Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Christopher G. Miller Date

Trace elements, inflammation, and rotavirus vaccine response among a cohort of infants in El Alto, Bolivia

By

Christopher G. Miller

Degree to be awarded: MPH

Global Epidemiology

_____

Juan S. Leon, PhD, MPH

Committee Chair

_____

Paulina Rebolledo, MD MSc

Committee Member

Trace elements, inflammation, and rotavirus vaccine response among a cohort of infants in El Alto, Bolivia

By

Christopher G. Miller

B.A.

Northwestern University

2012

Thesis Committee Chair: Juan S. Leon, PhD, MPH

An abstract of

A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University

in partial fulfillment of the requirements for the degree of
Master of Public Health
in Global Epidemiology

2018

Abstract

Trace elements, inflammation, and rotavirus vaccine response among a cohort of infants in El Alto, Bolivia

By Christopher G. Miller

Rotavirus infection is the most common cause of fatal diarrhea in infants despite the existence of effective rotavirus vaccines. Over 90% of rotavirus deaths occur in low- and middle-income countries (LMIC's). One such LMIC, Bolivia, experiences the highest under-5 mortality rate in Spanish speaking South America, with numerous deaths attributable to rotavirus infection. Yet, in LMIC's like Bolivia, rotavirus vaccines are less effective than in high-income countries. The Nutrition, Immunology, Diarrhea in Infants (NIDI) study in El Alto, Bolivia was created to explore the factors related to reduced immune response to the rotavirus vaccine in Bolivia. NIDI investigators enrolled 461 infants in the study. Blood was drawn up to three times – once pre-vaccination, at approximately 2 months, and twice post-vaccination, at approximately 6 months and 12-18 months – and analyzed for several biomarkers, including: serum concentrations of calcium, copper, iron, magnesium, and zinc; concentrations of systemic inflammatory markers α-1-acid glycoprotein (AGP) and C-reactive protein (CRP); and rotavirus-specific IgA antibodies. There is a need to understand how micronutrient levels play a role in the rotavirus vaccine's reduced performance in low income settings. To meet this need, a secondary analysis was completed on the NIDI data. Spearman's correlation analyses revealed moderate positive correlations between serum copper and both AGP ($\rho = 0.51$-0.71, $p < 0.01$) and CRP ($\rho = 0.46$-0.55, $p<0.01$). The distributions of values for trace element concentrations were described, with many values falling outside of reference ranges in the literature, especially values for serum magnesium and zinc (up to 55% and 67% of the time, respectively). There were no significant relationships between any serum element and rotavirus-specific IgA seroconversion using logistic regression analyses, but Spearman's analyses revealed inverse relationships between both serum copper ($\rho = -0.14$, $p=0.01$) and CRP ($\rho = -0.16$, $p<0.01$) and the fold-change in rotavirus-specific IgA following vaccination. These results indicate a need to account for inflammation when assessing copper status, as well as a need to refine the reference ranges for serum element concentrations in infants. Furthermore, the findings suggest that higher inflammatory burden may blunt the strength of the immune response to rotavirus vaccination.

Acknowledgements

Dr. Juan Leon – Dr. Leon, thank you for your dedication, patience, and guidance over the past two years. Your detail-orientated instruction was invaluable to me as I worked my way through this thesis. Thanks also for your support outside of the thesis process – from helping me through the residency application process to discussing life beyond Emory to supporting me at match day! I am so grateful to have had the opportunity to work with the NIDI dataset and I know I will take the lessons learned from this work with me as I pursue a career in global child health.

Dr. Parmi Suchdev – Dr. Suchdev, thanks again for helping me think through the micronutrient data and for helping me know what to care about. Thanks also for leveraging your contacts in the field to strengthen my thesis. Finally, thank you for letting me get more involved in global child health through GHOPE and the GH elective.

Dr. Paulina Rebolledo – Thank you for your work as a member of my thesis committee and for helping me understand the immunological markers in the NIDI study!

Dr. Rachel Burke and Dr. Jessica Prince-Guerra – Rachel, thanks again for walking me through the nitty-gritty of the dataset and for explaining the micronutrient correction process. Jessica, thank you for including me in your submission and for helping me contextualize my data.

Trace elements, inflammation, and rotavirus vaccine response among a cohort of infants in El Alto, Bolivia

By: Christopher G. Miller

B.A.

Northwestern University

2012

Thesis Committee Chair: Juan S. Leon, PhD, MPH

A thesis submitted to the Faculty of the

Rollins School of Public Health of Emory University

in partial fulfillment of the requirements for the degree of
Master of Public Health
in Global Epidemiology

2018

Table of Contents

Rotavirus Infection & Vaccination: A literature review of the role of trace elements and inflammation in vaccine response

Diarrhea is estimated to cause over 700,000 childhood deaths every year, making it the second leading cause of childhood mortality [1-3]. The most common cause of fatal diarrhea is rotavirus infection [4, 5], which accounts for up to 28% of fatal cases of diarrhea in children [1]. Most of the mortality burden of rotavirus diarrhea is borne by low- and middle-income countries (LMIC's) [1, 4, 6, 7]. Indeed, over 90% of rotavirus deaths occur in LMIC's [4]. Rotavirus deaths can be prevented with safe, effective vaccines [8-15] but these vaccines are seen to be less effective in LMIC's than they are in high-income countries [14-16]. The explanation for this remains unresolved [17, 18]. A more thorough understanding about why rotavirus vaccines are less effective in LMIC's than in high-income countries could guide interventions aimed at improving vaccine effectiveness in LMIC's.

I. Rotavirus

I.a. The Virus

Rotavirus diarrhea is caused by double-stranded RNA viruses in the Reoviridae family's *Rotavirus* genus (reviewed in [19, 20]). At least eight virus groups are recognized in the *Rotavirus* genus and substantial genetic diversity exists between and within these groups [19]. Three groups (groups A through C) are known to cause disease in humans, with the A group most commonly associated with gastroenteritis. Rotaviruses have traditionally been further classified based on the structure of two outer capsid proteins, VP7 (which determines the virus's G type) and VP4 (which determines the virus's P type).

I.b. Transmission and Pathogenesis

Transmission of rotavirus occurs via the fecal-oral route, and can take place following close contact with infected individuals or with contaminated surfaces, food, and water [19, 21]. Upon entry into the small intestine, the virus begins replication in the villous epithelium, leading to several histopathological changes including damage to and stunting of the intestinal villi, epithelial cell death, and crypt cell hyperplasia [19-21]. Diarrhea likely results from a combination of glucose, sodium, and water malabsorption, villous ischemia, increased enteric nervous system activity, and the presence of a viral enterotoxin (NSP4) produced by rotavirus, which promotes ion secretion.

I.c. Clinical Symptoms, Diagnosis, and Treatment

The clinical symptoms and disease course of rotavirus infection are similar to those of other types of viral gastroenteritis [22]. Rotavirus has an incubation period of one to three days and symptoms start acutely, usually as vomiting and fever with subsequent explosive, non-bloody diarrhea [19, 23, 24]. These symptoms are typically self-limiting and resolve in four to eight days, though reports of persistent diarrhea and lactose intolerance exist [19]. Infected infants and children undergo more severe disease courses than infected adults [25-27]. Among the most severe complications in rotavirus-infected infants and children are dehydration, metabolic acidosis, seizures, and death [19, 28]. While most children with rotavirus gastroenteritis experience mild disease courses, 7 to 18 percent have symptoms severe enough to necessitate hospital admission [19, 29].

Diagnosis and treatment of rotavirus gastroenteritis are based on clinical presentation [28]. Routine testing for rotavirus in children with gastroenteritis is not widespread, as it does not change disease management. Nevertheless, detection of the virus in stool can be accomplished

using enzyme-immunoassay (EIA) for rotavirus antigens, reverse transcription polymerase chain reaction (RT-PCR), electron microscopy, and polyacrylamide gel electrophoresis of rotavirus RNA excreted in stool. Evidence of infection can also be uncovered, with relatively high specificity and sensitivity, using enzyme linked immunosorbent assays (ELISA's) or immunochromatography to detect circulating antibodies against rotavirus in the serum. Treatment of rotavirus gastroenteritis revolves around prevention and management of dehydration. Supplemental treatment may include antiemetic medication, probiotics, and zinc supplementation, though there are no universal recommendations for such treatments [28, 30-32].

II. Immunity and Vaccination

II.a. Immunity

Infection with rotavirus confers natural immunity, with increased protection and less severe diarrhea accompanying each new rotavirus infection [33, 34]. Before widespread vaccination, it was estimated that over 95% percent of children experienced a primary rotavirus infection by age two, and that nearly all children had experienced a primary infection by age five [28, 33, 34]. Asymptomatic rotavirus infection confers a similar degree of immunity to symptomatic infection, and natural infection by one strain often confers immunity to other strains [34]. Nevertheless, not every infant with diagnosable rotavirus infection develops complete immunity following their first infection, suggesting that multiple doses of vaccine need to be given for vaccination to be as efficient as natural infection at producing immunity.

Development of immunity to rotavirus following infection or vaccination involves both components of the immune system: innate immunity and adaptive immunity (reviewed in [35]).

Briefly, the innate immune system is comprised of several different types of hematopoietic immune cells - including macrophages, neutrophils, eosinophils, natural killer cells, and dendritic cells – and non-hematopoietic cells – including epithelial cells lining the gut, skin, and airway (reviewed in [36]). The innate immune system was historically referred to as "non-specific" immunity, in part because it responds to structural components common to groups of different pathogens rather than to pathogen-specific structures. It acts rapidly to protect hosts from infection. Innate immune cells do not demonstrate "immunological memory"; that is, innate responses to invading pathogens are identical following each exposure to those pathogens. The innate immune system contrasts with the adaptive immune system, which mounts a specific protective response to pathogens encountered in the past. The adaptive immune system can be further categorized into humoral immunity – directed by B-lymphocytes which secrete antibodies against pathogens – and cellular immunity – directed by T-lymphocytes.

Immune responses to rotavirus infection and vaccination remain poorly understood. The innate immune response to rotavirus infection and vaccination likely involves upregulation of natural killer cells and increased expression of toll-like receptors (TLR's) by innate immune cells (reviewed in [35]). However, most data regarding the innate immune response to rotavirus are derived from animal studies, and data from human studies are lacking. Adaptive immune responses to infection and vaccination in humans have been more thoroughly studied, particularly humoral immune responses. Neutralizing IgG and IgA antibodies to the VP4 and VP7 rotavirus capsid proteins are associated with protection against rotavirus gastroenteritis. Accordingly, serum rotavirus-specific IgA is most often measured and used as a correlate of protection against future infections [37, 38]. Less information exists regarding the role of the cellular portion of the adaptive immune system in humans, though there is evidence that

rotavirus-specific T-helper cells circulate during the convalescent stage of infection (reviewed in [35]).

II.b. Rotavirus Vaccination

Three attenuated oral vaccines are available for the prevention of rotavirus gastroenteritis [8, 39]. The first vaccine Rotarix® (GlaxoSmithKline Biologicals) is a live, attenuated human monovalent vaccine against the G1P[8] strain of rotavirus, the most common human strain [12, 40]. Licensed in 2006, the monovalent vaccine is typically given in two doses, at 2 and 4 months of age, and it confers protection against most human strains of rotavirus [40], (reviewed in [35]). The other globally available vaccine, Rotateq® (Merck), is a pentavalent vaccine derived from a bovine rotavirus strain, WC3, and containing five human-bovine reassortant viruses [40]. The bovine WC3 strain of rotavirus is, by nature, weakly virulent in humans but the five reassortant viruses in the pentavalent vaccine contain genes encoding for outer capsid proteins found in the most common human rotavirus serotypes. Therefore, the pentavalent vaccine confers protection against most disease-causing rotavirus strains. Three doses of the pentavalent vaccine, given by 6-15 weeks of age and administered at least a month apart, are given to produce immunity. A third vaccine, Rotavac® (Bharat Biotech), designed in India and distributed there since 2014, recently received prequalification status from the World Health Organization (WHO) for use in national vaccination programs globally [41, 42]. A vaccine against the naturally occurring reassortant human-bovine 116E strain of rotavirus, Rotavac® is administered at 6, 10 and 14 weeks [41]. The WHO recommends that all infants receive one of the three vaccines to reduce rotavirus-associated diarrhea mortality [43].

II.c. Reduced Vaccine Effectiveness in Low and Middle-Income Countries

Numerous studies have demonstrated the rotavirus vaccine's reduced effectiveness in LMIC's [9, 14-16, 37, 44]. While the two oral vaccines show over 90% effectiveness against severe infections in high income countries, their effectiveness against severe infections in LMIC's is around 56% (reviewed in [44]). Phase III clinical trials have demonstrated vaccine efficacy of 81% in Latin America, 50% in Sub-Saharan Africa, and as low as 42.7% in high mortality regions of Asia. This is of particular concern given the fact that LMIC's bear the brunt of the rotavirus-associated mortality burden [1].

Several hypotheses have been proposed to account for the vaccines' decreased efficacy in LMIC's (reviewed in [18, 44]). Some suggest that LMIC's host a greater variety of rotavirus strain serotypes, many of which may not be cross-covered by the approved monovalent and pentavalent vaccines [18, 45]. Others posit that infants in LMIC's consume a relatively higher volume of neutralizing antibodies in breast-milk during vaccine administration (reviewed in [17, 18]). Further, some cite a higher degree of comorbidities, such as HIV/AIDS and environmental enteropathy (sub-clinical intestinal inflammation with disruption of the intestinal mucosal barrier and altered intestinal absorption) in low income countries leading to reduction of the vaccines' efficacy (reviewed in [17, 18, 46]). Additionally, it has been proposed that poor nutritional status among infants in LMIC's may play a role in the vaccine's reduced efficacy (reviewed in [17, 18]).

III. Nutrition and Trace Elements

Broadly speaking, malnutrition is divided into two categories: protein-energy malnutrition and micronutrient malnutrition (reviewed in [47, 48]). Protein-energy malnutrition

is defined as inadequate intake of calories or protein to meet the body's needs and it is typically detected in children using anthropometric measurements including weight and height (reviewed in [49]). Micronutrient malnutrition is characterized by deficiencies or excesses of minerals and vitamins that are essential to growth and normal functioning of body systems (reviewed in [47, 50]). It is ideally detected on the basis of biochemical measurements (reviewed in [47]). Micronutrient malnutrition leads to suppression of both the innate and adaptive immune responses (reviewed in [51] and in section II.a. above).

