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DEVELOPMENTAL EFFECTS OF EARLY LIFE EXPOSURE TO ENTERIC PATHOGENS AND OTHER ENVIRONMENTAL FACTORS

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INTRODUCTION

Almost fifty percent of the mortality of children under age five can be attributed to infectious diseases, with the majority of this global burden in Africa and South Asia (*Liu* et al., 2015). Additionally, childhood under-nutrition contributes to 30-40% of early childhood deaths and 70% of deaths due to diarrhoea (*UNICEF-WHO-World Bank Group*, 2015). Mortality rates have been declining over the past two decades due in part to the availability of vaccines against some respiratory and diarrhoeal pathogens, improved water quality and sanitation, the development and use of oral and intravenous rehydration solutions for dehydrating diarrhoea and an improvement in overall world economies. However, there are some areas of the world where these improvements have not been fully realised, and child mortality remains unacceptably high at rates three to four times those of developed countries (*Liu* et al., 2015).

Decline in the rates of morbidities associated with diarrhoeal disease have not been reduced to the same extent as those of mortality (*Kosek* et al., 2003). It has been recognized that children suffering from frequent episodes of diarrhoea may become malnourished as measured by stunted (length-for-age [LAZ] of ≤ -2), underweight (weight-for-age [WAZ] ≤ -2) or wasted (weight-for-length) [WLZ] ≤ -2) growth. Other longer-term morbidities such as reduced immune response to orally administered vaccines and impaired cognitive development and school performance have also been associated with the occurrence of diarrhoea in the first two years of life. The Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Project (MAL-ED) was initiated in 2008 to define the strength of association of enteric infection, undernutrition and other environmental exposures with the development of these longer-term morbidities. Such data are useful to better define the contribution of these exposures to the calculation of Disability Adjusted Life Years (DALY's) and in the hope that current and potential new interventions could be identified and prioritized.

MAL-ED is a prospective, longitudinal, observational birth cohort study. More than 200 new-borns were recruited from each of eight international sites with historically high rates of both diarrhoeal disease and stunted growth:

NOTE: This paper was originally presented at the 30th Old Herborn University Seminar in June 2016. Because some of the data presented at that meeting was unpublished, the print version of the presentation was embargoed until those data were published or accepted for publication in peer-reviewed journals. As of June of 2018, that has been accomplished. As a result, this manuscript has been updated so as to include that data and current literature citations.

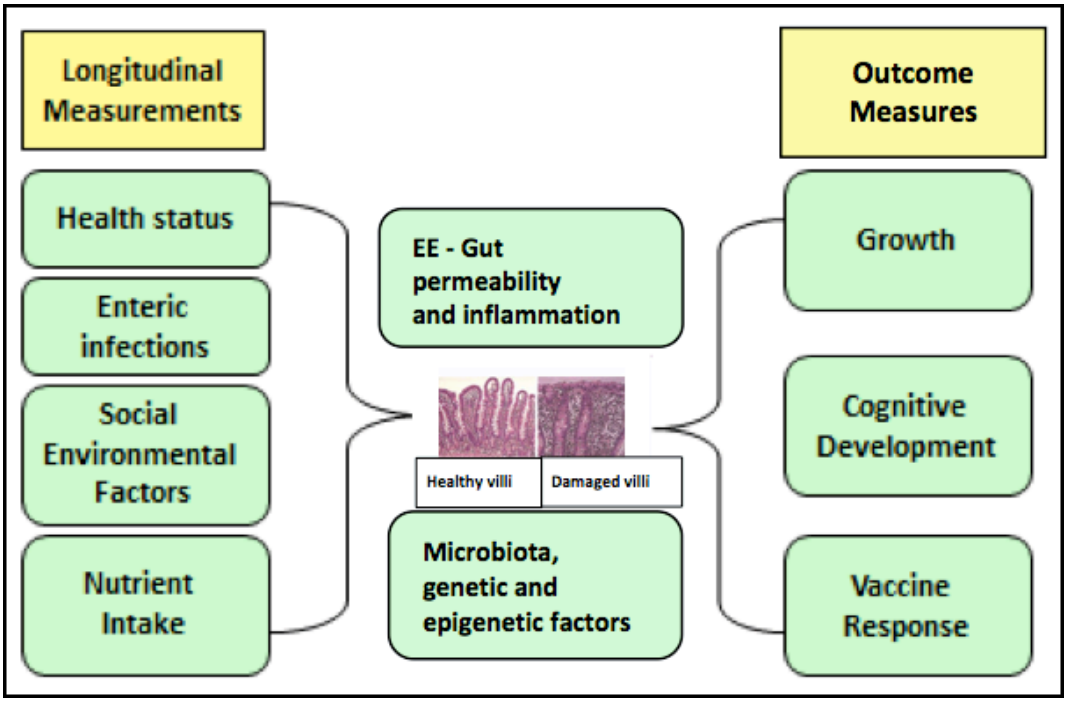

Figure 1: The MAL-ED hypotheses. Enteric infections were detected by examination of non-diarrhoeal surveillance and diarrhoeal stool throughout the study and were hypothesized to contribute to the development of Environmental Enteropathy (EE), characterized by measuring gut inflammation and increased permeability. A damaged gut was postulated to contribute to deficits in the major outcome measures of growth, cognitive development and immune response to routine scheduled childhood vaccines. Other variables including overall health status, social and environmental factors and nutrient intake were also considered as described previously (*MAL-ED Network Investigators*, 2014). This recognised the potential role for the gut microbiota and human genetic and epigenetic factors (which were not measured) that may also contribute to study outcomes.

four Asian sites located in Dhaka, Bangladesh (BGD); Vellore, India (INV); Bhaktapur, Nepal (NEB) and Nashero-Ferroze, Pakistan (PKN), two Southern African sites located in Venda, South Africa (SAV) and Haydom, Tanzania (TZH), and two South American sites located in Fortaleza, Brazil (BRF) and Loreto, Peru (PEL). MAL-ED collected information on the illness history of the cohort to include the incidence of diarrhoeal and respiratory disease identified through twice weekly home visits and caregiver reports. In contrast to other studies, we primarily identified cases of mild to moderate diarrhoeal disease that often go unrecorded due to lack of presentation to a health care facility**.** This study was also unique in that we collected monthly non-diarrhoeal stools in order to identify the enteric pathogens present in these samples as a way of assessing the overall rate of exposure of children.

