Breast milk prefusion F IgG as a correlate of protection against respiratory syncytial virus acute respiratory illness

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Running Title: Breast milk RSV pre-F antibodies and RSV ARI

Manuscript Summary

Low breast milk pre-F IgG antibodies before RSV ARI supports a potential role for pre-F IgG as a correlate of protection against RSV ARI. Induction of breast milk pre-F IgG may be a mechanism of protection for maternal RSV vaccines.

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Footnote Page

Potential Conflicts of Interest

All authors will submit the ICMJE Form for Disclosure of Potential Conflicts of Interest.

HYC: Institution receives research support from Novavax and Sanofi-Pasteur.

JAE: research support to my institution from Novavax, MedImmune, GlaxoSmithKline, Gilead

BG has a patent RSV pre-F candidate pending

JT reports grants from Bill and Melinda Gates Foundation, during the conduct of the study.

Dr. Katz reports grants from Bill and Melinda Gates Foundation, during the conduct of the study; grants from Bill and Melinda Gates, grants from Thrasher Research Fund, grants from National Institutes of Health, grants from Whole New World Foundation, grants from USAID, outside the submitted work.

Dr. Bont has regular interaction with pharmaceutical and other industrial partners. He has not received personal fees or other personal benefits. UMCU has received major funding (>€100,000 per industrial partner) for investigator initiated studies from AbbVie, MedImmune, Janssen, the Bill and Melinda Gates Foundation and MeMed Diagnostics. UMCU has received minor funding participation in trials by Regeneron and Janssen since 2015 (total annual estimate less than €20,000). He received minor funding for consultation and invited lectures by AbbVie, MedImmune, Ablynx, Bavaria Nordic, MabXience, Novavax, Janssen (total annual estimate less than €20,000).

https://www.umcutrecht.nl/en/Research/Research-programs/Infection-Immunity/Research-Groups/Group-Bont/Potential-Conflict-of-Interest-(2007-2017)

Funding Sources:

This work was supported by Netherlands Royal Academy for Medical Sciences (KNAW ter Meulen grant, Academy Medical Sciences Fund); the Bill & Melinda Gates Foundation (grant number 50274); and the National Institutes of Health (grant number K23).

Contributors

All co-authors contributed to the final manuscript.

MCS, JMT, JK, SKK, and JE contributed to the design of the original trial. MCS conceived of and secured funding for the original trial. SK, SL, JMT, and JK supervised the conduct of the study in the field for the original trial.

Meetings where the information has been presented

Poster presentation at RSV vaccines for the world meeting 2017 (29 Nov-1 Dec, 2017) Malaga, Spain (poster available upon request)

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Abstract

Background

Transplacental RSV antibody transfer has been characterized, but little is known about the protective effect of breast milk RSV-specific antibodies. Serum antibodies against the prefusion RSV fusion protein (pre-F) exhibit high neutralizing activity. We investigate protection of breast milk pre-F antibodies against RSV acute respiratory infection (ARI).

Methods

Breast milk at 1, 3, and 6 months postpartum and mid-nasal swabs during infant illness episodes were collected in mother-infant pairs in Nepal. 174 infants with and without RSV ARI were matched 1:1 by risk factors for RSV ARI. Pre-F IgA and IgG antibody levels were measured in breast milk.

Results

The median breast milk pre-F IgG antibody concentration before illness was lower in mothers of infants with RSV ARI (1.4, IQR 1.1–1.6 $\log_{10}$ ng/mL) than without RSV ARI (1.5, IQR: 1.3–1.8 $\log_{10}$ ng/mL, p=0.001). There was no difference in median maternal pre-F IgA antibody concentrations in cases (1.7, IQR: 0.0–2.2 $\log_{10}$ ng/mL) versus controls (1.7, IQR: 1.2–2.2 $\log_{10}$ ng/mL, p=0.58).

Conclusion

Low breast milk pre-F IgG antibodies before RSV ARI supports a potential role for pre-F IgG as a correlate of protection against RSV ARI. Induction of breast milk pre-F IgG may be a mechanism of protection for maternal RSV vaccines.