There is ongoing study into the role that micronutrition plays in determining immune responses not only to the rotavirus vaccine, but also to other licensed vaccines [52]. A 2009 Journal of Nutrition review, by Savy and associates, of the interactions between nutrition and responses to various vaccines concluded that "malnutrition has surprisingly little or no effect on vaccine responses" [52]. The authors evaluated a pool of over a hundred and thirty studies dealing with a range of live, inactivated, recombinant, conjugate, and polysaccharide vaccines and found limited evidence of relationships between selected micronutrients or protein-energy malnutrition and vaccine immunogenicity. Nevertheless, the studies included in the review only focused on protein-energy malnutrition, vitamin A, vitamin D, iron, and zinc because the authors found virtually no studies that dealt with other vitamins or trace elements. Further, no studies included in the review dealt with rotavirus vaccination.

Still, several studies not discussed in the review have evaluated the role of nutrition in rotavirus vaccine response. Studies exploring the role of macronutritional status (typically using weight-for-height or similar anthropometric measurements to assess for the presence of malnutrition) have found little evidence of reduced rotavirus vaccine effectiveness in the setting of malnourishment [53-55]. Indeed, one study of Bangladeshi infants found an increased risk of

developing rotavirus diarrhea associated with being well-nourished [56]. Multiple studies of the role micronutrients play in rotavirus vaccine response have focused on vitamins A and D. Vitamin A-deficient piglets were seen to have impaired innate and adaptive immune responses to rotavirus vaccines [57-59]. One study found an association between vitamin D deficiency and rotavirus diarrhea [60]. Fewer studies have evaluated the role of elemental micronutrients in influencing rotavirus vaccine response. Those that have done so have largely focused on zinc, with limited evidence of an association between zinc supplementation and rotavirus vaccine response [61]. There is an unmet need to understand how micronutrients – in particular, elemental micronutrients such as copper, zinc, calcium, magnesium, and iron – relate to rotavirus vaccine response and if this relationship can account for the reduced effectiveness of the vaccine in low-income settings.

IV.a. Copper

Although copper has a well-known role in several biological processes, including metabolism, thyroid function, anti-oxidation, and the maintenance of skin, blood vessels, and connective tissue, copper's role in the immune system is still being explored (reviewed in [62]) [63, 64]. Acquired copper deficiency has been implicated in increased incidence of infections and there is some evidence of cellular adaptive immune dysfunction, including decreased T-cell proliferation, in the setting of copper deficiency [65, 66] (reviewed in [51]). Furthermore, animal studies have demonstrated a link between copper deficiency and dysfunctional humoral adaptive immunity [67, 68]. Due to its pro-oxidative properties, copper is thought also to be recruited by the innate immune system as an antimicrobial agent in times of infection; cells of the innate immune system may capitalize on copper's toxic oxidative properties to kill pathogens [69]. For example, macrophages may leverage their high copper content to disrupt microbial enzymes or

create an environment high in reactive oxidative species (ROS), which are deadly to the microbe. Indeed, serum copper levels are seen to rise almost universally in the setting of acute or chronic infection, irrespective of the type of infection (fungal, bacterial, or viral) (reviewed in [69, 70]). Much of rise in copper in these settings is attributed to increases in ceruloplasmin, the major copper-carrying enzyme in blood and an acute phase reactant. Ceruloplasmin's transcription in the liver is upregulated in times of stress and it may play a role host defenses [71, 72]

While copper deficiency may be detrimental to the immune system, dietary copper overload may also increase susceptibility to infection (reviewed in [51, 69]). A small study of men fed long-term, high copper diets revealed associations between high copper intake and decreased percentage of circulating neutrophils, and between high copper intake and decreased serum interleukin 2R (IL-2R), a cytokine which upregulates lymphocyte proliferation [73]. Additionally, invasive microbes require copper for their own survival; these microbes often integrate the human host's copper into their own cellular mechanics (reviewed in [69]). Accordingly, the human host is believed to have developed mechanisms for copper limitation and sequestration during infection. Taken as a whole, the current body of research suggests that well-regulated copper balance is integral to immune function.

Nevertheless, there is limited evidence of a relationship between serum copper levels and vaccine response. Children chronically exposed to heavy metals, including copper, were seen to have lower antibody titers following vaccinations compared with unexposed children [74]. Similarly, a small study of the effect of high-copper diet on influenza vaccination found a lower fold-increase in circulating antibodies against influenza among men fed high-copper diets compared to control subjects [73]. However, studies have also linked higher copper levels to improved vaccine response. A prospective cohort study by Kaynar et al examined the

associations between plasma mineral levels and influenza vaccine immunogenicity and found a significant relationship between higher plasma copper and seroconversion following H1N1 vaccination [75]. A bovine study showed that dairy calves who received copper-containing injectable trace mineral supplements had a higher antibody titer following bovine herpes vaccine challenge compared unsupplemented calves [76]. Still other studies reveal no link between copper and vaccine response. For example, a human study of American cutaneous leishmaniasis (ACL) found no relationship between elevated or low copper levels and ACL vaccine response, as measured by Montenegro skin test diameter [77]. No studies have characterized the relationship between serum copper and rotavirus vaccine response.

IV.b. The Copper/Zinc Ratio

Researchers have speculated that the ratio of serum copper to zinc may also be a useful marker of mortality risk, inflammatory burden, immune system dysregulation, and nutritional status [78-84]. Elevated copper/zinc ratios have been associated with a range of diseases, including asthma [80], multiple sclerosis [83], and colorectal cancer [84]. The copper/zinc ratio has also been described as a valuable marker of response to treatment in patients with pulmonary tuberculosis [85] as well as a predictor of survival in HIV-positive men [81], with higher copper-zinc ratios associated with worse outcomes. Moreover, the copper/zinc ratio has been correlated with nutritional parameters (decreased BMI, creatinine, and albumin), inflammatory biomarkers, and markers of oxidative stress in individuals undergoing peritoneal dialysis [79]. It has been proposed that a high copper/zinc ratio may decrease proliferation of B-cells and CD3 and CD4 T-cells.

Zinc is one of the most studied of the micronutrients thought to impact the immune system [51]. Briefly, zinc deficiency has been associated with abnormal CD4/CD8 T-cell ratios,

decreased natural killer cell activity, and imbalance between cell-mediated and humoral immune mechanisms. Zinc deficiency has also been tied to immune system irregularities that contribute to increased vulnerability to infections, particularly among malnourished children. Zinc is an important cofactor for the secretion of thymulin, a immunoregulatory molecule involved in cellular immunity [86]. Zinc-deficient children who receive zinc supplementation have reduced incidence and duration of diarrhea and pneumonia [87]. Still, there are scarce data to indicate that zinc supplementation improves rotavirus vaccine immunogenicity. Indeed, a recent study found no effect of zinc supplementation on rotavirus vaccine immunogenicity in a low-income, urban community in India [61]. Plasma zinc is an appropriate measurement for estimation of zinc status on a population level [88].

IV.c. Magnesium, Calcium, and Iron

Magnesium is essential to virtually all major cellular functions and plays a central role in bone and muscle health [89]. Serum magnesium concentrations tend to be tightly regulated in humans in the absence of significant co-morbidities [89, 90]. Magnesium levels are maintained in homeostasis by intestinal and renal mechanisms; absorption and secretion can be up- or down-regulated depending on blood levels and bodily stores [89]. The majority of magnesium is stored in bone tissue and deficiency is rare. Magnesium deficiency is most commonly due to renal and gastrointestinal losses [91]. Furthermore, magnesium deficiency is rarely symptomatic in the absence of other biochemical or electrolyte abnormalities, making it difficult to ascribe symptoms solely to magnesium deficiency. Magnesium's role in the human immune system remains under study, though it has been implicated in inflammatory aging processes, cellular apoptosis, exercise-induced immunosuppression, and asthma pathophysiology (reviewed in [92]). In animal studies, magnesium deficiency has been associated with upregulation of

inflammatory mechanisms, including upregulation of certain cytokines, activation of macrophages and neutrophils, and local intestinal immune responses in mice [92, 93]. In *in vitro* studies, magnesium deficiency has been associated with deficient T-cell responses, without concurrent B-cell impairment [94]. Although serum magnesium concentration does not necessarily provide a complete picture of magnesium status on the individual level, it may be an appropriate measurement for assessments of magnesium status at the population level [95].

Like magnesium, calcium is necessary for virtually all cellular functions and calcium levels are tightly regulated in healthy humans [90, 96]. Taken alone, serum calcium is not thought to be a reliable, specific indicator of nutritional or immune status, though serum calcium levels may be elevated or decreased in many different settings, including malnutrition [90]. Beyond its ubiquitous role in cellular mechanisms, calcium is thought to be an important signaling molecule between cells of the immune system [97]. Moreover, body calcium is regulated by Vitamin D, which itself is an important immune system modulator [98].

Iron deficiency and abnormalities in iron metabolism can have profound effects on the immune system [99, 100]. However, considered in isolation, measurements of serum iron concentration do not adequately represent body iron stores or the presence of iron deficiency [101]. Further, serum iron concentration is dynamic and of limited clinical value [102, 103]. Nonetheless, it may be useful to consider serum iron concentrations when evaluating the relationship between elemental micronutrients and immunological or inflammatory phenomena; serum iron decreases in the setting of chronic disease and acute infection, due, in part, to the body's efforts to sequester iron from invasive organisms [104, 105]. The effect of iron deficiency on the immune system has been well-studied and is worth briefly reviewing. Iron is essential for hematopoiesis and it follows that successful generation and functioning of immune cells

depends on iron [50]. Among other things, iron deficiency has implicated in decreased T-cell proliferation, defective IL-2 production, impaired neutrophil function, and defective natural killer cell functioning (reviewed in [106]).

IV.d. Inflammation and Elemental Micronutrient Concentrations

Serum elemental concentrations may be affected by many factors. Among these factors is the presence or absence of inflammation. For instance, a positive association between serum copper concentrations and levels of circulating inflammatory markers has been documented in the literature [107]. Similarly, serum magnesium is negatively associated with circulating inflammatory markers [108]. A reduction in serum iron accompanying infection has also been observed [109]. Accordingly, it is important to characterize the relationship between elemental micronutrients and inflammation in order to better understand the role such micronutrients play in affecting vaccine response. For example, the presence of inflammation could conceivably impact the immune response to rotavirus vaccine while at the same time causing an increase in serum copper; if the presence of inflammation is not accounted for, then one may mistakenly attribute the altered immune response to increased serum copper. In such a situation, it is useful to have a way to account for inflammation.

C-reactive protein (CRP) and α_1-Acid Glycoprotein (AGP) are acute phase reactants which are elevated during inflammatory processes [110]. In addition to being relatively easy and inexpensive to measure, CRP and AGP have several other characteristics that make them ideal for use in population-level studies of inflammation (reviewed in [111]). Both markers allow researchers and clinicians to make objective observations about the presence of inflammation (as opposed to, say, estimating inflammation based on clinical exam). Moreover, both markers can be elevated in the setting of "sub-clinical" inflammation (i.e. in individuals with no outward

physical manifestations of inflammation) or after medical interventions to treat symptoms of inflammation have taken place (i.e. medications to combat fever). Measurements of CRP and AGP can be used to detect the presence of inflammation, to estimate the degree of inflammation, and to correct measurements of serum micronutrient concentrations accordingly [112, 113].

Briefly, researchers have proposed several ways to correct serum biomarker concentrations for inflammation based on AGP and/or CRP [114]. In particular, many efforts have been directed at describing methods to correct serum ferritin (a biomarker representing body iron stores, which also happens to be an acute phase reactant that rises in the setting of inflammation). Methods to correct ferritin for inflammation may be reasonably extrapolated for use with other biomarkers that are similarly affected by inflammation. Such methods include: 1) changing the cutoffs for a particular biomarker if inflammation is deemed to be present; 2) excluding from analyses those individuals whose CRP or AGP exceeds a certain threshold; 3) multiplying biomarker concentrations by a correction factor based on the presence of inflammation; and 4) using linear regression to determine the degree to which a biomarker changes with AGP and/or CRP and adjusting accordingly. Of these methods, the linear regression method may provide the most accurate and precise population-level measurements, because it includes all individuals (not just "uninflamed" individuals) and it best reflects the variability of a given biomarker based on the degree of inflammation [114].

Micronutrients, inflammation, and immunity are closely interrelated. Conceivably then, micronutrient status may affect infants' immune responses to rotavirus vaccine. Furthermore, differences in infants' micronutrient statuses between high income countries and LMIC's may explain the disparity in rotavirus effectiveness between high income countries and LMIC's. One such LMIC, Bolivia, began widespread rotavirus vaccination in 2008 [8]. Rotavirus vaccination

programs in Bolivia have been met with success, although the rate of protection conferred by vaccination remains lower in Bolivia than in high income countries [115].

IV. Context

IV.a. Bolivia

A landlocked country situated in the Western part of South America, Bolivia is one of the poorest Latin American countries [116]. It is home to approximately 1.24 million children under five years old with an all cause, under-5 morality rate of 66 deaths per 1000 live births [8], the highest under-5 mortality rate in Spanish speaking South America [117]. Chronic malnutrition and underweight in children under five years old are relatively common in Bolivia. At 18%, the rate of moderate to severe stunting is the second highest in South America, behind Ecuador, and the percentage of children who are underweight is among the highest in the region.

IV.b. Rotavirus Vaccination in Bolivia

Before the introduction of the rotavirus vaccine to Bolivia in 2008, diarrhea was projected to cause over 51,000 hospitalizations and 546 deaths annually in children under five years old [8]. The leading infectious cause of deadly diarrhea was rotavirus. In 2008, the country began vaccination with the monovalent oral rotavirus vaccine [115]. By 2011, officially reported rotavirus vaccine coverage was 80% and by 2016, officially reported coverage rates were as high as 87% [118]. The monovalent rotavirus vaccine has been estimated to confer 54%-84% protection to Bolivian children receiving two doses, a higher effectiveness than most low-income countries though still lower than that of high-income countries [115].

IV.c. El Alto

El Alto is the second most populous city in Bolivia [119]. Bordering La Paz, El Alto began as an “overflow” area that grew more populous as real estate in La Paz became limited.

The city is home to a large number of migrants from other parts of Bolivia and much of El Alto's growth has come in the shape of informal settlement. Poverty is widespread in most of El Alto's districts, with a growing gap between rich and poor residents. Children in El Alto are not immune to this poverty and face resultant rates of chronic malnutrition as high as 18% [120, 121].

