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Laura Calderwood Date

**ROTAVIRUS VACCINE PERFORMANCE:**
**TIMING OF DOSES AND SEROCONVERSION IN EL ALTO, BOLIVIA**

By

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Master of Public Health

Global Epidemiology

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## ABSTRACT

### ROTAVIRUS VACCINE PERFORMANCE:
### TIMING OF DOSES AND SEROCONVERSION IN EL ALTO, BOLIVIA

By Laura Calderwood

Rotavirus is a leading cause of diarrheal illness among children globally. Vaccination is the best line of protection against rotavirus infection; however, vaccine effectiveness is lower in low-income settings. One hypothesis states that higher levels of circulating rotavirus in these settings may increase maternal antibodies, which interfere with infants' immune response to the vaccine. Supporting this hypothesis, randomized controlled trials have found that vaccine protection may be improved through a delayed vaccine schedule, but results are inconsistent. There is a need to better understand the influence of timing on vaccine effectiveness in order to provide optimal protection against severe diarrheal illness in children. This analysis seeks to determine whether the timing (with respect to infant age) of each dose of RV1 affects the serological response in a cohort of vaccinated infants. This analysis uses data from a population of 309 infants who received two doses of the monovalent rotavirus vaccine, Rotarix (RV1), in the Infant Nutrition, Immunology, and Diarrhea study conducted in El Alto, Bolivia in 2013 – 15. Geometric mean titers (GMTs) for rotavirus-specific IgA were obtained from infant blood samples collected prior to the first dose of RV1 and ~2 months following the second dose. Seroconversion was defined as a 4-fold increase in GMT between blood draws. The effect of the timing of RV1 administration on immunogenic response was assessed using log-binomial models and Spearman's Rank correlations. There were no statistically significant associations between any of the timing exposures (infant age at Dose 1, infant age at Dose 2, and the length of interval between doses) and the immunological outcomes (seroconversion and the fold-change in GMT). However, as a general trend those infants with a longer interval and later second dose were less likely to seroconvert (Risk ratios and 95% Confidence Intervals for a one week increase in each timing variable were 0.96 [0.91, 1.00] for age at Dose 2 and 0.96 [0.92, 1.00] for the length of interval). These findings uphold the current guidelines for vaccine scheduling in Bolivia and do not indicate improved vaccine effectiveness through delayed vaccination.

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# REVIEW OF THE LITERATURE

Diarrhea, resulting in dehydration and malnutrition, is a leading cause of death, after neonatal causes and pneumonia, in children under 5 years of age (1, 2). Rotavirus infection is among the most common causes of diarrheal illness in developing countries (1). The GBD Diarrhoeal Diseases Collaborators estimated that in 2015, rotavirus accounted for approximately 146,480 mortalities in children <5, or 29.3% of all diarrhea-related deaths in this age group (3). Though high, these numbers represent an estimated 44% decline in child deaths due to rotavirus since 2005, before the use of rotavirus vaccines became widespread (3).

Unlike many other enteric diseases, rotavirus is not highly affected by interventions based on water, sanitation, and hygiene (WASH) (reviewed in (4)). In fact, the disease has been dubbed a "democratic virus" because it is ubiquitous in both rich and poor countries (5). Studies conducted in various regions have concluded that every child in the world will come into contact with rotavirus by their 5$^{th}$ birthday (reviewed in (5)). However, despite the fact that morbidity from rotavirus is common worldwide, low-income countries, where access to supportive treatments for diarrhea is limited, suffer higher mortality rates due to rotavirus (reviewed in (6)).

## Rotavirus Vaccines

Because of the challenges in controlling the rotavirus pathogen with WASH interventions, vaccination is considered the best defense against the disease (7). Three live, oral, attenuated rotavirus vaccines have been prequalified by the World Health Organization (WHO) and are currently available for use in national

immunization programs worldwide: Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium), RotaTeq (Merck & Co. Inc., West Point, PA, USA), and Rotavac (Bharat Biotech, Hyderabad, India). The monovalent human rotavirus vaccine, Rotarix (RV1), is the most widely used rotavirus vaccine (8), and was WHO prequalified in 2009 (9). RV1 is administered in two doses, 4 weeks apart. The manufacturer recommends infants receive both doses between 6 and 24 weeks of age (10). Pentavalent, human-bovine reassortant rotavirus vaccine, RotaTeq (RV5; prequalified in 2008 (9)), requires 3 doses, to be started at 6 – 12 weeks of age, and administered at intervals of 4 – 10 weeks, according to the manufacturer (11). The newest vaccine, Rotavac, has been used in India since 2016 and received WHO prequalification in January 2018 (12). Rotavac is a monovalent, liquid-frozen vaccine that comes in three recommended doses, four weeks apart, starting at 6 weeks of age (13).

The World Health Organization (WHO) recommends that any of the above described rotavirus vaccines be started as close as possible to 6 weeks of age, and subsequent doses should come no less than four weeks apart. However, the WHO warns that strict scheduling may exclude infants who would benefit from vaccination, and suggests the RV vaccine be administered alongside the diphtheria-tetanus-pertussis vaccine series (DTP1, DTP2, and if three doses are needed, DTP3), even if these fall after the upper limit of the manufacturer-recommended age window (7).

**Vaccine Safety**

One issue that has historically garnered some concern over rotavirus vaccines is their association with intussusception, a telescoping of the intestine that causes a blockage and severe pain (7). The baseline incidence of intussusception is low, and with prompt treatment there are no lasting effects; however, without access to proper medical care, the complication can be fatal (7). Following its 1998 approval, the first rotavirus vaccine, RotaShield, was found to be associated with an increased risk of intussusception, leading to RotaShield's discontinuation in 1999 (14). Although the increased risk of intussusception after vaccination with Rotarix and RotaTeq has been too small to detect in large in clinical trials (reviewed in (15)), some countries have seen increases in intussusception rates following the addition of the vaccines to the national immunization schedule, with case-control studies showing a period of increased risk during the week following vaccination (16). Non-vaccine-related intussusception is most common in young children aged 3-8 months (17), which has generated some concern that delaying vaccination later in infancy could exacerbate this risk.

Despite these concerns, the benefits of vaccination, even delayed vaccination, far outweigh the risk of intussusception (7). In a 2011 study published in the New England Journal of Medicine, Patel and colleagues found an annual excess risk of intussusception of approximately 1 case per 51,000 infants in Mexico and just 1 case per 68,000 infants in Brazil (or 96 excess cases in total) (18). The same study estimated that 80,000 hospitalizations and 1,300 deaths were prevented each year by RV1. Reviews of studies in other regions have similarly concluded that the

benefits of rotavirus vaccination dwarf the increased threat of intussusception (19, 20).

**Global Implementation**

The increasing number of countries with national rotavirus immunization programs is a promising development in the fight against childhood diarrhea. As of May, 2016, over 81 countries worldwide had incorporated rotavirus vaccination in their national schedules in accordance with WHO recommendations (21). Studies have found the introduction of the vaccine has a profound public health impact. Rates of rotavirus-related hospitalizations have decreased anywhere between 20 and 91%, depending on the country, with rotavirus mortality rates decreasing in the range of 22-88% after the addition of the vaccine to the national schedule, according to a 2017 review (22).