Key words:

Breast milk, maternal vaccination, IgG and IgA antibodies, respiratory syncytial virus, acute respiratory infection

Manuscript Summary

What is already known about this subject?

Maternal vaccines for RSV are in phase II and III clinical trials. Transplacental transfer of antibodies against RSV has been characterized but little is known about the protective role of antibodies in breast milk. Antibodies against the prefusion F (pre-F) protein of RSV account for the majority of neutralizing activity in human sera.

What does this article add to our knowledge?

This article examines the contribution of maternal pre-F antibodies in breast milk to protection against RSV acute respiratory illness in infants in the first 6 months of life.

How does this study impact current management guidelines?

There is an important gap in knowledge regarding immunological correlates of protection against RSV in infants. In the context of RSV maternal vaccine development in late phase clinical trials, it is important to understand such correlates of protection and the biological mechanisms underlying this effect.

Introduction

Maternal vaccination against respiratory syncytial virus (RSV) is a promising intervention to protect young infants against RSV infection through transfer of antibodies from mother to infant [1]. Transplacental transfer of RSV IgG antibodies via the neonatal Fc receptor (FcRn) has been characterized in mother-infant pairs in different populations[2–5]. Transplacental transfer ratio and decay kinetics of maternal IgG are considered cornerstones of protection of the infant through maternal vaccination[6]. However, other routes of antibody transfer may also be important to protect infants from RSV disease.

A novel route of RSV antibody transfer directly to the respiratory tract via RSV-specific IgG and IgA in amniotic fluid was recently described[7]. The acquired amniotic fluid antibodies show neutralizing activity against RSV and provide protection to the neonate for at least one week postpartum *in vivo*, demonstrating the role of mucosal immunity in protection of infants.

Postnatal antibody transfer to the mucosal surfaces occurs via breast milk[8–12]. A better understanding of the role of RSV-specific antibodies in breast milk may give further insight into mucosal antibody transfer from mother to infant in the context of maternal vaccination and may serve as a correlate of protection against RSV disease. Correlates of protection for RSV remain a knowledge gap and priority for RSV vaccine development[14]. Despite the lack of a clear correlate of protection [15], recent insights into the structure of viral envelope proteins have led to the distinction in antibody function on the basis of target epitopes. RSV F protein mediates RSV entry and fusion with the host cell membrane. Antibodies that target prefusion F (pre-F) protein account for the majority of neutralizing activity against RSV in human sera of infected individuals[16–18] and modify disease severity in young children[19]. Thus, antibodies directed against pre-F play an important role in protection against RSV infection. No previous studies have evaluated pre-F RSV antibody in breast milk in relationship to RSV disease risk in infants.

The aim of this study was to characterize the relationship between pre-F antibodies in breast milk and RSV acute respiratory infection (ARI) in infants.

Methods

Study Site, Design, and Population

From mid April 2011 to mid April 2013, 3,693 women in the second to third trimester of pregnancy were enrolled in a maternal influenza immunization trial in rural southern Nepal[20]. Weekly home-based visits were conducted until 180 days after birth for respiratory symptom surveillance of mother-infant pairs based on maternal report of symptoms each day in the past week. Nasal swabs were collected from infants if respiratory illness was noted was noted; samples from mothers were collected for febrile respiratory disease. Breast milk was collected from a subset of 827 women living in the three study regions closest to the study clinic. Within this subset of mother-infant pairs, infants who had RSV-confirmed respiratory illness in the first 6 months of life were matched 1:1 to controls (infants with no RSV ARI) based on the following risk factors for RSV ARI: maternal influenza vaccination, maternal education, infant month of birth, number of siblings, use of an indoor biomass cookstove, and preterm birth (less than 37 weeks gestation). Healthy infant controls were matched to have at least 4 months of respiratory surveillance.