IV.d. Nutrition, Immunology, Diarrhea in Infants Study

The Nutrition, Immunology, Diarrhea in Infants (NIDI) study sought to explore the factors associated with lower vaccine immune response in El Alto relative to that in high income settings. Among the factors hypothesized by the NIDI study team to relate to vaccine response were host genetics, infant antibodies, inflammation, presence of co-infections or environmental enteropathy, and nutritional status. To characterize the relationship between nutritional status and host response to rotavirus vaccine, the NIDI study team collected anthropometric data and measurements of various micronutrients in vaccinated infants' serum.

V. Conclusion

There is a need to understand how micronutrition plays a role in the rotavirus vaccine's reduced effectiveness in low income settings. The goal of the proposed study is to understand the importance of elemental micronutrients in influencing rotavirus vaccine immune response among a cohort of rotavirus-immunized infants living in El Alto Bolivia. The proposed study has three specific aims:

1) to evaluate the relationship between circulating inflammatory markers and serum trace element levels, and adjust trace element measurements for inflammation if necessary;

2) to describe serum concentrations of key trace elements – unadjusted and, if necessary, adjusted – in a cohort of rotavirus immunized infants in El Alto;
3) to evaluate the relationship between serum trace element concentrations and rotavirus vaccine immunogenicity.

VI. Significance

LMIC's experience the highest mortality from rotavirus diarrhea yet the effectiveness of the rotavirus vaccine is lowest in these settings. The reasons for the vaccine's reduced effectiveness in LMIC's are not fully understood but poorer micronutrient status among infants in LMIC's may play a role. In particular, elemental micronutrients such as calcium, copper, iron, magnesium, and zinc, may be associated with rotavirus vaccine response. The overarching goal of this research is to better characterize the relationship between elemental micronutrient status and rotavirus vaccine immune response among a cohort of rotavirus-immunized infants in El Alto, Bolivia. In so doing, this research could inform public health initiatives that aim to improve rotavirus vaccines' effectiveness in Bolivia and other LMIC's. For instance, if poor micronutrient status or specific elemental micronutrient deficiencies are associated with inferior vaccine response, public health stakeholders may focus efforts on designing programs to optimize infant nutrition. Likewise, if vaccines are seen to perform sub-optimally in areas with high prevalence of micronutrient deficiencies, rotavirus vaccination initiatives may be tailored to meet the specific needs of such situations (i.e. there may be a greater need for vaccine-boosters in areas with highly prevalent zinc deficiency). Similarly, any associations (or lack thereof) between elemental micronutrients and vaccine response may inform the directions of future research projects that evaluate rotavirus vaccination's reduced effectiveness in LMIC's. If no

compelling relationships between serum trace elements and rotavirus vaccine response are found, future research projects may explore other potential causes of the rotavirus vaccines' reduced effectiveness in LMIC's (i.e. environmental enteropathy, waning immunity needing additional booster doses of the vaccine).

Furthermore, achieving the specific aims of this research may yield secondary benefits. By describing the relationship between serum elemental concentrations and inflammation (Aim 1), this research may lead to improved interpretation of serum elemental concentrations in other clinical or biomedical settings. For example, serum copper measurements are helpful in uncovering diseases of disordered copper metabolism (i.e. Menke's disease) or severely copper deficient diets, yet such measurements are known to be influenced by the presence of inflammation and are, therefore, difficult to interpret [122, 123]; further exploration into the relationship between serum copper and circulating inflammatory markers may improve clinical interpretation of serum copper measurements. Likewise, description of the serum concentrations of trace elements in this population of Bolivian infants (Aim 2) could lead to a more thorough understanding of normal values based on clinical setting, age, sex, and other factors, as normal ranges for many trace elements remain poorly defined [124-126].

Analysis

VII. Methods

VII.a. The NIDI Study Population and Design

Secondary analysis was completed on data from the Nutrition, Immunology, and Diarrhea in Infants (NIDI) study. Briefly, the NIDI study was a comprehensive assessment of the factors associated with rotavirus vaccine immunogenicity, from nutrition, to inflammation, to human host genetics, to environmental enteropathy, to co-morbid infections, to maternal and socioeconomic conditions [127, 128]. The study took place in El Alto, Bolivia, the country's second largest city, home to a predominantly urban, indigenous population with relatively high rates of poverty [119-121]. Between May 2013 and March 2014, NIDI investigators enrolled 461 2-4-week-old infants and their mothers, whom they would follow for 12-18 months. Infants were scheduled to receive a dose of the Rotarix® oral monovalent rotavirus vaccine at 2 and 4 months of age. Informed, written consent of the mothers was required before study enrollment. Infants with documented or suspected immune deficiencies, acute illnesses during the period of study enrollment, congenital malformations, and those born to mothers unable to speak and understand Spanish or Aymara were excluded from the study. Investigators received permission to conduct the NIDI study from the Emory University IRB (IRB00056127) and the Research Ethics Committee of Bolivia.

Mother-infant pairs were surveyed at up to ten follow-up visits: as many as eight times at well-child clinical visits and one or two times following vaccine administration. Investigators collected blood from infants on up to three occasions: once (pre-vaccination) at approximately 2 months of age, again (post-vaccination) at approximately 6 months of age, and, optionally, at 12-18 months of age. Biochemical assays were conducted to determine, among other things, the

serum concentrations of inflammatory markers AGP and CRP, serum element concentrations, and serum rotavirus-specific IgA titers. Serum AGP and CRP were detected using sandwich enzyme-linked immunosorbent assay (ELISA) as described in Burke, et al. 2017 [127]. Serum element concentrations were detected using inductively-coupled plasma optical emission spectrometry by Children's Hospital Oakland Research Institute (CHORI) Elemental Analysis Facility, as described by Cvijanovich, et al., 2009 [129]. Serum rotavirus-specific IgA titers were detected using ELISA by the Gastroenteritis & Respiratory Viruses Laboratory, Division of Viral Diseases, CDC, as described in Moon et al., 2010 [130].

VII.b. Statistical Methods

Statistical analysis of the NIDI dataset was performed using SAS® Statistical Analysis Software Version 9.4 (Cary, NC). Normality of serum elements and inflammatory biomarkers (AGP and CRP) was assessed using Kolmogorov-Smirnov testing. Because neither the values for serum element concentrations nor the values for inflammatory biomarkers were normally distributed, the correlations between serum elements and inflammatory biomarkers were calculated using Spearman's rank order correlation for non-parametric data. To assess for significant differences in serum element concentrations across the three blood draws, the Kruskal-Wallis for non-parametric data was used. The Wilxocon Two-Sample t-Test was used to evaluate for significant differences in serum element concentrations between males and females and between preterm and term infants. Logistic regression analyses were used to investigate the relationships between natural-log transformed serum biomarkers and seroconversion (defined as a four-fold or higher increase in serum rotavirus-specific IgA titers between the first and second blood draws, with a positive rotavirus-specific IgA titer being defined as greater than or equal to 40), controlling for breastfeeding and prematurity. Both linear regression analyses and

Spearman's testing were used to investigate the relationships between serum elements and the fold-change in rotavirus-specific IgA between the first and second blood draws.

VII.c. Linear Regression to Correct Copper for AGP and CRP

Serum copper has a well-known, direct relationship with inflammation [69, 70, 107, 131]. Indeed, this relationship was borne out by our analyses. The decision was made, a priori, to correct element concentrations for a particular inflammatory marker (AGP, CRP, or both) with which they had at least moderate correlations ($|\rho| > 0.4$) that were present across multiple blood draws. Based on this decision, serum copper was corrected for AGP and CRP (table 2) using the linear regression method [127]. In brief, to determine how serum copper (natural-log transformed, to better meet the assumptions of linear regression) varied with AGP and CRP (both also natural-transformed), the following linear regression was used:

$$\ln(Cu) = \beta_{0+} \beta_1(\ln(AGP)) + \beta_2(\ln(CRP))$$

In the above equation, the beta-coefficients β_1 and β_2 represent the degree to which ln(Cu) changed based on unit increases in ln(AGP) or ln(CRP), respectively. We then determined the differences between an individual's observed ln(AGP) or ln(CRP) from a reference ln(AGP) or ln(CRP) (in this case, the 10^{th} percentiles for AGP and CRP in the study population). The differences between observed and reference values for ln(AGP) and ln(CRP) were multiplied by their respective beta-coefficients from the initial linear regression. These products were ultimately subtracted from an individual's observed ln(Cu) to calculate his or her corrected ln(Cu):

$$\ln(Cu_{corr}) = \ln(Cu_{obs}) - \beta_1(\ln(AGP_{obs}) - \ln(AGP_{10th\%})) - \beta_2(\ln(CRP_{obs}) - \ln(CRP_{10th\%}))$$

AGP or CRP were only accounted for in copper corrections when their respective values were above the 10^{th} percentile for the study population (i.e. if an infant had a serum AGP below the 10^{th} percentile for the study population, but a CRP above the 10^{th} percentile, only CRP was considered).

VIII. Results

Before beginning analyses, it was important to describe the study population (Table 1). Initially, 461 infants were enrolled in the study. However, data regarding serum element concentrations were only available for 346 infants at the first blood draw, 324 at the second blood draw, and 175 at the third, optional blood draw. Reasons for drop out between study enrollment and completion included refusal of blood draw, off-schedule vaccination, and loss to follow-up. Additionally, many blood draws did not yield sufficient blood volume for analyses. Infants' ages at the first blood draw ranged from 1-5 months old, with an average age of 2.11 months. At the second blood draw, infants' ages ranged from 6-10 months old with an average age of 6.73 months, and at the third blood draw, ages ranged from 11-17 months old, with an average age of 12.08 months. Among those infants for whom serum elemental data were available, the majority were male, most were born following full-term pregnancies, and the minority were exclusively breastfed for at least 6 months. The majority of mothers were younger than 26 years. Most mothers had received at least some secondary education. Although some participants did not return for the second blood draw or the third, optional blood draw, the demographic makeup of the study population remained relatively stable across blood draws.

To characterize the relationships between inflammatory markers and serum element concentrations, the investigators calculated the Spearman correlation coefficients between each element concentration and AGP and CRP (Table 2). The following cut-offs were used to define the strength of the Spearman correlations: weak correlations were defined as those with absolute values greater than 0 and less than 0.4; moderate correlations were defined as those with absolute values between 0.4 and 0.8; strong correlations were defined as those with absolute values greater than 0.8 [132].

When values across all three blood draws were included in calculations, serum calcium concentrations were significantly weakly negatively correlated with AGP and CRP. Serum calcium concentrations were also significantly weakly negatively correlated with CRP during the second blood draw. Serum copper concentrations were significantly moderately positively correlated with AGP and CRP when values from all three blood draws were included in calculations. There was a significant moderate positive correlation between serum copper concentration and AGP at the first, second, and third blood draws. There was also a significant moderate positive correlation between serum copper concentration and CRP at the first, second and third blood draws. Serum iron concentrations were significantly weakly negatively correlated with AGP and CRP when values from all three blood draws were included in calculations. Serum iron concentration was also significantly weakly negatively correlated with AGP at the second blood draw and the third blood draw, and with CRP at the second blood draw. Serum magnesium concentrations were significantly weakly negatively correlated with AGP when values from all three blood draws were included in calculations, at the first blood draw, and at the second blood draw. Serum magnesium concentrations were not significantly correlated with CRP. Serum zinc concentration was significantly weakly negatively correlated with CRP using data from all three blood draws, and with both CRP and AGP at the second blood draw. The copper/zinc ratio was significantly moderately positively correlated with AGP using values from all three blood draws, weakly positively correlated at the first blood draw, and moderately positively correlated at the second and third blood draws. The copper/zinc ratio was significantly moderately positively correlated with CRP using values from all three blood draws, at the first blood, at the second blood draw, and at the third blood draw.

To summarize, concentrations of calcium, iron, magnesium, and zinc in the serum were significantly negatively correlated with either AGP, CRP, or both at some time point during the study, but these correlations were weak and inconsistent across all three blood draws. Conversely, serum copper concentrations and the serum copper/zinc ratio were consistently significantly positively correlated with both AGP and CRP at every blood draw (for graphical representations of the relationships between serum element concentrations and inflammatory biomarkers, see Appendix Figure 1). Given that consistent, moderately strong correlations existed between copper and AGP and CRP, the decision was made to correct copper concentrations for AGP and CRP.

To describe the serum concentrations of each element in the study population, the investigators calculated the median and interquartile range (IQR) of each element concentration at each blood draw. Median values and IQR's were calculated for all infants as a whole, for term and preterm infants, and for male and female infants (Table 3). Median values and IQR's were calculated because the distributions of the serum element concentrations at each blood draw were non-normal by Kolmogorov-Smirnov testing. For every element, serum concentrations were significantly different across all three blood draws by Kruskal-Wallis test with an $\alpha=0.05$; serum copper tended to increase over time while serum iron and magnesium tended to decrease. Serum elemental concentrations did not significantly differ between term and preterm infants for any element at any blood draw. Serum copper concentrations calculated by correcting for AGP and CRP were significantly higher in males compared with females at the second and third blood draws, using the Wilcoxon Two Sample t-Test and an $\alpha=0.05$. Serum iron concentrations were significantly higher in females at the third blood draw, using the Wilcoxon Two Sample t-Test and an $\alpha=0.05$. In conclusion, serum element concentrations differed across blood draws for each

element, but not between term and preterm infants. Furthermore, corrected serum copper and serum iron inconsistently differed between male and female infants, depending on the time-point of blood collection.

Serum concentrations for each element were compared with conventional ranges found in the literature (Table 4). Given that such reference ranges are often ill-defined in infants, attempts were made to use the most precise ranges available for each serum element concentration based on age, sex, or both [133-137]. The reference range used for calcium was 9-11 mg/dL for all infants [134]. The reference ranges used for serum copper were 40-140 µg/dL for infants younger than 2 months, 40-160 µg/dL for infants 3-6 months old, 40-170 µg/dL for infants 7-9 months old, 80-170 µg/dL for infants 10-12 months old, and 80-180 µg/dL for children older than a year [135]. The reference ranges used for iron were 72-203 µg/dL for male infants 90 days or younger, 75-235 µg/dL for female infants 90 days or younger, 23-142 µg/dL for male infants 91 days to 12 months old, 60-192 µg/dL for female infants 91 days to 12 months old, 25-126 µg/dL for male children 13 months or older, and 55-162 µg/dL for female children 13 months or older [136]. The reference range used for serum magnesium was 1.6-2.4 mg/dL The reference range used for serum zinc concentration was 64-124 µg/dL [137]. Values for serum calcium, copper, and iron fell within the reference ranges over 70% of the time, while serum magnesium concentrations were frequently above range and serum zinc concentrations were frequently below range. When corrected for AGP and CRP, serum copper concentrations were more likely to be within the reference range.