The MAL-ED study hypothesised that frequent enteric infections (with or without diarrhoea) lead to the development of environmental enteropathy (EE), a condition characterized by intestinal inflammation, damaged gut architecture and decreased absorptive

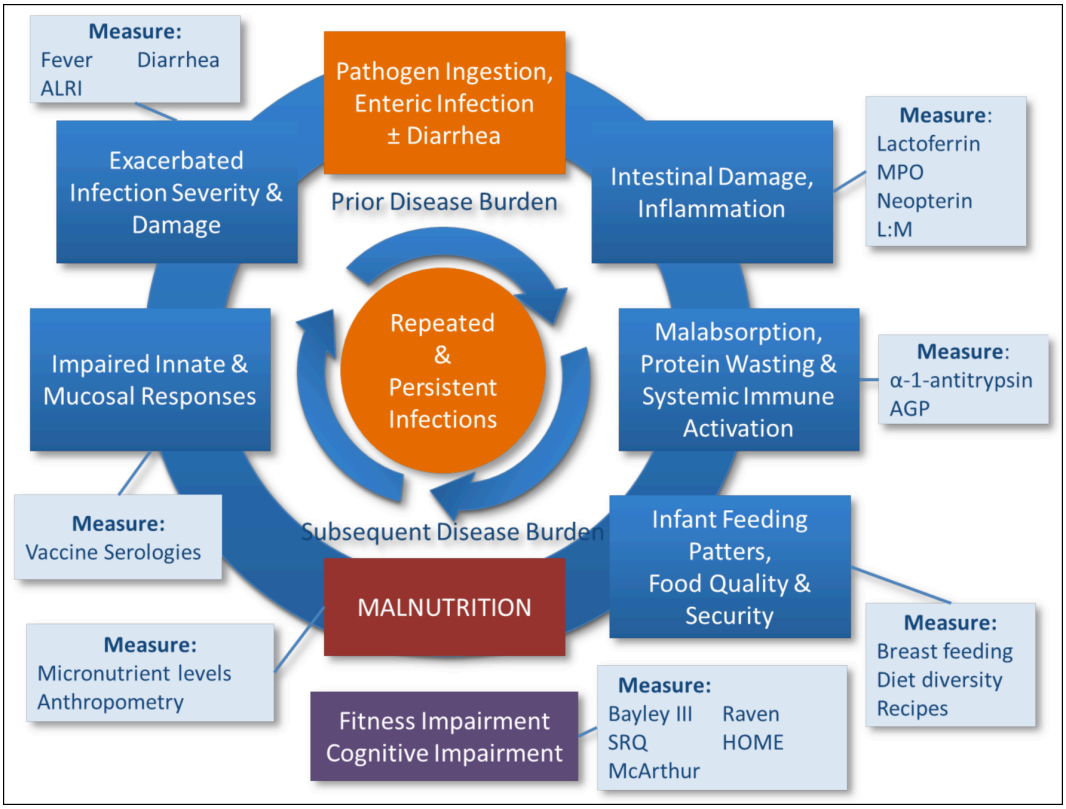

Figure 2: A depiction of the "vicious cycle of poverty" (*Guerrant* et al., 2008) of enteric infections leading to enteropathy and malabsorption of nutrients which, together with an inadequate diet, contributes to the development of malnutrition (growth deficits), cognitive impairment and decreased immune responses which, in turn, increase the child's susceptibility to more infections. The light blue boxes indicate some of the MAL-ED measures used to assess each step in this cycle.

capacity that was initially described histologically using gut biopsies. This condition, originally called tropical enteropathy, was first identified as altered intestinal architecture visualized in gut biopsies in individuals who travelled to or resided in areas that have high environmental burdens of enteropathogens and who lacked access to clean water and adequate sanitation (*Lindenbaum* et al., 1966; *Colwell* et al., 1968; *Fagundes-Neto* et al., 1984). MAL-ED further hypothesised that development of EE, in combination with an inadequate diet, leads to malnutrition manifested as growth deficits and the other morbidities as depicted in Figure 1. In children living in poverty, the progression of events from initial enteric infection to the development of enteropathy, undernutrition, impaired growth and cognitive development and suppressed immune response to certain vaccines has been proposed as a "vicious cycle of poverty" (*Guerrant* et al., 2008). Figure 2 depicts this cycle and indicates the data types that were collected throughout MAL-ED to evaluate the strength of evidence supporting this model.

