th percentile), or 2 (> =
131 75th percentile) (14).
132
133 **Diet and socioeconomic status**
134 Food intake was assessed by 24h recall, with separate forms for recipes. To calculate nutrient
135 intake, the FAO International Network of Food Data Systems (INFOODS) database for Asia
136 (22) was mainly used. Parity and socioeconomic status were assessed by questionnaire at 12
137 months. Socioeconomic status was assessed by a WAMI (**W**ater, **A**ssets, **M**aternal education
138 and **I**ncome) index, with scores ranging from 0 to 1 (23).
139
140 **Nutrient density adequacy**
141 Context specific desired nutrient density (DND) and nutrient density adequacy (NDA) of
142 complementary foods was calculated for 10 micronutrients: thiamin, riboflavin, niacin,
143 vitamin B_6, folate, vitamin C, vitamin A, calcium, iron and zinc, based on methodology by
144 Dewey and Brown (24). Mean nutrient density adequacy (MNDA) was calculated as the mean
145 of individual NDAs for all ten micronutrients each capped at 100%. Further details on
146 assessment of dietary intake and calculations of MNDA are reported elsewhere (16).
147
148 **Statistical analysis**
149 Statistical Package for Social Science (SPSS) version 24.0 was used to analyze data.
150 Continuous data were presented as mean and standard deviation (SD) if normally distributed,

and as median and inter quartile range (IQR) if not normally distributed. Growth velocity was calculated based on the WHO 2009 standards (25).

Multiple linear regression models were constructed to assess the associations between EE score, AAT, MPO and NEO (independent variables) and LVZ (dependent variable) respectively. Models were constructed both for 3 and 6-month growth periods starting at the time of fecal marker sampling. Models were adjusted for baseline (at the beginning of each time slot) HAZ, child's gender, WAMI and diarrhea (proportion of days in time slot). Only regression models including the growth period 21-24 months were additionally adjusted for breastfeeding status (yes or no). Then models were adjusted for MNDA in addition to the above mentioned variables. We tried adjusting for stool consistency, but due to very little variation among participants, it was excluded. Apart from child's gender and breastfeeding status all variables were continuous.

Generalized estimating equations (GEE) models with first order autoregressive (AR-1) covariance matrix were used to assess these associations for the entire follow-up period. Similar adjustments to those used in multiple linear regression models were included.

Associations between quartile groups (< = 25, 25-75 and >= 75th percentile) of AAT, MPO and NEO and 3-month LVZ were also assessed. Models were adjusted for baseline (at the beginning of the time slot) HAZ and diarrhea (proportion of days in time slot).

Results

Baseline characteristics of the mother and child pairs are presented in Table 1. The median (IQR) number of years of education among mothers was 9 (6, 10), and the median (IQR) monthly household income (in USD) was 157 (100, 248). Further, the median (IQR) WAMI-

score was 0.72 (0.63, 0.81), and all households had access to improved water and sanitation. Mean (SD) length was 50 (2.1) cm, and 12% of children were stunted at birth. The majority (53%) of children were male.

EE-score, biomarker concentrations, LVZ and child feeding practices are presented in Table 2. Median (IQR) EE-score remained stable at 5 (3, 7) throughout follow-up. Concentrations of fecal markers decreased gradually through time slots (with the largest decrease seen for MPO), apart from an increase in NEO at 12-15 months. About 70% of our participants in the first three and 57% in the last time slot had AAT concentrations above reference values for healthy populations (0.27 mg/g) (26). For MPO, 80% had concentrations above reference value (2000 ng/mL) (27) in the first two, and 50% in the last time slot, while for NEO, all participants in all time slots had concentrations well above (from 14 to 30 times) the reference value (70 nmol/L) (28). Mean LVZ was lowest at 15-18 months (-0.72, SD 1.12) while median (IQR) MNDA was stable at about 40 (35, 50) % up to 21 months and was 49 (40, 56) % in the final time slot. Virtually all children were breastfed up to 21 months.

Results from multiple linear regression models and GEE models for associations between EE-score, fecal biomarkers and length velocity z-scores for 3-month and 6-month growth periods are presented in Table 3 and 4, respectively. For 3-month growth periods, associations for separate time slots were overall weak with few significant findings. EE-score (-0.03, CI -0.05, 0) and MPO (-0.03, CI -0.06, 0) were significantly associated with LVZ for the whole follow-up period when MNDA was not adjusted for. Adjusting for MNDA made no changes to the estimates for individual time slots, but slightly weakened the negative association between EE-score and MPO and LVZ for the whole follow-up period. For 6-month growth periods, only MPO was significantly associated with lower LVZ for the whole follow-up period. Very

little variation was explained in our models, with the highest adjusted R^2 found for AAT (5%) at 18-21 months.

For MPO, children in the high quartile had significantly lower LVZ (-0.47, CI -0.86, -0.07) than children in the low quartile in the 12-15-month time slot. Otherwise, no consistent relationship between quartile groups for fecal marker concentrations and LVZ was found either for separate time slots or throughout follow-up with 3-month growth periods (See Table, Supplemental Digital Content 1, which shows associations between quartile groups for fecal markers and LVZ).

Discussion

We found that associations between EE-score or fecal markers for EE and LVZ were generally weak and few reached statistical significance. EE-score and MPO for 3-month growth periods and MPO for 6-month growth periods were significantly associated with LVZ for the whole follow-up period. Adjusting for micronutrient adequacy made minor changes to the estimates for the entire follow-up period.

Our findings are in accordance with a study from the MAL-ED Bangladesh site where the EE-score and MPO were significantly associated with 3 months ΔHAZ in children 12-21 months (18), and findings from the MAL-ED Brazil case-cohort study where MPO and AAT were associated with 2-6 months ΔHAZ in children 6-26 months old (11). However, it contrasts previous findings by Kosek et al. (14) based on all MAL-ED sites where EE-score and all three fecal markers were significantly associated with 6 months ΔHAZ in children 3-15 months old. In our study, only MPO for the entire period of follow-up was significantly associated with linear growth over a 6-month period, while in the MAL-ED Bangladesh site,

no significant associations were found. The study by Kosek et al. reported fecal marker concentrations similar to our higher values (first 2 time slots), with MPO especially elevated, and had more participants and higher statistical power than our study (14), while fecal marker concentrations in the study from the MAL-ED Bangladesh site resembled the lower values in our sample (measured after 18 months age). Importantly, the associations are generally weak, similar to our findings. Finally, a lack of association between NEO and growth was also found in a recent study based on all MAL-ED sites (29). LVZ as a longitudinal growth indicator differs conceptually from ΔHAZ since it accounts for different variability of growth rates at different ages. However, differences in estimates with the two methods are expected to be small and substantial deviations from previous findings in our study population are unlikely.

Although especially EE-score and MPO seem to be associated with longitudinal growth in the second year of life, independent of growth indicator used, the proportion of variance in growth explained by the fecal markers is low. This is likely in part caused by high variability in the fecal markers assessed (29). A study by Campbell et al. found that biomarker scores differed in their associations with sociodemographic characteristics, recent morbidities and prior anthropometry suggesting that they reflect multiple underlying biological processes underlining the numerous etiologies of EED (30). In line with this, McCormick et al. (13) , investigating environmental exposures and feeding practices influencing AAT, MPO and NEO in MAL-ED, found that the variability in biomarker concentrations mainly seemed to be attributable to other child or environmental factors than those examined in the study (i.e SES, breastfeeding, season, birthweight). Large inter-individual variation combined with small effect sizes for growth and strong influences by age on biomarker concentrations limit the prognostic value of these fecal markers (31). Recent research also implies that the relationship

between NEO and growth may be modified by MPO so that while NEO alone may reflect normal intestinal immune function, NEO in the presence of MPO indicate EED and possibly constrained growth (11, 30). Further, numerous biomarkers linked to intestinal- and systemic inflammation or microbial translocation are used to assess EE (6). Including more biomarkers in our analysis would likely have provided a more comprehensive assessment of the association between EE and growth. Finally, the utility of the fecal markers in describing EED has also been questioned since they correlate with the prevalence, activity and severity of other GI diseases and consequently are not specific to EED (12).