To characterize the relationships between serum element concentrations, copper/zinc ratios, and inflammatory biomarkers with seroconversion, the investigators performed logistic regression analyses (Table 5, Table 6). Continuous independent variables were natural-log

transformed for fit. Seroconversion was defined as a four-fold or higher increase in serum rotavirus-specific IgA titers between the first and second blood draws (IgA titers of 0 were assigned a value of 1 for the purposes of this calculation). Elemental values from the second blood draw were used in calculations (for information on logistic regression analyses using data from other blood draws, see Appendix Table 1). No serum element concentration, copper/zinc ratio, or inflammatory marker was a significant predictor of seroconversion (Table 5). Controlling for prematurity and exclusive breastfeeding at least than 6 months (Table 6), uncorrected serum copper concentration was inversely associated with the odds of seroconversion (OR=0.329, 95% CI [0.116, 0.936]), while serum iron concentration was directly associated with the odds of seroconversion (OR=1.634, 95% CI [1.034, 2.580]).

To further characterize the relationships between serum element concentrations, copper/zinc ratios, and inflammatory biomarkers with the immune response to rotavirus vaccine, linear regression analyses were performed (Table 7). To quantify the strength of the immune response, the fold change in serum rotavirus-specific IgA GMT between the first and second blood draws was calculated. The predictor variables and the outcome variable were natural-log transformed for fit. Fold change in IgA GMT was negatively associated with uncorrected serum copper, the uncorrected copper/zinc ratio, AGP, and CRP. Fold change in IgA GMT was positively associated with serum iron. Spearman correlation coefficients between elements and fold change in IgA GMT were also calculated, given the non-normal distribution of variables (Table 8). Fold change in IgA GMT was significantly negatively associated with uncorrected serum copper, the uncorrected copper/zinc ratio, and serum CRP. Fold change in IgA GMT was also significantly positively correlated with serum iron. In summary, higher concentrations of uncorrected serum copper, a high uncorrected copper/zinc ratio, and elevated levels of

inflammatory markers were significantly associated with a smaller change between pre- and post-vaccination rotavirus-specific IgA GMT. Conversely, higher serum iron concentrations were associated with a larger change between pre- and post-vaccination rotavirus-specific IgA GMT (for more information about the relationships between serum biomarkers and the change in IgA GMT, see Appendix Table 2).

Logistic regression analyses were performed to characterize the relationships between serum biomarkers and various morbidities among the population, including fever, diarrhea, and cough (Table 9). Data from the second blood draw were used. Uncorrected copper, uncorrected copper/zinc ratio, and AGP were associated with higher odds of having had a recent history of fever, diarrhea, and cough/respiratory problem. Serum CRP was associated with higher odds of having a recent history of diarrhea and cough/respiratory problem. Corrected copper and corrected copper/zinc ratio were only associated with higher odds of recent cough/respiratory problem. Serum iron was associated with lower odds of recent diarrhea. To summarize, serum copper, the copper/zinc ratio, and serum inflammatory markers tended to be elevated in the setting of recent illness while uncorrected serum iron was lower in the setting of recent illness (in particular, diarrhea).

IX. Discussion

Despite facing the highest mortality rates from rotavirus diarrhea, children in low- and middle-income countries such as Bolivia receive less protection from the oral rotavirus vaccine than children in high-income countries [14-16]. Children's nutritional status in low- and middle-income settings may play a role. The goal of this project was to characterize the relationships between serum elemental micronutrient concentrations and the immune response to rotavirus vaccine among a population of rotavirus-vaccinated infants in El Alto, Bolivia. To do so, the investigators aimed to assess the associations between serum elements and circulating inflammatory biomarkers, to describe the range of serum element concentrations in the study population (correcting for inflammatory markers if necessary), and to evaluate the relationship between serum element concentrations and measures of vaccine immune response. Analyses revealed a moderate positive correlation between serum copper concentrations and the circulating inflammatory markers AGP and CRP, a relationship that was consistent across multiple blood draws. Also, of note were the findings that serum concentrations of every element varied across the three blood draws and that the serum element concentrations in this population frequently fell outside of reference ranges found in the literature. Finally, while no serum element concentrations from the first and second blood draws predicted seroconversion following rotavirus vaccine, serum copper, the copper/zinc ratio, AGP, and CRP predicted a smaller change between pre- and post-vaccination rotavirus-specific IgA GMT, while serum iron predicted a larger change.

The finding that serum copper concentrations rose with elevations in AGP and CRP (Table 2) is consistent with the literature, where the elevations in serum copper that accompany inflammation, infection, and stress responses have been well-described [69, 70, 107, 131]. One

explanation for this may be that the copper-carrying oxidase enzyme ceruloplasmin is an acute phase reactant, known to rise in times of stress or trauma [71]. Ceruloplasmin carries 95% of copper in the serum and its transcription in the liver is upregulated by proinflammatory cytokines, leading some to hypothesize that ceruloplasmin plays a role in host immune defense [71, 72]. In the present study, the observed elevation in serum copper accompanying elevated AGP and CRP may be reflective of elevated ceruloplasmin levels induced by some inflammatory insult. Alternatively, it may be reasonable to hypothesize that having elevated serum copper predisposes one to an inflammatory state (though this is less likely, given that elevated serum copper was observed in the setting of cough, fever, and diarrhea; see Table 6). Whatever the explanation, the observation of a direct, moderately strong correlation between copper and both AGP and CRP over multiple blood draws suggests that it is appropriate to account for inflammation when assessing infants' copper status.

In the study population, serum element concentrations differed significantly across blood draws, for every element (Table 3), with copper tending to increase with time, and iron and magnesium tending to decrease with time. This finding was not surprising, as the normal ranges for serum element concentrations are known to differ with age [134-137]. Perhaps more surprising were the high percentages of infants and children with serum element concentrations outside the reference ranges documented in the literature (Table 4), with magnesium frequently falling above range and zinc frequently falling below range. For example, at the first blood draw, more than half of the serum magnesium values were above range, while at the third blood draw, more than half of the serum zinc values were below range. These findings may be the result of a systematic error inherent to the study – for instance, an error with the biochemical assays used to determine serum elemental concentrations or an error in the data analysis steps. It is also possible

that this specific population of infants in El Alto has a different biochemical profile than those populations from which the serum elemental reference ranges were inferred (i.e. higher rates of zinc deficiency). A more likely explanation, perhaps, is that age- and sex-based reference ranges for many of the studied elements in children younger than 1 year are ill-defined or imprecise; the fact that the observed values fell out of range so frequently may highlight the need for further studies that more precisely define the normal range of serum element concentrations in infants. Lastly, once serum copper was corrected for AGP and CRP, fewer values fell above range. This is also consistent with the hypothesis that serum copper may be overestimated if the presence of an inflammatory state is not accounted for.

Logistic regression analyses revealed no significant relationships between serum element concentrations or inflammatory markers and seroconversion (Table 5). However, uncorrected serum copper, uncorrected copper/zinc ratios, AGP, and CRP were all related to a lower fold-change in rotavirus-specific IgA GMT in linear regression and Spearman's correlation analyses (Table 7, Table 8). This may indicate that infants with a higher inflammatory burden do not mount as thorough an immune response to vaccination as infants with a lower inflammatory burden. Indeed, when serum copper and the copper/zinc ratio were corrected based on AGP and CRP, their relationships with the degree of change in post-vaccine, rotavirus-specific IgA GMT became non-significant. This suggests that the presence of inflammation, not serum copper itself, is driving the observed relationship with IgA GMT fold-change. Similarly, serum iron was directly related to the fold-change in IgA GMT. Serum iron is known to decrease in the setting of inflammation and, in fact, we observed a weak, inconsistent negative correlation between serum iron and circulating inflammatory markers in our study (Table 2) [104, 105]. Thus, the finding that lower serum iron, which could result from increased inflammatory burden, was associated

with lower fold-change in IgA GMT may be further evidence that the strength of the immune response to rotavirus vaccine is blunted in the setting of inflammation.

The binary seroconversion measurement was obtained by calculating whether post-vaccination IgA titers increased by a certain amount from pre-vaccination IgA titers (in this case, fourfold). Fold-change in IgA was calculated simply by dividing post-vaccination IgA by pre-vaccination IgA. Neither measurement perfectly represents protection from rotavirus infection and, indeed, there is no universal definition for the IgA fold-change threshold that indicates seroconversion following vaccination [38]. Furthermore, there are instances where the seroconversion measurement used in our study indicates little about the strength of the immune response following vaccination. For example, an infant whose IgA had increased from 1 pre-vaccination to 40 post-vaccination would receive the same value for the seroconversion measurement as an infant whose IgA had increased from 1 pre-vaccination to 2560 post-vaccination. In this scenario, both infants may be protected from rotavirus infection but, arguably, the second infant mounted a greater response to the vaccine. This difference in the ways of measuring immune response to vaccination could explain the seemingly disparate conclusions that could be drawn from the results in tables 5-8. Conceivably, the fold change measurement is capable of detecting subtler changes in the immune response to rotavirus vaccine due to the presence of inflammation.

Other studies have found evidence of an association between inflammation and vaccine underperformance. In particular, oral vaccines have been shown to work less well than parenteral vaccines in inflamed individuals. For example, Naylor et al., 2015 found that oral polio and rotavirus vaccines underperformed in Bangladeshi infants who had evidence of systemic inflammation or environmental enteropathy[46]. Similarly Becker-Dreps and associates (2017)

found an association between biomarkers of environmental enteropathy and seroconversion after one does of the pentavalent rotavirus vaccine in Nicaraguan infants [138]. Elevated CRP may signal underlying environmental enteropathy [139] and the association we observed between CRP and fold-change in rotavirus IgA GMT could indicate a blunted immunological response mediated by the presence of environmental enteropathy.

This study was not without limitations. For one, the study population may not be representative of infants in other low- and middle-income countries. Infants in such other settings may be exposed to different genetic strains of rotavirus, have different feeding practices, or receive the other licensed rotavirus vaccine (i.e. the pentavalent vaccine); the major findings of this study may not be generalizable. Another limitation of this study is that the measurements used to represent the exposures and the outcomes (i.e. serum element or biomarker concentrations and rotavirus IgA titers, respectively) are not perfect correlates of the conditions they are meant to represent. For example, while serum copper may be an appropriate measure of copper status at a population level, it does not provide a perfect estimate of individual copper status [140]. Similarly, there is ongoing research into the best way to measure immunity to rotavirus; the presence of rotavirus-specific IgA in the serum may not always signify immunity, especially in individuals with borderline titers [141]. Moreover, the presence of rotavirus-specific IgA antibodies in serum may reflect natural immunity following exposure to rotavirus, not necessarily immunity following vaccination; it is not always possible to differentiate between natural- and vaccine-induced immune response to rotavirus based on IgA. A third limitation of the study was participant attrition, incomplete participation in blood draws, or unavailable data for serum element concentrations, the latter due primarily to blood draws with insufficient volume for analysis. Initially, 461 infants enrolled in the study but data on serum element

concentrations were only available for 346 infants at the first blood draw, 324 infants at the second blood draw, and 175 infants at the third, optional blood draw.

Nevertheless, the study had several strengths. Chief among these was the high statistical power of the study. Despite some attrition, serum elemental information was available for nearly 350 infants, improving the chance that any true relationships between serum elements, inflammatory markers, and rotavirus vaccine immunogenicity would be uncovered. A second strength of the study was its longitudinal nature. Much can change for infants and their mothers during the first year of life. The longitudinal nature of the data allowed investigators the see, for example, how serum element concentrations varied over the course infancy, at a level of precision rarely found in the literature. Another strength was the richness of the study dataset. Perhaps a major reason why rotavirus vaccination remains less effective in low- and middle-income settings, is the fact that conducting research in such settings is often difficult. Ethical study execution in resource-limited settings poses a unique set of challenges, from ensuring participant retention, to maintaining a functional "cold-chain" of biomaterials, to promoting sustainable, respectful cross-cultural interactions [128]. NIDI investigators established a collaborative framework to create a robust dataset that allows researchers to examine the complex relationships between nutrition, inflammation, human genetics, environment, and rotavirus vaccine effectiveness.

X. Conclusion

The goal of the proposed study was to understand the importance of elemental micronutrients in influencing rotavirus vaccine effectiveness among a cohort of rotavirus-immunized infants living in El Alto Bolivia. The proposed study had three specific aims: 1) to evaluate the relationship between circulating inflammatory markers and serum trace element levels, and adjust trace element measurements for inflammation if necessary; 2) to describe serum concentrations of key trace elements – unadjusted and, if necessary, adjusted – in a cohort of rotavirus immunized infants in El Alto; 3) to evaluate the relationship between serum trace element concentrations and rotavirus vaccine immunogenicity. In achieving the first aim, serum copper was found to have persistently significant, direct correlations of moderate strength with both AGP and CRP. From this finding, we concluded that the presence of inflammation must be accounted for when assessing infant copper status. Completion of the second aim revealed that many values for serum element concentrations fell outside of reference ranges in the literature, particularly values for serum magnesium and zinc. This finding could perhaps lead to future refinement of the accepted reference ranges in infants based on age. Finally, in completing the third aim, we found that seroconversion, per se, was not related to serum element concentrations, but that the strength of the immune response to rotavirus vaccination may be blunted in the setting of inflammation.

