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FREQUENT SYMPTOMATIC OR ASYMPTOMATIC INFECTIONS MAY HAVE LONG-TERM CONSEQUENCES ON GROWTH AND COGNITIVE DEVELOPMENT

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THE IMPORTANCE OF DIARRHOEA

Diarrhoeal disease represents a continuing public health challenge. This is based on two combined issues: in terms of mortality it ranks as the second most common cause of death in children under 5 years old (*Liu* et al., 2012) and in terms of morbidity it has been associated with long-term deficits in physical (Checkley *et al.* 2008) and cognitive development (*Fischer Walker* et al., 2012).

Diarrhoea as a clinical symptom is defined as increased stool mass due to excess fluid in stool (*Field*, 2003). Most commonly this is assessed by the frequency of loose stools (by convention ≥3 loose stools per day), where "loose" is when a stool takes the shape of its container. Disruption to normal functioning of the gut, where water would normally be reabsorbed (*Woods*, 1990; *Field*, 2003), results in loss of fluids in stool and hence risk of dehydration and death. Whilst there are many causes of diarrhoea the most common – oft assumed cause – is enteric infection (WHO, 1988; *Thapar* and *Sanderson*, 2004). In fact, enteric pathogens were deemed to be responsible for between 12.5-94.3% of the moderate to severe diarrhoea (Kotloff *et al.* 2012) in the first year of life in the recent Global Enteric Multicenter Study (Kotloff *et al.* 2013).

Diarrhoeal symptoms that continue over a number of days is classed into one of three syndromic diarrhoeal diseases (Keusch *et al.* 2006): acute diarrhoeal disease (<7 days), prolonged (7≤ days <14) and persistent (≥14 days) (Moore *et al.* 2010). Mortality and morbidity are associated with higher rates of diarrhoeal disease episodes and in particular with persistent diarrhoea. In general, the burden of diarrhoeal disease is highest in low income countries, though it remains a universal health challenge (*Black* et al., 2003).

Research into diarrhoeal disease as a non-specific syndrome has a long history and correspondingly has produced non-specific health interventions (*Sedgwick* and *Macnutt*, 1910; *Blaise* et al., 2007). Considerable progress has been made in establishing the role of specific enteric pathogens as causes of diarrhoeal symptoms, but perhaps more important is the lack of detail regarding entero-pathogens as a cause of the long-term morbidity in the *absence* of overt diarrhoea.

Here we present preliminary findings based on incomplete data from The Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Project (MAL-ED). Through the analysis of both symptomatic and asymptomatic stool samples we are beginning to quantify the role of asymptomatic infection as a driver of the long-term morbid consequence of the diarrhoeal disease syndrome.

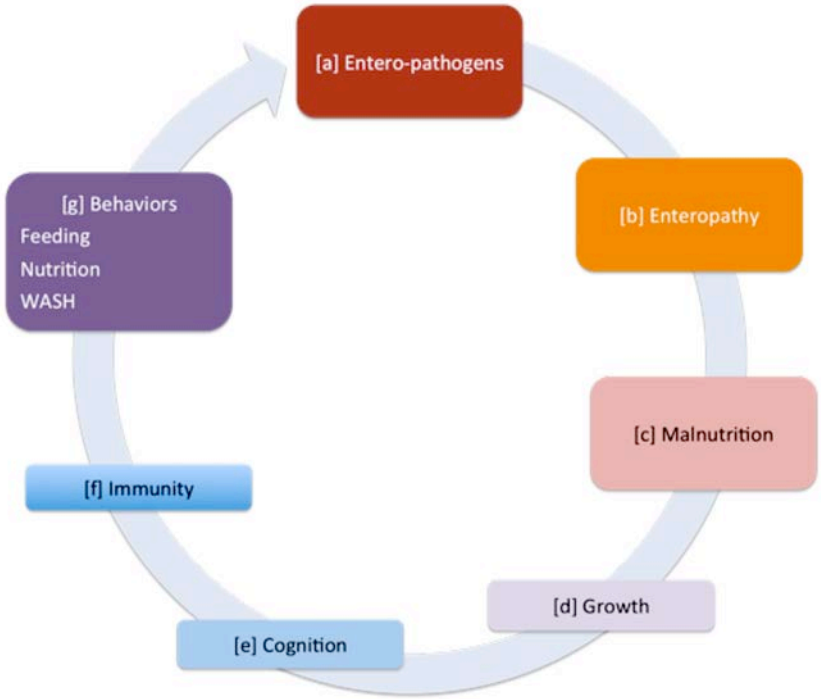

Figure 1: The "vicious cycle" of enteric infection-malnutrition and long-term consequences.