Data Collection and Case Definition

A respiratory illness was defined as fever, cough, wheeze, rapid breathing or a draining ear on any one day in the past week. Breastfeeding was not exclusive if anything other than breast milk was given to the baby. Illness episodes were considered distinct when separated by seven symptom-free days. Clinical and sociodemographic data were collected at enrollment, birth, and weekly respiratory surveillance visits. Mid-nasal swabs were collected from infants who met criteria for respiratory illness in the past 7 days and were tested for RSV by RT-qPCR[21]. Breast milk was collected at 1, 3 and 6 months postpartum. Participants were asked to wash their hands and self-express 15 mL of breast milk into a sterile container. Samples were transported on wet ice to the field laboratory and centrifuged to remove the lipid layer, aliquoted and frozen at -80°C prior to shipment to the University of Washington, Seattle, WA for testing.

Laboratory Testing

Breast milk IgA and IgG antibody concentrations against RSV stabilized pre-F (DS-Cav1) protein were quantified by ELISA. DS-Cav1 is an RSV F protein that is stabilized by a T4 fibritin-trimerization domain (foldon) at the C-terminus, S155C and S290C cysteine mutations to form an additional disulfide bond, and S190F and V207L cavity filling mutations. DsCav-1 is expressed by transient transfection of HEK293F cells and purified by affinity purification (NTA resin and StrepTactin resin) and a Superose 200 gel filtration column[22]. Nunc MaxiSorp 96-well plates (Thermo Scientific, Roskilde, Denmark) were coated overnight at 4°C with either pre-F (100 ng/mL, DS-Cav1, for pre-F IgA or pre-F IgG ELISA). In between steps plates were washed 3 times with PBS containing 0.05% Tween-20 (Sigma Aldrich) (PBS-T) using a microplate washer (Biotek 405 LS). Plates were blocked for 1 hour at room temperature (RT) with 1% bovine serum albumin (Roche Diagnostics) in PBS-T. Breast milk was added (100µl/well) in duplicate, at 2 to 3 dilutions and incubated for 1.5 hours at RT. Recombinant palivizumab-IgA1 and recombinant palivizumab-IgA2 were synthesized by cloning variable heavy and light chain sequences of palivizumab into Lonza expression vector, followed by production in HEK293T cells, and purification by Kappa-select and HP-SEC[23]. Recombinant palivizumab-IgA1 and IgA2, palivizumab (Synagis; MedImmune, Gaithersbug, MD). Horseradish peroxidase-labeled goat-anti-human IgA (Jackson, West Grove, PA) and horseradish-peroxidase-labeled goat-anti-human IgG (Jackson, West Grove, PA) were added at a concentration of 0.5 µg/mL and 0.16 µg/mL, respectively, as detection antibodies and incubated 1 hour at RT. Plates were developed with

ABTS substrate (Roche, Darmstadt, Germany) and absorbance was measured at 415nm with a microplate spectrophotometer (Biotek Epoch, Winooski VT). Data were captured and exported using Gen5 software (Biotek, Winooski VT).

Statistical Analyses

Continuous variables were described using mean (standard deviation) or median (interquartile range). Differences in the mean or median of continuous variables were tested with a two-sided t-test or a non-parametric Mann-Whitney test when appropriate. Log_{10} transformation was performed for all antibody measurements. For our primary analysis we compared antibody titers prior to infection using a Mann-Whitney test; for infections that occurred before 1 month we used the antibody titer at 1 month. We used the corresponding time point for controls as used for the matched cases. A linear mixed model analysis was performed to compare the difference of antibody titers over time for cases and controls. We included time of breast milk collection (month 3 or 6 versus month 1) as covariates and RSV status of children in the first six months of life, as well as the interaction terms of collection time by RSV positivity, to test the hypothesis of whether RSV antibody levels in breast milk increased or decreased differently by RSV status of the infant. We used a Spearman's correlation to perform a correlation of RSV antibody titer to time of infection in cases only as well as a correlation of pre-F antibody to total antibody by isotype and pre-F IgA to pre-F IgG in both cases and controls. The statistical analysis was performed using STATA/SE 13.1 (Statacorp, College Station TX) and sinusoid function to examine seasonal variation using SPSS Statistics 25 (IBM, Armonk NY).

Ethical Considerations

Ethical approval for the primary trial (Clinicaltrials.gov #NCT01034254) was obtained from the institutional review boards at Institute of Medicine at Tribhuvan University, the Nepal Health Research Council, John Hopkins University Bloomberg School of Public Health, Seattle Children's Hospital, and Cincinnati Children's Medical Center.