XI. Public Health Implications

- The findings revealed a need to correct copper for inflammation (Table 2, Appendix Figure 1). While serum copper is infrequently tested in clinical settings, it can be useful in detecting diseases of copper metabolism, such as Wilson's disease and Menke's kinky hair disease. Serum copper increases with inflammation, and it is possible to correct copper based on AGP and CRP using the linear regression method.
- Other elements exhibited some weak correlations with AGP and/or CRP (often with a coefficient of around 0.1 or less) but these did not hold for every blood draw (Table 2). Further, the scatter plots of these elements against AGP and CRP (all natural-log transformed for fit) did not suggest a strong, linear relationship (Appendix Figure 1).
- I believe that the high number of serum element concentrations that fell outside of the reference ranges in the literature highlights the need for more precision in defining "normal" serum element ranges in infants. This is particularly true for magnesium and zinc.
- In uncontrolled logistic regression analyses, none of the elements were associated with seroconversion as it was measured (greater than 4-fold change between pre- and post-vaccine IgA GMT, with a post-vaccine IgA GMT at least 40). However, in linear regression and Spearman's analyses, uncorrected serum copper, serum iron, AGP, and CRP were all associated with the fold change in IgA GMT. The fold change was smaller when copper, AGP, and CRP were higher. The fold change was larger when iron was higher. Because copper increases and iron decreases in the setting of inflammation, I concluded that the presence of inflammation may lead to a blunted immune response. I

do not believe that the elemental micronutrients themselves were responsible for reducing the fold change in IgA GMT.

- Based on the above conclusion, I believe that, to improve vaccine immunogenicity, it is less important for public health campaigns to optimize or "normalize" infants' copper and iron statuses and more important to reduce their inflammatory burdens. The increased inflammatory burden experienced by children in LMIC's may contribute to the vaccine's reduced effectiveness in such settings.
- Uncorrected copper was also increased in the setting of illness (recent fever, diarrhea, or cough). Similarly, AGP and CRP were increased. I hypothesize that the presence of illness/infection causes an up-regulation of the inflammatory response, which, in turn, affects the concentrations of copper in the blood (i.e. copper increases because ceruloplasmin is an acute phase reactant). I see these illnesses (fever, diarrhea, and cough) as adding to the inflammatory burdens experienced by infants in LMIC's. Based on my other findings, I concluded that the immune response to rotavirus vaccination may be blunted in the setting of inflammation. Thus, public health interventions in LMIC's aimed at reducing illnesses in infancy (i.e. diarrhea and respiratory infections) may have the added benefit of improving infants' responses to rotavirus vaccines.

XII. Tables

Table 1: Demographic information for a cohort of Bolivian infants and children in El Alto

	First Blood Draw (n=346[1]) (1-5 months old)[2]		Second Blood Draw (n=324[1]) (6-10 months old)[3]		Third Blood Draw (n=175[1]) (11-17 months old)[4]	
Characteristics	Frequency	Percent	Frequency	Percent	Frequency	Percent
Male	188	54.34	173	53.40	94	53.71
Preterm (<37 weeks gestational age)	65[5]	18.79	57[5]	17.59	30[6]	17.14
Exclusively Breastfed At Least 6 months[7]	-	-	114[8]	35.19	61[9]	34.86
Maternal Age[10]						
Less than 22 Years	107	30.92	92	28.40	40	22.86
22-25 Years	87	25.14	77	23.77	50	28.57
26-30 Years	85	24.57	81	25.00	40	22.86
More than 30 Years	63	18.21	72	22.22	43	24.57
Maternal Education[11]						
Primary or Less	56	16.18	48	14.81	25	14.29
At least Some Secondary	210	60.69	206	63.58	107	61.14
At least Some Superior	78	22.54	69	21.30	43	24.57

[1]Number of subjects for whom data regarding serum elemental concentrations were available
[2]Average age = 2.11 months, standard deviation = 0.29 months
[3]Average age = 6.73 months, standard deviation = 0.87 months
[4]Average age = 12.08 months, standard deviation = 0.73 months
[5]Information regarding gestational age missing for 8 subjects
[6]Information regarding gestational age missing for 7 subjects
[7]Includes infants who had received formula on no more than one occasion
[8]Information regarding breastfeeding practices missing for 2 subjects
[9]Information regarding breastfeeding practices missing for 3 subjects
[10]Information regarding maternal age missing for 4 subjects
[11]Information regarding maternal education missing for 2 subjects

Table 2: Summary of correlations between serum elemental concentrations and inflammatory markers

			AGP[1]		CRP[2]	
		n	Spearman's Correlation Coefficient	*p*	Spearman's Correlation Coefficient	*p*
Calcium	All Blood Draws	842	**-0.08508[3]**	**0.0135**	**-0.08212[3]**	**0.0172**
	Visit 2 (1-5 months old)	344	0.06019	0.2656	-0.03094	0.5674
	Visit 6 (6-10 months old)	324	-0.0637	0.2529	**-0.12882[3]**	**0.0204**
	Visit 8 (11-17 months old)	174	-0.12577	0.0982	0.03023	0.6922
Copper	All Blood Draws	842	**0.71254[3]**	**<.0001**	**0.55385[3]**	**<.0001**
	Visit 2 (1-5 months old)	344	**0.51521[3]**	**<.0001**	**0.46563[3]**	**<.0001**
	Visit 6 (6-10 months old)	324	**0.5401[3]**	**<.0001**	**0.48133[3]**	**<.0001**
	Visit 8 (11-17 months old)	174	**0.68109[3]**	**<.0001**	**0.53759[3]**	**<.0001**
Iron	All Blood Draws	842	**-0.33535[3]**	**<.0001**	**-0.25715[3]**	**<.0001**
	Visit 2 (1-5 months old)	344	-0.07644	0.1572	-0.0644	0.2335
	Visit 6 (6-10 months old)	342	**-0.18751[3]**	**0.0007**	**-0.25765[3]**	**<.0001**
	Visit 8 (11-17 months old)	174	**-0.19885[3]**	**0.0085**	-0.04766	0.5323
Magnesium	All Blood Draws	842	**-0.26753[3]**	**<.0001**	0.00732	0.8321
	Visit 2 (1-5 months old)	344	**-0.3487[3]**	**<.0001**	0.04924	0.3626
	Visit 6 (6-10 months old)	324	**-0.18809[3]**	**0.0007**	0.0575	0.3022
	Visit 8 (11-17 months old)	174	-0.07197	0.3453	0.01973	0.7961
Zinc	All Blood Draws	840	-0.01926	0.5772	**-0.09819[3]**	**0.0044**
	Visit 2 (1-5 months old)	343	0.09502	0.0789	-0.0178	0.7425
	Visit 6 (6-10 months old)	323	**-0.14678[3]**	**0.0082**	**-0.20669[3]**	**0.0002**
	Visit 8 (11-17 months old)	174	-0.11879	0.1185	-0.13861	0.0682
Copper/Zinc Ratio	All Blood Draws	840	**0.61571[3]**	**<.0001**	**0.5256[3]**	**<.0001**
	Visit 2 (1-5 months old)	343	**0.37642[3]**	**<.0001**	**0.4056[3]**	**<.0001**
	Visit 6 (6-10 months old)	323	**0.47555[3]**	**<.0001**	**0.49718[3]**	**<.0001**
	Visit 8 (11-17 months old)	174	**0.63295[3]**	**<.0001**	**0.51673[3]**	**<.0001**

[1]α-1-acid glycoprotein
[2]C-reactive protein
[3]Statistically significant correlation at $\alpha = 0.05$

Table 3: Median serum elemental concentrations among Bolivian infants and children in El Alto

	First Blood Draw (1-5 months old)		Second Blood Draw (6-10 months old)		Third Blood Draw (11-17 Months Old)	
	Median (n)	IQR (25%, 75%)	Median (n)	IQR (25%, 75%)	Median (n)	IQR (25%, 75%)
Serum Calcium (mg/dL)[1]						
All Infants	9.79 (346)	(9.41, 10.31)	9.84 (324)	(9.40, 10.33)	9.33 (175)	(8.97, 9.76)
Term Infants[2]	9.78 (273)	(9.41, 10.27)	9.80 (259)	(9.38, 10.33)	9.32 (138)	(8.93, 9.73)
Preterm Infants	9.93 (65)	(9.48, 10.61)	9.92 (57)	(9.47, 10.53)	9.50 (30)	(9.14, 9.83)
Male	9.85 (188)	(9.43, 10.36)	9.84 (173)	(9.42, 10.29)	9.33 (94)	(9.02, 9.83)
Female	9.75 (158)	(9.36, 10.29)	9.83 (151)	(9.36, 10.40)	9.36 (81)	(8.95, 9.67)
Unadjusted Serum Copper (µg/dL)[1]						
All Infants	84.4 (346)	(72.0, 103.1)	120.2 (324)	(106.3, 143.8)	134.4 (175)	(118.8, 153.1)
Term Infants[2]	84.4 (273)	(71.9, 100.0)	121.9 (259)	(106.3, 140.6)	134.3 (138)	(115.6, 156.3)
Preterm Infants	86.4 (65)	(75.0, 103.1)	131.3 (57)	(109.4, 153.1)	135.9 (30)	(121.9, 150.0)
Male	85.0 (188)	(71.9, 101.5)	125.0 (173)	(109.4, 146.9)	135.5 (94)	(118.8, 156.3)
Female	84.4 (158)	(75.0, 103.1)	118.9 (151)	(100.2, 140.6)	131.3 (81)	(115.6, 149.3)
Adjusted Serum Copper (µg/dL)[1]						
All Infants	71.7 (346)	(61.8, 80.7)	105.0 (324)	(92.7, 117.5)	113.8 (175)	(103.6, 127.2)
Term Infants[2]	70.5 (273)	(61.8, 79.9)	104.5 (259)	(92.2, 115.7)	113.7 (138)	(103.8, 127.0)
Preterm Infants	72.5 (65)	(62.7, 82.9)	109.0 (57)	(93.3, 130.4)	112.5 (30)	(101.1, 126.0)
Male	70.3 (188)	(61.8, 78.6)	105.9 (173)[3]	(95.8, 118.8)	116.1 (94)[3]	(106.4, 130.6)
Female	72.6 (158)	(61.8, 81.5)	101.2 (151)	(87.4, 117.1)	110.4 (81)	(101.5, 122.1)
Serum Iron (µg/dL)[1]						
All Infants	134.3 (346)	(109.4, 164.8)	100.7 (324)	(74.8, 140.6)	75.0 (175)	(53.1, 100.0)
Term Infants[2]	134.4 (273)	(107.6, 162.5)	100.0 (259)	(74.0, 143.8)	75.0 (138)	(53.1, 96.9)
Preterm Infants	128.1 (65)	(112.5, 168.8)	109.6 (57)	(75.0, 129.0)	82.8 (30)	(53.1, 140.6)
Male	125.0 (188)[1]	(103.9, 156.1)	93.8 (173)	(68.6, 138.9)	65.6 (94)[3]	(43.8, 90.6)
Female	139.0 (158)	(112.5, 168.8)	105.5 (151)	(81.3, 143.8)	87.5 (81)	(62.5, 109.4)
Serum Magnesium (mg/dL)[1]						
All Infants	2.45 (346)	(2.08, 2.69)	2.31 (324)	(2.00, 2.61)	2.28 (175)	(2.12, 2.44)
Term Infants[2]	2.44 (273)	(2.06, 2.68)	2.26 (259)	(1.99, 2.58)	2.28 (138)	(2.08, 2.43)
Preterm Infants	2.47 (66)	(2.10, 2.79)	2.41 (57)	(2.03, 2.67)	2.27 (30)	(2.12, 2.49)
Male	2.46 (188)	(2.08, 2.71)	2.36 (173)	(2.02, 2.64)	2.28 (94)	(2.10, 2.46)
Female	2.44 (158)	(2.06, 2.66)	2.26 (151)	(1.94, 2.57)	2.27 (81)	(2.13, 2. 43)
Serum Zinc (µg/dL)[1]						
All Infants	63.7 (345)	(56.3, 71.9)	66.3 (323)	(56.7, 77.7)	59.4 (175)	(56.3, 68.8)
Term Infants[2]	65.4 (272)	(56.3, 71.9)	65.6 (258)	(56.7, 75.4)	59.4 (138)	(55.6, 68.8)
Preterm Infants	62.5 (65)	(56.3, 69.9)	67.1 (57)	(59.4, 80.6)	65.6 (30)	(56.3, 68.8)
Male	64.9 (188)	(55.9, 71.9)	68.4 (173)	(56.9, 78.1)	59.4 (94)	(56.3, 68.8)
Female	63.0 (157)	(56.3, 71.9)	65.6 (150)	(56.7, 75.0)	62.5 (81)	(53.1, 68.8)
Copper/Zinc Ratio[1]						
All Infants	1.37 (345)	(1.09, 1.67)	1.83 (323)	(1.52, 2.28)	2.16 (175)	(1.84, 2.56)
Term Infants[2]	1.36 (272)	(1.08, 1.36)	1.84 (258)	(1.52, 2.24)	2.17 (138)	(1.86, 2.59)
Preterm Infants	1.37 (65)	(1.12, 1.74)	1.81 (57)	(1.49, 2.39)	2.14 (30)	(1.81, 2.53)
Male	1.36 (188)	(1.08, 1.63)	1.82 (173)	(1.52, 2.33)	2.22 (94)	(1.87, 2.63)
Female	1.37 (157)	(1.11, 1.71)	1.84 (150)	(1.50, 2.25)	2.06 (81)	(1.82, 2.45)

[1]Significantly different serum concentrations across blood draws, using Kruskal-Wallis Test and $\alpha = 0.05$

[2]No significant difference in serum concentrations between term and preterm infants for row, using Wilcoxon Two Sample t-Test and $\alpha = 0.05$

[3]Significantly different serum concentrations compared to females during the same blood draw, using Wilcoxon Two Sample t-Test and $\alpha = 0.05$

Table 4: Distribution of Bolivian infants' serum element concentrations relative to reference ranges documented in the literature

	First Blood Draw (1-5 months old)			Second Blood Draw (6-10 months old)			Third Blood Draw (11-17 Months Old)		
	Below reference range n (%)	Within reference range n (%)	Above reference range n (%)	Below reference range n (%)	Within reference range n (%)	Above reference range n (%)	Below reference range n (%)	Within reference range n (%)	Above reference range n (%)
Serum Calcium[1]	34 (9.83%)	262 (75.72%)	50 (14.45%)	40 (12.35%)	237 (73.15%)	47 (14.51%)	44 (25.15%)	129 (73.71%)	2 (1.14%)
Unadjusted Serum Copper[2]	-	329 (95.09%)	17 (4.91%)	-	285 (88.24%)	38 (11.76)	-	154 (89.02%)	19 (10.98%)
Adjusted Serum Copper[2]	6 (1.73%)	339 (97.98%)	1 (0.29%)	-	323 (99.69%)	1 (0.31%)	-	173 (98.86%)	2 (1.14%)
Serum Iron[3]	7 (2.02%)	303 (87.57%)	36 (10.40%)	10 (3.09%)	256 (79.01%)	58 (17.90%)	20 (11.43%)	148 (84.57%)	7 (4.00%)
Serum Magnesium[1]	2 (0.58%)	155 (44.8%)	189 (54.62%)	4 (1.23%)	176 (54.32%)	144 (44.44%)	-	117 (66.86%)	58 (33.14%)
Serum Zinc[4]	173 (50.14%)	168 (48.70%)	4 (1.16%)	138 (42.72%)	183 (56.66%)	2 (0.62%)	108 (61.71%)	66 (37.71%)	1 (0.57%)