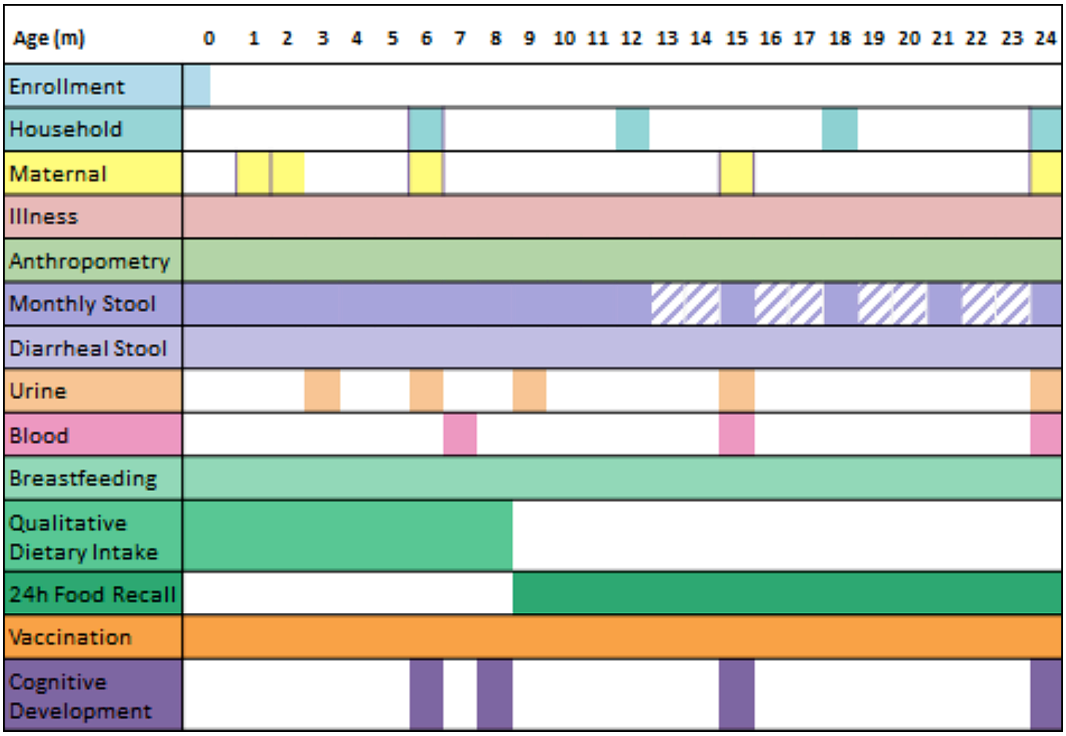

Figure 3: Data collection schedule. Colours refer to the different data types that were collected at the indicated months of age of the MAL-ED cohort children. For example, household assessments were done when the child was 6, 12, 18 and 24 months old and anthropometry measurements were schedule monthly for the duration of the study. The hashed months indicated as part of the monthly stool collections reflect samples that were collected but not analysed for presence of pathogens. These samples are maintained as study sample archives at the field sites where they were collected.

METHODS

Descriptions of the MAL-ED field sites, study design and methods have been previously described (*Lang*, 2010; *MAL-ED Network Investigators*, 2014) and are only briefly summarized here. Children were followed from enrolment near birth through the first 24 months of life. Variables of interest included enteropathogen exposure, illness history, dietary intakes and the home environment.

The protocol was approved by each local Institutional/Ethical Review Board (I/ERB) and by the IRBs at collaborating institutions in the United States, Norway and Thailand. Enrolment followed signed informed consent. In order to capture seasonal effects of exposures, monthly enrolment of between 15-18 new-borns was attempted at each site. The data collection schedule used in the study is shown in Figure 3. A data-coordinating centre (DCC) was established at the Fogarty International Center (NIH, Bethesda, MD, USA).

Biologic specimens are maintained at the site where they were collected and frozen at -80° C. All analyses were performed using de-identified data. Figure 4 illustrates the magnitude and breadth of some of the data types that were collected in the study.

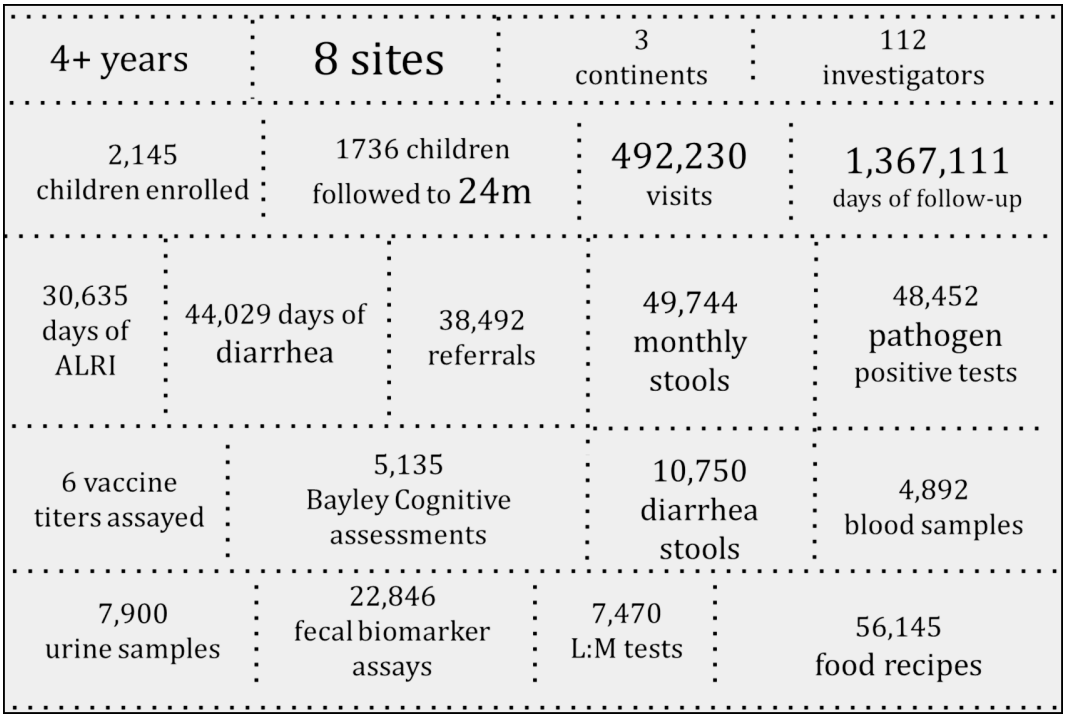

Figure 4: The magnitude and breadth of data types and samples collected within MAL-ED. This figure indicates the number and variety of a partial list of the data types that were collected during the study. Abbreviations used: ALRI = Acute Lower Respiratory Infection, L:M = Lactulose : Mannitol ratio.

Data were collected during twice weekly home visits by trained field workers who executed a common protocol that included physical measurement of children's length, weight and head circumference, and collection of stool samples, illness and dietary histories. A number of questionnaires were used to measure home environment, family socio-economic status (SES), a child's health, vaccination history, use of antibiotics and oral rehydration solution, extent of breastfeeding, weaning food composition, child temperament and the care-givers' education, parity, use of alcohol and tobacco products during pregnancy. Mother's reasoning ability was assessed by the Raven's Progressive Matrices (*Raven*, 2000).