Results

Clinical and Sociodemographic Characteristics

Clinical and sociodemographic characteristics were compared for 174 children (87 cases and 87 controls). A total of 106 (61%) of 174 children were female. No significant differences for clinical or sociodemographic characteristics of mother or infants were observed between cases and controls [Table 1]. The mean age at primary RSV ARI in cases was 3.1 months (SD: 1.5 months).

Quantification of pre-F IgA, pre-F IgG, total IgA, total IgG

Pre-F IgA, pre-F IgG, total IgA, and total IgG antibodies were measured in 454 breast milk samples from 174 mothers at 1 (n=150), 3 (n=151), and 6 (n=153) months postpartum. The median concentration of pre-F IgA (77.7 ng/mL, IQR: 22.3–200.7) was higher than the median concentration of pre-F IgG, (36.5 ng/mL, IQR: 21.0–62.8). Likewise, the median concentration of total IgA was higher (0.2 mg/mL, IQR: 0.15–0.27) than total IgG (0.04 mg/mL IQR: 0.03–0.05) [Table S1]. In Table 2 the $\log_{10}$ median concentrations of pre-F IgA, pre-F IgG, total IgA and total IgG for all breast milk samples for both cases and controls are described in addition to the raw values in Table S1.

Correlation between specific and total antibody levels

Pre-F IgG concentration showed a moderate positive correlation to total IgG at 1 month (r_s: 0.38, $p<0.0001$) [Supplementary Figure 2], 3 months (r_s: 0.38, $p<0.0001$) and 6 months postpartum (r_s: 0.40, $p<0.0001$). Pre-F IgA showed a lower positive correlation to total IgA at 1 month (r_s: 0.22, p=0.007)[Supplementary Figure 2], and at 3 months (r_s: 0.27, p=0.0009), but not at 6 months (r_s: 0.09, p=0.29). Pre-F IgG was positively correlated with pre-F IgA at 1 month (r_s: 0.18, p=0.03) [Supplementary Figure 3], at 3 months (r_s: 0.37, p<0.0001) and at 6 months postpartum (r_s: 0.22, p=0.008).

Pre-F IgG and pre-F IgA in cases and controls before infection

We compared pre-F antibody titers at the time point prior to RSV ARI in cases and matched controls. If there was no breast milk sample before infection, then we used the closest time point after RSV ARI. The median time gap between antibody measurement used and RSV ARI was 1.1 months (IQR: 0.45–1.6 months). Eight infants had RSV ARI before 1 month of age, and the median time at RSV ARI in these infants was age 0.64 months (IQR: 0.49–0.80). The median $\log_{10}$ pre-F IgG antibody titer before infection was significantly lower in breast milk of mothers of cases (median (IQR): 1.4 $\log_{10}$ ng/mL (1.1–1.6)) than in mothers of controls (median (IQR): 1.5 $\log_{10}$ ng/mL (1.3–1.8), p=0.001) [Figure 1A]. The effect was more pronounced after excluding eight children who had RSV ARI before 1 month of age and their matched controls: $\log_{10}$ pre-F IgG antibody titer was significantly lower in breast milk of mothers of cases (median (IQR): 1.4 $\log_{10}$ ng/mL (1.1–1.5) compared to mothers of controls (median (IQR): 1.6 $\log_{10}$ ng/mL (1.3–1.8), p=0.0002) [Figure S5A].

The median $\log_{10}$ pre-F IgA antibody titer of the breast milk sample at the latest time point prior to infection did not differ significantly in breast milk of mothers of cases (median (IQR): 1.7 $\log_{10}$ ng/mL (0.0–2.2)) compared with mothers of controls (median (IQR): 1.7 $\log_{10}$ ng/mL (1.2–2.1), p=0.58) [Figure 1B]. Similarly, when excluding children with RSV ARI under 1 month of age, there was no significant difference in pre-F IgA antibody in breast milk of mothers of cases (median (IQR): 1.7 log_{10} ng/mL (0.0–2.1)) compared to mothers of controls (median (IQR): 1.7 log_{10} ng/mL (1.1–2.1), p=0.50) [Figure S5B].

