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THE MAL-ED PROJECT: DECIPHERING THE RELATIONSHIPS AMONG NORMAL GUT FLORA, ENTERIC INFECTION AND MALNUTRITION AND THEIR ASSOCIATION WITH IMMUNE RESPONSE TO VACCINES

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SUMMARY

It has been estimated that malnutrition affects 20% of children in the developing world (*Black* et al., 2008) and that poor nutrition is linked to more than half of all deaths worldwide in children under the age of five (*Caulfield* et al., 2004). In addition to its role in childhood deaths, malnutrition in early childhood may lead to cognitive and physical deficits and may cause similar deficits in future generations as malnourished mothers give birth to low birth weight children (*Victora*, et al., 2008). Malnutrition increases both susceptibility to and incidence of infections and mortality due to diarrhoea and other infectious diseases (*Campbell* et al. 2003); these effects may be mediated through a depressed immune response to natural infection or to administered vaccines.

The MAL-ED Consortium has been established in eight countries with a high incidence of both diarrhoeal disease and malnutrition. We are investigating the hypothesis that infection or co-infection with certain enteropathogens contributes to malnutrition by causing intestinal inflammation and/or by altering intestinal barrier and absorptive function which, in turn, leads to growth faltering, stunting, and deficits in cognitive development. In addition, the relationship of repeated enteric infection and malnutrition on the diminished protective immunity in children given orally delivered childhood vaccines is being examined at these sites. Our study also aims to shed light on relevant questions such as: (1) which pathogens or which combination of infectious agents are most frequently associated with growth faltering and poor development, and (2) at what periods during the first two years of life do specific infections produce the largest effect on growth and development? Among the factors being evaluated for their effects are: Enteric infections, micronutrient levels, diet, socio-economic status, composition of the gut microbial flora (the gut microbiome) and human genetic factors. Based on these new data we hope to be able to better define the problem and make both site specific and more generalized recommendations regarding the nature and timing of interventions aimed at improving child health in these resource poor settings.