Enteric pathogens were detected by standard microbiologic assays, microscopy, ELISA and molecular methods. EE biomarkers were determined by ELISA. As the study did not collect gut biopsies (the gold standard for assessing EE) from these very young children, we chose a panel of non-invasive biomarkers of inflammation (myeloperoxidase [MPO] and neopterin [NEO]) and gut permeability (alpha-1-antitrypsin [AAT]) assayed in stool. Urine samples were used to measure gut permeability by the lactulose : mannitol test and for some micronutrients. Plasma was used to measure vaccine immune response and systemic inflammation.

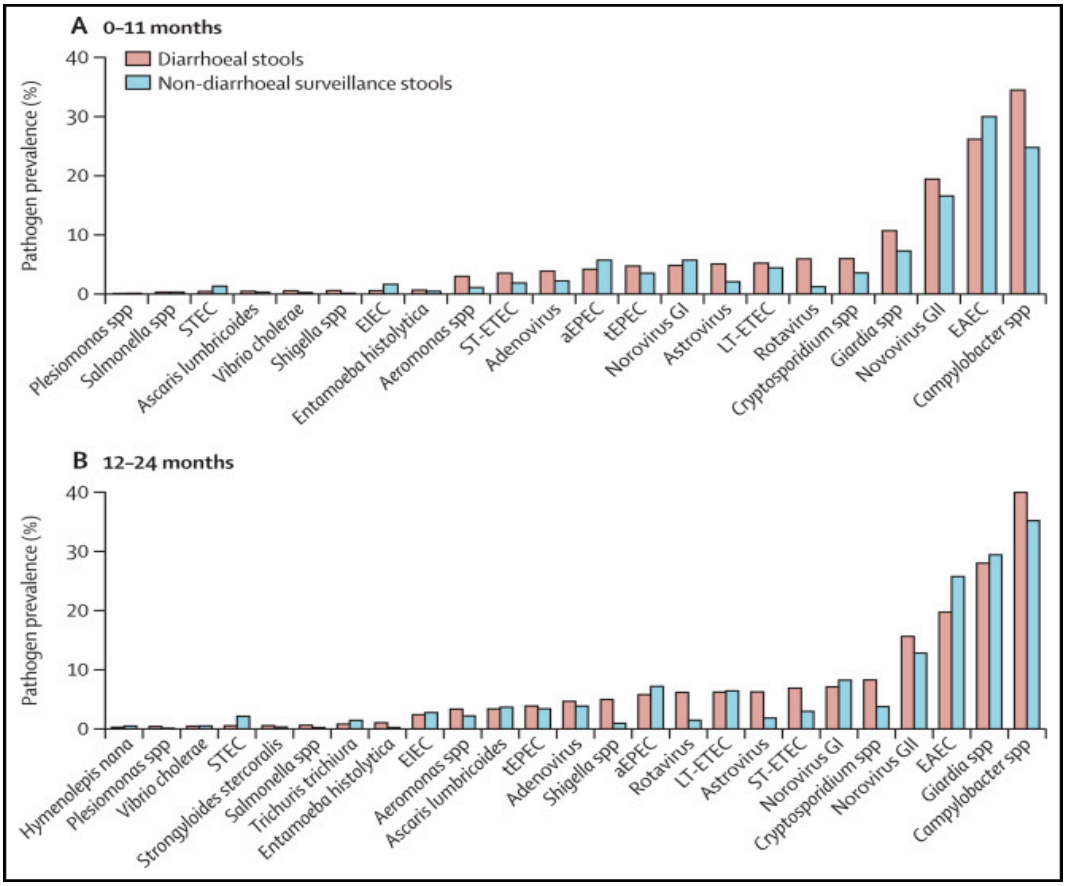

Figure 5: Pathogens detected in diarrhoeal (red bars) and non-diarrhoeal stools (blue bars), 0-11 months (A) and 12-24 months (B). EAEC=enteropathogenic *E. coli*; EIEC=enteroinvassive *E. coli*; aEPEC=atypical enteropathogenic *E. coli*; tEPEC=typical enteropathogenic *E. coli*; LT-ETEC=LT-producing enterotoxigenic *E. coli*; ST-ETEC=ST-producing enterotoxigenic *E. coli*; STEC=Shiga toxin-producing *E. coli*. Pathogens present in less than 0.1% of stool samples are not shown. Figure from *Platts-Mills* et al. (2015).

RESULTS

Enteric pathogen burden

We examined diarrhoeal stools (n = 7,318) and non-diarrhoeal surveillance stools collected monthly (n = 24,310) from cohort children for enteric pathogens (bacteria, viruses and parasites) as previously described (*Houpt* et al., 2014). The frequency of detection of the majority of these pathogens was higher in diarrhoeal than in non-diarrhoeal specimens (*Platts-Mills* et al., 2015). However, detection of some pathogens in non-diarrhoeal stools was higher, and for others nearly equivalent, to what was found in diarrhoeal stools during the first 24 months of life (Figure 5). The most frequently identified organisms in both diarrhoeal and non-diarrhoeal surveillance stools were *Campylobacter*, entero-aggregative *E. coli* (EAEC), *Giardia*, Norovirus and *Cryptosporidium* (*Platts-Mills* et al., 2015; Figure 5) although there was site-to-site heterogeneity in the rank order.