Much of the increase in rotavirus vaccine use in the developing world has been achieved thanks to Gavi, the Global Alliance for Vaccines and Immunizations, an international organization that promotes health equality by working with both public and private sectors to increase vaccine access in low-income countries (23). So far, the organization has supported the incorporation of rotavirus vaccines into the national immunization schedules of 38 low-income countries eligible for Gavi support (21). According to a Gavi report, over 50% of the 36 million courses of rotavirus vaccine used globally in 2015 were administered in Gavi-supported countries (8). The demand for the vaccine in low-income countries is expected to grow to around 80% of the projected 107 million courses to be administered globally in 2025 (8).

**Vaccine Efficacy**

While the global burden of rotavirus has substantially decreased over the past decade with the widespread use of rotavirus vaccine (reviewed in (22, 24)), the vaccine's efficacy varies greatly by world region (reviewed in (25, 26)). Estimates range from over 90% vaccine efficacy in high-income regions to <50% in Southern Asia and sub-Saharan Africa (25). In general, vaccination against rotavirus has been shown to be less effective in low-income countries compared to high-income settings (26).

A number of hypotheses for these differences in vaccine efficacy have been put forth, including genetic variations in either the host (27) or the virus (28), nutritional deficiencies in the host ((29), reviewed in (30)), imbalances in the gut microbiota of the host (31), and maternal factors in the host ((32, 33), reviewed in (34)). One hypothesis is that maternal antibodies, transferred to the infant through the placenta or during breastfeeding, interfere with infant immune response to the vaccine (32-38). Because the presence of maternal antibodies wanes with infant age, this hypothesis has raised the question of whether delayed vaccination has the potential to increase the vaccine's impact in places where vaccine efficacy is low.

**Studies of RV1 Dosing Schedules**

Randomized controlled trials (RCTs) investigating the relationship between alternative vaccine schedules and the efficacy of the rotavirus vaccine in Africa and Asia have provided some support for the hypothesis that delayed vaccination improves vaccine performance. While most countries follow a 6 and 10 week schedule in conjunction with other routine vaccines, trends show better rotavirus

protection from a later dosing schedule, where the two doses are given at around 10 and 14 weeks of age (39-42). All RCTs on the subject to date have focused on the RV1 vaccine. In 2015, RV1 made up 78% of the rotavirus vaccine market globally, and 92% in GAVI-supported countries (8), so the focus on RV1 by RCTs in low-income countries is justified.

In the studies detailed below, the outcomes used to demonstrate vaccine efficacy are rotavirus-specific IgA geometric mean titers (GMTs) or concentrations (GMCs) and seroconversion to RV1. To determine seroconversion status, blood draws taken before and after vaccination are assessed for rotavirus-specific IgA concentrations (measured in standardized international units (43, 44) and reported in units per milliliter). As defined in the studies below, this concentration must be below the threshold of 20 units/mL before vaccination, and above 20 units/mL after vaccination for an infant to be considered "seroconverted."

*Steele et al., 2010*

In 2010, Steele and colleagues published one of the first RCTs to explore RV1 vaccine efficacy with a varied dosing schedule. Healthy South African infants were randomized to receive two doses of RV1 or a placebo, coadministered with polio vaccines (either IPV or OPV) at either 6 and 10 (6/10) or 10 and 14 (10/14) weeks. All infants were seronegative at the start of the study, although there was some evidence of wild rotavirus in the population during the study. After 2 doses of RV1, the researchers found that among infants in the 6/10 group, only ~39% seroconverted, while among those in the 10/14 group, ~58% seroconverted (39). GMC of anti-rotavirus IgA was 30.3 units/mL after 2 doses for the 6/10 group, and

52.6 units/mL for the 10/14 group. Some (<5%) of the placebo group did seroconvert after the second dose, suggesting this difference may be partially attenuated, though not necessarily nullified, by the presence of wild type rotavirus in the study population (39).

*Anh et al., 2011*

In a 2011 study by Anh et al., Vietnamese and Filipino infants aged 5 – 10 weeks were given three doses of vaccine or placebo (either 2 RV1 and 1 placebo, or 3 placebo) spaced one month apart. Infants in Vietnam received RV1 doses at an average age of 9 and 13 weeks (9/13) or 9 and 17 weeks (9/17). In the Philippines, one group received the vaccine at an average age of 7 and 15 weeks (7/15), and the other at 11 and 15 weeks (11/15). Serum samples were taken pre-vaccination and one month after the second dose, and tested for anti-rotavirus IgA. In the 9/13 group, 63% of infants seroconverted, compared to 59% in the 9/15 group, 82% in the 9/17 group, and 70% in the 11/15 group. Rotavirus-specific IgA GMTs in the 9/13 and 9/17 groups from Vietnam were 77.4 units/mL and 176.3 units/mL respectively. In the 9/15 and 11/15 groups from the Philippines, rotavirus-specific IgA GMTs were 68 and 75.6 units/mL respectively. Although the study was not designed to evaluate statistical differences by schedule, older infants had stronger immunological responses to the vaccine as an overall trend (40).

*Ali et al., 2014*

A study of Pakistani infants by Ali et al. in 2014 compared rotavirus-specific IgA GMTs and rates of seroconversion to RV1 in infants who received two doses of RV1

at 6 and 10 weeks of age (6/10) or 10 and 14 weeks (10/14), or three doses at 6, 10, and 14 weeks (6/10/14). Serological vaccine response to RV1 at 18 weeks was low across all study groups, with anti-rotavirus IgA GMTs of just 24.0 in the 6/10 group, 24.4 in the 10/14 group, and 25.8 units/mL in the 6/10/14 group. Seroconversion rates were 36.1%, 38.5%, and 36.7%, respectively, with no statistical difference across groups. In a comparison group of unvaccinated infants from the source population, background seroconversion rates were 13.3% at 18 weeks of age (41).

*Armah et al., 2016*

A 2016 RCT by Armah et al. used the same RV1 dosing schedule combinations as the previous study by Ali (6/10, 10/14, and 6/10/14), but found a modest improvement in vaccine efficacy with a third dose in a population of infants in rural Ghana. Infants who received Rotarix on the earlier 6/10 schedule had a seroconversion rate of 29%, while infants on the delayed 10/14 schedule showed 37% seroconversion. Of those who received a third dose, 43% seroconverted. Only the 6/10/14 versus 6/10 groups were statistically significantly different; however, these data lend some support for the delayed schedule, as IgA seroconversion rates against RV1 were numerically (though not significantly) higher for the 10/14 schedule, compared to the 6/10 schedule (42).

*Overview*

A 2017 systematic review by Gruber and colleagues confirms that the general pattern of RCT data suggests the 6/10 week schedule is not optimal (45). Across the trials conducted to date, risk differences indicating the protective effect of the later

schedule ranged from 9 to 25% (45). In general, however, evidence of the benefits of a delayed schedule is not strong, with few trials claiming statistically significant results. Thus, continued assessment of vaccine timing is warranted.