The slight tendency for MNDA to weaken the negative associations between intestinal inflammation and LVZ supports studies showing improvements in EED in Zambean adults (32), and transient improvements in EED with micronutrient (33) supplementation in 12-35 months old Malawian children (34). Micronutrient supplementation in combination with growth monitoring and health education and/or supplementary food and psychosocial stimulation also improved intestinal permeability in a study among severely undernourished 6-24 months old Bangladeshi children (35). In children with EED, nutrient absorption may decrease due to reduced intestinal absorptive surface (36). To our knowledge, the extent to which a scarce pool of micronutrients is allocated into combatting systemic inflammation, gut maturation and repair or growth is largely unknown. Although the children included in the study from Malawi received the WHO micronutrient requirements daily with good compliance, a 24-week follow-up only showed only modest improvements in EED, which led the authors to conclude that MN supplements alone is insufficient to unequivocally ameliorate EED (34). Further, in the study from Bangladesh, the authors attributed the main improvements in EED to weight gain (35), which may partly be linked to gut microbiota immaturity seen in children with low WHZ. This immaturity increases susceptibility to

intestinal inflammation (37), reduces growth hormone (GH) sensitivity and growth rate in the host (38). It is thus possible that energy sufficiency plays a greater role than micronutrient sufficiency in the relationship between EE and growth.

The main advantage of this study was frequent measurements of environmental enteropathy, anthropometry and diet across a critical time point in children's lives, allowing longitudinal analyses of relationships between EE, micronutrient adequacy and growth. Also, detailed assessment of diarrhea incidence (several times per week) was a major strength, and allowed for improved quality of data for fecal marker concentrations. Growth velocity is suggested to be more sensitivity in capturing influencing factors such as previous growth experience (39) and season (40), and has a greater potential to assess short-term consequences (41) than measures of attained growth. Further, recruitment was from a relatively homogenous setting. Although fewer fecal samples were available for the 9-12 and 15-18-month time slots, likely due to an increased burden of diarrhea, attrition was relatively low (15%) in the MAL-ED Nepal cohort.

Growth velocities have drawbacks such as a higher instability in subsequent short-time periods (42). However, 3-month periods were chosen because they represent a balance between the influence of measurement error and the ability to represent current growth (43). Six-month growth periods were added to enable comparisons with other main publications on the subject (14, 18), where it has been argued that assessing longer growth periods produce more clinically relevant results than shorter growth periods (14). However, these did not change the association to a great extent. Further, although P-value adjustments such as the Bonferroni correction may not be required when conducting exploratory analysis, multiple regression models, such as in our study, increases the likelihood of significant results

occurring by chance (44). Finally, due to the multifactorial aetiology of malnutrition and varying importance of different risk factors, the generalizability of our study is limited to populations with similar characteristics.

In summary, associations between EE-score, fecal markers and LVZ were weak and varied with biomarkers and age. EE-score and MPO were significantly associated with 3-month growth and MPO with 6-month growth in Nepali children 9-24 months of age. Further studies to establish the usefulness of these biomarkers in assessing environmental enteropathy and risk of growth retardation in different settings and for different age groups are warranted.

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Online supplementary materials:

Table, Supplemental Digital Content 1: Linear models for fecal biomarker quartile groups and 3-month length velocity z-scores, children 9-24 months, Bhaktapur, Nepal

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Table 1 Baseline characteristics, mother-child pairs, Bhaktapur, Nepal

Characteristic[a] (n=207)	
Mother's age in years, mean (SD)	27.4 (3.7)
Parity	
One child, %	47
Two children, %	42
Three or more children, %	11
Mother's education in years, median (IQR)	9 (6, 10)
Household income per month (USD)[b], median (IQR)	157 (100, 248)
Improved water and sanitation, %	100
WAMI, median (IQR)	0.72 (0.63, 0.81)
Birth weight (kg), mean (SD)[c]	3.0 (0.4)
Length at birth, mean (SD)[c]	50.1 (2.1)
Stunted at birth, %	11.6
Stunted at 9 months, %	7.7
Wasted at 9 months, %	1.9
Stunted at 24 months, %	21.7[d]
Wasted at 24 months, %	3.9[d]
Child's gender, male (%)	52.7

[a] Measured at 12 months age
[b] Exchange rates from Oanda.com
[c] Measured within 17 days
[d] n= 203

Table 2 Environmental enteropathy (EE) score, fecal biomarker[a] concentrations, length velocity z-scores (LVZ) and feeding practices, children 9-24 months, Bhaktapur, Nepal

	9-12 mo n= 162	**12-15 mo** n= 207	**15-18 mo** n= 143	**18-21 mo** n=198	**21-24 mo** n= 203
EE-score, median (IQR)[b]	5 (3, 7)	5 (3, 7)	5 (3, 7)	5 (3, 7)	5 (3, 7)
AAT (mg/g), median (IQR)	0.43 (0.26, 0.84)	0.39 (0.21, 0.67)	0.42 (0.22, 0.87)	0.36 (0.24, 0.60)	0.30 (0.14, 0.61)
MPO (ng/mL), median (IQR)	7204.6 (2443.8, 18335)	5714.7 (2513.9, 13508,3)	3866.4 (1874.2, 6692.4)	3270.4 (1312.4, 5805.1)	2001.7 (854.3, 3981.1)
NEO (nmol/L), median (IQR)	1736.3 (1104.2, 2460.8)	2385.5 (1454.2, 3464.3)	1524.9 (818.4, 2432.5)	1369.6 (687.1, 2315.7)	951.2 (480, 1938.2)
LVZ, mean (SD)	-0.56 (1.05)	-0.69 (1.02)	-0.72 (1.12)	-0.50 (0.97)	-0.45 (0.95)
MNDA (%), median (IQR)[c]	41.8 (33.3, 49.2)	38.5 (32, 44.5)	40.6 (33.4, 46.7)	42.9 (36.3, 51)	48.8 (40.1, 56))
Children being breastfed, %[d]	100	100	100	98	90

[a] AAT: alpha-1-antitrypsin; MPO: myeloperoxidase; NEO: neopterin, measured at the beginning of each growth period.
[b] Calculated based on methodology by Kosek et al. (15)
[c] Mean nutrient density adequacy
[d] Measured at the beginning of the growth period

Table 3 Linear models for associations between environmental enteropathy (EE) score, fecal biomarkers[a] and 3-month length velocity z-score (LVZ), children 9-24 months, Bhaktapur, Nepal