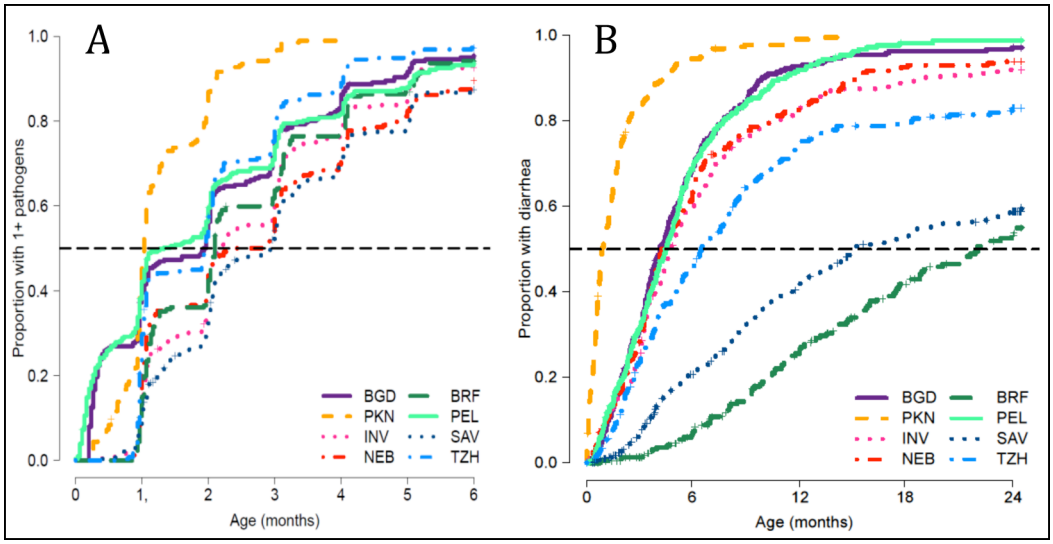

Figure 6: Proportion of children (vertical-axis) who have been infected with an enteric pathogen (A) and the proportion that have experienced their first episode of diarrhoea (B) during the first two years of life. The horizontal dotted line on both graphs indicates 50 % of children. Range of ages by which 50% of children have their first diarrhoea episode: 1 month in PKN, about 5-7 months in BGD, INV, PEL, NEB, and TZH, about 15 months in SAV and about 23 months in BRF.

Children experienced their first enteric infection early in life (Figure 6), with 50% of children at all sites infected by the time they were three months old and over 85% of these children had been infected at least once by the time they were six months of age, depending on the site. Despite this early high rate of infection, many children had not experienced diarrhoea until later in life, with the time to first diarrhoeal episode ranging from 1 month (PKN) to 22 months (BRF).

Several children at the BRF and SAV sites did not develop diarrhoea at all during their first two years of life, despite frequent enteric infections during that time. The extent of this enteric infectious burden is shown in Figure 7. Of note, is the percentage of diarrhoeal and non-diarrhoeal surveillance stools containing at least one pathogen that continued to increase throughout the study until between 80 and 100% of diarrhoeal stools and 70 to 90% of non-diarrhoeal surveillance stools contained at least one pathogen. Approximately 30-40% of all stool samples contain two or more pathogens (*Platts-Mills* et al, 2015*)* with as many as eight pathogens having been identified as co-infections in the same sample.

Growth of children

Between 10-20% of children at all sites were born stunted (<-2 LAZ), as illustrated in Figure 8. Only the BRF site observed a decline in the rate of stunting over the first two years where only 3.6% were stunted at 24 months of age. This is in contrast to the findings at other sites that exhibited an increased proportion of children stunted at two years of age, from between 23% in NEB to 70.6% in TZH. Slower linear growth was associated with the presence of enteric pathogens (cumulative burden of all measured pathogens) in non-diarrheal stool. Individual pathogens that had a negative association