The definition of seroconversion used in these studies has some limitations as a correlate of protection. The cutoff point of 20 units/mL was established because it is well above the minimum detection limit of 4 units/mL (46), and it was previously used to determine a history of natural infection (47). However, it often takes multiple rotavirus infections in an individual to confer complete immunity (48), so while the cutoff of 20 units/mL for rotavirus-specific IgA GMTs may demonstrate previous infection, it is not indicative of full protection on the individual level. Studies have found a strong correlation between anti-rotavirus IgA concentration and clinical correlates of protection but place the threshold of strong protection much higher than 20 units/mL (reviewed in (49, 50)). Of note, not all rotavirus vaccine studies have used the same definition of seroconversion. The current study will use an alternative definition, common elsewhere in the rotavirus vaccination literature (e.g. (33)), that bases seroconversion status on a ≥4-fold increase in IgA concentrations, with the cutoff for seropositivity at a serum titer of 40 units/mL.

If there is truly a modest gain in vaccine efficacy from a delayed dosing schedule, there are additional considerations for weighing the risks and benefits changing RV1 schedule recommendations. For example, a recent study found that the burden of rotavirus gastroenteritis is shifted earlier in life in low-income versus high-income countries, and in Africa, incidence peaks at around 4-7 months of age, with some cases developing as early as the first month of life (51). This suggests that

many children are at high risk of natural infection before they receive their vaccinations. In other words, it is not clear that a 9-25% gain in vaccine efficacy would avert enough extra cases to make up for the extended unprotected time period. Therefore, it is important to establish a better understanding of the protective benefits of a delayed schedule before making any universal recommendations.

**Rotavirus Vaccination in Bolivia**

In Latin America and the Caribbean, rotavirus vaccines have been introduced in 18 countries, 5 of which are Gavi-eligible (21). Vaccine effectiveness estimates in Latin American countries have fallen in the range of 64-72% (52), although results have been heterogeneous among countries (53).

Bolivia ranks among the lowest on health and development measures in Latin America, with a mortality rate among children under 5 years of age of 36 per 1,000 live births and an estimated 45% of the population living below the international poverty line of $2/day (54). However, as the first Gavi-supported country to introduce rotavirus vaccination to its National Immunization Program, Bolivia is a success story (55). In the post-introduction period, coverage rose from 16% in 2008 to 86% in 2014, with a 41% reduction in rotavirus related hospitalizations in the same period (56). As of 2015, Bolivia has assumed full financial responsibility for the rotavirus vaccine, and continues to offer RV1 to the public, free-of-charge (55, 57).

Estimates of vaccine effectiveness from case-control studies in Bolivia fall in the range of 69-80% protection against severe diarrhea (58, 59). To the author's

knowledge, no RCT has evaluated the role of timing in RV1 efficacy in Latin America. A recent case-control analysis by Burke and colleagues found that vaccine effectiveness tended to be higher among infants who received the first dose early in a population of Bolivian infants (60). Though not conclusive, this result contradicts the overall pattern of delayed vaccination improving RV1 efficacy, and underscores the importance of using real-world data and examining the relationship in multiple populations.

There is a need to better understand the influence of timing on vaccine effectiveness in order to provide optimal protection against severe diarrheal illness in children. The goal of this analysis is to determine whether the timing of vaccine administration for each of two RV1 doses affects the serological response of vaccinated infants living in El Alto, Bolivia. The results of this study will add to the body of knowledge on RV1 scheduling and vaccine response, and may lend support to the current recommended RV1 schedule in Bolivia or provide insights into a potential avenue for improving vaccine effectiveness in the source population.

# METHODS

## Study Background

The data for this analysis comes from the Infant Nutrition, Immunology, and Diarrhea (or, Nutrición, Inmunología y Diarrea Infantil [NIDI]) study conducted in El Alto, Bolivia from June 2013 through March 2015 (61, 62). The primary aim of the NIDI study was to assess the role of chronic undernutrition in the immunogenic response to RV1 in infants.

### *Population/setting*

Bolivia was selected as the study setting in part for its high prevalence of undernutrition and high incidence of diarrheal disease (63). Bolivia also has an established rotavirus vaccine program and has offered RV1 vaccines free-of-charge since 2008 (55). Two hospitals, Los Andes and Corea, were selected for recruitment in the mostly indigenous city of El Alto, named for its high elevation of over 4,000m (64). Additionally, a partner laboratory at the University Mayor de San Andres (UMSA) was available in the neighboring city of La Paz.

### *Data Collection*

Detailed descriptions of participant recruitment and data collection have been published previously (61, 62). Briefly, 461 infants and their mothers were recruited during well-child or vaccination visits, prior to the first dose of RV1. Infants were excluded from enrolment if they had acute illness, known HIV exposure, or congenital malformations. Infant-mother pairs were followed for a target of at least 12 months, with 7 scheduled hospital-based visits throughout the study period, and

a home visit 4-7 days after the first dose of RV1 (**Figure 1**). Infant blood samples were collected at Visit 2 (immediately prior to the first dose of RV1; around 2 months of age) and again at Visit 6 (approximately two months after the second dose of RV1; around 6 months of age). Infant vaccination cards were presented by mothers at each study visit, and data were recorded by study staff. Morbidity and clinical data were recorded at each visit, and sociodemographic data were collected at Visits 1 and 8. At the time of data collection, all information was recorded on paper records. Data were later computerized through double-data entry in REDcap. Biologically implausible points and discrepancies were rechecked against the paper records, and corrected or set to missing. Laboratory data were double-entered and reconciled in Microsoft Excel.

*Blood collection and processing*

Trained hospital phlebotomists drew blood using sterile and disposable Safety-Lok™ 23-gauge winged needles and trace-metal-free EDTA Vacutainers® (BD, Franklin Lakes, NJ). Study staff isolated blood plasma per Vacutainer® instructions. The supernatant was stored in microtubes at 2-8°C for up to 24 hours in the hospital, transferred on ice to the UMSA laboratory where samples were stored at −70 °C, and then transported on dry ice (following International Air Transport Association and CDC regulations) to Emory University. Plasma samples aliquoted for rotavirus serology were transferred on dry ice to the Gastroenteritis & Respiratory Viruses Laboratory, in the Division of Viral Diseases, at CDC. Plasma was analyzed for RV-specific Immunoglobulins A (IgA) and G (IgG) by enzyme-linked immunosorbent assay (ELISA) as described in Moon et al., 2010 (38).

*Ethics*

The NIDI study was conducted with approval by the Emory University IRB (IRB00056127) and the Bolivian Research Ethics Committee. Mothers provided informed consent during study enrollment on behalf of themselves and their infants.

## Analysis

Infants were included in the analysis if they had successful blood draws at both Visits 2 and 6, and recorded vaccination dates for Doses 1 and 2. Two infants were excluded for invalid vaccination dates (age at Dose 2 greater than 365 days).

*Primary Analysis: RV1 timing vs. Seroconversion*

Exposure Definitions

Age in days at each dose was calculated by subtracting infant's date of birth from the date of vaccination. The interval between doses was calculated by subtracting the date of Dose 1 receipt from the date of Dose 2. All three vaccination timing variables were treated as continuous in the models.

Outcome Definitions

Seroconversion was previously defined in the NIDI database. Rotavirus-specific IgA levels were converted to GMTs by taking the antilog of the base 10 log of each infant's measurement for Visits 2 and 6 (to convert all zeros to ones in the serological data). The fold-change was then calculated by dividing GMT at Visit 6 by GMT at Visit 2. Seroconversion was defined as a 4-fold change in anti-rotavirus IgA GMT from Visit 2 to Visit 6, with a minimum final GMT of 40 U/mL. Infants with less

than a 4-fold change, or with an anti-rotavirus IgA GMT less than 40 U/mL were therefore considered "not seroconverted".