	9-12 months[b] n=162			12-15 months n= 207			15-18 months n=143			18-21 months n=198			21-24 months n=203			9-24 months[c] n=231		
	B	CI	ß	B	CI	ß	B	CI	ß	B	CI	ß	B	CI	ß	B	CI	ß
EE-score[d]																		
Model 1[e]	0.01	-0.06, 0.07	0.01	**-0.06**	-0.12, -0.002	-0.14	-0.04	-0.12, 0.04	-0.09	-0.01	-0.07, 0.04	-0.03	-0.04	-0.10, 0.02	-0.09	**-0.03**	-0.05, -0.01	-0.06
Model 2[f]	0.01	-0.06, 0.08	0.01	-0.05	-0.11, 0.01	-0.12	-0.03	-0.10, 0.05	-0.06	-0.01	-0.07, 0.05	-0.02	-0.05	-0.11, 0.01	-0.11	**-0.03**	-0.05, -0.001	-0.06
Model 3[g]	0.01	-0.06, 0.08	0.02	-0.05	-0.11, 0.01	-0.12	-0.04	-0.12, 0.05	-0.08	-0.01	-0.07, 0.05	-0.03	-0.05	-0.11, 0.01	-0.12	-0.02	-0.05, 0.001	-0.04
N samples																	917	
AAT																		
Model 1	0.10	-0.02, 0.22	0.13	-0.08	-0.19, 0.03	-0.10	-0.01	-0.12, 0.10	-0.01	**-0.14**	-0.23, -0.05	-0.21	-0.01	-0.11, 0.08	-0.02	-0.03	-0.07, 0.01	-0.04
Model 2	0.11	-0.02, 0.23	0.14	-0.06	-0.17, 0.04	-0.08	-0.01	-0.11, 0.10	-0.01	**-0.13**	-0.22, -0.0[g]	-0.20	-0.03	-0.13, 0.06	-0.05	-0.03	-0.07, 0.01	-0.04
Model 3	0.11	-0.02, 0.23	0.14	-0.06	-0.17, 0.05	-0.08	-0.01	-0.12, 0.11	-0.01	**-0.13**	-0.22, -0.04	-0.20	-0.04	-0.14, 0.05	-0.06	-0.03	-0.07, 0.01	-0.04
N samples																	929	
MPO																		
Model 1	0.07	-0.03, 0.16	0.11	**-0.09**	-0.16, -0.01	-0.15	-0.06	-0.18, 0.06	-0.08	0.02	-0.06, 0.11	0.04	-0.03	-0.11, 0.05	-0.06	**-0.03**	-0.06, -0.003	-0.05
Model 2	0.06	-0.03, 0.16	0.11	**-0.09**	-0.16, -0.01	-0.15	-0.05	-0.17, 0.07	-0.07	0.02	-0.06, 0.11	0.04	-0.04	-0.11, 0.04	-0.06	**-0.03**	-0.06, -0.003	-0.05
Model 3	0.07	-0.03, 0.16	0.11	**-0.09**	-0.16, -0.01	-0.15	-0.06	-0.19, 0.07	-0.08	0.02	-0.06, 0.11	0.04	-0.03	-0.11, 0.04	-0.06	-0.02	-0.05, 0.01	-0.04
N samples																	921	
NEO																		
Model 1	0	-0.17, 0.17	0	-0.04	-0.18, 0.10	-0.04	-0.04	-0.20, 0.13	-0.04	0.05	-0.05, 0.16	0.08	0.01	-0.08, 0.11	0.02	-0.01	-0.05, 0.03	-0.01
Model 2	0	-0.18, 0.17	0	-0.04	-0.18, 0.11	-0.04	-0.02	-0.19, 0.15	-0.02	0.06	-0.04, 0.17	0.09	-0.01	-0.11, 0.09	-0.01	-0.01	-0.06, 0.03	-0.01
Model 3	0	-0.18, 0.18	0	-0.03	-0.18, 0.11	-0.03	-0.02	-0.19, 0,16	-0.02	0.06	-0.04, 0.17	0.09	-0.01	-0.11, 0.09	-0.01	0	-0.04, 0.05	0
N samples																	923	

[a] AAT: Alpha-1-antitrypsin; MPO: myeloperoxidase; NEO: neopterin. Concentrations were Log_2 transformed, the beta equals the change LVZ seen with a two-fold increase in biomarker concentrations. Significant associations in bold.

[b] Multiple linear regression models used

[c] Generalized estimating equations (GEE) models with first order autoregressive covariance matrix used

[d] Calculations based on Kosek et al. (2013), measured at the beginning of each time slot

[e] Model 1 was unadjusted

[f] Model 2 was adjusted for HAZ at the beginning of the time slot, WAMI (measured at 12 months), gender, diarrhea (proportion of days in time slot) and BF status (only relevant at 21-24 months)

[g] Model 3 was adjusted for the same covariates as in model 2 and mean nutrient density adequacy (MNDA)

Table 4 Linear models for associations between environmental enteropathy (EE) score, fecal biomarkers[a] and 6-month length velocity z-score (LVZ), children 9-24 months, Bhaktapur, Nepal

	9-15 months[b] n=162			**12-18 months** n= 199			**15-21 months** n=141			**18-24 months** n=197			**9-24 months[c]** n=228		
	B	CI	ß	B	CI	ß	B	CI	ß	B	CI	ß	B	CI	ß
EE-score[d]															
Model 1[e]	-0.06	-0.13, 0.01	-0.13	-0.04	-0.11, 0.03	-0.08	-0.03	-0.11, 0.06	-0.05	-0.03	-0.09, 0.04	-0.06	-0.03	-0.06, 0.01	-0.06
Model 2[f]	-0.06	-0.13, 0.01	-0.13	-0.03	-0.10, 0.04	-0.06	-0.02	-0.11, 0.06	-0.05	-0.02	-0.08, 0.04	-0.05	-0.02	-0.05, 0.01	-0.04
Model 3[g]	-0.06	-0.13, 0.01	-0.13	-0.03	-0.09, 0.04	-0.05	-0.03	-0.12, 0.06	-0.05	-0.03	-0.09, 0.04	-0.06	-0.02	-0.05, 0.01	-0.04
N samples														703	
AAT															
Model 1	0.03	-0.09, 0.16	0.04	0.01	-0.12, 0.13	-0.01	0.06	-0.06, 0.18	0.08	**-0.10**	-0.21, -0.003	-0.14	0.02	-0.04, 0.08	0.03
Model 2	0.03	-0.11, 0.16	0.03	0.02	-0.10, 0.14	0.02	0.06	-0.06, 0.18	0.08	-0.09	-0.19, 0.02	-0.12	0.03	-0.03, 0.09	0.04
Model 3	0.02	-0.11, 0.15	0.03	0.02	-0.11, 0.14	0.02	0.06	-0.06, 0.19	0.09	-0.09	-0.19, 0.02	-0.12	0.03	-0.03, 0.09	0.04
N samples														712	
MPO															
Model 1	-0.09	-0.19, 0.01	-0.14	-0.08	-0.17, 0.01	-0.13	-0.03	-0.16, 0.11	-0.04	-0.02	-0.11, 0.07	-0.03	**-0.06**	-0.10, -0.02	-0.11
Model 2	-0.08	-0.18, 0.01	-0.14	-0.08	-0.17, 0.01	-0.13	-0.03	-0.16, 0.11	-0.03	-0.02	-0.11, 0.07	-0.03	**-0.05**	-0.10, -0.01	-0.09
Model 3	-0.09	-0.18, 0.01	-0.14	-0.08	-0.17, 0.01	-0.13	-0.03	-0.17, 0.11	-0.04	-0.03	-0.12, 0.07	-0.05	**-0.05**	-0.09, -0.01	-0.09
N samples														707	
NEO															
Model 1	0.02	-0.17, 0.20	0.01	0.10	-0.07, 0.27	0.08	-0.14	-0.32, 0.04	-0.13	0.06	-0.06, 0.17	0.07	-0.01	-0.08, 0.06	-0.01
Model 2	0.02	-0.17, 0.21	0.02	0.11	-0.06, 0.28	0.09	-0.15	-0.33, 0.03	-0.14	0.06	-0.06, 0.17	0.07	-0.01	-0.07, 0.06	-0.01
Model 3	0.03	-0.16, 0.21	0.02	0.11	-0.06, 0.28	0.09	-0.16	-0.35, 0.03	-0.15	0.06	-0.06, 0.17	0.07	0.01	-0.06, 0.08	0.01
N samples														709	

[a] AAT: Alpha-1-antitrypsin; MPO: myeloperoxidase; NEO: neopterin. Concentrations were Log_2 transformed, the beta equals the change LVZ seen with a two-fold increase in biomarker concentrations. Significant associations in bold.