MORTALITY FROM DIARRHOEA AND ENTERO-PATHOGENS

A key detail of the studies that estimate the worldwide burden of diarrhoeal mortality is that they are based, not on measures of diarrhoea as the name of the syndrome might imply, but on enteric infection (*Black* et al., 2010). International Classification of Diseases certificates (WHO, 1990) are restricted just to the range of codes corresponding to 'intestinal infectious disease' (A00-A09). Intestinal infection is one, albeit probably the first, among many possible causes of diarrhoea, but other causes of diarrhoeal symptoms are (for these purposes) ignored. The estimated burden of mortality is, therefore, specifically an estimate of the mortality attributable to enteric infection.

Identifying an enteric infection is considerably harder than observing a visible physiological symptom like diarrhoea. Inevitably with collated certificates of death there is considerable variability in the robustness of reporting and specificity of the cause of death (*Murray* et al., 2007). For example, one classification included in the estimation of the burden of mortality is "presumed infectious origin" and upon closer inspection, some certificates are based on 'verbal autopsy' without laboratory confirmation, which has been estimated to have a positive predictive value of just 0.39 for diarrhoeal disease (*Setel* et al., 2006). Diarrhoea then, in some cases at least, is presumed to be the result of enteric infection in the absence of laboratory confirmation. To make full use of the estimates of mortality, diarrhoea must be assumed to be a reliable indicator of enteric infection.

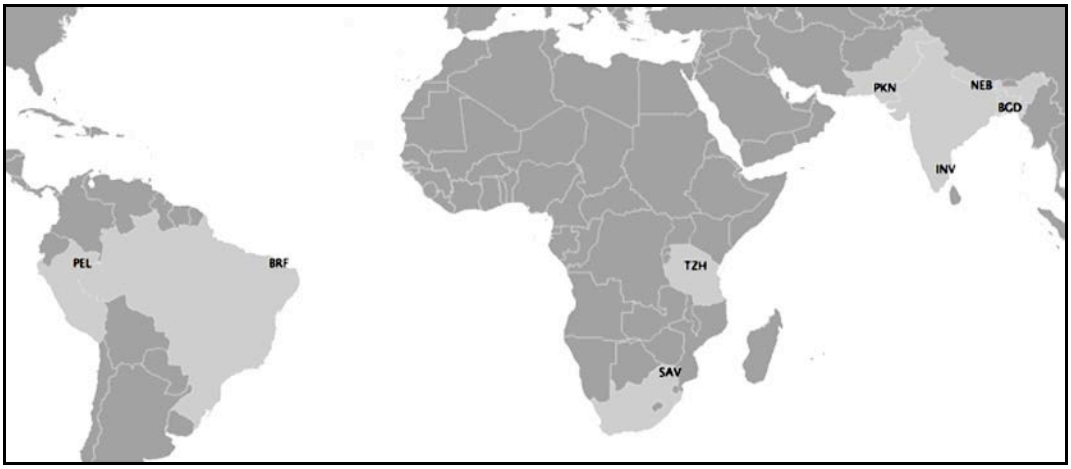

Figure 2: The location of the 8 cohorts of children who make up the MAL-ED study population. PEL Iquitos, Peru; BRF Fortaleza, Brazil; SAV Limpopo, S. Africa; TZH Haydom, Tanzania; PKN Naushahro Feroze, Pakistan; INV Vellore, India; NEB Bhaktapur, Nepal; BGD Mirpur, Bangladesh.

MORBIDITY FROM DIARRHOEA

Only in the most severe cases does diarrhoea lead to dehydration and risk of death (*Snyder* and *Merson*, 1982; *Glass* et al., 1991). Far more common are self-limiting episodes of diarrhoea (*Baqui* et al., 1991). The importance of diarrhoeal disease is not, therefore diarrhoea *per se*, so much as more insidious and cryptic morbidity that is considered so common as to be overlooked (*de Wit* et al., 2001; *MacDougall* et al., 2008). A considerable body of evidence suggests that enteric infection in children can result in a 'vicious cycle of poverty' (*Guerrant* et al., 2008) (Figure 1): (a) infection with enteric pathogens is associated with (b) impaired gut-function(*Salazar-Lindo* et al., 2004; *Petri* et al., 2008; *Viswanathan* et al., 2009; *Costa* et al., 2011) that can then (c) exacerbate malnutrition (*Guerrant* et al., 2008) and restrict the processing of nutrients necessary for (d) physical (*Mondal* et al., 2012) and (e) cognitive development (*Guerrant* et al., 1999; *Niehaus* et al., 2002; *Lorntz* et al., 2006) on top of (f) suppressing immune responses (*Schaible* and *Kaufmann*, 2007), thereby impairing a child's ability to resist (a) recurrent infections (*Moore* et al., 2010) and illness (*DeBoer* et al., 2012) from (g) increasingly 'risky' behaviours/environments. Indeed, it is the exploration of this cycle that has formed the essential hypotheses of the MAL-ED study:

1. Infection with specific entero-pathogens contributes to stunting, wasting, and/or micronutrient deficiencies causing intestinal inflammation and/or by altering the barrier and adsorptive functions of the gut; and,
2. The combination of enteric infections and malnutrition results in growth and cognitive impairments in young children and may lead to impaired immunity as measured by responses to childhood vaccines.

This cycle is predicated on the role of enteric pathogens. Enteric infection results both in physical damage to the gut and to a diversion of metabolic activity away from growth and towards combating infection(s). What is unclear is the relative degree to which infections are reflected by diarrhoeal symptoms.

Table 1: Diagnostic accuracy of diarrhoea as an indicator of enteric infection, using data from all 8 field sites and children from 0-6 months of age

	Mean	CI	N
Sensitivity	0.18	(0.15,0.21)	6,203
Specificity	0.91	(0.89,0.93)	5,966
Accuracy	0.61	(0.58,0.64)	12,169

THE MAL-ED NETWORK

The Malnutrition and Enteric Infections (MAL-ED) network ("Mal-ED") was formed to investigate the aetiology, risk factors, and interactions of enteric infections and under-nutrition and their consequences for child health and development (*Lang*, 2011; The MAL-ED Network, 2013a). This prospective field, clinical and laboratory-based observational study follows cohorts of children from (approximately) birth to 24 months. The population studied come from eight sites in countries with a historic high incidence of undernutrition and diarrhoeal disease (Figure 2).

Enrolment of new-borns began in November 2009 and ended in February 2012. Each MAL-ED Network site has enrolled more than 200 study subjects at regular intervals from near birth (prior to 17 days after birth) to 24 months of age. To reach each site's goal of following 200 children over two years and allowing for dropouts, each site enrolled approximately 10-12 children per month. Active surveillance for illness symptoms is accomplished by visiting each home two times per week. Using harmonized protocols, each site performs a number of tests and assessments, including those for gut function, monthly anthropometry, nutritional intake, cognitive development, micronutrient status, environmental assessment, and vaccine response. Follow-up will cease in February 2014, when the last child enrolled at each site will have turned two years of age.

The data presented here are from the early stages of the MAL-ED project – at the time of writing, data and sample collection has not yet ended – and the analyses here represent tentative preliminary findings based on incomplete data and should be viewed in that context. Given the staggered enrolment and substantial quantity of data being generated through longitudinal surveillance, new data are still being added and at different rates depending both on the nature of the data and on the site. One early finding from MAL-ED is the considerable heterogeneity in the biological data among the field sites – a finding that is consistent across many aspects of the study and requires considerable attention and caution both in analysis and translation into general actionable conclusions.

SYMPTOMATIC INFECTION

The assumption that diarrhoea is a useful indicator of enteric infection is implicit in the majority of public health surveillance. Diarrhoea is logistically easy to identify, though with some variability as to what rate of loose