Modeling Strategy

Potential covariates were selected based on a directed acyclic graph (DAG) and included variables representative of socioeconomic status (maternal education, hospital, and indigenous ethnic identity), recent infant morbidities (diarrhea preceding Visits 1 and 2, fever preceding Visit 2), and general infant health (preterm birth, stunting). Maternal education was defined in three categories: primary or less, at least some secondary, and at least some university. All other covariates were dichotomous. Comorbidities were based on a maternal recall of two weeks for diarrhea and 48 hours for fever. Stunting was defined as a length for age Z-score below -2 based on WHO reference charts (65) at any visit during the study period, and low birth weight as a weight of less than 2500g at birth. Preterm birth was defined as delivery before 37 weeks of estimated gestational age.

The effect of vaccination timing on seroconversion was assessed using log-risk regression. Confounding was assessed though a change-in-estimate evaluation. The final model produced estimates with no detectable change from the full model, and included maternal education, infant diarrhea (two week recall at Visit 2), preterm birth, and hospital of recruitment (see DAG, **Figure 2**). Five infants missing data for preterm birth were assigned the mean value of the cohort. All models were assessed for collinearity using the Collinearity Diagnostic Macro from SAS-L by Matthew Zack (66). Models were additionally run with a subset of data, in which those with IgA

GMT at Visit2 >40 or fold-change in GMT <1 (indicators of high levels of rotavirus exposure at the time of Dose 1) were excluded, and estimates were compared to those for the larger cohort.

*Secondary Analysis: RV1 timing vs. Geometric Mean Titers*

Assessment of Differences in GMT

To assess whether infants with earlier vaccinations had higher final anti-rotavirus IgA levels compared to those who received RV1 later, infants were divided into two age groups based on the median age at vaccination in the cohort for each dose. The same groups were formed whether the Dose 1 median age or Dose 2 median age was used (i.e., infants were either below or above the median age for both doses) so these age groups are representative of the two doses together.) Geometric means were calculated for rotavirus-specific IgA titers for both vaccination age groups for each blood draw. Differences between groups at Visits 2 and 6 were assessed using Wilcoxon-Mann-Whitney tests.

Assessment of Fold-Change in GMT

The fold-change in anti-rotavirus IgA GMT from Visit 2 to Visit 6 (fold-change) was examined as a continuous variable as an indication of the strength of immune response. An adjusted linear regression of the fold-change versus each timing variable was not appropriate due to the skewness of the fold-change data, even after log transformation. Therefore, scatter-plots of the fold-change versus age at Dose 1, age and Dose 2, and the interval were used to visually assess the relationships, and

non-parametric Spearman's Rank Tests were used to statistically assess correlations.

Data cleaning and analysis were conducted in SAS 9.4. Figures and tables were created in Microsoft Excel.

## RESULTS

Of the 461 infants enrolled in the NIDI study, 67 were lost-to-follow-up and 27 were excluded (3 were of inadequate age, 22 received RV1 before the first blood draw, and 1 did not receive the second dose of RV1) by Visit 6. Of the 367 infants who completed Visit 6, 325 had two successful blood draws (with sufficient blood for laboratory analysis). An additional 16 infants were missing dates for at least one dose of RV1. A total of 309 infants were included in the analysis. A subset of 285 infants had GMTs of ≤40 at Visit 2, and fold-change in GMT of ≥1 from Visit 2 to Visit 6.

Information on population characteristics is provided in **Table 1**. A little under half of the infants in the cohort were female. The majority of study mothers self-identified as belonging to an indigenous ethnic group, and maternal education was low overall, with less than a quarter of mothers advancing beyond secondary school. Infant enrollment was almost evenly split between Corea (N= 145) and Los Andes (N= 164) Hospitals. Fifty-five infants were born preterm and ninety-three experienced stunting by Visit 6. At the time of Visit 2, 39 infants had a reported history of diarrhea in the previous two weeks. The average ages at Dose 1 and 2 of RV1 were 64.3 days and 132.5 days, respectively. Infants received the two doses 68.2 days apart on average. By Visit 6, 64% of infants showed evidence of seroconversion. Details of population characteristics by seroconversion status and early versus late vaccination groups are shown in **Supplementary Table 1.**

The distribution of RV1 timing was strongly right skewed for all three timing variables (**Figure 3)**. Age at Dose 1 had a range of 58 – 105 days, and an

interquartile range (IQR) of 61 – 65 days (Panel A). Age at Dose 2 had a range of 116 – 241 days and an IQR of 123 – 136 days (Panel B). The interval between doses had a range of 38 – 179 days, and an IQR of 61-70 days (Panel C). **Visually,** boxplots show a slight shift in the distribution of those who did not seroconvert towards later vaccination, compared to those who did seroconvert, across all timing variables.

**Age at Dose 1 vs Seroconversion**

There was no significant relationship between the timing of the first dose of RV1 and seroconversion in either crude or adjusted analyses (**Table 2**).

**Age at Dose 2 vs seroconversion**

Older age at the second dose of RV1 was associated with a decreased likelihood of seroconversion in the crude analysis (RR (95%CI)=0.95 (0.91, 1.00), $p$=0.03; Table 2). After adjustment for maternal education, preterm birth, hospital of recruitment, and recent diarrhea, infants were 4% less likely to seroconvert for each one week increase in age at Dose 2 (RR=0.96 [95% CI: 0.91, 1.00]). However, the adjusted estimate was not statistically significant at the 0.05 alpha level ($p$=0.06).

**Interval**

A longer interval between doses of RV1 also tended towards a lower likelihood of seroconversion in both crude and adjusted analyses (Table 2). After adjustment for maternal education, preterm birth, hospital of recruitment, and recent diarrhea, infants were 4% less likely to seroconvert for each one week increase in the time between doses (RR=0.96 [95% CI: 0.92, 1.00]). Neither crude nor adjusted estimates were statistically significant at the 0.05 alpha level ($p$=0.07 for both).

No changes to the results of the log-risk models were observed in a subset of 285 infants after excluding those with evidence of high rotavirus exposure at Visit 2

**GMT analysis**

To evaluate whether the same trend would be observed using a continuous serological outcome, additional analyses were conducted using geometric mean titers of anti-rotavirus IgA (GMTs) measured at baseline (Visit 2) and after two doses of RV1 (Visit 6), as well as the fold-change in GMTs between the two visits.