[b] Multiple linear regression models used

[c] Generalized estimating equations (GEE) models with first order autoregressive covariance matrix used

[d] Calculations based on Kosek et al. (2013), measured at the beginning of each time slot

[e] Model 1 was unadjusted

[f] Model 2 was adjusted for HAZ at the beginning of the time slot, WAMI (measured at 12 months), gender, diarrhea (proportion of days in time slot) and BF status (only relevant at 21-24 months)

[g] Model 3 was adjusted for the same covariates as in model 2 and mean nutrient density adequacy (MNDA)

Supplemental table

Linear models for fecal biomarkers quartile groups and 3-month length velocity z-scores, children 9-24 months, Bhaktapur, Nepal

Supplemental table 1 Linear models for fecal biomarkers[a] quartile groups and 3-month length velocity z-scores, children 9-24 months, Bhaktapur, Nepal

	9-12 months[b] n=162			12-15 months n=207			15-18 months n=143			18-21 months n=202			21-24 months n=201			9-24 months[c] n= 231		
	B	CI	ß	B	CI	ß	B	CI	ß	B	CI	ß	B	CI	ß	B	CI	ß
AAT																		
Low	Ref			Ref			Ref			Ref			Ref			Ref		
Medium	-0.03	-0.43, 0.37	-0.02	0.02	-0.33, 0.36	0.01	-0.40	-0.84, 0.06	-0.18	0.06	-0.28, 0.40	0.03	-0.17	-0.49, 0.16	-0.09	-0.11	-0.27, 0.06	-0.06
High	0.17	-0.30, 0.64	0.07	-0.13	-0.53, 0.27	-0.05	-0.12	-0.65, 0.40	-0.05	-0.33	-0.72, 0.07	-0.14	-0.10	-0.47, 0.28	-0.04	-0.15	-0.32, 0.03	-0.07
HAZ[d]	0.02	-0.15, 0.19	0.02	-0.07	-0.23, 0.09	-0.06	0	-0.21, 0.21	0	0.07	-0.07, 0.22	0.07	-0.04	-0.18, 0.10	-0.04	0.02	-0.03, 0.08	0.02
Diarrhea[e]	-0.80	-4.42, 2,81	-0.04	1.20	-1.43, 3.81	0.06	-2.57	-6.83, 1.69	-0.10	-1.27	-4.08, 1.54	-0.06	-0.81	-4.42, 2.79	-0.03	-0.59	-1.80, 0.63	-0.03
Constant	-0.55			-0.76			-0.43			-0.36			-0.39			-0.46		
MPO																		
Low	Ref			Ref			Ref			Ref			Ref			Ref		
Medium	0.11	-0.30, 0.51	0.05	0.03	-0.31, 0.36	0.01	-0.26	-0.72, 0.19	-0.12	0.18	-0.17, 0.52	0.09	-0.06	-0.38, 0.26	-0.03	0.01	-0.14, 0.15	0.01
High	0.03	-0.43, 0.50	0.01	**-0.47**	-0.86, -0.07	-0.20	-0.29	-0.81, 0.24	-0.11	0.31	-0.09, 0.70	0.13	-0.31	-0.68, 0.06	-0.14	-0.15	-0.32, 0.03	-0.15
HAZ	0.02	-0.15, 0.19	0.02	-0.07	-0.22, 0.09	-0.06	-0.03	-0.24, 0.18	-0.02	0.08	-0.07, 0.22	0.08	-0.03	-0.17, 0.10	-0.03	0.02	-0.03, 0.08	0.02
Diarrhea	-0.72	-4.37, 2.92	-0.03	0.85	-1.72, 3.43	0.05	-2.58	-6.86, 1.71	-0.10	-0.79	-3.58, 2	-0.04	-0.82	-4.41, 2.76	-0.03	-0.60	-1.85, 0.64	-0.03
Constant	-0.59			-0.68			-0.48			-0.58			-0.38			-0.51		
NEO																		
Low	Ref			Ref			Ref			Ref			Ref			Ref		
Medium	0.18	-0.22, 0.58	0.09	-0.04	-0.39, 0.31	-0.02	0.15	-0.31, 0.60	0.06	0.07	-0.27, 0.41	0.04	-0.01	-0.34, 0.32	-0.01	0.08	-0.06, 0.22	0.05
High	-0.25	-0.71, 0.20	-0.11	-0.13	-0.53, 0.27	-0.05	0	-0.53, 0.54	0	0.10	-0.30, 0.50	0.04	0.07	-0.31, 0.44	0.31	0.01	-0.16, 0.17	0.01
HAZ	0.06	-0.12, 0.23	0.05	-0.07	-0.23, 0.09	-0.06	-0.02	-0.23, 0.19	-0.02	0.07	-0.08, 0.22	0.07	-0.04	-0.18, 0.10	-0.04	0.03	-0.03, 0.08	0.03
Diarrhea	-0.54	-4.09, 3.01	-0.02	1.20	-1.42, 3.81	0.06	-2.42	-6.81, 1.96	-0.09	-0.92	-3.77, 1.93	-0.05	-0.76	-4.39, 2.88	-0.03	-0.57	-1.80, 0.67	-0.03
Constant	-0.53			-0.73			-0.75			-0.48			-0.51			-0.59		

[a] AAT: Alpha-1-antitrypsin; MPO: myeloperoxidase; NEO: neopterin. Low: <= 25th percentile, Medium 25-75th percentile, High >= 75th percentile. Significant associations in bold.
[b] Multiple linear regression models used
[c] Generalized estimating equations (GEE) models with first order autoregressive covariance matrix used
[d] Height-for-age z-score measured at the beginning of the growth period.
[e] Proportion of days with diarrhea in growth period

Paper 4

Nutrient intake and environmental enteric dysfunction among Nepalese children 9-24 months old – the MAL-ED birth cohort study

Status: Submitted to Paediatric Research

IV

Nutrient intake and environmental enteric dysfunction among Nepalese children 9-24 months old– the MAL-ED birth cohort study

Running title: Nutrient intake and enteric dysfunction

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Disclosure

The authors declare no conflict of interest

Category of study: population study

Abstract

Background: Nutrient deficiencies limit the growth and turnover of intestinal mucosa, but studies assessing whether specific nutrients protect against or improve environmental enteric dysfunction (EED) are scarce. We aimed to investigate associations between nutrient intake and EED assessed by lactulose:mannitol ratio, anti-1-antitrypsin, myeloperoxidase and neopterin among children 9-24 months in Bhaktapur, Nepal.

Methods: Among 231 included children, nutrient intake was assessed monthly by 24 hour recalls, and 3-month usual intake was estimated using Multiple Source Method. Associations between nutrient intake and L:M ratio (measured at 15 months) were assessed using multiple linear regression, while associations between nutrient intake and fecal markers (measured quarterly) were assessed using Generalized Estimating Equations (GEE) models.

Results: We found that associations between nutrient intake from complementary food and lactulose-mannitol (L:M) ratio, alpha-1-antitrypsin (AAT), myeloperoxidase (MPO) and neopterin (NEO) were generally negative but weak. The only significant associations between nutrient intake (Potassium, Magnesium, Phosphorous, Folate and Vitamin C) and markers for intestinal inflammation were found for MPO.

Conclusion: Negative but weak associations between nutrient intake and markers of intestinal inflammation were found. Significant associations between several nutrients and MPO might merit further investigation.

Introduction

Environmental enteric dysfunction (EED) refers to a highly prevalent condition affecting populations in low- and middle income countries (LMICs) with increased gut inflammation, increased intestinal permeability and reduced absorption of nutrients due to villous atrophy (1) and loss of enzymatic activity (2). EED is established during infancy and is associated with poor sanitation, gut infections, home births, micronutrient deficiencies and breastfeeding practices (3). Possible consequences include infectious disease, stunting, impaired cognitive development and reduced vaccine efficacy (4).

The biomarker most commonly used to diagnose EED in previous studies is the lactulose:mannitol (L:M) ratio. While mannitol is passively absorbed proportional to intestinal absorptive capacity, lactulose is a disaccharide which is not absorbed by the healthy intestine. Increased L:M ratio thus indicates intestinal damage demonstrated by reduced absorptive capacity and increased permeability (2). Among newer, less invasive biomarkers used to assess EED are the fecal markers alpha-1-antitrypsin (AAT) measuring intestinal permeability, myeloperoxidase (MPO) measuring neutrophil activity and neopterin (NEO) representing Th-1 immune stimulation (5).

Increased dietary diversity may enhance gut microbiota (1), which reduces the risk of intestinal inflammation (6). Further, generalized malnutrition, protein depletion and deficiencies of specific nutrients including essential fatty acids, folate, zinc, vitamin A, and vitamin B_{12} has been shown to inhibit the growth and turnover of the intestinal mucosa (7). Meanwhile, studies assessing improvements in EED with micronutrient supplementation either alone (8) or in combination with other interventions (9) show mixed results. For specific nutrients, zinc (10) and vitamin A (11) have been associated with reduced L:M ratio

in children and alanyl-glutamin intake improved trans-mucosal resistance in mice (12). However, studies assessing whether specific nutrients protect against or improve EED are scarce. Also, studies investigating associations between nutrient intake and fecal markers for EED are mainly lacking.