[1]Reference range by age from Wu, 2006
[2]Reference range by age from Mayo Clinic
[3]Reference range by age and sex from Soldin, et al. 2009
[4]Reference range by age from Lin, et al. 2012

Table 5: Summary of logistic regression analysis for serum element concentrations[1,2], element ratios, and inflammatory markers[1,2], predicting seroconversion[3]

Biomarker	n	OR (95% CI)
Calcium	305	0.324 (0.048, 2.198)
Copper	305	0.362 (0.129, 1.011)
Corrected Copper	305	0.417 (0.120, 1.451)
Iron	305	1.557 (0.996, 2.434)
Magnesium	305	0.817 (0.203, 3.287)
Zinc	305	1.111 (0.372, 3.324)
Copper/Zinc Ratio	304	0.806 (0.558, 1.163)
Corrected Copper/Zinc Ratio	304	0.832 (0.483, 1.434)
AGP	320	0.717 (0.443, 1.161)
CRP	320	0.883 (0.764, 1.020)

[1]Natural log-transformed for fit
[2]Values from second blood draw (6-10 months)
[3]Defined as a four-fold or higher increase in serum rotavirus-specific IgA geometric mean titer between first and second blood draws

Table 6: Summary of logistic regression analyses for serum element concentrations[1,2], element ratios[2], and inflammatory markers[1,2] predicting seroconversion[3], controlling for prematurity and exclusive breastfeeding for the first 6 months, n=298

Biomarker	OR (95% CI)
Calcium	0.275 (0.040, 1.898)
Copper	**0.329 (0.116, 0.936)[4]**
Corrected Copper	0.365 (0.103, 1.292)
Iron	**1.634 (1.034, 2.580)[4]**
Magnesium	0.731 (0.180, 2.973)
Zinc	1.096 (0.357, 3.366)
Copper/Zinc Ratio	0.792 (0.546, 1.151)
Corrected Copper/Zinc Ratio	0.804 (0.462, 1.400)
AGP	0.730 (0.449, 1.186)
CRP	0.882 (0.762, 1.019)

[1]Natural log-transformed for fit
[2]Values from second blood draw (6-10 months old)
[3]Defined as a four-fold or higher increase in serum rotavirus-specific IgA geometric mean titer between first and second blood draws
[4]95% confidence interval excludes 1

Table 7: Summary of linear regression analysis for serum element concentrations[1, 2], element ratios[2], and inflammatory markers[1,2] predicting fold change in IgA geometric mean titers[1] between blood draws 1 and 2

Biomarker	Parameter Estimate	Standard Error	*p*
Calcium	-2.21	1.3217	0.0951
Copper[3]	**-1.74084**	**0.687**	**0.0118**
Corrected Copper	-1.21722	0.83735	0.1471
Iron[3]	**0.58497**	**0.29246**	**0.0464**
Magnesium	0.10455	0.95221	0.9126
Zinc	0.34771	0.75106	0.6437
Copper/Zinc Ratio[3]	**-0.53652**	**0.25233**	**0.0343**
Corrected Copper/Zinc Ratio	-0.47696	0.37364	0.2027
AGP	**-0.72421**	**0.32840**	**0.0282**
CRP	**-0.31867**	**0.09793**	**0.0013**

[1]Natural log-transformed for fit
[2]Values from second blood draw (6-10 months old)
[3]Statistically significant at α=0.05

Table 8: Summary of Spearman correlations between serum element concentrations[1], element ratios[1], inflammatory markers[1], and fold change in rotavirus-specific IgA geometric mean titers between blood draws 1 and 2

Biomarker	n[2]	Spearman's correlation coefficient	*p*
Calcium	305	-0.04919	0.392
Copper	305	**-0.14001**	**0.0144[3]**
Corrected Copper	305	-0.08477	0.1397
Iron	305	**0.11415**	**0.0464[3]**
Magnesium	305	0.01807	0.7533
Zinc	304	0.04218	0.4637
Copper/Zinc Ratio	304	**-0.13845**	**0.0157[3]**
Corrected Copper/Zinc Ratio	304	-0.09576	0.0956
AGP	320	-0.10947	0.0504
CRP	320	**-0.15829**	**0.0045[3]**

[1]Values from second blood draw
[2]Of the subjects with data for elemental concentrations from blood draw 2, 19 did not have data for IgA
[3]Statistically significant at α=0.05

Table 9: Summary of logistic regression analysis of serum element concentrations[1], element ratios, and inflammatory markers[1] predicting comorbidities[2]

	Fever in the past 2 weeks		Diarrhea in the past 2 weeks		Cough or respiratory problem in the past 2 weeks	
Biomarker	OR(95%CI)	n	OR(95%CI)	n	OR (95% CI)	n
Calcium	0.238 (0.035, 1.623)	324	0.183 (0.011, 3.021)	323	0.501 (0.078, 3.204)	324
Copper	**3.689 (1.379, 9.868)[3]**	**324**	**4.239 (1.127, 15.949)[3]**	**323**	**17.920 (5.966, 53.828)[3]**	**324**
Corrected Copper	1.730 (0.548, 5.459)	324	1.348 (0.278, 6.520)	323	**11.282 (3.317, 38.377)[3]**	**324**
Iron	0.781 (0.516, 1.181)	324	**0.338 (0.176, 0.647)[3]**	**323**	1.015 (0.674, 1.528)	324
Magnesium	4.270 (1.097, 16.628)	324	0.298 (0.050, 1.775)	323	1.825 (0.484, 6.877)	324
Zinc	0.551 (0.195, 1.554)	323	0.563 (0.135, 2.351)	322	0.607 (0.216, 1.702)	323
Copper/Zinc Ratio	**1.619 (1.136, 2.307)[3]**	**323**	**1.628 (1.050, 2.523)[3]**	**322**	**2.579 (1.709, 3.892)[3]**	**323**
Corrected Copper/Zinc Ratio	1.604 (0.962, 2.672)	323	1.473 (0.748, 2.899)	322	**2.792 (1.609, 4.846)[3]**	**323**
AGP	**2.325 (1.456, 3.713)[3]**	**342**	**2.747 (1.504, 5.019)[3]**	**341**	**2.835 (1.736, 4.629)[3]**	**342**
CRP	1.144 (1.000, 1.310)	342	**1.396 (1.170, 1.666)[3]**	**341**	**1.214 (1.055, 1.396)[3]**	**342**

[1]Natural log-transformed for fit
[2]Values from second blood draw (6-10 months old)
[3]95% confidence interval excludes 1

XIII. Appendices

Appendix Table 1: Summary of logistic regression analysis for serum element concentrations[1], element ratios, and inflammatory markers[1] predicting seroconversion[2]

Element	Blood Draw	n	OR (95% CI)
Calcium	1 (1-5 months old)	297	0.336 (0.051, 2.241)
	2 (6-10 months old)	305	0.324 (0.048, 2.198)
	3 (11-17 months old)	160	0.701 (0.007, 71.907)
Copper	1 (1-5 months old)	296	0.508 (0.211, 1.225)
	2 (6-10 months old)	305	0.362 (0.129, 1.011)
	3 (11-17 months old)	160	0.234 (0.045, 1.224)
Corrected Copper	1 (1-5 months old)	296	0.357 (0.117, 1.090)
	2 (6-10 months old)	305	0.417 (0.120, 1.451)
	3 (11-17 months old)	160	0.129 (0.014, 1.202)
Iron	1 (1-5 months old)	296	0.857 (0.460, 1.596)
	2 (6-10 months old)	305	1.557 (0.996, 2.434)
	3 (11-17 months old)	160	1.503 (0.786, 2.875)
Magnesium	1 (1-5 months old)	297	0.492 (0.140, 1.726)
	2 (6-10 months old)	305	0.817 (0.203, 3.287)
	3 (11-17 months old)	160	0.764 (0.043, 13.592)
Zinc	1 (1-5 months old)	296	0.385 (0.123, 1.209)
	2 (6-10 months old)	305	1.111 (0.372, 3.324)
	3 (11-17 months old)	**160**	**0.136 (0.020, 0.927)[3]**
Copper/Zinc Ratio	1 (1-5 months old)	296	0.955 (0.609, 1.498)
	2 (6-10 months old)	304	0.806 (0.558, 1.163)
	3 (11-17 months old)	160	0.992 (0.585, 1.680)
Corrected Copper/Zinc Ratio	1 (1-5 months old)	296	0.908 (0.456, 1.808)
	2 (6-10 months old)	304	0.832 (0.483, 1.434)
	3 (11-17 months old)	160	1.285 (0.551, 2.998)
AGP	1 (1-5 months old)	319	0.897 (0.532, 1.513)
	2 (6-10 months old)	320	0.717 (0.443, 1.161)
	3 (11-17 months old)	168	0.815 (0.437, 1.520)
CRP	1 (1-5 months old)	319	1.003 (0.827, 1.216)
	2 (6-10 months old)	320	0.883 (0.764, 1.020)
	3 (11-17 months old)	168	1.055 (0.857, 1.298)

[1]Natural log-transformed for fit
[2]Defined as a four-fold or higher increase in serum rotavirus-specific IgA geometric mean titer between first and second blood draws, with second blood draw rotavirus-specific IgA geometric mean titer at least 40
[3]95% confidence interval excludes 1

Appendix Table 2: Summary of Spearman correlations between serum element concentrations[1], element ratios[1], and inflammatory markers[1], and difference in rotavirus-specific IgA geometric mean titers between blood draws 1 and 2

Biomarker	n[2]	Spearman's correlation coefficient	*p*
Calcium	305	-0.05495	0.3389
Copper	305	**-0.13654**	**0.0170**[3]
Corrected Copper	305	-0.09896	0.0845
Iron	305	**0.11402**	**0.0466**[3]
Magnesium	305	0.01810	0.7529
Zinc	304	0.02683	0.6412
Copper/Zinc Ratio	304	**-0.12960**	**0.0238**[3]
Corrected Copper/Zinc Ratio	304	-0.09697	0.0915
AGP	320	-0.09602	0.0863
CRP	320	**-0.11972**	**0.0323**[3]

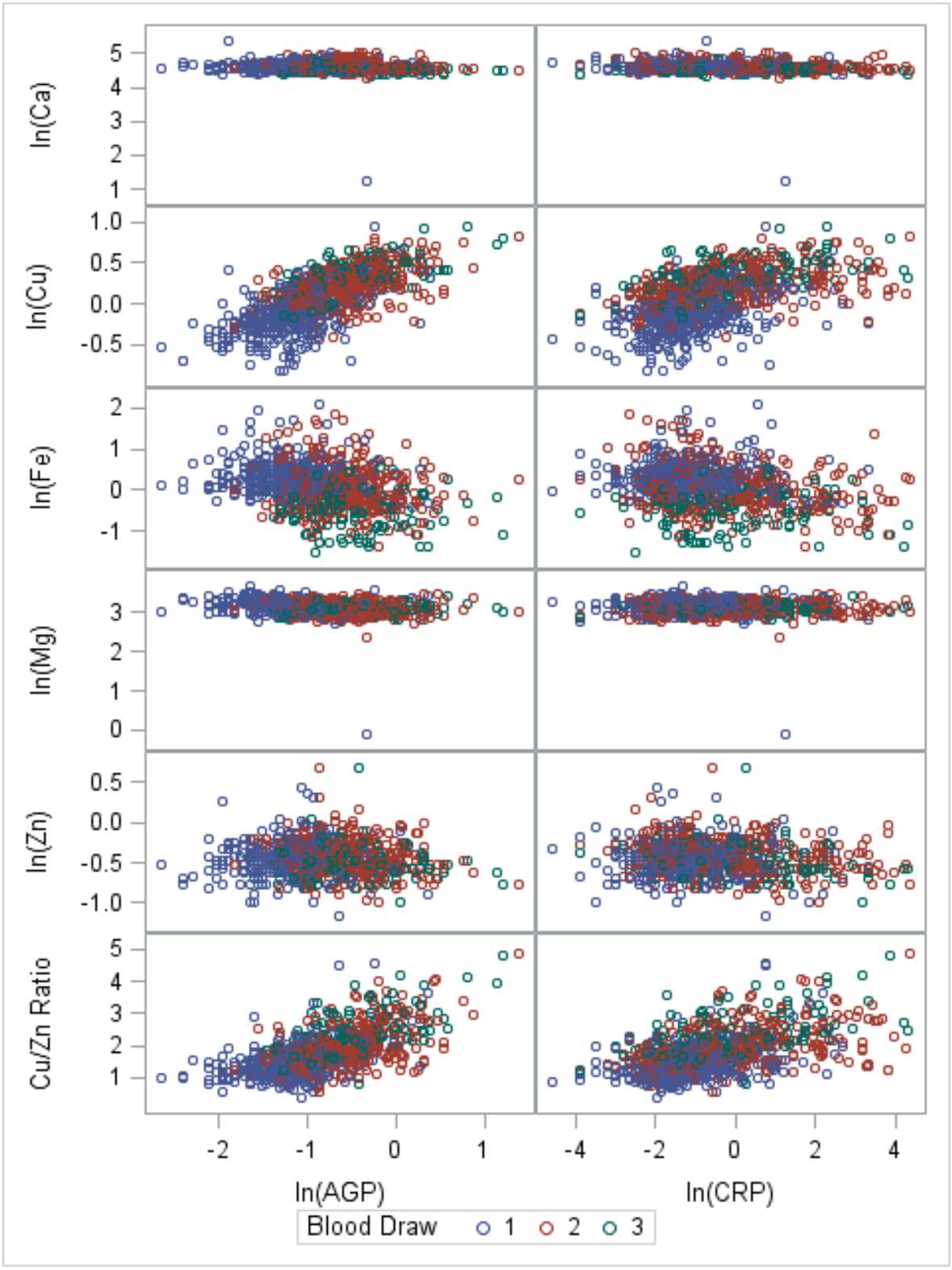
[1]Values from blood draw 2
[2]Of the subjects with data for elemental concentrations from blood draw 2, 19 did not have data for IgA
[3]Statistically significant at α=0.05