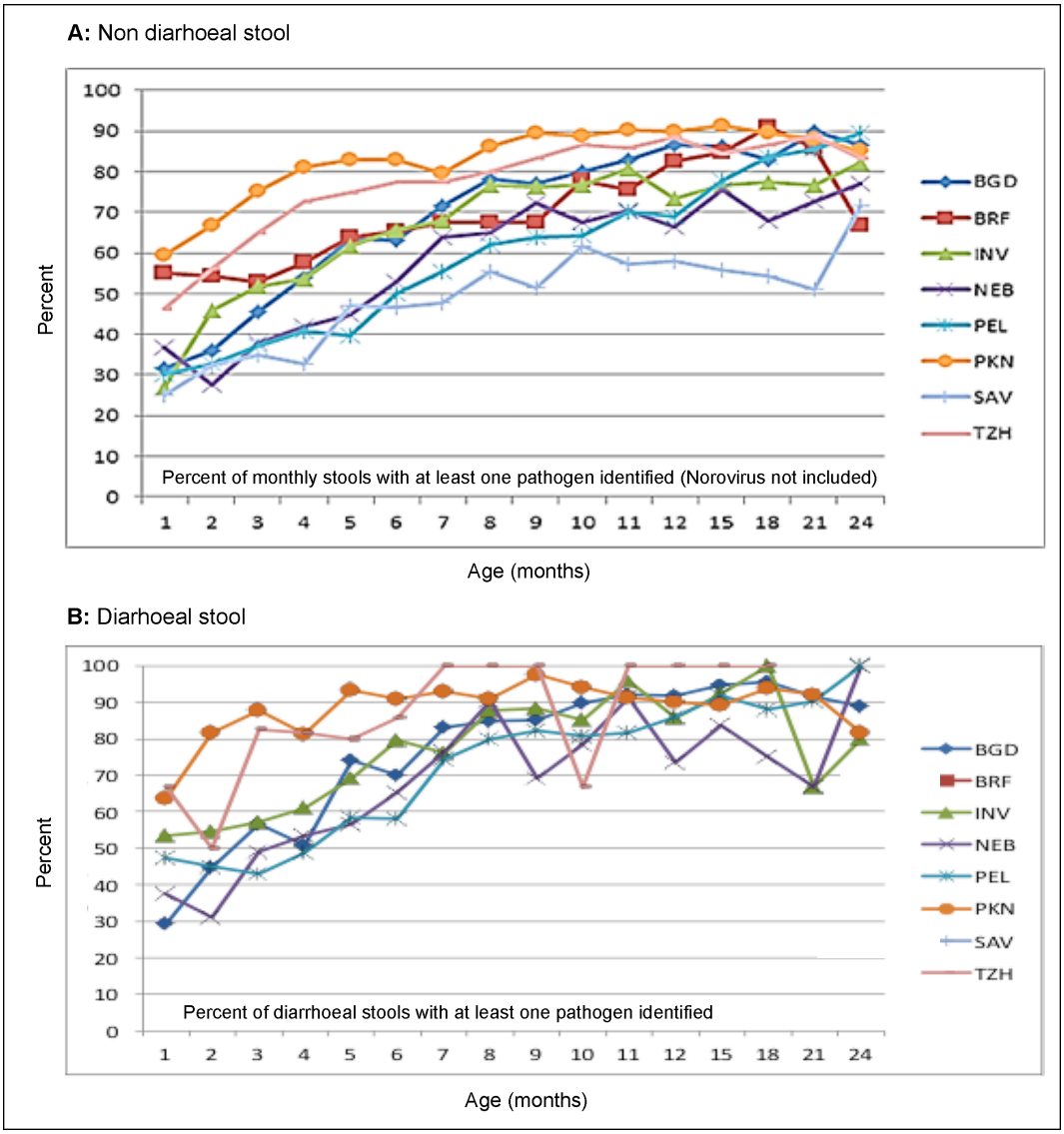

Figure 7: Percent of collected non-diarrhoeal surveillance (A) and diarrhoeal (B) stools containing at least one pathogen during the first two years of life, by site. Approximately 70-90% of all non-diarrhoeal surveillance stool samples collected from 8-24 month old children contain at least one enteric pathogen (bacteria, virus or parasite). Likewise, but perhaps more predictable, 80-100% of diarrhoeal specimens from 8-24 month old children also contained at least one enteric pathogen. Brazil and South Africa sites are not included in (b) due to their low frequency of diarrhoea.

were *Campylobacter* and enteroaggregative *E. coli.* Additionally, *Cryptosporidium*, LT-ETEC and atypical EPEC tended toward an association with slower growth rate. Diet also affected growth. Lower quality (lower energy and protein density) complementary diets from 9-24 months were associated with a slower growth rate and lower attained length at 24 months of age (*MAL-ED Investigators,* 2017a).

In a separate analysis of growth (*MAL-ED Network Investigators*, 2017b) we determined that maternal

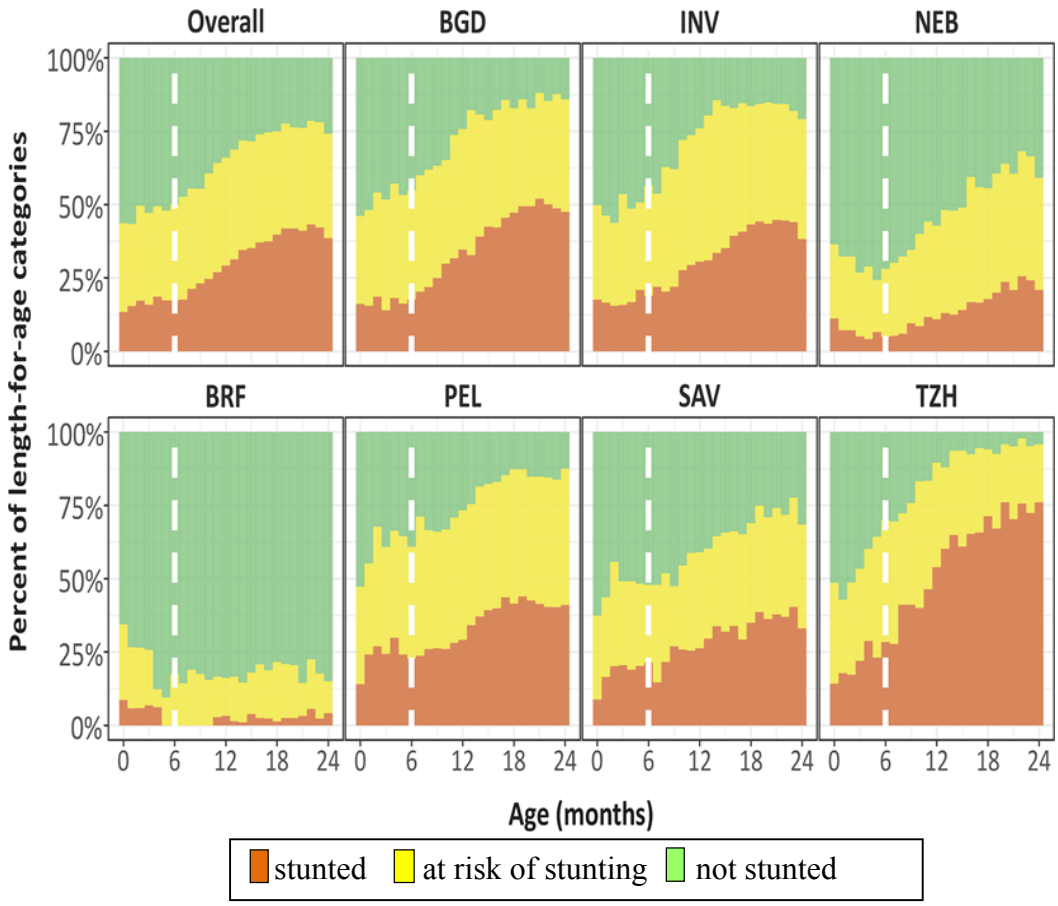

Figure 8. Categories of length-for-age stratified by exact month of age and site. In this figure, not stunted (length-for-age *Z*-score [LAZ] > −1) is represented in green, at risk of being stunted (−2 < LAZ < −1) is represented in yellow, and stunted (LAZ < −2) is represented in orange. Sites include BGD, Dhaka, Bangladesh; BRF, Fortaleza, Brazil; INV, Vellore, India; NEB, Bhaktapur, Nepal; PEL, Loreto, Peru; SAV, Venda, South Africa; TZH, Haydom, Tanzania. The vertical broken line represents 6 months of age. (Figure from *MAL-ED Network Investigators,* 2017b).

and prenatal factors were also important in early child growth. Maternal short stature and lower enrolment weight of the child were negatively associated with growth attained at 24 months of age. This analysis also identified enteropathogen burden in non-diarrheal stool, lower percent of energy from protein in the diet and lower household SES to be negatively associated with LAZ at 24 months. We have previously reported an association of elevated levels of the stool biomarkers of intestinal and systemic inflammation (MPO, MEO and AGP) and gut permeability with reduced growth (LAZ) over the six months following the biomarker measurements (*Kosek* et al., 2013, 2016).

As has been noted, reaching the stunting threshold is not the only measure that should be used to define suboptimal child growth (*Richard,* 2018). Even though there are many children in LMICs whose growth attainment does not reach that definition (< -2 LAZ), they are still likely to be growing at less than their full potential.