The population of Bhaktapur, Nepal, has high socioeconomic status compared to national averages (13). Meanwhile, micronutrient adequacy, especially for iron, zinc, vitamin A and niacin, among children in the MAL-ED Nepal cohort was very low (14), and the prevalence of anemia and zinc deficiency at 24 months was 29 and 23 % respectively (15). National governmental programs to improve micronutrient status are ready to use therapeutic food (RUTF) to children with severe malnutrition (16), a biannual vitamin A supplementation program for children 6-59 months and zinc supplementation to children with diarrhea (17). Finally, the main enteric pathogens causing diarrhea after 12 months age in the MAL-ED Nepal cohort were campylobacter and enterohaemorrhagic e-coli (EHEC), norovirus GII and rotavirus (18).

The aim of this study was to investigate by exploratory analysis associations between nutrient intake and environmental enteric dysfunction assessed by lactulose:mannitol ratio, anti-1-antitrypsin, myeloperoxidase and neopterin among children 9-24 months in Bhaktapur, Nepal.

Methods

Design and subjects

The MAL-ED Nepal site provided data for the analyses. The data collection took place in Bhaktapur, a peri-urban, agriculture-based community located 15 km east of Kathmandu. Children were enrolled within 17 days from birth and followed at least until 24 months. The data collection period for age 9-24 months was February 2011 to November 2012. Out of 240 enrolled children, 229 had complete nutrition data at 24 months, the number of urine samples (collected at 15 months) was 218, while the number of fecal samples varied throughout follow-up. Data was divided into five time slots (9-12, 12-15, 15-18, 18-21 and 21-24 months respectively). The study received ethical approval from Nepal Health Research Council (NHRC) and the Walter Reed Institute of Research (Silver Springs, Maryland) and all caregivers signed informed consent forms. Further details on design and methodology are reported elsewhere (4).

Dietary intake and socioeconomic status

Trained local fieldworkers conducted monthly 24-hour recall interviews to collect data on foods and amounts consumed the previous day. A separate form was used to collect details about recipes. Amounts were estimated using household utensils, portion size booklets and play dough. The FAO International Network of Food Data Systems (INFOODS) database for Asia (19) was the main food composition database, but supplementary nutrient values from other databases were also used.

The Multiple Source Method (20) was used to calculate individuals' usual intake of energy, animal source protein, fiber, poly-unsaturated fatty acids (PUFA), iron, zinc, calcium, sodium, potassium, magnesium, phosphorous, thiamin, riboflavin, niacin, pantothenic acid,

vitamin $B_{6,}$ folate, vitamin $B_{12,}$ A, C and E. Usual intake was calculated based on three 24h recalls in each time slot or four recalls in time slots with secondary recalls. Socioeconomic status was assessed by questionnaire at 12 months by a WAMI (**W**ater, **A**ssets, **M**aternal education and **I**ncome) index, with scores ranging from 0 to 1 (21). The 8 assets included were separate room for a kitchen, household bank account, mattress, refrigerator, TV, people per room (mean), table and chair or bench.

Nutrient density adequacy

The nutrient density (ND) was defined as the amount of nutrient consumed per 100 kcal of complementary food and calculated for 10 micronutrients: thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin C, vitamin A, calcium, iron and zinc. Context specific desired nutrient density (DND) and nutrient density adequacy (NDA) of complementary foods was calculated based on methodology by Dewey and Brown (22) for the same micronutrients. For each time slot and for each nutrient, context specific DNDs were calculated in the following way:

[Recommended nutrient intake (RNI) of nutrient χ – (concentration of nutrient χ in breastmilk x median breast milk intake in time slot)]/median energy intake from complementary food in time slot*100.

For iron, FAO/WHO micronutrient requirements corresponding to low absorption (5%), while for zinc low or middle absorption (23) (depending on the phytate:zinc ratio measured) was used. For nutrients where the levels in breast milk are negatively affected by maternal status (thiamin, riboflavin, vitamin B_6 and vitamin A), we used concentrations based on studies conducted among women in low income countries (24, 25). Otherwise, WHO values based on breast milk from western women (26) were used. Breast milk intake was not

assessed, but calculated the following way:

[Total energy requirements (body weight measured monthly * FAO energy requirement per kg body weight for the appropriate age) (27) - energy intake from complementary food] /energy density of breast milk (LMICs) (26)

For non-breastfed children, desired nutrient densities were calculated as FAO/WHO micronutrient requirements (23) divided by median energy intake in the non-breastfed group.

NDA was calculated for each nutrient and for each observation as the ND as percentage of the DND. Finally, mean nutrient density adequacy (MNDA) was calculated as the mean of individual NDAs for all ten micronutrients each capped at 100%. Mean MNDA based on three months measurements (i.e. measured at 9, 10 and 11 months for time slot 1) was used in the analysis. A more detailed description of calculations of MNDA are reported elsewhere (14).

L:M ratio and fecal markers for EED

Children were instructed to fast 2 hours prior to and 30 minutes after the L:M test and recommended to void before administration of the sugar dose. The L:M ratio was assessed in urine collected during voiding (5-hour follow-up period) following the administration of 250 mg/mL lactulose and 50 mg/mL mannitol at a dose of 2 mL/kg to a maximum administered dose of 20 mL at a concentration of 1002 mOsm/L. Aliquots were stored at -70°C until testing and concentrations of lactulose and mannitol measured by high-performance liquid chromatography (HPLC) and either pulsed amperometric detection or iron chromatography. Results were presented as molar ratio of lactulose to mannitol (5). During our follow-up

period L:M ratio was only measured at 15 months. Due to skewed values, the variable was log-transformed.

Stool samples were collected monthly for children < 12 months, then quarterly up to 36 months age (4). Samples collected at 12, 15, 18, 21 and 24 months were used in the analysis. The samples were stored for processing at -70°C without fixative (5). The concentrations of AAT, MPO and NEO were measured by ELISA tests at Walter Reed/AFRIMS Research Unit Nepal (WARUN) with initial dilutions of 1:500 ng/mL for MPO (ALPCO, Salem, NH), AAT (BioVendor, Candler, NC) and NEO (GenWay Biotech, San Diego, CA). Tests showing out of range values were run again at a 2-fold higher or lower (as appropriate) concentration (5). To avoid overly diluting the samples, stool samples collected either during or ≤ 7 days after a diarrheal episode (3 semi-liquid stools in a 24h period separated by ≥ 2 days without diarrhea) or at the same time as the urine sample for the lactulose:mannitol test of intestinal permeability inherent in MAL-ED protocol (4) were excluded. Due to skewed distributions, the variables were log transformed to obtain normality and ease interpretation of results.

Statistical analysis

Continuous data are presented as mean and standard deviation (SD) if normally distributed, and as median and interquartile range (IQR) if not normally distributed. Variables to be included in regression models were selected based on a theory-based approach. Models for associations between nutrient intake and MNDA and L:M ratio, AAT, MPO and NEO, respectively, are presented. Models for nutrient intake and L:M ratio and fecal markers were adjusted for energy intake from complementary food, WAMI, gender, season and age (only for fecal markers), while models with MNDA were not adjusted for energy intake. We also tried adjusting for stool consistency, but due to very little variation this made no changes to

the estimates and was excluded. Models describing associations between nutrient intake and L:M ratio were assessed with multiple linear regression analysis. All other analysis was performed using GEE with autoregressive (AR-1) covariance structure. Season was coded according to the date when the fecal sample was taken as pre-monsoon (March-May), monsoon (June-August), post-monsoon (September-November) and winter (December-January). Apart from season and gender, all variables were continuous. The statistical package for the social sciences (SPSS) version 24.0 was used for data analysis.

Results

The baseline characteristics of mother and child pairs are presented in Table 1. The mean (SD) age of mothers was 27 (4) years and 11% had 3 children or more. The median (IQR) number of assets (out of 8 assessed) was 6 (5, 7). Median (IQR) WAMI score was 0.7 (0.6, 0.8), where all participants had access to improved water and sanitation. The majority of participants (53%) were male.