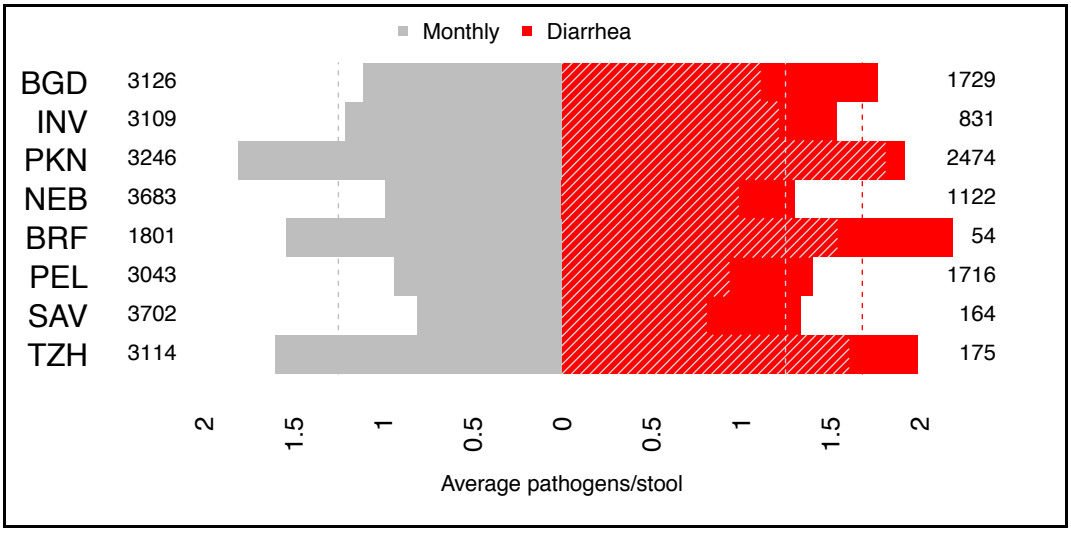

Figure 3: The average number of positive tests for pathogens per stool. Monthly "control" stools are shown in grey and reflected in the hashed bars that overlay the diarrhoeal stools in red. The number of stools tested is given in numbers at the end of each bar. Each bar corresponds to a MAL-ED site (see Figure 1 for site names). This figure is based on stools from children aged 0-12 months.

stools constitutes 'unusual' (*Kosek* et al., 2003). What is consistent, though, is the use of diarrhoea as an indicator for the pernicious morbidity associated with the syndrome.

Experience to date from the MAL-ED network suggests that diarrhoea is, at best, a poor substitute for laboratory confirmed enteric infection. Stools are collected: 1) on a monthly schedule to represent background carriage of pathogenic gut flora in the absence of overt symptoms, and 2) *ad hoc* when a mother declares that a child has had diarrhoea, defined as ≥3 loose stools within 24 hours as recommended by WHO (2006).

A panel of some 57 enteric pathogens is tested in the MAL-ED study including common viruses, bacteria and parasites (The MAL-ED Network, 2013b). Using a generalized estimating equation to account for repeated sampling of the same children and to control for such factors as breast-feeding and age, these tests were used to assess the utility of diarrhoea as a diagnostic test for the presence of enteric infection (*Sternberg* and *Hadgu*, 2001; *Murakami*, 2010). To simplify this assessment, all the various tests including ELISA, microscopy and PCR confirmation were treated as a binary variable such that any test being positive was used to indicate infection. What has emerged from a preliminary assessment in cohort children from 0-6 months of age is a sobering observation that the sensitivity and specificity of diarrhoea as an indicator of enteric infection were 0.18 and 0.91 respectively (Table 1). The low sensitivity reflects a large number of asymptomatic infections that would be classed as false negatives (i.e. no diarrhoeal symptoms despite at least one positive laboratory test for an enteric pathogen). However, the high specificity is the result of the small number of cases with a diarrhoeal (indeed any) stool that did not have a corresponding positive test for a pathogen.

Indeed the paucity of diarrhoea as a diagnostic indicator of enteric infection is evident in the average number of pathogens detected in stools that have been tested so far in MAL-ED (Figure 3). The number of pathogens detected is remarkably similar between monthly and diarrhoeal stools, with an overall average 1.7 pathogens identified per diarrhoeal stool and 1.3 pathogens per monthly "control" stool.

Diarrhoea consequently appears to be an under-estimate of enteric infection. One possible reason for this is that symptoms are a product not of the mere presence of pathogens – a limitation imposed by traditional testing methods – but of the absolute number of any given pathogen or the quantitative combinations of pathogens present. With regard to this latter case, emerging technologies offer exciting possibilities in quantifying pathogen burden based on quantitative PCR (*Operario* and *Houpt*, 2011; *Platts-Mills* et al., 2012).

INFECTION AND GROWTH

Enteric pathogens theoretically have a twofold impact on growth and development: first they damage the intestine by inducing inflammation, decreasing barrier function, decreasing absorptive capacity or combinations of all three (*Field*, 2003); and second, they can divert metabolic energy towards an immune response and away from optimal growth. Evidence of an association between enteric infection and deficits in growth or cognition are somewhat ambiguous – more so considering that many studies have used diarrhoea as a surrogate of infection.

In a recent study, *Lee* et al., (2013) present a simple model of the growth of a child in relation to *Campylobacter* infection. Specifically, they distinguish between those infections that were from symptomatic (diarrhoeal) stools and those from regularly collected asymptomatic ('control') stools. This is an interesting analysis, not least because they make a rare attempt to explicitly differentiate the two types of carriage (another example being changes of weight in relation to *Cryptosporidium* infection (*Checkley* et al., 1997)).