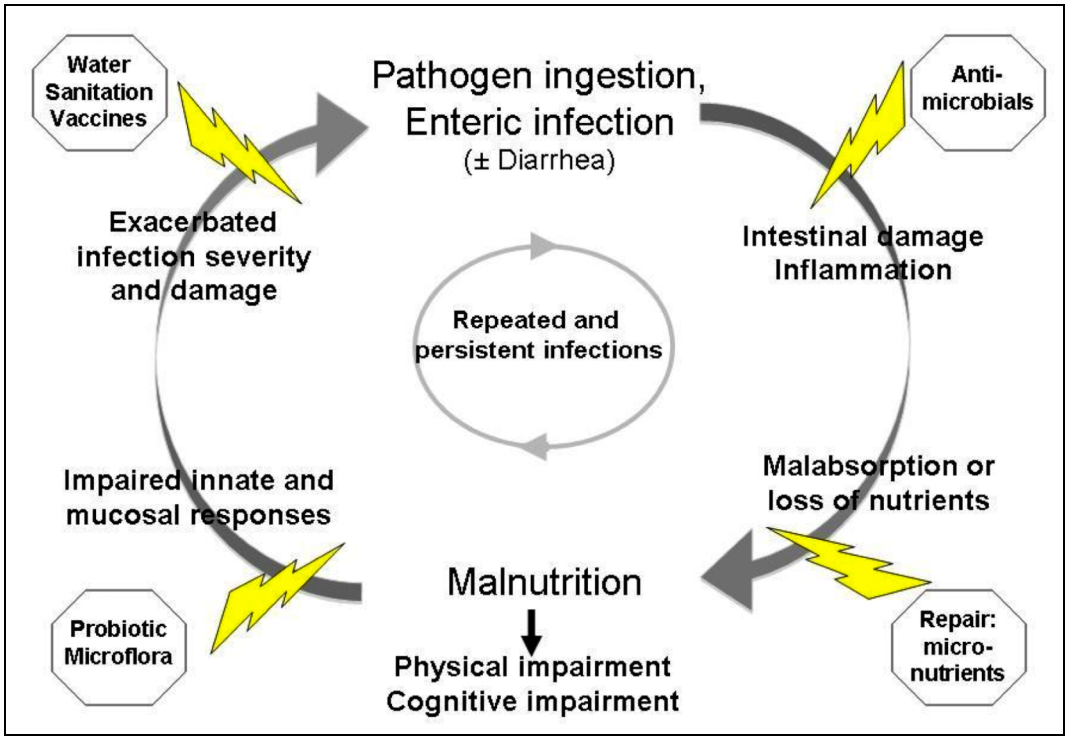

Figure 1. The cycle of malnutrition and enteric disease.
This figure, adapted from *Guerrant*, et al. 2008, depicts the cyclical nature of the synergistic relationship between infection with enteric pathogens and the development of malnutrition (undernutrition). Around the outer circle are indicated the expected physiological effects on children. The arrow pointing from "malnutrition" indicates the resulting impairment of both physical and cognitive development observed in other studies during the first two years of life that may extend into adulthood. Potential interventions that may be capable of interrupting this "vicious cycle" are depicted by the lightning bolts.

INTRODUCTION

It has been estimated that malnutrition affects 20% of children in the developing world (*Black* et al., 2008) and that poor nutrition is linked to more than half of all deaths worldwide in children under the age of five (*Caulfield* et al., 2004). Moreover, it is recognized that malnutrition increases susceptibility to and incidence of infections and is associated with diminished response to vaccines. Diarrhoeal infections and undernutrition synergistically contribute to morbidity and mortality. The combination of diarrhoeal infections and undernutrition has been demonstrated to have significant detrimental effects on growth during the first two years of life which can be observed as early as three months of age (*Victora* et al., 2010). This relationship between diarrhoea and malnutrition can be depicted as a "vicious cycle" (*Guerrant* et al., 2008) and is illustrated in Figure 1.

Among the factors that may lead to undernourishment in young children are: the lack of adequate amounts of food, insufficient breastfeeding, inadequate diversity of complementary foods which may lead to specific micronutri-

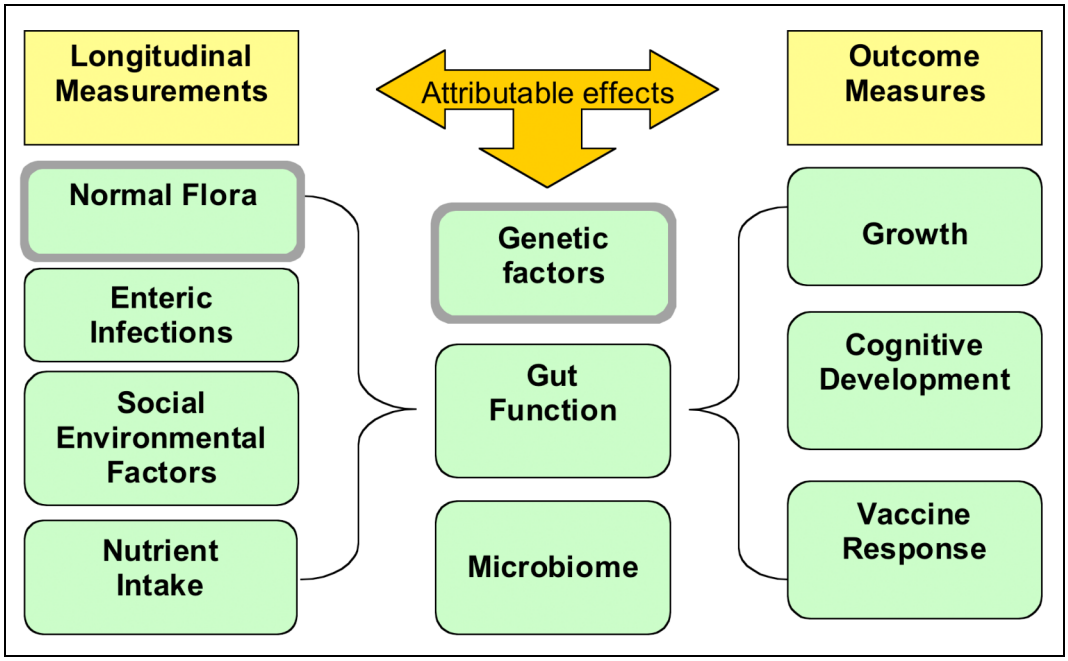

Figure 2: Proposed association among the factors being assessed during the MAL-ED study. We believe that the factors measured longitudinally will have their effects through alteration of gut function as indicated. We also recognize that gut function will be influenced by both genetic and epigenetic factors and by the composition of the gut normal flora (microbiome). These factors in combination, lead to effects on growth, cognitive development and immune response to vaccines indicated as outcome measures.