Cognitive development

Diarrhoea, enteric pathogens and subsequent malnutrition have been linked to impaired cognitive development (*Berkman* et al., 2002; *Patrick* et al., 2005; *Oria* et al., 2016). We examined the association of several environmental exposures with cognitive development as measured by the Bayley Scales of Infant and Toddler Development (BSID-III) (*Bayley*, 2005) at 24 months (*MAL-ED Network Investigators, 2018a)*. Factors that associated with lower BSID-III scores included illnesses indicators (vomiting, fever, and acute lower respiratory infection), higher frequencies of enteric pathogen detection both in diarrhoeal and non-diarrhoeal stools, and lower scores on the Raven's test of mothers reasoning ability. Illness and enteric pathogen burden effects were at least partially mediated through lower haemoglobin concentration. Factors associated with higher age-appropriate cognitive development scores included both physiologic factors such as higher levels of B vitamins (B6 and folate), haemoglobin, and social factors including a more nurturing home environment as measured by the Home Observation for the Measurement of the Environment (HOME) instrument (*Bradley* et al., 1996), and the mother's ability to form comparisons, reason by analogy, and organize spatial perceptions as assessed with the RCM.

Immune response to vaccines

Previous research has shown that enteric diseases and malnutrition also lead to reduced immune responses to certain childhood vaccines (*Myaux* et al., 1996; *Parker* et al., 2014). We assessed the immune responses to a number of childhood vaccines administered as part of the routine immunisation schedule in each country. These included two oral vaccines (oral polio in seven sites, oral rotavirus in three sites) as well as parenteral vaccines (measles, pertussis, and tetanus at all eight sites).

Our findings on factors effecting performance of the oral polio vaccine (OPV) have recently been published (*MAL-ED Network Investigators,* 2018b). Plasma was obtained and titres determined at 7 and 15 months of age. Failure to seroconvert to OPV administration was associated with high detection rates of enteric bacterial and parasitic pathogens in non-diarrhoeal surveillance stool samples during the neonatal period. This was especially true for poliovirus serotypes 2 and 3. Interestingly, viral pathogen burden did not exhibit similar negative effects. Biomarkers of gut function (increased L:M ratio and lower AAT) in stool were also weakly associated with poorer response to serotypes 2 and 3 of OPV. Factors associated with higher levels of seroconversion included receiving more and early vaccine doses and a higher SES, particularly having more household assets (an indicator of longer-term wealth) including improved water supply and improved sanitation in the home (Table 1).

DISCUSSION

The MAL-ED study examined the relative contributions of a number of environmental exposures to major outcomes of child development. It is unprecedented in the breadth of data types collected and the number of geographically dispersed sites studied using a common protocol. While many studies have examined the effect of diarrhoea on growth, cognitive development or immune response, MAL-ED was the first to intensively examine the

Table 1: Factors positively or negatively associated with the major study outcomes in MAL-ED children. Growth outcome was assessed as LAZ at 24 months of age, cognitive development was assessed with Bayley III at 24 months, and immune response to OPV (serotypes 1,2, and 3) were assessed at 7 and 15 months of age.

Outcome	Negative associations	Positive associations	References
Linear growth attainment at 24 months	1. Sub-clinical enteric infections 2. Infection with *Campylobacter* and EAEC (*Cryptosporidium*, LT-ETEC and aEPEC tended toward negative) 3. Elevated levels of gut inflammatory biomarkers in stool	1. Energy and protein from animal milk and dairy (3-8 months) 2. Protein from complimentary food (9-24 months) 3. Higher SES 4. Higher LAZ and WAZ at birth	*MAL-ED Investigators* (2016a,b) *Kosek* et al., (2013, 2016) *Rogawski* et al., (2017) *Amour* et al., (2016) *Korpe* et al., (2018)
Cognitive development at 24 months	1. Illness indicators (fever, vomiting, ALRI) 2. Enteropathogen detection in both diarrhoea and sub-clinical stool 3. Lower haemoglobin levels 4. Lower weight at enrolment	1. B vitamins (B6 and folate), higher haemoglobin 2. Home environment (HOME) 3. Mother's reasoning ability (RCM)	*MAL-ED Investigators* (2018a)
Immune response to oral polio vaccine (OPV)	1. Sub-clinical bacterial and parasitic infection during the neonatal period was a predictor of OPV failure, especially for serotypes 2 and 3. Viral infections did not exhibit similar relationships 2. Elevated L:M ratio and lower AAT were weakly associated with failure for serotypes 2 and 3 only.	1. Receiving more and early OPV doses improved response 2. Four doses maximized seroconversion of serotype 1 3. Early dosing and receiving five or more doses maximized response for serotype 3 4. Higher SES (household assets and improved water and sanitation) were the strongest predictors of seroconversion	*MAL-ED Investigators* (2018b)