We are developing a similar model though rather than just *Campylobacter*, we are assessing the role of those pathogens that, to date, have been detected in >0.5% of all stools collected (either normal monthly or diarrhoeal samples) amongst the total MAL-ED study population (Figure 4).

The change in a child's linear growth can then be calculated over a defined period. *Lee* et al. (2013) reported a 9-month period in their final model, though we are analysing our data over a range of from 2 to 18 months. Over the corresponding interval, the numbers of positive pathogen tests are summed. This model, thereby, attributes a portion of the change in length over a period of time to infections with a particular enteric pathogen. In the case of infections that result in transient perturbation in growth, with growth deficits that can readily be caught up, the effect of infection is likely to be noted over short duration lags. Pathogens with longer, more persistent effects or repeated infections with one or more pathogens are more likely to be identified in longer intervals over which their cumulative impact becomes apparent.

Individually, each pathogen is comparatively rare and consequently the impact on the change in height over 9

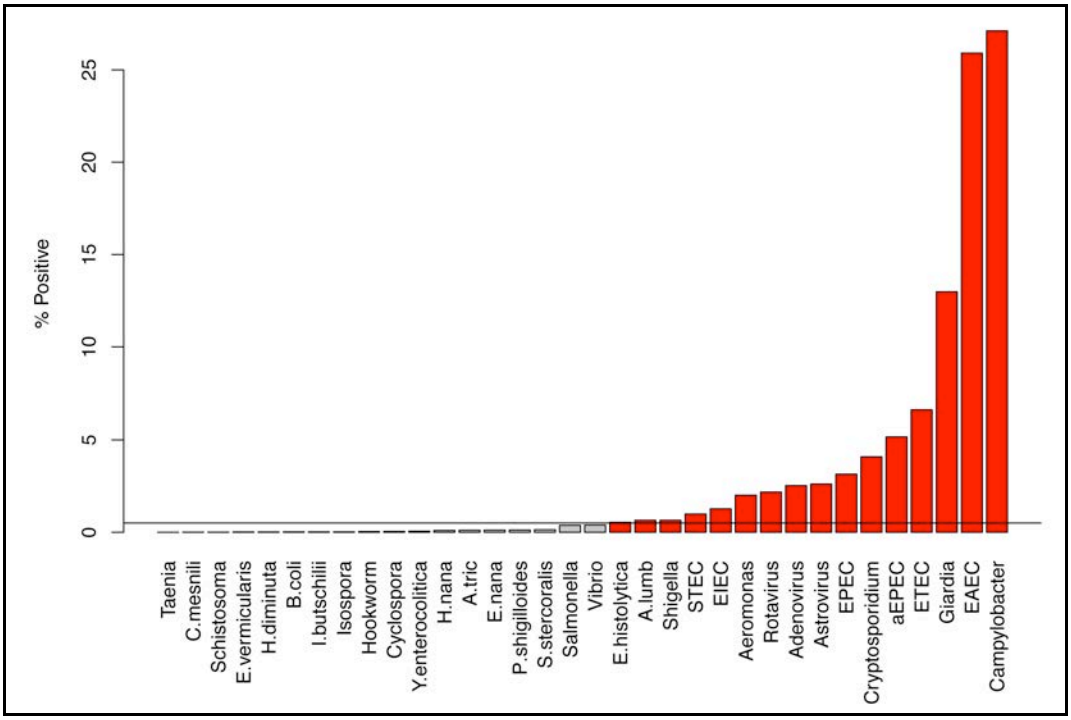

Figure 4: The percentage of stools with positive test results for pathogens in the MAL-ED study. The horizontal line distinguishes those pathogens (in red) that were found in >0.5% of all stool samples and those that were not (in grey). This figure is based on microbiology data from children 0-24 months old, aggregating the currently available data from all 8 field sites.

months was both negligible and statistically non-significant (at 5%). In preliminary analyses, there appear to be four exceptions: (i) STEC is negatively associated with a change in height when detected in diarrhoeal samples (-0.34cm/infection/9 months) as are (ii) symptomatic infections with giardia (-0.05cm/infection/9 months), while (iii) EIEC and (iv) *Campylobacter* in asymptomatic stools appear to be associated with positive (+0.14 cm/infection/9 months) and negative (-0.03 cm/infection/9 months) growth respectively.

When pathogens are pooled, however, both symptomatic (-0.03 cm/infection/9 months) and asymptomatic (-0.01 cm/infection/9 months) infections were statistically significantly associated with decreased growth. Despite the fact that the effect is greater in symptomatic infections, the overall impact of asymptomatic infection is likely to be greater as they are considerably more frequently represented in the data. Similarly, aggregating all infections, regardless of provenance, appears to be statistically significantly associated with negative growth and is just under the mean of the effect of the two stool types.