ent deficiencies, diets that are high in inhibitors of micronutrient absorption, catabolic states due to infection, and inadequate response of the host and the host's gut microbiome to caloric insufficiency. Pathogenic bacteria, viruses, and parasites in the gut also impact nutritional status through multiple mechanisms. First, enteric pathogens impair nutrient absorption by damaging the absorptive capacity of the intestine, causing protein-energy and micronutrient malnutrition. Second, enteric infections can compromise the intestinal barrier, causing increased intestinal permeability to pathogens, endotoxins, and other macromolecules that can result in the chronic stimulation of the immune system. Importantly, both micronutrient deficiencies and chronic immune stimulation have been found to impair growth and increase susceptibility to infectious diseases (*Black* et al., 2008). Additionally, altered gut flora and pathogens may also influence the effectiveness of orally-delivered vaccines. Understanding the complex and synergistic relationships between enteric infections and malnutrition, therefore, is fundamental to the design of better intervention strategies capable of reducing the infectious disease burden and improving the nutritional status of children born in these settings.

While it is likely that enteric infections contribute to malnutrition, data on the role of particular enteropathogens are limited by small sample sizes, limited geographic locales, and robustness of diagnostic tests employed. Additionally, there has been relatively little study of morbidity and mortality due to

Table 1a: MAL-ED network field site institutions and principal investigators

Performance sites	Principal investigators
Aga Khan University, Karachi, Pakistan[1]	Dr. Zulfiqar Bhutta
Christian Medical College Vellore, Vellore, India[1]	Drs. Gagandeep Kang, Sushil John
JHSPH Satellite Laboratory, Iquitos, Peru[1]	Dr. Margaret Kosek
Federal University of Ceara, Fortaleza, Ceará, Brazil[1, 2]	Drs. Aldo A. M. Lima, Reinaldo Oria
Walter Reed/AFRIMS Research Unit, Kathmandu, Nepal[1]	Dr. Sanjaya Kumar Shrestha
Institute of Medicine, Kathmandu, Nepal[1]	Dr. Prakash Sunder Shrestha
University of Venda, Limpopo, South Africa[1]	Dr. Pascal Bessong
International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh[1, 2]	Drs. Tahmeed Ahmed, Rashidul Haque
Haydom Lutheran Hospital, Haydom, Tanzania[1]	Dr. Erling Svensen

[1]Location of birth cohort studies.
[2]Location of case-control studies.

chronic and recurrent enteric microorganisms and parasites, their contribution to the global burden of disease in children under five, and the consequential long-term effects in adulthood. To date, there have been no systematic studies that help define particular windows of vulnerability in early childhood when specific pathogens or mixed infections could lead to greater deficits in developmental outcomes. Furthermore, there have not been conclusive studies that define the associations between enteric infections and undernutrition with intermediary indicators, such as measures of gut function, which would help explain the effects of enteric infections on growth and development. Likewise, there are also limited studies looking at the effects of normal gut flora, particular infectious agents, mixed infections, or micronutrient levels on the immune response in children. If we are to develop more effective vaccination strategies, it is important to elucidate how these factors interact to reduce the immune response to oral vaccines in particular, that is observed in the developing world.

ESTABLISHMENT OF THE MAL-ED NETWORK

The MAL-ED (pronounced mal-a-dee) Network has been established in order to better define the interrelationships among exposure to enteric pathogens, infection and diarrhoeal disease, diet, and socio-economic status (SES) affecting gut physiology, growth, immune response to vaccines and cognitive development in birth cohorts from eight resource poor communities (Figure 2).

MAL-ED's Scientific and Administrative Core was established at the Fogarty International Center, NIH (FIC) and at the Foundation for the National Institutes of Health (FNIH) to provide scientific and organizational management. Dr. Mark Miller, FIC and

Table 1b: MAL-ED collaborating institutions and investigators

Collaborating institutions	Principal investigators
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA[1]	Drs. Laura Caulfield, Laura Murray-Kolb, Robert Black
University of Virginia, Charlottesville, VA, USA[2,4]	Drs. Richard Guerrant, William Petri, Patrick Concannon, Stephen Rich Eric Houpt, Rebecca Dillingham
Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand[3]	Drs. Carl Mason, Ladaporn Bodhidatta
Washington University School of Medicine, St. Louis, MO, USA[4]	Dr. Jeffrey I. Gordon
University of Colorado at Boulder, Boulder, CO, USA[4]	Dr. Rob Knight

[1]Johns Hopkins Bloomberg School of Public Health is collaborating with the Peru field site.
[2] University of Virginia is collaborating with the Bangaladesh, Brazil, South Africa, and Tanzania field sites.
[3]Armed Forces Research Institute of Medical Sciences is collaborating with the Nepal field site.
[4]Location of Companion project.