*Assessment of Differences in GMT*

Infants were divided into two age groups based on the median age at vaccination in the cohort for each dose (63 days for Dose 1 and 126 days for Dose 2). Because all infants fell either below the median age for both doses, or above the median age for both doses, these age groups are representative of age differences in the 2-dose RV1 schedule as a whole. At Visit 2, geometric mean anti-rotavirus IgA titers were 2.02 among infants who received rotavirus vaccinations at or before the median age, and 2.27 among those who received RV1 after the median age. Rotavirus-specific IgA GMTs at Visit 6 were 102.75 and 79.19 for those who received RV1 before and after the median age, respectively. While GMTs were similar between age groups at baseline, those who received the vaccine later had numerically lower GMTs on average after two doses of RV1, compared to those who received the vaccine earlier. However, the difference between age groups was not statistically significant at either time point**.**

*Assessment of Fold-Change in GMT*

To assess whether a linear or log linear relationship exists between age at either dose or the interval between doses and the change in GMT from Visit 2 to Visit 6, the log of the fold-change in anti-rotavirus IgA GMT was plotted against each of the vaccine timing exposures, as shown in **Figure 4.** Exponential trendlines suggest that the log of the fold-change in anti-rotavirus IgA GMT may be negatively related to age at Dose 1 (panel A), age at Dose 2 (panel B), and the interval between doses (panel C). However, Spearman's Rank Tests assessing the correlation between fold-change in anti-rotavirus IgA GMT between visits with age at Dose 1, age at Dose 2, and the length of interval between doses found no significant relationship (**Table 4**).

## DISCUSSION

This analysis sought to determine whether RV1 timing, with respect to infant age, was associated with infant serological response to RV1 in a cohort of infants from El Alto, Bolivia. We examined RV1 timing through three exposure variables: age at Dose 1 of RV1, age at Dose 2 of RV1, and the length of the interval between the two doses. The primary outcome of interest was seroconversion, defined as a 4-fold change in anti-rotavirus IgA geometric mean titers from Visit 2 to Visit 6. Overall, this study did not find strong statistical evidence that age at RV1 vaccination is associated with immune response in the NIDI cohort. However, multiple analyses consistently showed a statistically non-significant trend suggesting that later vaccination reduced the likelihood of a strong immunogenic response. The crude association between age at Dose 2 and seroconversion was the only statistically significant result found in the log binomial analysis. Nonetheless, both crude and adjusted risk ratios showed a statistically non-significant trend of decreased likelihood of seroconversion for increasing age at each dose, as well as the interval between doses. Similarly, unadjusted log linear models showed non-significant negative relationships between the fold-change in rotavirus-specific IgA GMT and each of the three timing variables. It is necessary to acknowledge, however, that the low magnitude of the point estimates across analyses suggest these negative trends, though consistent, may not be clinically meaningful.

Age at the first dose of RV1 followed the overall trend of decreased immune response with increased age, though estimates were unstable with wide confidence intervals and non-significant. It is possible that the negative association between age

at Dose 1 and seroconversion (RR[95%CI]=0.93[0.83,1.04]) was due to chance alone. However, due to the highly skewed distribution of age at Dose 1 in the NIDI cohort, this analysis may have lacked sufficient power to statistically detect a relationship, even if one exists. While the distribution of age at Dose 1 ranged from 58 to 105 days (a spread of 6.7 weeks), three quarters of the infants included in the analysis received their first dose of RV1 between 58 and 65 days of age. The fact that the majority of available data fell into just a 7-day period may have prevented the precise extrapolation of the model over a longer time period. In contrast, RCTs designed to statistically assess differences by vaccine schedule have used set schedules (typically 6/10 versus 10/14 week schedules) with a 4 week difference in age at each dose (39, 41, 42). (The findings of these RCTs are discussed in the Literature Review, above, and their agreement with the present analysis is discussed below.) Of note, the NIDI study was not designed to examine vaccine timing, and mothers were reminded and encouraged to have infants vaccinated on time (according to the Bolivian schedule of 8 and 12 weeks for the two RV1 doses); therefore, this cohort likely demonstrated unusually punctual RV1 administration for the source population.

In the analyses of age at Dose 2 and the interval between doses, there was a marginal, non-significant, negative relationship between each of these timing variables and seroconversion after adjusting for maternal education, diarrhea within 2 weeks prior to Visit 2, hospital of recruitment, and preterm birth (RR[95% CI]= 0.96 [0.91, 1.00] for age at Dose2; RR[95% CI]= 0.96 [0.92, 1.00]). Correlations between these exposures (age at Dose 2 and the interval between doses) and the

fold-change in anti-rotavirus IgA GMT provided further support for the pattern of a negative relationship with negative trendlines and correlation coefficients.

It is difficult to determine whether the age at which an infant receives the second dose of RV1 or the length of time between doses plays a more important role in RV1 response in these data. It is possible that both relationships are due to chance; however, if a true relationship exists, it may be that one factor is driving the relationship, rather than two independent associations. Due to the issue of the distribution of age at Dose 1 (discussed above), there was a strong correlation between the age at Dose 2 and the length of interval (Pearson's R-Square=0.88). This interdependence precludes any determination of whether age at Dose 2 or the length of interval between doses is responsible for the observed trend of decreased immune response in older infants in this analysis. Additionally, from this author's review of the literature, no studies have specifically assessed the effect of the length of interval, separate from the age at vaccination, on RV1 response. It may be that the effects of age at each dose and the length of interval between doses cannot be examined independently, regardless of study design, due to the mathematical relationship between them.

If there is truly a decreased likelihood of seroconversion with increased age at the second dose, it is possible that these changes are attributable to changes in the gut microbiota of the infant over time. Evidence suggests that the microbiome of the gut influences RV1 response (31), and co-infections with enteric bacteria and viruses have been found to impair immune response to other live oral vaccines (reviewed in (67, 68)). As older infants have had more time to encounter enteric

pathogens, they may be more likely than younger infants to have co-infections or imbalanced microbiota that interfere with RV1 (as suggested in (60, 67, 68)), which could explain why older infants were less likely to seroconvert in this analysis.

Another potential explanation for the observed negative age-vaccine response relationships is that the commonly proposed mechanisms involving maternal antibodies may apply differently in the NIDI cohort due to its overall older age distribution compared to the schedules tested in other studies (e.g. the RCTs reviewed in (45)). The general hypothesis is that anti-rotavirus IgG antibodies from the mother are passed to the infant transplacentally or through breastfeeding, and these antibodies interfere with the infant's immune response to RV1 (33-38). The majority of the body of literature on RV1 timing supports this hypothesis: overall trends in RCT data suggest that a delayed vaccine schedule improves RV1 vaccine efficacy (39, 40, 45) (although some RCTs have found non-significant (40, 42) or inconclusive results (41)). However, studies have shown that IgG levels only decrease with age for the first 3-5 months of life, then begin to increase with age due to natural rotavirus exposure (69). Given that the minimum age at Dose 2 in this analysis was 116 days (3.8 months), it may be that the youngest infants in the distribution had already reached their lowest levels of IgG antibodies, while infants who were older at the second dose of RV1 had already encountered natural rotavirus and synthesized their own anti-rotavirus antibodies. Thus, if there is an increasing trend in IgG's with age in our data, the hypothesis of IgG interference in RV1 response could explain the small observed decreases in seroconversion with age. To summarize, while maternal IgG's may interfere with RV1 in very young

infants, it is possible that the infants in our study had already aged out of this phenomenon, and instead were subject to increasing IgG levels with age (due to natural exposure), which could have hindered RV1 response among older NIDI infants. Therefore, while the results of the current study may seem to contradict the findings of RCTs, they may simply suggest there is an upward limit on the delay in vaccination that would be beneficial.