It is surprising how few analyses have explicitly considered asymptomatic infection in relation to growth, particularly given the number of infections that are unlikely to result in symptoms (*Hellard* et al., 2000) or continued infection once symptoms have resolved (*Tangermann* et al., 1991). Despite this, a model of early MAL-ED data that ignores the type of stool in

favour of a binary 'infected or not', is the most informative model based on the Akaike Information Criterion (AIC) goodness of fit (*Akaike*, 1974) suggesting that it is the presence of pathogen and not symptoms that are of greatest importance to growth.

What remains to be assessed in this type of analysis is the consequence of simultaneous co-infections. In theory the co-incidence of pathogens may have greater impact than pathogens in isolation. From the MAL-ED data, aggregating across the field sites, some 37% of diarrhoeal stools and 17% of the monthly stools have had more than one pathogen detected (most of which had two positive tests, but a maximum of 9 pathogens have been detected in the same stool so far).

INFECTION AND COGNITION

As well as being required for physical growth, nutrients and growth factors regulate brain development during foetal and early postnatal life. Over this period, the brain demonstrates its greatest plasticity, but also its greatest vulnerability to nutrient insufficiency, hence early brain development is crucial for later cognitive development and learning (*Rice* and *Barone*, 2000; *Thompson* and *Nelson*, 2001). Over 200 million children (worldwide) under five years old have been estimated to fail to reach their full cognitive potential, which cascades into poorer school performance and a subsequent reduction in economic productivity (*Grantham-McGregor* et al., 2007). Many factors contribute to this period of cognitive development including, and most relevant here, are nutrient deficiencies and infectious disease (*Walker* et al., 2007).

Through a suite of tests, MAL-ED aims to assess various aspects of cognitive development at several time points over the first two years of life (The MAL-ED Network, 2013c). The developmental assessments are performed at periodic intervals to capture the progression of child development across a range of domains including: a general cognitive assessment (*Bayley*, 2005), memory, motor development, language (*Fenson* et al., 2006), learning, temperament (*Wachs* and *Desai*, 1993), social interactions and the immediate environment (*Caldwell* and *Bradley* 1984). Alongside these child metrics, maternal reasoning (*Raven* and *Court*, 1996) and depressive symptoms (*Beusenberg* and *Orley* 1994) are also assessed.

In much the same fashion as the physiological stages of the vicious cycle, there are conceptual routes by which symptomatic infection (diarrhoea) might influence opportunities for cognitive development beyond the direct interference with nutrition (*Murray-Kolb* and *Beard*, 2009; *Armony-Sivan* et al., 2010; *Lima* et al., 2013) since diarrhoea may also alter infant-caregiver interactions in terms of both quality and quantity (*Lamb* et al., 1999). In addition, nutritional status itself may feedback into the infant-caregiver interaction (*Gardner* et al., 2005).

Cognition is hard to assess, because it is difficult to define a specific measurement(s) (*Siegler*, 1989; *Richardson* and *Richardson*, 2002). This is made more complex because conceptually, cognitive development is a function of other factors that are themselves ambiguous constructs (*Walker* et al., 2007) – for example parental engagement, the child's environment and nutrition. Though intuitively important, these constructs are hard to analyse because they are troublesome to quantify consistently and to pin down to specific

observable metrics. A solution that is often used is to combine observable measurements (for example, individual items on surveys or responses to questionnaires) as multi-dimensional representations of unobserved 'latent' variables (*Hoyle* and *Smith*, 1994; *MacCallum* and *Austin*, 2000).

An example of one solution to examining these thematic variables is given by Amorim et al. (2010). Individual measurements were combined into themes: the sum of sections from the Home Observation for the Measurement of the Environment (HOME) Scale (*Caldwell* and *Bradley*, 1984) represent "parenting style" and the child's "environment" and anthropometric measures as surrogates of "nutritional status". These unobservable constructs were then used to estimate the observed cognitive score measured as the sum of the cognitive and language subscales from the Bayley scales of infant development, 3rd edition (BSID-III) (*Bayley*, 2005).

Amorim et al. (2010) found the nutritional scores to be statistically significant predictors of the cognitive development score. This relationship appears also to hold in early MAL-ED analyses; the enrolment weight and length-for-age, which is an indicator of long-term nutritional status (WHO Working Group, 1986), both appear significantly related to the Bayley score.