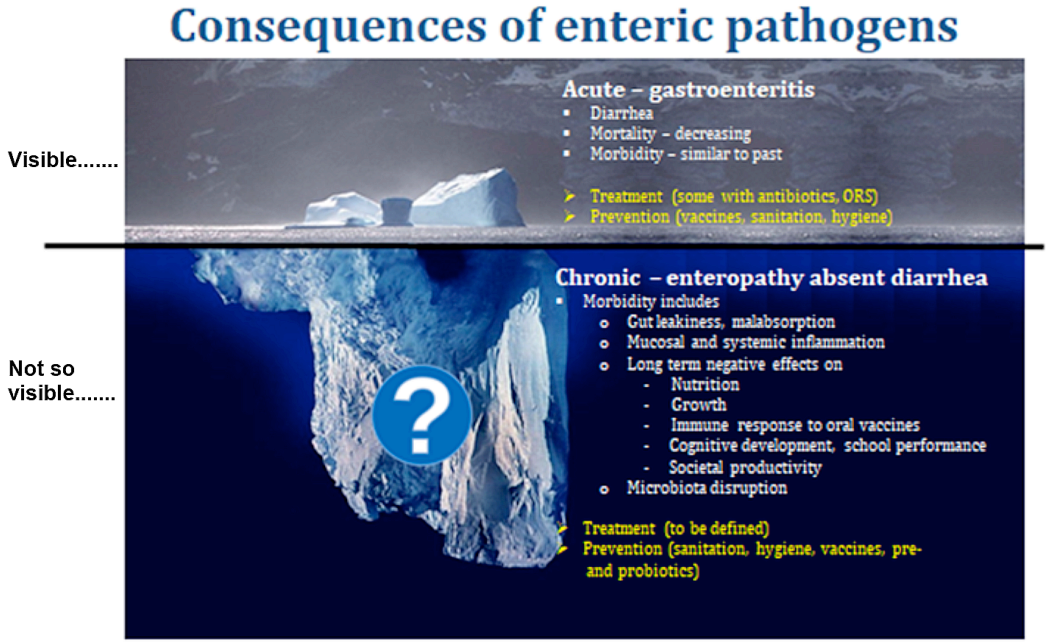

Figure 9: Depiction of both readily observed acute disease (diarrhoea and consequent mortality and morbidity) and the cryptic subclinical chronic effects (enteropathy, malnutrition, stunted growth, decreased cognitive development, impaired immune response, reduced school achievement and societal productivity) of enteric infections. The question mark highlights the currently unrecognized factors that could also contribute additional burden (e.g. new pathogens, dietary components, environmental xenobiotic chemicals, loss of beneficial commensal gut flora, and host genetic and epigenetic factors).

cumulative extent of enteric infections, both in diarrhoeal and non-diarrhoeal stools, within the context of multiple other environmental factors and their association with these developmental outcomes. We believe it was the first study to comprehensively characterize the longitudinal exposure of children, within a community setting, to enteric pathogens by recording their presence in non-diarrhoeal surveillance stool samples.

The observation that a high pathogen burden is negatively associated with all of the major child developmental outcomes - growth, cognitive development and immune response to vaccination - is, we believe, a critical outcome of this study. We have documented the extent to which children in these settings are infected with enteric pathogens; that these high infection rates persist over at least the first two years of life; and because most occur subclinically, largely go unrecognized. Therefore, they were not considered in calculations of the global burden of enteric diseases (*Global Burden of Disease Pediatrics Collaboration*, 2015).

It should be noted that in this study, we used classical microbiologic detection methods of culture, ELISA, microscopy. Our data implicate a number of bacterial and parasitic pathogens, though not viral pathogens, as having the most significant negative associations with the major study outcomes. When more sensitive and quantitative methods, e.g. quantitative-PCR (*Liu* et al., 2013) are used with MAL-ED samples (in progress) it is expected that

even more pathogens will be identified in both diarrhoeal and non-diarrhoeal samples as has been demonstrated in another study (*Liu* et al., 2016). Because this method is semi-quantitative, it may be possible to more accurately ascribe aetiology of diarrhoea or developmental deficits to particular pathogens.

MAL-ED data have revealed that the burden of subclinical (i.e. non-diarrhoeal) enteric infections on child morbidity is actually greater than that due to diarrhoea (Table 1 and Figure 9). A child with diarrhoea is easily recognized and treated. A child with few, if any, outward signs of morbidity but burdened none-the-less with enteric pathogens, is much more difficult to identify and to define pathologically. Clearly, validated biomarkers capable of detecting developing enteropathy are needed; markers that can be easily and inexpensively used to monitor very young infants in field studies. More work is needed to further evaluate the biomarkers used in MAL-ED and others (*Naylor* et al., 2015; *Guerrant* et al., 2016; *Kosek* et al., 2016) for their usefulness as indicators of clinically relevant pathology and for their potential as indicators of successful therapeutic intervention. Another biomarker of promise is a gut microbiota signature consisting of a limited number of microbial taxa found in children growing relatively well but lacking in children with severe malnutrition (*Subramanian* et al., 2014). If it were easy to identify the strength of this "signature" profile in stool samples collected longitudinally, it might allow the identification of at risk children and the ability to monitor improvement following intervention.

With any biomarker, it would be helpful to have ranges of values obtained from comparably aged children in developed countries. Such data would help in the interpretation of the values observed in studies in LMICs. However, the use of periodic child length measurements may remain the cheapest, quickest and most reproducible method to detect at-risk children and to monitor the success of any intervention.

We have identified the early exposure to and asymptomatic carriage of enteropathogens and dietary deficiencies as important contributors to less than optimal growth in this cohort of children (*MAL-ED Network Investigators, 2017a, 2017b)*. Reduction in the exposure to enteric pathogens could reduce the level of growth faltering in young children. When combined with efforts to improve the quality, quantity and diversity of complementary food may yield improvement in growth velocity during the critical first two years of life. Our observations that low maternal height, low neonatal weight and low SES also have deleterious effects on child growth attainment throughout the first 24 months suggest that earlier pre-natal interventions could be helpful (*MAL-ED Network Investigators*, 2017B). The observed negative association of asymptomatic infections extended to the other long-term outcomes of cognitive development (*MAL-ED Network Investigators,* 2018a*)* and OPV immune response (*MAL-ED Network Investigators,* 2018b*)* (also see Table 1), emphasizes the importance of reducing environmental exposure to these pathogens, improved nutrition and higher SES as predictable effectors of improved child development.

Despite our efforts to account for many of the factors contributing to child development shortfalls, substantial inter-site variability remained unaccounted for. As indicated by the question mark in Figure 9, we acknowledge that there are unknown factors that also

may contribute to developmental shortfalls that are likely to occur in different quantitative combinations in different locations. Among these factors could be new, as yet unidentified enteric pathogens, environmental xenobiotic chemical contaminants, dietary components or toxins, genetic and epigenetic factors and the absence or imbalance of certain gut microbes.

The fact that impaired development is negatively associated with multiple factors, suggests that a combination of interventions, applied in concert, may be needed in order to see significant improvement, improvements typically found when public health improves and poverty decreases. A first step is recognizing that early exposure to enteric pathogens is common in these settings, and that the ensuing repeated subclinical infections during the first few months and years of life is an important contributor to the subsequent delayed development of children.

ACKNOWLEDGEMENTS

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