Appendix Table 3: Summary of Spearman correlations between serum element concentrations[1], element ratios[1], inflammatory markers[1], and fold change in rotavirus-specific IgA geometric mean titers between blood draws 1 and 2

Biomarker	n	Spearman's correlation coefficient	*p*
Calcium	295	-0.02361	0.6863
Copper	295	**-0.014549**	**0.0124**[2]
Corrected Copper	295	-0.10344	0.0761
Iron	295	-0.06880	0.2388
Magnesium	295	0.00569	0.9225
Zinc	295	-0.11268	0.0532
Copper/Zinc Ratio	295	-0.06211	0.2877
Corrected Copper/Zinc Ratio	295	-0.00419	0.9428
AGP	318	**-0.10947**	**0.0504**[2]
CRP	318	-0.10004	0.0749

[1]Values from first blood draw
[2]Statistically significant at α=0.05

Appendix Figure 1: Graphical representations of the relationships between serum elements and inflammatory markers



References

1. Walker, C.L.F., et al., *Global burden of childhood pneumonia and diarrhoea.* The Lancet. **381**(9875): p. 1405-1416.
2. Bryce, J., et al., *WHO estimates of the causes of death in children.* Lancet, 2005. **365**(9465): p. 1147-52.
3. Liu, L., et al., *Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000.* The Lancet, 2012. **379**(9832): p. 2151-2161.
4. Tate, J.E., et al., *Global, Regional, and National Estimates of Rotavirus Mortality in Children <5 Years of Age, 2000-2013.* Clin Infect Dis, 2016. **62 Suppl 2**: p. S96-s105.
5. Troeger, C., et al., *Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015.* The Lancet Infectious Diseases.
6. Leung, D.T., M.J. Chisti, and A.T. Pavia, *Prevention and Control of Childhood Pneumonia and Diarrhea.* Pediatr Clin North Am, 2016. **63**(1): p. 67-79.
7. Fischer Walker, C.L., et al., *Diarrhea incidence in low- and middle-income countries in 1990 and 2010: a systematic review.* BMC Public Health, 2012. **12**: p. 220.
8. De Oliveira, L.H., et al., *Temporal trends in diarrhea-related hospitalizations and deaths in children under age 5 before and after the introduction of the rotavirus vaccine in four Latin American countries.* Vaccine, 2013. **31**: p. C99-C108.
9. Santos, V.S., et al., *Effectiveness of rotavirus vaccines against rotavirus infection and hospitalization in Latin America: systematic review and meta-analysis.* Infect Dis Poverty, 2016. **5**(1): p. 83.
10. Pringle, K.D., et al., *Sustained Effectiveness of Rotavirus Vaccine Against Very Severe Rotavirus Disease Through the Second Year of Life, Bolivia 2013-2014.* Clin Infect Dis, 2016. **62 Suppl 2**: p. S115-20.
11. Araujo, E.C., et al., *Safety, immunogenicity, and protective efficacy of two doses of RIX4414 live attenuated human rotavirus vaccine in healthy infants.* J Pediatr (Rio J), 2007. **83**(3): p. 217-24.
12. Salinas, B., et al., *Evaluation of safety, immunogenicity and efficacy of an attenuated rotavirus vaccine, RIX4414: A randomized, placebo-controlled trial in Latin American infants.* Pediatr Infect Dis J, 2005. **24**(9): p. 807-16.
13. Vesikari, T., et al., *Efficacy of a pentavalent rotavirus vaccine in reducing rotavirus-associated health care utilization across three regions (11 countries).* Int J Infect Dis, 2007. **11 Suppl 2**: p. S29-35.
14. Santos, V.S., E.N. Berezin, and R.Q. Gurgel, *Rotavirus in Latin America: Current Situation and Perspectives.* J Pediatric Infect Dis Soc, 2017. **6**(1): p. 1-2.
15. Soares-Weiser, K., et al., *Vaccines for preventing rotavirus diarrhoea: vaccines in use.* Cochrane Database Syst Rev, 2012. **11**: p. Cd008521.
16. Leshem, E., et al., *Distribution of rotavirus strains and strain-specific effectiveness of the rotavirus vaccine after its introduction: a systematic review and meta-analysis.* The Lancet Infectious Diseases, 2014. **14**(9): p. 847-856.
17. Ustrup, M., et al., *Outstanding challenges for rotavirus vaccine introduction in low-income countries--a systematic review.* Dan Med Bull, 2011. **58**(10): p. A4323.

18. Patel, M., et al., *Oral Rotavirus Vaccines: How Well Will They Work Where They Are Needed Most?* The Journal of infectious diseases, 2009. **200**(0 1): p. S39-S48.
19. Staat, M.A., M.M. McNeal, and D.I. Bernstein, *Rotavirus*, in *Feigin and Cherry's Textbook of Pediatric Infectious Diseases*, J.D. Cherry, et al., Editors. 2014, Elsevier Saunders: Philadelphia, PA.
20. Desselberger, U., *Rotaviruses.* Virus Res, 2014. **190**: p. 75-96.
21. Bernstein, D.I., *Rotavirus overview.* The Pediatric infectious disease journal, 2009. **28**(3): p. S50-S53.
22. Blacklow, N.R. and H.B. Greenberg, *Viral gastroenteritis.* N Engl J Med, 1991. **325**(4): p. 252-64.
23. Staat, M.A., et al., *Clinical presentations of rotavirus infection among hospitalized children.* Pediatr Infect Dis J, 2002. **21**(3): p. 221-7.
24. Rodriguez, W.J., et al., *Clinical features of acute gastroenteritis associated with human reovirus-like agent in infants and young children.* J Pediatr, 1977. **91**(2): p. 188-93.
25. Rodriguez, W.J., et al., *Longitudinal study of rotavirus infection and gastroenteritis in families served by a pediatric medical practice: clinical and epidemiologic observations.* Pediatr Infect Dis J, 1987. **6**(2): p. 170-6.
26. Wenman, W.M., et al., *Rotavirus infection in adults. Results of a prospective family study.* N Engl J Med, 1979. **301**(6): p. 303-6.
27. Hrdy, D.B., *Epidemiology of rotaviral infection in adults.* Rev Infect Dis, 1987. **9**(3): p. 461-9.
28. Parashar, U.D., E.A. Nelson, and G. Kang, *Diagnosis, management, and prevention of rotavirus gastroenteritis in children.* Bmj, 2013. **347**: p. f7204.
29. Raul Velazquez, F., et al., *Cohort study of rotavirus serotype patterns in symptomatic and asymptomatic infections in Mexican children.* Pediatr Infect Dis J, 1993. **12**(1): p. 54-61.
30. Fedorowicz, Z., V.A. Jagannath, and B. Carter, *Antiemetics for reducing vomiting related to acute gastroenteritis in children and adolescents.* Cochrane Database Syst Rev, 2011(9): p. Cd005506.
31. Allen, S.J., et al., *Probiotics for treating acute infectious diarrhoea.* Cochrane Database Syst Rev, 2010(11): p. Cd003048.
32. Lazzerini, M. and L. Ronfani, *Oral zinc for treating diarrhoea in children.* Cochrane Database Syst Rev, 2013(1): p. Cd005436.
33. Gladstone, B.P., et al., *Protective effect of natural rotavirus infection in an Indian birth cohort.* N Engl J Med, 2011. **365**(4): p. 337-46.
34. Velazquez, F.R., et al., *Rotavirus infection in infants as protection against subsequent infections.* N Engl J Med, 1996. **335**(14): p. 1022-8.
35. Desselberger, U. and H.-I. Huppertz, *Immune Responses to Rotavirus Infection and Vaccination and Associated Correlates of Protection.* The Journal of Infectious Diseases, 2011. **203**(2): p. 188-195.
36. Turvey, S.E. and D.H. Broide, *Chapter 2: Innate Immunity.* The Journal of allergy and clinical immunology, 2010. **125**(2 Suppl 2): p. S24-S32.
37. Tissera, M.S., et al., *Options for improving effectiveness of rotavirus vaccines in developing countries.* Hum Vaccin Immunother, 2017. **13**(4): p. 921-927.
38. Patel, M., et al., *A Systematic Review of Anti-Rotavirus Serum IgA Antibody Titer as a Potential Correlate of Rotavirus Vaccine Efficacy.* The Journal of Infectious Diseases, 2013. **208**(2): p. 284-294.

39. Neuzil, K.M., K. Zaman, and J.C. Victor, *A proposed framework for evaluating and comparing efficacy estimates in clinical trials of new rotavirus vaccines.* Vaccine, 2014. **32**: p. A179-A184.
40. Glass , R.I. and U.D. Parashar *The Promise of New Rotavirus Vaccines.* New England Journal of Medicine, 2006. **354**(1): p. 75-77.
41. Bhandari, N., et al., *Efficacy of a monovalent human-bovine (116E) rotavirus vaccine in Indian infants: a randomised, double-blind, placebo-controlled trial.* The Lancet, 2014. **383**(9935): p. 2136-2143.
42. Davidson, K., *India-made rotavirus vaccine achieves World Health Organization prequalification*, in *ROTAVAC® will now be available for procurement by United Nations agencies and Gavi, the Vaccine Alliance, for use in low-resource countries*. 2018, PATH: PATH.
43. World Health Organization. *Rotavirus*. Immunization, Vaccines and Biologicals [Webpage] 2010 12 April 2010 [cited 2017 June 30]; Available from: http://www.who.int/immunization/topics/rotavirus/en/.
44. Fischer Walker, C.L. and R.E. Black, *Rotavirus vaccine and diarrhea mortality: quantifying regional variation in effect size.* BMC Public Health, 2011. **11 Suppl 3**: p. S16.
45. Santos, N. and Y. Hoshino, *Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine.* Rev Med Virol, 2005. **15**(1): p. 29-56.
46. Naylor, C., et al., *Environmental Enteropathy, Oral Vaccine Failure and Growth Faltering in Infants in Bangladesh.* EBioMedicine, 2015. **2**(11): p. 1759-66.
47. Ibrahim, M.K., et al., *Impact of Childhood Malnutrition on Host Defense and Infection.* Clin Microbiol Rev, 2017. **30**(4): p. 919-971.
48. Chandra, R.K., *Nutrition and the immune system: an introduction.* The American journal of clinical nutrition, 1997. **66**(2): p. 460S-463S.
49. Grover, Z. and L.C. Ee, *Protein energy malnutrition.* Pediatr Clin North Am, 2009. **56**(5): p. 1055-68.
50. Bhaskaram, P., *Micronutrient malnutrition, infection, and immunity: an overview.* Nutrition reviews, 2002. **60**(s5).
51. Wintergerst, E.S., S. Maggini, and D.H. Hornig, *Contribution of selected vitamins and trace elements to immune function.* Ann Nutr Metab, 2007. **51**(4): p. 301-23.
52. Savy, M., et al., *Landscape analysis of interactions between nutrition and vaccine responses in children.* J Nutr, 2009. **139**(11): p. 2154s-218s.
53. Perez-Schael, I., et al., *Efficacy of the Human Rotavirus Vaccine RIX4414 in Malnourished Children.* The Journal of Infectious Diseases, 2007. **196**(4): p. 537-540.
54. Ahmed, F., D.B. Jones, and A.A. Jackson, *Effect of undernutrition on the immune response to rotavirus infection in mice.* Ann Nutr Metab, 1990. **34**(1): p. 21-31.
55. Bar-Zeev, N., et al., *Population Impact and Effectiveness of Monovalent Rotavirus Vaccination in Urban Malawian Children 3 Years After Vaccine Introduction: Ecological and Case-Control Analyses.* Clin Infect Dis, 2016. **62 Suppl 2**: p. S213-9.
56. Verkerke, H., et al., *Malnutrition Is Associated with Protection from Rotavirus Diarrhea: Evidence from a Longitudinal Birth Cohort Study in Bangladesh.* Journal of Clinical Microbiology, 2016. **54**(10): p. 2568-2574.

57. Vlasova, A.N., et al., *Prenatally acquired vitamin A deficiency alters innate immune responses to human rotavirus in a gnotobiotic pig model.* Journal of immunology (Baltimore, Md. : 1950), 2013. **190**(9): p. 4742-4753.
58. Chattha, K.S., et al., *Vitamin A deficiency impairs adaptive B and T cell responses to a prototype monovalent attenuated human rotavirus vaccine and virulent human rotavirus challenge in a gnotobiotic piglet model.* PloS one, 2013. **8**(12): p. e82966.
59. Kandasamy, S., et al., *Prenatal vitamin A deficiency impairs adaptive immune responses to pentavalent rotavirus vaccine (RotaTeq®) in a neonatal gnotobiotic pig model.* Vaccine, 2014. **32**(7): p. 816-824.
60. Bucak, I.H., et al., *Is there a relationship between low vitamin D and rotaviral diarrhea?* Pediatr Int, 2016. **58**(4): p. 270-3.
61. Lazarus, R.P., et al., *The effect of probiotics and zinc supplementation on the immune response to oral rotavirus vaccine: A randomized, factorial design, placebo-controlled study among Indian infants.* Vaccine, 2018. **36**(2): p. 273-279.
62. Osredkar, J. and N. Sustar, *Copper and zinc, biological role and significance of copper/zinc imbalance.* J. Clinic. Toxicol. S, 2011. **3**: p. 001.
63. Percival, S.S., *Copper and immunity.* The American journal of clinical nutrition, 1998. **67**(5): p. 1064S-1068S.
64. Lukasewycz, O.A. and J.R. Prohaska, *The immune response in copper deficiency.* Annals of the New York Academy of Sciences, 1990. **587**(1): p. 147-159.
65. Olivares, M. and R. Uauy, *Copper as an essential nutrient.* Am J Clin Nutr, 1996. **63**(5): p. 791s-6s.
66. Harbige, L.S., *Nutrition and immunity with emphasis on infection and autoimmune disease.* Nutr Health, 1996. **10**(4): p. 285-312.
67. Lukasewycz, O., *Copper deficiency suppresses the immune response of mice.* Science, 1981. **213**(4507): p. 559-561.
68. Stabel, J., J. Spears, and T. Brown, *Effect of copper deficiency on tissue, blood characteristics, and immune function of calves challenged with infectious bovine rhinotracheitis virus and Pasteurella hemolytica.* Journal of animal science, 1993. **71**(5): p. 1247-1255.
69. Besold, A.N., E.M. Culbertson, and V.C. Culotta, *The Yin and Yang of copper during infection.* J Biol Inorg Chem, 2016. **21**(2): p. 137-44.
70. Cartwright, G., et al., *The anemia associated with chronic infection.* Science (Washington), 1946. **103**: p. 72-73.
71. Hellman, N.E. and J.D. Gitlin, *Ceruloplasmin metabolism and function.* Annu Rev Nutr, 2002. **22**: p. 439-58.
72. Gitlin, J., *Transcriptional regulation of ceruloplasmin gene expression during inflammation.* Journal of biological chemistry, 1988. **263**(13): p. 6281-6287.
73. Turnlund, J.R., et al., *Long-term high copper intake: effects on indexes of copper status, antioxidant status, and immune function in young men.* Am J Clin Nutr, 2004. **79**(6): p. 1037-44.
74. Lin, X., et al., *Decreased vaccine antibody titers following exposure to multiple metals and metalloids in e-waste-exposed preschool children.* Environ Pollut, 2017. **220**(Pt A): p. 354-363.
75. Kaynar, A.M., et al., *Are plasma mineral levels related to antibody response to influenza vaccination in older adults?* Hum Vaccin Immunother, 2016. **12**(4): p. 1003-8.