The illness and infection variables have not yet been demonstrated to be statistically related to the cognitive score, with the exception that the total days of maternally reported fever that may be associated with a diminished Bayley score. That these variables were not related to cognition does not necessarily mean that they are not important factors. In the context of a structural equation (as used by *Amorim* et al., 2010), measured variables explicitly feed into latent variables, thus specifying the direct and indirect relationships between variables. In this particular model, there are likely to be strong correlations between the non-significant illness factors and the highly significant nutrition factors (for example see the above association between height and infection). As such, there are putative indirect, though not shown here, associations between infection and cognition. It is perhaps noteworthy that the mean estimate of the association with cognition is stronger for the symptomatic infections, which again may (though not demonstrated here) reflect the additional associations expected between diarrhoeal symptoms and caregiver interactions.

THE LEGACY OF DIARRHOEA

Despite the weakness of diarrhoea as an indicator of enteric infection and the lack of studies quantifying the impact of asymptomatic infection, there remains a persistent literature that discusses diarrhoea as a 'cause' of physical and cognitive shortfalls. The reason for this apparent paradox may lie in the naming of the syndrome. Diarrhoea is used as a short-hand for both the overall "diarrhoeal disease syndrome" (indicative of serious gut dysfunction) as well as loose stools. This confusion allows the persistence of language that suggests that diarrhoea (the symptom) is a useful indicator of the syndrome despite the lack of evidence that "loose stools" *per se* are a robust metric of enteric infection. Indeed this language under-estimates the importance of the largely asymptomatic syndrome.

An additive Bayesian network (abn) (*Heckerman* et al., 1995; *Lewis* and *McCormick*, 2012) using the earliest

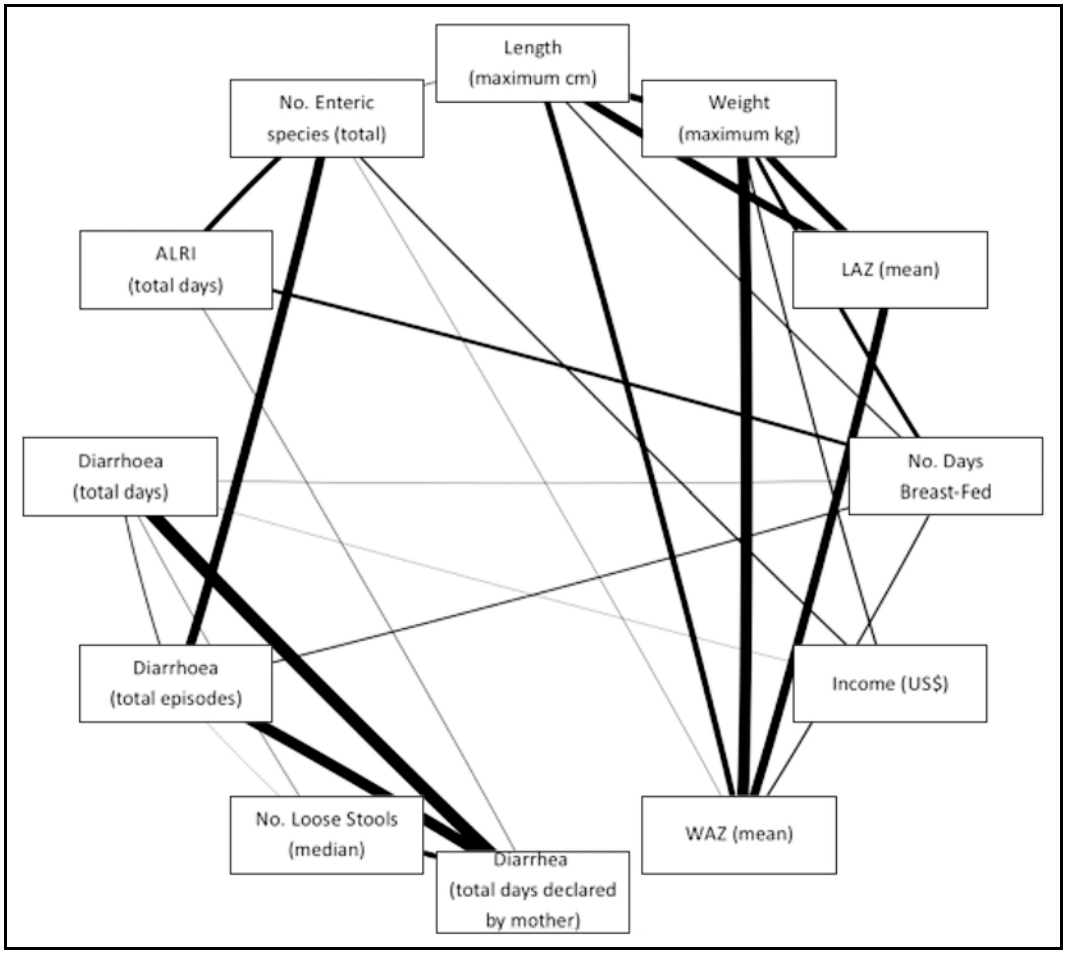

Figure 5: The statistical relationships between variables using an additive Bayesian network (abn). MAL-ED data from all 8 field sites for children at 6 months of age were used, with random effects for the field site. Line width indicates the strength of statistical association.