Dr. Michael Gottlieb, FNIH serve as co-Principal Investigators of the project. This core also provides a data-coordinating centre (DCC) which receives de-identified data from all sites and will conduct, with participation of study investigators, a comprehensive analysis of the pooled data. The DCC may also assist sites in their analysis of site-specific data. We anticipate that these analyses will yield informative site-specific results and conclusions as well as ones that may transcend sites and geographic areas. The science/administrative core also conducts budget management and oversight activities and manages the activities and meetings of the advisory committees that have been established. The core also conducts site visits, organizes annual meetings and teleconference meetings with study investigators and has worked with our investigators, organized into technical sub-committees, to develop the common protocols used in the study.

Study sites from both urban and rural communities were chosen, in part, because of the high incidence of both diarrhoeal disease and malnutrition experienced by children less than five years of age and because of the scientific experience of the investigators at those sites. A list of the MAL-ED study sites and the Principal Investigators at each site are indicated in Table 1a while Table 1b indicates the collaborating institutions and investigators.

All participating institutions have agreed to abide by the terms and conditions of a Research Consortium Agreement (RCA) that provides the governance structure and decision making authority of the Network and its associated advisory committees. The RCA also provides guidance on publication, intellectual property, and data sharing and release policies. The intent of these policies is to ensure that any benefit resulting from the study is for those most in need in the developing world. Clearly delineating these issues with input from the participating institutions and investigators prior to study

initiation was very helpful; having them in place will facilitate the addition of other study sites and related projects in the future.

MAL-ED STUDY HYPOTHESES

1. Infection (and/or co-infection) with certain enteropathogens leads to malnutrition by causing intestinal inflammation and/or by altering the barrier and absorptive functions of the gut.
2. The combination of enteric infections and malnutrition results in growth and cognitive impairments in children and may lead to impaired immunity as measured by vaccine response.
3. Particularly sensitive periods exist during early childhood when environmental exposure, infection, and malnutrition lead to exacerbated and lasting effects on development.

STUDY DESIGN

The MAL-ED study employs standardized and harmonized study protocols. Use of a shared Manual of Procedures and common data collection forms by all eight field sites ensures that comparable data will be collected and that the data can be pooled for analysis. Each site has received approval of study protocols from their local Institutional Review Board (IRB) and from the IRBs of collaborating U.S. institutions.

Prior to enrolment, each site conducted an extensive census to determine the location of women of childbearing age and pregnancies that served to identify potential enrolees. Recruitment plans were developed in collaboration with science/administrative core staff to ensure that enrolees would be representative of the overall population in the community. In addition, each site conducted a pilot study in 100 households in an area representative of the study population in order to determine socio-economic status, food security, and anthropometric base line characteristics of the community.

Each field site will enrol a minimum of 200 children before they are 17 days old in a birth cohort study that will follow children from birth to two years of age. Recruitment of the cohorts will be distributed over two years in order to control for, and allow analysis of, seasonal variation in infectious disease burden, or the quantity and nature of the food supply, for example. Further details of the birth cohort study design are presented in Tables 2 and 3.

Although only a cohort study design can adequately assess sequential events that precede the onset of persistent diarrhoea and/or malnutrition or growth shortfalls, close biweekly household follow-up (as is necessary for obtaining accurate data on diarrhoeal illnesses) may have a "Hawthorne Effect" that dramatically reduces diarrhoea rates and malnutrition, as well as mortality (*Ricci* et al., 2006). Hence, in order to understand the aetiologies, pathogenesis and interventions of moderate or severe malnutrition, two of our sites (Brazil and Bangladesh) are also conducting case control studies of malnutrition with 500 cases and 500 control children (cases (HAZ >-2) and controls (HAZ <-1) identified at community clinics.