L:M ratio, fecal markers for EED, nutrient intake, nutrient adequacy and information about breast feeding is presented in Table 2. All outcome variables were skewed with some very high values. The median (IQR) L:M ratio was 0.07 (0.05, 0.12), where 26% had values above the reference (0.12) (28). All fecal markers decreased gradually with age. The largest reductions between the first and the last time slot were seen for MPO (74%) and NEO (72%).

Associations between intake of individual nutrients and MNDA and L:M ratio, AAT, MPO and NEO are presented in Table 3. Associations were generally negative and weak with few significant findings in view of the number of models presented. Significant negative associations were found between intake of potassium (-0.33, C.I -0.61, -0.05), magnesium (-2.81, C.I -5.36, -0.26), phosphorous (-0.58, C.I -1.14, -0.02), folate (-2.08, C.I -3.90, -0.25), vitamin C (-0.01, C.I -0.001, 0) and MNDA (-0.01, C.I -0.01, 0) and log MPO. Weak but significant negative associations were also found between intake of zinc, calcium, potassium, magnesium, phosphorous and lactulose (data not shown), while for L:M ratio, AAT, NEO and mannitol no significant associations were found.

Discussion

We found that associations between nutrient intake from complementary food and L:M ratio, AAT, MPO and NEO were generally negative but weak and few reached statistical significance. The only significant associations between nutrient intake and markers for intestinal inflammation were found for MPO.

The weak associations found between nutrient intake and intestinal inflammation are comparable to a cross-sectional study among 18 months old children in Bangladesh (29), and likely has several explanations. Firstly, associations between nutrient intake and EED are hard to assess since they are bidirectional or cyclical in nature, with malnutrition being both a cause and a consequence of EED. The level of severity of EED in our population might also be questioned since most participants had L:M ratios below the reference standard. Intestinal permeability is mediated by inflammation (30), and effects of enteroaggressive pathogens on fecal markers are cumulative (31). Meanwhile, a murine study by Brown et al. (2017) showed that increased permeability due to enteropathogens was only present in mice who had consumed a malnourished (in energy and protein) diet (6). Although the severity of intestinal lesions associated with elevated MPO, to our knowledge, is unknown, our data suggest that in this population EED may be moderate and the demand (additional to daily requirements) for typical "repair nutrients", such as folate, zinc and vitamin B_{12} (7), relatively limited. Further, nutrient intake in our study was assessed only from complementary food, whereas estimates of breast milk intake performed in a previous study suggested that this population were high breast milk consumers (14). Adjusting for energy intake from complementary food in our analysis did not account for the favorable absorption of many nutrients (i.e zinc and iron) (32) from breast milk compared to complementary food with low bioavailability (14). As a result, associations between nutrient intake, vitamin status and EED may be distorted.

Other important aspects likely to weaken associations between nutrient intake and EED in our study is the length of follow-up and the age of the included children (9-24 months). The gut microbiota matures and becomes more stable during the first 3 years of life (33). This process is negatively influenced by malnutrition and frequent use of antibiotics (15) and likely positively influenced by increased dietary diversity (34). Improved microbiota maturity is in turn associated with increased resistance to pathogens (34). In addition, it is hypothesized that increased levels of the biomarkers assessed may be side-effects of self-limiting natural processes (intestinal immune maturation) up to a certain "turning-point" where after elevated levels indicate EED (30). If this turning point occurs within our period of follow-up but at different time points for each participant, it could further complicate interpretation of results. In the end, dividing the data into 3-month time slots may not be sufficiently refined to assess the complex temporal interplay between nutrient intake, pathogen exposure and markers of EED in our age group. Finally, correlations between the fecal markers assessed and between the fecal markers and L:M ratio are low (35), indicating that they reflect different biological processes. For this reason, more comprehensive scores may be needed to adequately describe EED and assess risk factors associated with EED (31).

Although the number of models performed in our study suggests that some significant associations likely are spurious, those found between several nutrients, MNDA and MPO may still be of importance. MPO is the fecal marker most strongly affected by the most prevalent enteroaggressive pathogen (Campylobacter) in this population and in MAL-ED overall (18). It was the only fecal marker out of the three assessed here which was significantly associated with length velocity among children in the Bhaktapur cohort (36) and may thus be the marker most indicative of EED in our population. The usefulness of MPO as a biomarker for

inflammatory bowel disease (IBD) is currently being investigated, and has been shown to increase both with onset and severity of the disease (37). Although studies assessing associations between nutrient intake and MPO are lacking, both magnesium, vitamin C, potassium and fruit- and vegetable intake have been associated with decreased risk of IBD (38). Although the pathways at present seem unclear, these foods and nutrients may protect against intestinal inflammation, which supports our findings. However, potential associations between intake of specific nutrients and MPO need to be corroborated by future studies.

The main strength of the study is the longitudinal design with monthly measurements of nutrient intake enabling calculations of within- and between subject variance and likely more valid assessment of nutrient intake than in a cross-sectional study. The level of detail of nutrient data collected was high and included estimation of amounts. Fecal markers were assessed from asymptomatic stool samples. Frequent assessment of diarrhea incidence (several times per week) was a major advantage which improved the quality of data for fecal marker concentrations. Both L:M ratio and fecal markers were assessed according to strict guidelines in laboratories undergoing regular quality checks and standardization of tests between MAL-ED sites (5). The sample was drawn from a relatively homogenous population. Finally, retention was favorable (85% in the final time slot) in the MAL-ED Nepal cohort.

The main limitation of our study was uncertainty about the reliability of the outcome variables assessed. The L:M ratio may be affected by mannitol believed to be present naturally in urine, and HPLC may lack sensitivity for determining low concentrations of lactulose (35) while both MPO and NEO are non-specific markers of intestinal immunity (2). Also, assessing only nutrient intake from complementary food in this group who are high breast milk consumers (14), weakens the strength of the inferences made from our study.

Further, since all participants had access to improved water and sanitation, it was impossible to assess differences between exposed and unexposed participants regarding water, sanitation and hygiene believed to be of major importance in the development of EED (31). The vast number of regression models increases the likelihood of spurious significant associations. Meanwhile, correction for multiple comparisons is not required in explorative studies (39). Finally, the lack of international reference standards for biomarkers for EED, complicates the interpretation of results.

Conclusions

We found that associations between nutrient intake from complementary food and L:M ratio, AAT, MPO and NEO were generally negative but weak in this group of children aged 9-24 months in Bhaktapur, Nepal. The only significant associations were found for intake of Potassium, Magnesium, Phosphorous, Folate, Vitamin C and MNDA and MPO. General approaches, such as improving dietary diversity, might have beneficial effects on microbiota and gut maturation and would likely be advantageous in reducing EED in our population and in similar settings.

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Disclosure

The authors declare no conflict of interest

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Table 1 Baseline characteristics, mother-child pairs, Bhaktapur, Nepal

Characteristic[a] (n=211)	
Mother's age in years, mean (SD)	27.4 (3.7)
Parity	
One child, %	47
Two children, %	42
Three or more children, %	11
Improved water and sanitation, %	100
Number of assets, median (IQR)	6 (5, 7)
Mother's education in years, median (IQR)	9 (6, 10)
Monthly household income (USD)[b], median (IQR)	157 (101, 248)
WAMI, median (IQR)	0.7 (0.6, 0.8)
Birth weight (kg), mean (SD)[c]	3.0 (0.4)
Child's gender, male (%)	53.1

Parts of the table has been presented previously.
[a]Measured at 12 months
[b] Exchange rates from Ouanda.com
[c] n=207

Table 2 Markers of environmental enteric dysfunction, nutrient intake from complementary food and prevalence of breastfeeding, children 9-24 months, Bhaktapur, Nepal*