In addition to differences in tested RV1 schedules, the results of RCT studies may also vary from those of the current analysis due to key differences in the study design. That is, the effect of vaccine timing as detected by observational studies may reflect interaction with real-world, outside influences not present in controlled trials. For instance, it is possible that other proposed vaccine-response predictors, such as nutrient deficiencies (29) or co-infections (67) in the host, are effect modifiers in the vaccine timing-immune response relationship. Thus, both RCT and observational studies from the same population will be necessary to understand the true impact of RV1 timing on rotavirus protection.

Interestingly, the negative trends between vaccination age and immune response in the NIDI cohort do agree with recent findings from a post-hoc analysis of data from a case-control study of infants hospitalized for diarrheal disease in Bolivia (60). Burke et al. analyzed data from two post-licensure RV1 evaluations in four cities across the country, and found that vaccine effectiveness (defined as [1 – odds ratio] x 100%) was highest among those who received their first dose early, as compared to on-time or late. The study found no detectable differences by age at second dose. However, the practical implications of the Burke study and the present

analysis are consistent despite major differences in study design (case-control vs. cohort) and outcome (clinical vs. serological). This result underscores the need for future studies to examine the RV1 timing-response relationship in multiple populations.

**Strengths and Limitations**

The current analysis has several limitations. Primarily, the NIDI study was not designed to examine the relationship between dose timing and vaccine response, and therefore did not have ideal exposure distributions to assess the research question. As previously discussed, the narrow, right-skewed distribution in the age at which infants received the first dose of RV1 led to low precision in the assessment of the influence of age at Dose 1 on immune response, and made the effects of age at Dose 2 and the length of interval indistinguishable from one another. A second limitation is that the population studied in El Alto (a mostly indigenous, high altitude, urban center) is quite distinct with respect to environment, culture, and socioeconomic factors, and results are not expected to be fully generalizable to outside populations. Additionally, only 67% of enrolled infants were included in this analysis, so there is some potential for selection bias. For example, selection bias could result if those who were lost to follow-up were less likely to seroconvert and had a different distribution of age at vaccination.

Despite these limitations, this analysis contributes additional data to the question of vaccine timing and effectiveness, an area of study that is thus far largely inconclusive. The observational nature of the NIDI study reflects real-world conditions, which provides new insight into the effect of RV1 timing on

immunogenic response in El Alto, Bolivia. Additionally, prospective data were available for a wide range of covariates, limiting concerns about uncontrolled confounding in the analysis.

**Conclusions**

This analysis of data from the NIDI study suggests that delays in the RV1 schedule do not necessarily lead to better vaccine protection. On the contrary, non-significant trends in this analysis suggest increased age at Dose 2 and increased length of interval between doses are associated with decreased seroconversion rates. Our results support the current Bolivian 8/12 week RV1 schedule. Late vaccination can still benefit those who miss the recommended schedule; however, we recommend that families be encouraged to vaccinate on time to maximize vaccine protection.

Additional research on this topic is warranted. Future studies should seek to determine optimal RV1 timing, accounting for both the potential influence of age on the immunogenic response to the vaccine, as well as the corresponding window of opportunity for natural infection. These relationships should also be studied across multiple regions to determine whether the association differs by population or environment.

## PUBLIC HEALTH IMPLICATIONS

- This analysis found no statistically significant associations between rotavirus vaccine timing and serological vaccine response based on anti-rotavirus specific IgA titers. However, as a general trend, RV1 response was slightly lower for older infants compared to younger infants in the NIDI cohort. In combination with the fact that a delayed schedule would only increase the window for unvaccinated children to be exposed to natural rotavirus (51, 70), these results suggest that families should be encouraged to vaccinate children on time.
- Point estimates for associations between vaccine timing and RV1 seroresponse were close to the null values across analyses and may not be clinically meaningful. While vaccine response was slightly better on average among infants who received RV1 on time compared to those who received the vaccine late, the results of this analysis do not suggest that older infants do not benefit from the vaccine. Therefore, this study does not contradict the WHO recommendation (7) to avoid strict scheduling that may exclude infants who could benefit from vaccination.

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# TABLES

**Table 1.** Characteristics of the NIDI cohort

| **Characteristics** | N=309 |
|---|---|
| **General, N (%)** | |
| Sex (Female) | 145 (46.9) |
| Indigenous ethnicity† | 191 (61.8) |
| Maternal education | |
| Primary or less | 45 (14.6) |
| Secondary | 192 (62.1) |
| University | 72 (23.3) |
| Hospital | |
| Los Andes | 164 (53.1) |
| Corea | 145 (46.9) |
| Diarrhea At Visit 2 | 39 (12.6) |
| Preterm birth* | 55 (18.2) |
| Stunted¥ | 93 (33.6) |
| **Exposures, Mean days (SD)** | |
| Age at dose 1 | 64.5 (6.2) |
| Age at dose 2 | 132.9 (17.4) |
| Length of dose interval | 68.4 (16.6) |
| **Outcome, N (%)** | |
| Seroconverted | 197 (63.8) |

† Maternal self-identification of membership to any indigenous ethnic group
§ 2 week recall
* Preterm defined as < 37 weeks gestational age
¥ Stunting at any time up to the second blood draw defined as a length-for-age Z-score < -2

**Table 2.** Relative risks of IgA seroconversion to RV1 for a 1 week increase in the exposure.

| | Crude Analysis | | Adjusted Analysis* | |
|---|---|---|---|---|
| Exposure | RR (95%CI) | *P*-Value | RR (95%CI) | *P*-Value |
| Age at Dose 1 | 0.92 (0.82, 1.04) | 0.17 | 0.93 (0.83, 1.04) | 0.19 |
| Age at Dose 2 | 0.95 (0.91, 1.00) | 0.03 | 0.96 (0.91, 1.00) | 0.06 |
| Length of Interval | 0.96 (0.92, 1.00) | 0.07 | 0.96 (0.92, 1.00) | 0.07 |

* All models adjusted for maternal education, diarrhea at visit 2, hospital of recruitment, and preterm birth. Age at dose 2 additionally adjusted for age at dose 1.

**Table 3.** Mean anti-rotavirus IgA GMTs pre-RV1 (Visit 2) and ~2 months following 2 doses of RV1 (Visit 6) by vaccination age groups.

| | Early¥ (N=167) | Late¥ (N=142) | *P*-value* |
|---|---|---|---|
| Visit 2 | 2.02 | 2.27 | 0.74 |
| Visit 6 | 102.75 | 79.19 | 0.20 |

* Wilcoxon-Mann-Whitney test

¥ Age groups are based on the median age at vaccination in the cohort for each dose (63 days for Dose 1 and 126 days for Dose 2). "Early" infants received both doses before the median age for each dose, while "late" infants received each dose after the median age.