Table 2: Overview of sample collection for the MAL-ED cohort study

Objectives		Measurement	Sample type
Gut functional capacity:	Gut integrity	Lactulose-mannitol	Urine
	Gut inflammation	Quantitative lactoferrin, A-1-antitrypsin	Stool
Enteric infection assessment:	Incidence and prevalence of enteric pathogens	Microbiological assays[a]	Stool
	Diarrheal disease[b] history	Frequency, severity, and duration	Interview
Growth and development:	Anthropometry	Length, weight, head circumference	Examiner administered
	Nutrition	Breastfeeding status (exclusivity, partial/full cessation)	Interview
		Child feeding practices (e.g. introduction of solids, feeding patterns, key foods)	Interview
	Micronutrients	Iron (hemoglobin, trans-ferrin receptor, ferritin)	Blood
		Zinc (plasma)	Blood
		Vitamin A (plasma retinol)	Blood
		Plasma protein (α-1-acid glycoprotein)	Blood
		Others (e.g. iodine, lead, glutamine, arginine)	Blood
	Cognitive function	Global	Examiner administered
		Language (verbal fluency)	Interview
		Others (e.g. child temperament)	Interview
	Assessments on mother/household	Home environment SES/demographic	Examiner administered
		Maternal IQ	Examiner administered
		Other (e.g. depressive symptoms)	Interview
Vaccine response:	Vaccine immunogenicity	Antibody titers to mucosal vaccines: - Rotavirus vaccine - Oral Polio vaccine	Blood
		Antibody titers to EPI vaccines: - tetanus, measles, pertussis	Blood
Other illness surveillance (syndrome):	Incidence of respiratory and other illnesses	Frequency, severity, and duration	Case report form

[a] Microbiological assays include bacterial culture, microscopy, and PCR for identification of site-specific bacterial, viral, and parasitic pathogens.
[b] Diarrhea defined as 3 or more unformed stools in a 24 hour period; episodes separated by 2 diarrhea-free days.

Table 3: Timing of measurements conducted during the two years of obseervation in the MAL-ED cohort study

Sample	Measured	When
Blood	Haemoglobin, ferritin, zinc, vitamin A, lead, α-1 acid glycoprotein, transferrin receptor, amino acids	7, 15 months
	Immune response to pertussis, tetanus, polio, measles, rotavirus	7, 15 months
Urine	Gut integrity: Lactulose-mannitol permeability test	3, 6, 9, 15 months
	Iodine	6, 15 months
Stool	Lactoferrin, α-1-antitrypisn	Monthly 0- 24m, AND one time during each diarrhea episode
	Enteric pathogens	Monthly 0- 24m, AND one time during each diarrhea episode
General survey	Length, weight, head circumference	Monthly 0- 24 months
	Comprehensive diet	Monthly 0- 24 months
	Cognitive function	6, 15, 24 months
	Demographic/ SES/ medical history	0, 6, 15 months
	Household/ maternal assessment	0, 8, 15 months
	Incidence of diarrhea and other illness	2x per week until 2 years
	Breastfeeding, supplemental diet	2x per week until 2 years

COMPANION PROJECTS

The MAL-ED Network provides both a scientific and administrative platform from which to launch additional related projects. These projects could include hypothesis driven research and targeted interventional trials. We anticipate that such opportunities will present themselves as our analysis proceeds over the next four years and the clinical situation at each site becomes better defined. Currently, the Network collaborates with three associated companion projects which, together with the cohort and case control studies, constitute the larger MAL-ED Consortium. Companion project institutions and investigators have agreed to abide by the same Research Consortium Agreement, as have all Network investigators. The three companion projects are briefly described below:

1) Studies of the human gut microbiome and its role in nutrition, is being conducted at Washington University, St. Louis, by Dr. Jeff Gordon, and at the University of Colorado, Boulder by Dr. Rob Knight.
2) Genome wide studies aimed at identifying candidate human genes associated with undernutrition and growth impairment are being conducted at the University of Virginia by Dr. William Petri, Dr. Pat Con-

cannon and Dr. Steve Rich. The University of Virginia investigators are working with the Bangladesh site.

3) Development of a multiplex PCR assay capable of detecting all of the bacterial, viral and parasitic pathogens being studied in the MAL-ED project is headed by Dr. Eric Houpt at the University of Virginia in collaboration with Dr. Jim Nataro at the University of Maryland.

CURRENT STATUS

The MAL-ED Network and Consortium has been operational for $1^1/_2$ years. All field sites are actively recruiting subjects, the earliest having started in November, 2009. Enrolment of new subjects will proceed evenly paced over a two-year period in order to capture seasonal variation in exposure to pathogens, disease aetiology and food availability. We look forward to the comprehensive analysis of the data and applying the findings to the improvement of the public health in the participating sites and in the rest of the developing world. We envision that the Network will accommodate expansion to include additional performance sites and companion projects related to the study objectives of the Network. By thoroughly characterizing the populations under study, and improving the local infrastructure and capacity, these sites will be ready to test appropriate intervention strategies by conducting clinical trials relevant in their setting.

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