	Recommended	9-12 mo n=211[a]	12-15 mo n=144	15-18 mo n=205	18-21 mo n=207	21-24 mo n=192
L:M ratio, median (IQR)	<0.12[b]	NA	0.07 (0.05, 0.12)[c]	NA	NA	NA
AAT (mg/g), median (IQR)	<0.27[d]	0.40 (0.21, 0.70)	0.42 (0.23, 0.86)	0.35 (0.23, 0.60)	0.31 (0.15, 0.61)	0.27 (0.14, 0.53)
MPO (ng/mL), median (IQR)	<2000[e]	5935.2 (2524.5, 14267)	3883.8 (1880, 6660.3)	3162.5 (1184.5, 5693)	2001.7 (854.3, 3976)	1541.6 (592.9, 2983.5)
NEO (nmol/L), median (IQR)	<70[f]	2439.7 (1476.3, 3511.2)	1549.5 (822.5, 2438.8)	1378.1 (678.3, 2318.5)	957.1 (496.4, 1874.9)	683.1 (375.9, 1266.3)
Energy (kcal)	516[g]	235.8 (170, 323.8)	285.5 (214.8, 375)	370.4 (266.4, 467.9)	456.7 (355.3, 582.6)	596.4 (449.7, 729.3)
ASP (g)[h]	NA	4.1 (2.6, 5.8)	4.6 (3.2, 7)	6.2 (4.3, 9.2)	7 (5.1, 9.6)	8.9 (6.3, 12.4)
PUFA (g)	NA	1.2 (0.9, 1.7)	1.7 (1.3, 2.3)	2 (1.6, 2.9)	2.8 (1.9, 2.6)	3.3 (2.8, 4.2)
Fiber (g)	NA	1.6 (1.3, 2.2)	2 (1.5, 2.9)	2.5 (1.9, 3.3)	3.3 (2.7, 4.6)	4 (2.9, 5)
Iron (mg)	12[i]	0.81 (0.6, 1.07)	1.03 (0.75, 1.39)	1.28 (0.99, 1.67)	1.7 (1.28, 2.2)	1.91 (1.47, 2.56)
Zinc (mg)	5.6/2.7[j]	0.86 (0.57, 1.14)	1.01 (0.71, 1.33)	1.21 (0.93, 1.67)	1.55 (1.14, 2.1)	2.01 (1.44, 2.63)
Calcium (mg)	500	66.8 (35.8, 138.4)	81.7 (41.3, 157.3)	111.6 (54.6, 190.1)	134.4 (91.5, 227.6)	187.5 (114.5, 335.3)
Potassium (mg)	3000[k]	228.5 (148.9, 330.1)	284 (184.6, 384.3)	366.7 (254.9, 501.8)	458.9 (364.9, 598.8)	566.4 (395, 768.1)
Magnesium (mg)	60	29.9 (21, 40)	36.9 (25.2, 47.3)	44.3 (32.6, 57.3)	58.6 (42.7, 72.2)	69 (50.4, 90)
Phosphorous (mg)	460[k]	121.4 (78.6, 189.1)	144.5 (94.7, 203.5)	184.2 (124.5, 253.9)	227.5 (175.8, 305.7)	291.7 (202.5, 410.8)
Thiamin (mg)	0.5	0.09 (0.07, 0.15)	0.11 (0.08, 0.17)	0.16 (0.11, 0.22)	0.20 (0.14, 0.26)	0.24 (0.16, 0.33)
Riboflavin (mg)	0.5	0.15 (0.09, 0.24)	0.18 (0.12, 0.28)	0.24 (0.15, 0.37)	0.29 (0.19, 0.44)	0.38 (0.24, 0.55)
Niacin (mg)	6	0.92 (0.69, 1.17)	1.25 (0.94, 1.65)	1.52 (1.19, 2.01)	2.01 (1.58, 2.61)	2.51 (1.87, 3.15)
Pantothenic acid (mg)	2	0.68 (0.5, 0.96)	0.84 (0.59, 0.14)	1 (0.79, 1.43)	1.22 (0.98, 1.63)	1.63 (1.20, 2.13)
Vitamin B_6 (mg)	0.5	0.14 (0.10, 0.19)	0.17 (0.12, 0.24)	0.22 (0.16, 0.29)	0.28 (0.22, 0.37)	0.35 (0.25, 0.44)
Folate (mg)	160	26.4 (19.8, 34.8)	31 (24.9, 44.6)	39.2 (28.7, 52.3)	53.1 (38, 66.3)	62.8 (46.8, 82.4)
Vitamin B_{12} (µg)	0.9	0.31 (0.16, 0.58)	0.38 (0.23, 0.69)	0.52 (0.31, 0.83)	0.62 (0.38, 0.98)	0.85 (0.48, 1.37)
Vitamin A (µg)	400[l]	43.1 (26.2, 76.7)	56.7 (33.8, 86.8)	79.6 (50.7, 119.7)	99.1 (64.1, 146.2)	125.1 (84.3, 201.4)
Vitamin C (mg)	30	3.6 (2.6, 5.3)	4.9 (3.6, 7.5)	7.1 (4.6, 9.8)	9.9 (7.5, 12.6)	10.7 (7.8, 15.8)
Vitamin E (mg)	5[k]	0.65 (0.46, 0.90)	0.9 (0.65, 1.17)	1.09 (0.71, 1.44)	1.27 (0.98, 1.66)	1.56 (1.13, 1.93)
MNDA (%), median (IQR)	100	41.8 (33.3, 49)	39.2 (33.4, 44.7)	40.1 (33.5, 45.7)	42.1 (36.3, 49.6)	48.8 (40.4, 55.7)
Children being breastfed (%)[m]	NA	100	100	98	90	61

*Lactulose:mannitol (L:M) ratio, alpha-1-antitrypsin (AAT), myeloperoxidase (MPO) and neopterin (NEO). Nutrient intakes are median (IQR) usual intakes estimated by Multiple Source Method (19). Recommended nutrient intakes from WHO/FAO for children 1-3 years (22). MNDA: mean nutrient density adequacy. Parts of the table has been presented previously.

[a] Number of participants with both dietary data and fecal sample collected

[b] Lunn PG, Northrop-Clewes CA & Downes RM (1991) (26)

[c] n=218

[d] Beckmann (2000) (28)

[c] Saiki (1998) (38)

[f] Ledjeff (2001) (39)

[g] Energy requirement from complementary food for children with high breast milk intake (40)

[h] Only participants with intake of ASP included (n= 193, 136, 192, 197 and 183 respectively)

[i] Corresponding to low (5%) absorption

[j] Corresponding to low (5%) and medium (10%) absorption

[k] Adequate intake
[l] Recommended safe intake
[m] Measured at the end of the time slot (12, 15, 18, 21 and 24 months)

Table 3 Associations between nutrient intake from complementary foods and L:M ratio and fecal markers for environmental enteric dysfunction (EED), children 9-24 months, Bhaktapur, Nepal