**Table 4.** Unadjusted correlation coefficients for exposures and fold-change in rotavirus-specific IgA GMT.

| | Spearman's ρ | *P*-value |
|---|---|---|
| Age at dose 1 | -0.02 | 0.71 |
| Age at dose 2 | -0.10 | 0.09 |
| Interval | -0.07 | 0.25 |

# FIGURES

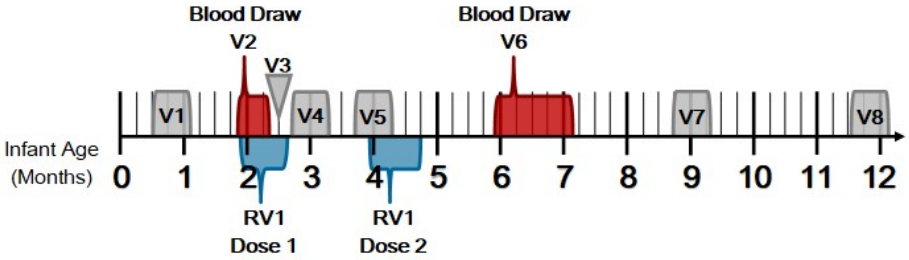


**Figure 1.** Timeline of study visits and rotavirus vaccination relative to infant ages**.** RV1 Dose indicates the approximate range of ages in which the majority of participants received the rotavirus vaccine. V1-8 represent study visits. Visits 1, 4, 5, 7, and 8 are shown at target ages per study protocol, while Visits 2 and 6 show approximate ages from the data. Visit 3 was a home visit following 4-7 days after Dose 1.

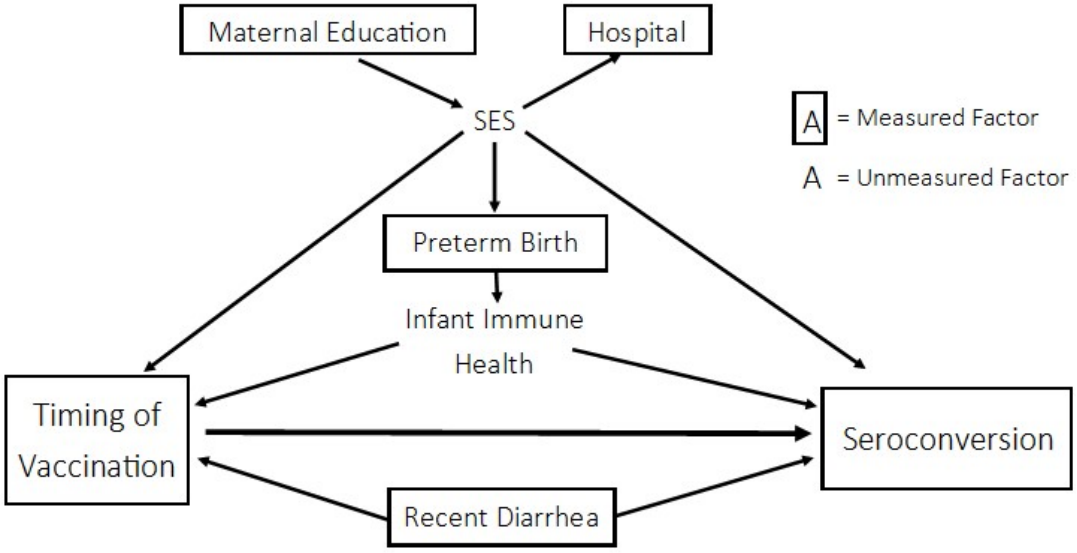


**Figure 2.** Directed acyclic graph showing proposed relationships between variables in the final model. The direction of arrows represents the direction of a hypothesized causal relationship. Timing of vaccination and seroconversion represent the exposure and outcome, respectively. The remaining boxed variables are measured and included in the final model to control for confounding directly, or by proxy for unmeasured confounding variables (SES and infant immune health).

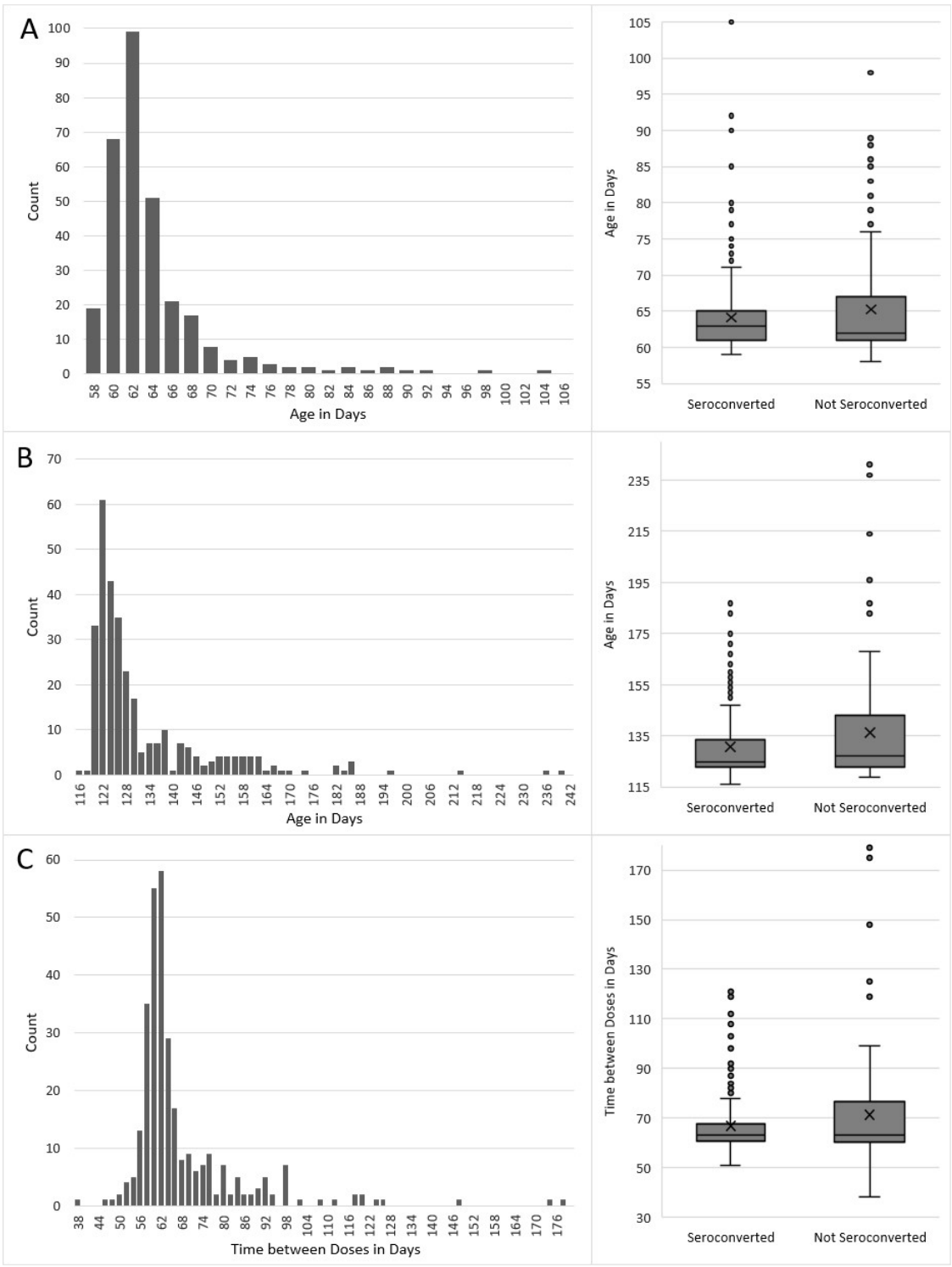


**Figure 3.** Histograms and boxplots of (A) age at dose 1, (B) age at dose 2, and (C) the interval between doses. Histograms show the distribution of timing variables for all infants included in the analysis. Side-by-side boxplots for timing variables by seroconversion status show mean age (x), as well as range (whiskers), IQR (upper and lower limits of box), and median (center line through box) of each variable. The presence of points above the whiskers indicates potential outliers in the data.