available data was constructed in an effort to agnostically identify relationships among variables being studied in MAL-ED. The abn was used for structure discovery, identifying a single globally optimal directed acyclic graph (DAG) that describes the data (*Koivisto* and *Sood*, 2004; *McCormick* et al., 2013). The abn is a multivariate model, treating each variable as both a potential predictor of every other variable included in the analysis as well as a response. Unlike traditional models (such as a generalized linear model), the abn avoids the temptation to fallaciously combine the results of individual regression models that have a single (presumed independent response variable) (*Hand* et al., 1997). Furthermore it better reflects the co-dependency of the many interacting components of a system that is by definition a collection of related variables. This allows a statistical structure to emerge from the data themselves that identifies, not only the immediate relationship between a suite of variables and a single outcome, but also the relationships between the

many variables that are both directly and indirectly associated within a system (*Lewis* and *Ward*, 2013).

What emerges from MAL-ED data of the first 6 months of life is that, within these preliminary data, there appear to be no direct associations between diarrhoeal symptoms and anthropometric measures (Figure 5). This makes sense in the context of the analyses above. What also makes sense is the presence of indirect statistical associations between diarrhoeal symptoms, enteric pathogens (not distinguishing between symptomatic and asymptomatic) and anthropometry (mean WAZ and maximum length by 6 months old). This putative pathway is particularly interesting because it reflects what is widely expected from the diarrhoeal disease syndrome even if the disease symptoms are not reliable indicators.

What were missing from the available data at the time of the analysis, were measures of cognitive development and nutrition. The latter would appear central to the manifestation of the diarrhoeal disease – the assumption is that the syndrome impacts the efficient processing of nutritional input, for example through physical damage to the intestine, the rapid egestion of food before nutrients are absorbed. Such data are being collected within the MAL-ED study, however, and when available, will be added to this emerging model. Based on other studies, it is perhaps unlikely that the impacts on growth become detectable (above the between-site heterogeneity) until children are older (*Checkley* et al., 1998; *Lima* et al., 2000; *Moore* et al., 2001). As additional data become available it will be possible to reassess this early observation.

CONCLUSIONS

There is a considerable body of evidence supporting a conceptual model in which growth and cognitive development are negatively affected by undernutrition. A prime cause, aside from poor quality or low quantity of food, is the interference with optimal gut function by enteric infection. Within this cycle, enteric infection is frequently referred to as diarrhoeal disease, however this belies the importance of asymptomatic infection.

Enteric infection resulting in diarrhoea is relatively uncommon compared to an overwhelming number of asymptomatic infections or continued carriage (whether or not deleterious) observed in the settings studied in MAL-ED. The question is whether or not symptoms are important contributors to growth outcomes? As indicators of gut dysfunction and to indicate the risk of dehydration leading to hospitalization or death, symptomatic diarrhoea is clearly useful. However, preliminary evidence from this longitudinal prospective study suggests that any enteric infection– symptomatic or not – contributes to decreased growth. The impact of each infection is relatively small and slow to emerge, with the significance of each infection becoming more apparent as the lag since the infection increases. These results may change when examining the complete data from MAL-ED and in the broader context of growth outcomes over the entire 24 months of the study are modelled.

Likewise, any association between enteric infection and cognitive development may become clearer as more data become available from older children. Even so, early data from MAL-ED hint

that the role that entero-pathogens play is indirect - acting on nutrition and thereby influencing cognitive development. Such a hierarchy of interactions is likely to be most clearly identified by accounting for the many interlinked associations in the wider syndrome.

The diarrhoeal disease syndrome is something of a misnomer that has distorted the expectations that diarrhoea (the symptom) is a reliable indicator of gut infection and/or dysfunction. In fact, enteric infection is what is commonly understood when referring to "diarrhoea" and that seems to, based on preliminary observations noted here, have long-term consequences for growth and cognitive development, whether or not those infections are associated with overt diarrhoea.

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