76. Palomares, R.A., et al., *Effects of injectable trace minerals on humoral and cell-mediated immune responses to Bovine viral diarrhea virus, Bovine herpes virus 1 and Bovine respiratory syncytial virus following administration of a modified-live virus vaccine in dairy calves.* Vet Immunol Immunopathol, 2016. **178**: p. 88-98.
77. Araujo, A.P., et al., *The influence of copper, selenium and zinc on the response to the Montenegro skin test in subjects vaccinated against American cutaneous leishmaniasis.* Trans R Soc Trop Med Hyg, 2008. **102**(1): p. 64-9.
78. Mocchegiani, E., et al., *Cu to Zn ratio, physical function, disability, and mortality risk in older elderly (ilSIRENTE study).* Age (Dordr), 2012. **34**(3): p. 539-52.
79. Guo, C.H., et al., *Cu/Zn ratios are associated with nutritional status, oxidative stress, inflammation, and immune abnormalities in patients on peritoneal dialysis.* Clin Biochem, 2011. **44**(4): p. 275-80.
80. Kadrabová, J., et al., *Plasma Zinc, copper and copper/zinc ratio in intrinsic asthma.* Journal of Trace Elements in Medicine and Biology, 1996. **10**(1): p. 50-53.
81. Lai, H., et al., *Plasma zinc, copper, copper: zinc ratio, and survival in a cohort of HIV-1-infected homosexual men.* JAIDS Journal of Acquired Immune Deficiency Syndromes, 2001. **27**(1): p. 56-62.
82. Malavolta, M., et al., *Plasma copper/zinc ratio: an inflammatory/nutritional biomarker as predictor of all-cause mortality in elderly population.* Biogerontology, 2010. **11**(3): p. 309-19.
83. Socha, K., et al., *Dietary habits; concentration of copper, zinc, and Cu-to-Zn ratio in serum and ability status of patients with relapsing-remitting multiple sclerosis.* Nutrition, 2017. **39-40**: p. 76-81.
84. Stepien, M., et al., *Pre-diagnostic copper and zinc biomarkers and colorectal cancer risk in the European Prospective Investigation into Cancer and Nutrition cohort.* Carcinogenesis, 2017.
85. Ciftci, T.U., et al., *Changes in serum selenium, copper, zinc levels and Cu/Zn ratio in patients with pulmonary tuberculosis during therapy.* Biological trace element research, 2003. **95**(1): p. 65-71.
86. Saha, A.R., E.M. Hadden, and J.W. Hadden, *Zinc induces thymulin secretion from human thymic epithelial cells in vitro and augments splenocyte and thymocyte responses in vivo.* International Journal of immunopharmacology, 1995. **17**(9): p. 729-733.
87. Bhutta, Z.A., et al., *Prevention of diarrhea and pneumonia by zinc supplementation in children in developing countries: pooled analysis of randomized controlled trials. Zinc Investigators' Collaborative Group.* J Pediatr, 1999. **135**(6): p. 689-97.
88. Lowe, N.M., K. Fekete, and T. Decsi, *Methods of assessment of zinc status in humans: a systematic review.* Am J Clin Nutr, 2009. **89**(6): p. 2040s-2051s.
89. de Baaij, J.H.F., J.G.J. Hoenderop, and R.J.M. Bindels, *Regulation of magnesium balance: lessons learned from human genetic disease.* Clinical Kidney Journal, 2012. **5**(Suppl 1): p. i15-i24.
90. Chang, W.T., B. Radin, and M.T. McCurdy, *Calcium, magnesium, and phosphate abnormalities in the emergency department.* Emerg Med Clin North Am, 2014. **32**(2): p. 349-66.
91. Agus, Z.S., *Hypomagnesemia.* Journal of the American Society of Nephrology, 1999. **10**(7): p. 1616-1622.

92. Tam, M., et al., *Possible roles of magnesium on the immune system.* European journal of clinical nutrition, 2003. **57**(10): p. 1193.
93. Zimowska, W., et al., *Morphological and immune response alterations in the intestinal mucosa of the mouse after short periods on a low-magnesium diet.* British Journal of Nutrition, 2002. **88**(5): p. 515-522.
94. Flynn, A., *Control of in vitro lymphocyte proliferation by copper, magnesium and zinc deficiency.* J Nutr, 1984. **114**(11): p. 2034-42.
95. Witkowski, M., J. Hubert, and A. Mazur, *Methods of assessment of magnesium status in humans: a systematic review.* Magnes Res, 2011. **24**(4): p. 163-80.
96. Sutton, R.A.L. and J.H. Dirks, *Renal Handling of Calcium: Overview*, in *Phosphate Metabolism*, S.G. Massry and E. Ritz, Editors. 1977, Springer US: Boston, MA. p. 15-27.
97. Feske, S., *Calcium signalling in lymphocyte activation and disease.* Nature reviews. Immunology, 2007. **7**(9): p. 690.
98. Cantorna, M.T., et al., *Vitamin D status, 1, 25-dihydroxyvitamin D3, and the immune system.* The American journal of clinical nutrition, 2004. **80**(6): p. 1717S-1720S.
99. Walker, E.M. and S.M. Walker, *Effects of iron overload on the immune system.* Annals of Clinical & Laboratory Science, 2000. **30**(4): p. 354-365.
100. Ekiz, C., et al., *The effect of iron deficiency anemia on the function of the immune system.* The Hematology Journal, 2005. **5**(7): p. 579-583.
101. Punnonen, K., K. Irjala, and A. Rajamäki, *Serum transferrin receptor and its ratio to serum ferritin in the diagnosis of iron deficiency.* Blood, 1997. **89**(3): p. 1052-1057.
102. Romslo, I. and I. Talstad, *Day-to-day variations in serum iron, serum iron binding capacity, serum ferritin and erythrocyte protoporphyrin concentrations in anaemic subjects.* Eur J Haematol, 1988. **40**(1): p. 79-82.
103. Dale, J.C., M.F. Burritt, and A.R. Zinsmeister, *Diurnal variation of serum iron, iron-binding capacity, transferrin saturation, and ferritin levels.* Am J Clin Pathol, 2002. **117**(5): p. 802-8.
104. Cherayil, B.J., *The role of iron in the immune response to bacterial infection.* Immunologic research, 2011. **50**(1): p. 1-9.
105. Weiss, G. and L.T. Goodnough, *Anemia of chronic disease.* New England Journal of Medicine, 2005. **352**(10): p. 1011-1023.
106. Oppenheimer, S.J., *Iron and Its Relation to Immunity and Infectious Disease.* The Journal of Nutrition, 2001. **131**(2): p. 616S-635S.
107. Bo, S., et al., *Associations of dietary and serum copper with inflammation, oxidative stress, and metabolic variables in adults.* The Journal of nutrition, 2008. **138**(2): p. 305-310.
108. Rodríguez-Morán, M. and F. Guerrero-Romero, *Serum magnesium and C-reactive protein levels.* Archives of disease in childhood, 2008. **93**(8): p. 676-680.
109. Locke, A., E. Main, and D. Rosbash, *The copper and non-hemoglobinous iron contents of the blood serum in disease.* Journal of Clinical Investigation, 1932. **11**(3): p. 527.
110. Heinrich, P.C., J.V. Castell, and T. Andus, *Interleukin-6 and the acute phase response.* Biochemical Journal, 1990. **265**(3): p. 621-636.
111. Wander, K., E. Brindle, and K.A. O'Connor, *Sensitivity and specificity of C-reactive protein and α(1)-acid glycoprotein for episodes of acute infection among children in Kilimanjaro, Tanzania.* American Journal of Human Biology, 2012. **24**(4): p. 565-568.

112. Thurnham, D.I., C.A. Northrop-Clewes, and J. Knowles, *The use of adjustment factors to address the impact of inflammation on vitamin A and iron status in humans.* J Nutr, 2015. **145**(5): p. 1137s-1143s.
113. Thurnham, D.I., et al., *Adjusting plasma ferritin concentrations to remove the effects of subclinical inflammation in the assessment of iron deficiency: a meta-analysis.* Am J Clin Nutr, 2010. **92**(3): p. 546-55.
114. Namaste, S.M.L., et al., *Adjusting ferritin concentrations for inflammation: Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project.* The American Journal of Clinical Nutrition, 2017. **106**(Suppl 1): p. 359S-371S.
115. Patel, M.M., et al., *Effectiveness of monovalent rotavirus vaccine in Bolivia: case-control study.* Bmj, 2013. **346**: p. f3726.
116. UNICEF. *Statistics*. Bolivia, Plurinational State of 2013 December 18 2013 [cited 2017 July 18]; Available from: https://www.unicef.org/infobycountry/bolivia_statistics.html.
117. UNICEF, *The State of the World's Children 2016 Statistical Tables*. 2016, UNICEF: UNICEF.org.
118. UNICEF, W.a., *WHO and UNICEF estimates of national immunization coverage*. 2017, World Health Organization: World Health Organization.
119. Crabtree, J., *Bolivia Processes of Change*. Bolivia, ed. A. Chaplin. 2013, London: London : Zed Books.
120. Inchauste, L., et al., *Impact of rotavirus vaccination on child mortality, morbidity, and rotavirus-related hospitalizations in Bolivia.* International Journal of Infectious Diseases, 2017. **61**: p. 79-88.
121. Reynoso, H.P.-L.M.T., et al., *Mejorando la Nutrición del Niño Pequeño en El Alto, Bolivia: Resultados Utilizando la Metodología de ProPAN.*
122. González-Tarancón, R., et al., *Serum copper concentrations in hospitalized newborns.* Journal of Trace Elements in Medicine and Biology, 2017. **39**: p. 1-5.
123. Vulpe, C., et al., *Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper–transporting ATPase.* Nature genetics, 1993. **3**(1): p. 7-13.
124. Rukgauer, M., J. Klein, and J.D. Kruse-Jarres, *Reference values for the trace elements copper, manganese, selenium, and zinc in the serum/plasma of children, adolescents, and adults.* J Trace Elem Med Biol, 1997. **11**(2): p. 92-8.
125. Versieck, J. and R. Cornelis, *Normal levels of trace elements in human blood plasma or serum.* Analytica chimica acta, 1980. **116**(2): p. 217-254.
126. Versieck, J., *Trace elements in human body fluids and tissues.* Crit Rev Clin Lab Sci, 1985. **22**(2): p. 97-184.
127. Burke, R.M., et al., *Early deterioration of iron status among a cohort of Bolivian infants.* Maternal & child nutrition, 2017. **13**(4).
128. Aceituno, A.M., et al., *Using a monitoring and evaluation framework to improve study efficiency and quality during a prospective cohort study in infants receiving rotavirus vaccination in El Alto, Bolivia: the Infant Nutrition, Inflammation, and Diarrheal Illness (NIDI) study.* BMC Public Health, 2017. **17**: p. 911.
129. Cvijanovich, N.Z., et al., *Zinc homeostasis in pediatric critical illness.* Pediatr Crit Care Med, 2009. **10**(1): p. 29-34.
130. Moon, S.S., et al., *Inhibitory effect of breast milk on infectivity of live oral rotavirus vaccines.* Pediatr Infect Dis J, 2010. **29**(10): p. 919-23.

131. Beshgetoor, D. and M. Hambidge, *Clinical conditions altering copper metabolism in humans.* The American Journal of Clinical Nutrition, 1998. **67**(5): p. 1017S-1021S.
132. McSeveny, A.C., Rob; Wilkes, Steve; Smith, Michael, ed. *nternational Mathematics for the Middle Years 5*. ed. F. Brodribb. Vol. 2. 2011, Pearson.
133. Arcara, K.M. and M.M. Tschudy, *The Harriet Lane handbook: a manual for pediatric house officers*. 2012: Elsevier/Mosby.
134. Wu, A.H., *Tietz Clinical Guide to Laboratory Tests-E-Book*. 2006: Elsevier Health Sciences.
135. Mayo Medical Laboratories. *Copper, Serum*. [cited 2018 March 23]; Available from: https://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/8612.
136. Soldin, O.P., et al., *Serum iron, ferritin, transferrin, total iron binding capacity, hs-CRP, LDL cholesterol and magnesium in children; new reference intervals using the Dade Dimension Clinical Chemistry System.* Clinica chimica acta, 2004. **342**(1-2): p. 211-217.
137. Lin, C.N., et al., *Pediatric reference intervals for serum copper and zinc.* Clin Chim Acta, 2012. **413**(5-6): p. 612-5.
138. Becker-Dreps, S., et al., *The association between fecal biomarkers of environmental enteropathy and rotavirus vaccine response in Nicaraguan infants.* The Pediatric infectious disease journal, 2017. **36**(4): p. 412-416.
139. Guerrant, R.L., et al., *Biomarkers of environmental enteropathy, inflammation, stunting, and impaired growth in children in northeast Brazil.* PloS one, 2016. **11**(9): p. e0158772.
140. Harvey, L.J., et al., *Methods of assessment of copper status in humans: a systematic review.* Am J Clin Nutr, 2009. **89**(6): p. 2009s-2024s.
141. Velazquez, F.R., et al., *Serum antibody as a marker of protection against natural rotavirus infection and disease.* J Infect Dis, 2000. **182**(6): p. 1602-9.