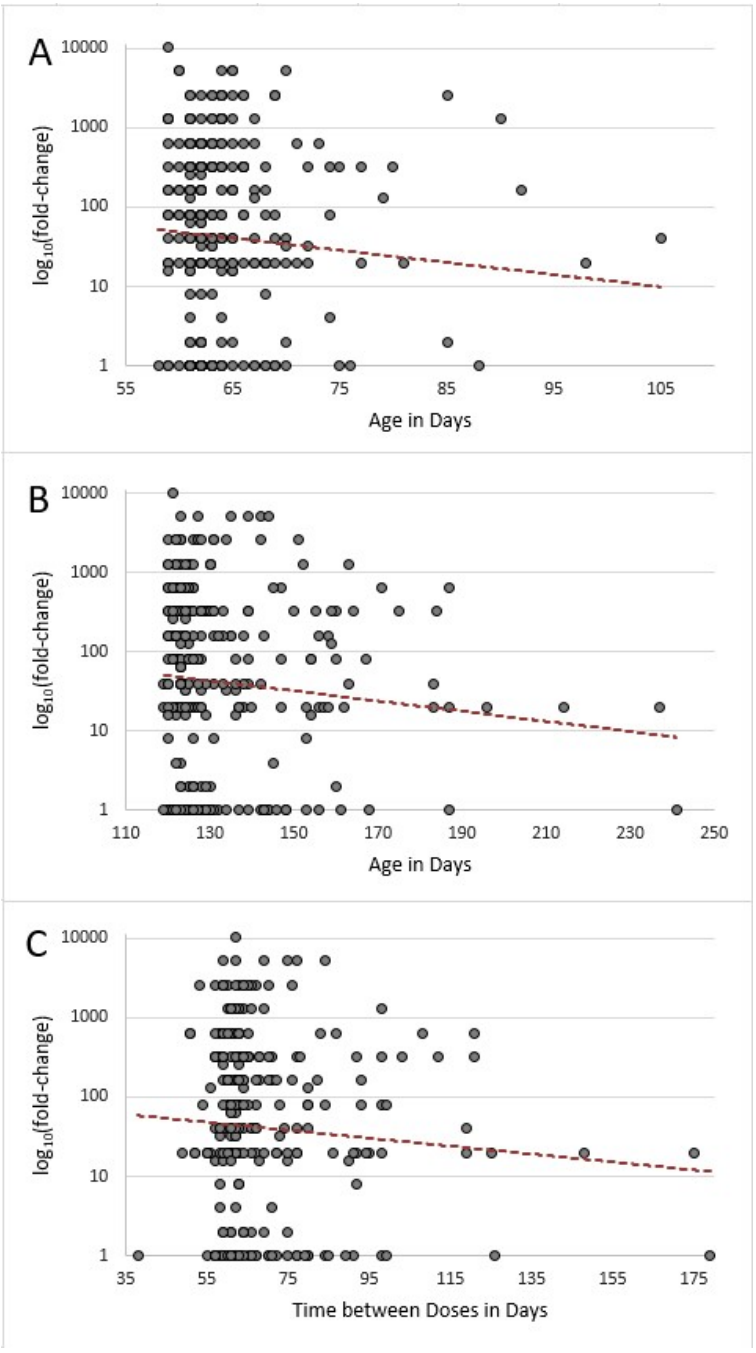


**Figure 4.** Scatterplots and exponential trendlines for (A) age at dose 1, (B) age at dose 2, and (C) length of interval versus the log of the fold-change in anti-rotavirus IgA GMT.

**Supplemental Table 1.** Detailed breakdown of cohort characteristics by age categories and seroconversion status.

| | Total (N=309) | Early¤ (N=167) | Late¤ (N=142) | *P*-value | Seroconverted (N=197) | Not seroconverted (N=112) | *P*-value |
|---|---|---|---|---|---|---|---|
| Exposures, Mean (SD) | | | | | | | |
| Age at dose 1 | 64.5 (6.2) | 62.7 (3.4) | 66.6 (7.8) | | 64.1 (5.6) | 65.2 (7.0) | 0.40‡ |
| Age at dose 2 | 132.9 (17.4) | 123 (2.1) | 144.5 (20.3) | | 130.9 (13.4) | 136.4 (22.6) | 0.06‡ |
| Length of dose interval | 68.4 (16.6) | 60.3 (3.5) | 77.8 (20.4) | | 66.7 (12.4) | 71.2 (21.9) | 0.06‡ |
| Outcome, N (%) | | | | | | | |
| Seroconverted | 197 (63.75) | 112 (67.1) | 85 (59.9) | 0.19 | | | |
| Covariates, N (%) | | | | | | | |
| Sex (Female) | 145 (46.9) | 78 (46.7) | 67 (47.2) | 0.93 | 92 (46.7) | 53 (47.3) | 0.92 |
| Indigenous ethnicity£ | 191 (61.8) | 106 (63.5) | 84 (59.9) | 0.51 | 120 (60.9) | 71 (63.4) | 0.67 |
| Maternal education | | | | | | | |
| Primary or less | 45 (14.6) | 28 (16.8) | 17 (12.0) | | 24 (12.2) | 21 (18.8) | |
| Secondary | 192 (62.1) | 95 (56.9) | 97 (68.3) | 0.12 | 123 (62.4) | 69 (61.6) | 0.21 |
| University | 72 (23.3) | 44 (26.3) | 28 (19.7) | | 50 (25.4) | 22 (19.6) | |
| Morbidities | | | | | | | |
| Diarrhea at Visit 1* | 13 (4.2) | 9 (5.4) | 4 (2.8) | 0.26 | 9 (4.6) | 4 (3.6) | 0.67 |
| Diarrhea at Visit 2* | 39 (12.7) | 24 (14.4) | 15 (10.6) | 0.32 | 27 (13.7) | 12 (10.7) | 0.45 |
| Fever at Visit 2† | 16 (5.2) | 7 (4.2) | 9 (6.3) | 0.46 | 10 (5.1) | 6 (5.4) | 0.92 |
| Hospital | | | | | | | |
| Los Andes | 164 (53.1) | 96 (57.8) | 67 (47.2) | 0.06 | 111 (56.4) | 53 (47.3) | 0.13 |
| Corea | 145 (46.9) | 70 (42.2) | 75 (52.8) | | 86 (43.6) | 59 (52.7) | |
| Preterm birth§ | 55 (18.2) | 31 (18.9) | 24 (17.4) | 0.77 | 36 (18.8) | 19 (17.3) | 0.74 |
| Stunted¥ | 93 (33.6) | 44 (28.8) | 49 (39.5) | 0.06 | 62 (34.4) | 31 (32.0) | 0.68 |

¤ Age groups based on the median age at vaccination in the cohort for each dose

£ Maternal self-identification of membership to any indigenous ethnic group

* 2 week recall

† 48 hour recall

§ Preterm birth defined as < 37 weeks gestational age

¥ Stunting at any time up to the second blood draw defined as a length for age Z-score <-2

‡ Wilcoxon-Mann-Whitney test *p*-values. All other *p*-values from Pearson Chi-Square tests