	Lactulose:mannitol (L:M) ratio			**Alpha-1-antitrypsin (AAT)**			**Myeloperoxidase (MPO)**			**Neopterin (NEO)**		
	B	CI	ß	B	CI	ß	B	CI	ß	B	CI	ß
Nutrients[a]												
ASP (g)	0.013	-0.046, 0.072	0.035	0	-0.009, 0.009	0	-0.005	-0.015, 0.005	-0.036	-0.001	-0.011, 0.008	-0.009
PUFA (g)	-0.143	-0.373, 0.087	-0.110	-0.029	-0.064, 0.006	-0.088	0.009	-0.025, 0.043	0.022	-0.017	-0.048, 0.014	-0.055
Fiber (g)	-0.037	-0.163, 0.090	-0.045	-0.005	-0.024, 0.015	0.020	-0.019	-0.046, 0.007	0.060	-0.022	-0.045, 0	-0.092
Iron (mg)	-0.098	-0.435, 0.238	-0.051	-0.017	-0.066, 0.033	-0.032	-0.046	-0.107, 0.016	-0.069	-0.018	-0.079, 0.044	-0.036
Zinc (mg)	-0.224	-0.833, 0.386	-0.108	-0.013	-0.106, 0.081	-0.023	-0.094	-0.205, 0.016	-0.136	-0.004	-0.105, 0.097	-0.008
Calcium (g)	-0.944	-3.006, 1.119	-0.141	0.037	-0.269, 0.342	0.010	-0.354	-0.744, 0.036	-0.080	0.022	-0.285, 0.328	0.007
Potassium (g)	-0.956	-2.518, 0.607	-0.145	0.008	-0.199, 0.215	0.004	-0.329	-0.609, -0.048	-0.145*	-0.137	-0.358, 0.083	-0.080
Magnesium (g)	-5.763	-18.6, 7.075	-0.105	0.422	-1.583, 2.426	0.027	-2.814	-5.363, -0.264	-0.144*	-1.173	-3.619, 1.272	-0.079
Phosphorous (g)	-1.934	-4.976, 1.107	-0.168	0.060	-0.408, 0.528	0.018	-0.581	-1.144, -0.017	-0.141*	-0.023	-0.493, 0.448	-0.007
Thiamin (mg)	1.390	-1.193, 4.694	0.090	0.271	-0.197, 0.740	0.065	-0.251	-0.774, 0.272	-0.048	-0.149	-0.570, 0.272	-0.038
Riboflavin (mg)	-0.958	-2.408, 0.492	-0.126	0.069	-0.136, 0.274	0.030	-0.255	-0.544, 0.035	-0.091	0.068	-0.162, 0.298	0.032
Niacin (mg)	-0.006	-0.376, 0.363	-0.003	-0.016	-0.069, 0.036	-0.034	0.007	-0.054, 0.068	0.012	-0.048	-0.109, 0.012	-0.108
Pantothenic acid (mg)	-0.551	-1.235, 0.134	-0.225	0.007	-0.092, 0.105	0.010	-0.080	-0.176, 0.017	-0.096	0.061	-0.027, 0.148	0.097
Vitamin B_6 (mg)	-0.555	-2.756, 1.645	-0.049	0.150	-0.169, 0.470	0.048	-0.200	-0.597, 0.198	-0.051	-0.256	-0.539, 0.028	-0.087
Folate (g)	-5.767	-15.817, 4.282	-0.097	-0.479	-1.917, 0.960	-0.029	-2.077	-3.901, -0.253	-0.102*	-1.037	-2.850, 0.849	-0.065
Vitamin B_{12} (mg)	-0.116	-0.385, 0.153	-0.060	-0.007	-0.074, 0.059	-0.008	-0.027	-0.102, 0.048	-0.026	0.011	-0.054, 0.076	0.014
Vitamin A (mg)	-2.284	-6.273, 1.705	-0.093	-0.075	-0.659, 0.509	-0,011	-0.409	-1.124, 0.307	-0.048	0.080	-0.532, 0.693	0.013
Vitamin C (mg)	-0.023	-0.064, 0.019	-0.079	-0.002	-0.008, 0.004	-0.025	-0.008	-0.014, -0.002	-0.082*	-0.004	-0.010, 0.002	-0.054
Vitamin E (mg)	-0.295	-0.745, 0.154	-0.107	-0.010	-0.090, 0.069	-0.013	-0.036	-0.123, 0.051	-0.038	-0.004	-0.069, 0.060	-0.006
MNDA[b]	-0.005	-0.021, 0.010	-0.048	-0.001	-0.004, 0.002	-0.014	-0.004	-0.007, -0.001	-0.076*	0	-0.003, 0.002	0
N Samples		218			959			960			962	

Dependent variables were log- transformed, associations between nutrient intake and L:M ratio were assessed by multiple linear regression, other associations were assessed by GEE models with auto-regressive (AR-1) covariance structure, significant associations are marked by *

[a] Models adjusted for energy intake, WAMI, season, gender and age (in days) (only fecal markers)

[b] Models adjusted for WAMI, season, gender and age (only fecal markers)

Appendixes

Appendix 1

MAL-ED 24-hour food recall questionnaire (FRQ)

MAL-ED

24 HOUR FOOD RECALL QUESTIONNAIRE (FRQ)

1. Participant ID: ☐☐☐☐☐☐☐☐ **2. Date (DD/MMM/YY):** ☐☐/☐☐☐/☐☐ **3. Fieldworker ID:** ☐☐☐

Instructions: Options for *Home*: Food consumed at home=1, Elsewhere = 0; Options for *R/C: Raw or Cooked*: Raw =1; Cooked = 2.

					Recipe		Food Item			Food Served		Food Leftover	
4	5	6	7	8	9	10	11	12	13	14	15	16	17
Line#	Food #	Meal	Home	Time	Description	Code	Description	Code	R/C	Portion Size Description	Amt (g)	Portion Size Description	Amt (g)
☐☐	☐☐	☐☐	☐	☐☐:☐☐	☐ NA	(4 digits)	☐ NA	(6 digits)		☐ NA	☐ NA	☐ 0 .0 ☐ NA	☐ 0.0 ☐ NA
☐☐	☐☐	☐☐	☐	☐☐:☐☐	☐ NA		☐ NA			☐ NA	☐ NA	☐ 0 .0 ☐ NA	☐ 0.0 ☐ NA
☐☐	☐☐	☐☐	☐	☐☐:☐☐	☐ NA		☐ NA			☐ NA	☐ NA	☐ 0 .0 ☐ NA	☐ 0.0 ☐ NA
☐☐	☐☐	☐☐	☐	☐☐:☐☐	☐ NA		☐ NA			☐ NA	☐ NA	☐ 0 .0 ☐ NA	☐ 0.0 ☐ NA
☐☐	☐☐	☐☐	☐	☐☐:☐☐	☐ NA		☐ NA			☐ NA	☐ NA	☐ 0 .0 ☐ NA	☐ 0.0 ☐ NA

MAL-ED

Instructions: Options for *Home*: Food consumed at home=1, Elsewhere = 0; Options for *R/C: Raw or Cooked*: Raw =1; Cooked = 2.

					Recipe		Food Item			Food Served		Food Leftover	
4	5	6	7	8	9	10	11	12	13	14	15	16	17
Line#	**Food #**	**Meal**	**Home**	**Time**	**Description**	**Code**	**Description**	**Code**	**R/C**	**Portion Size Description**	**Amt (g)**	**Portion Size Description**	**Amt (g)**
☐☐	☐☐	☐☐	☐	☐☐:☐☐	☐ NA	(4 digits)	☐ NA	(6 digits)		☐ NA	☐ NA	☐ 0 .0 ☐ NA	☐ 0.0 ☐ NA
☐☐	☐☐	☐☐	☐	☐☐:☐☐	☐ NA		☐ NA			☐ NA	☐ NA	☐ 0 .0 ☐ NA	☐ 0.0 ☐ NA
☐☐	☐☐	☐☐	☐	☐☐:☐☐	☐ NA		☐ NA			☐ NA	☐ NA	☐ 0 .0 ☐ NA	☐ 0.0 ☐ NA
☐☐	☐☐	☐☐	☐	☐☐:☐☐	☐ NA		☐ NA			☐ NA	☐ NA	☐ 0 .0 ☐ NA	☐ 0.0 ☐ NA
☐☐	☐☐	☐☐	☐	☐☐:☐☐	☐ NA		☐ NA			☐ NA	☐ NA	☐ 0 .0 ☐ NA	☐ 0.0 ☐ NA

18. How many times was the child nursed during the daytime: ☐☐

9. How many times was the child nursed during the nighttime: ☐☐

20. Total times nursed: ☐☐

21. **Comments**:

Appendix 2

24-hour recall form part 2 (FRS) (recipes)

MAL-ED

1. Participant ID: ☐☐☐☐☐☐☐☐

2. Date (DD/MMM/YY): ☐☐ / ☐☐☐ / ☐☐

3. Fieldworker ID: ☐☐☐

4. Recipe Name: _____ 5.Recipe Code: ☐☐☐☐

24 HOUR RECALL FORM PART 2 (FRS)

Instructions:

6. line #	7. Ingredient	8.Code	9.Amount	10.Weight (g)	11.Remarks
1		(6 digits)		☐☐☐☐☐	
2		(6 digits)		☐☐☐☐☐	
3		(6 digits)		☐☐☐☐☐	
4		(6 digits)		☐☐☐☐☐	
5		(6 digits)		☐☐☐☐☐	
6		(6 digits)		☐☐☐☐☐	
7		(6 digits)		☐☐☐☐☐	
8		(6 digits)		☐☐☐☐☐	
9		(6 digits)		☐☐☐☐☐	
10		(6 digits)		☐☐☐☐☐	
11		(6 digits)		☐☐☐☐☐	
12		(6 digits)		☐☐☐☐☐	
13		(6 digits)		☐☐☐☐☐	
14		(6 digits)		☐☐☐☐☐	

12.Cooking Method Notes: _____