We evaluated the ratio of pre-F antibody titers to total antibody titer by IgG or IgA isotype. For the ratio of pre-F IgG to total IgG and pre-F IgA levels to total IgA, the same trends were observed [Figure 1C-D]. Pre-F IgG/ total IgG was lower in cases than in controls (0.89 log_{10} ng/mL (0.58–1.1) versus 1.0 log_{10} ng/mL (0.83–1.2); p=0.001) whereas pre-F IgA/ total IgA did not differ between cases and controls (2.4 log_{10} ng/mL (0.0–2.9) versus 2.4 log_{10} ng/mL (1.8 – 2.8), p=0.72). We performed a sensitivity analysis for infants who were exclusively breastfed in the first few days of life (n=106), and found pre-F IgA prior to infection did not differ significantly between cases and controls (1.7 (1.1–2.2) versus 1.9 (0.1–2.1), p=0.85) but did differ for pre-F IgG (1.3 log_{10} ng/mL (1.1 – 1.6) versus 1.6 log_{10} ng/mL (1.3–1.8), p=0.01).

Mixed Model Analysis of pre-F antibodies over time

We used a mixed-effects linear regression model to compare pre-F IgG and pre-F IgA antibody concentrations in breast milk over time in mothers of cases compared to controls. The mean log_{10} difference of pre-F IgG concentration in breast milk of mothers of cases compared to controls was -0.21 (95%CI: -0.35 to -0.06, p=0.004) at 1 month postpartum, –0.12 (95%CI: -0.26 to 0.02, p=0.09) at 3 months postpartum, and 0.00 (95%CI: -0.14 to 0.14, p=0.99) at 6 months postpartum [Figures 2A – B]. The mean log_{10} difference of pre-F IgA in breast milk of mothers of cases compared to controls was -0.10 (95%CI: -0.38–0.17, p=0.46) at 1 month postpartum, 0.11 (95%CI: -0.17–0.38, p=0.44) at 3 months postpartum, and 0.28 (95%CI: 0.01–0.55, p=0.046) at 6 months postpartum [Figure 2C – D]. Antibody level was found to increase at 6 months relative to month 1 only for cases (0.27 log_{10} ng/mL increase in titer for pre-F IgG, p=0.001; 0.44 log_{10} ng/mL increase for pre-F IgA, p=0.003). There was no evidence of increase over time for either antibody level among controls (p=0.45 and p=0.21 for pre-F IgA and pre-F IgG respectively). Consequently, for pre-F IgG, the difference found at 1 month between cases and controls was no longer present at 6 months of age (p=0.99).

Antibody Concentration and time to infection

Among cases, there was a low negative correlation between pre-F IgG concentration in breast milk at 1 month postpartum and time to RSV ARI in cases which is marginally significant (r_s: –0.22, p=0.06), indicating that higher antibody levels may be associated with shorter time to infection. However, there was no detected correlation between pre-F IgA antibody concentration at 1 month postpartum and time to RSV ARI in cases (r_s:0.10, p=0.40).

Seasonal Fluctuation of pre-F IgA and pre-F IgG titers

We applied a sinusoidal model to the pre-F IgG and pre-F IgA concentrations in breast milk of all mothers at one month postpartum. All RSV-infected infants in this substudy were born between June and September, as were the controls who were matched by birth month. Therefore, no children in this substudy were born in October through January, corresponding to the peak of the RSV season in Nepal [24] which resulted in a poor fit of the model (goodness of fit measure: r=0.008 for pre-F IgG; r=0.02 for pre-F IgA) [Figures S4A–B].

Discussion

We provide evidence that IgG antibodies in breast milk against RSV pre-F are lower in mothers of children who develop RSV ARI in the first months of life compared to children who do not. In the context of RSV maternal vaccine development with no established correlate of protection against RSV [25], we conclude that breast milk pre-F IgG antibodies may be a correlate of protection against RSV ARI. The importance of these findings is underscored by the fact that premature infants, who are disproportionately affected by RSV disease[26] and have reduced transplacental antibody transfer, may still be potentially protected by maternal immunization via breast milk[11].

One strength of this study was the development of a novel breast milk RSV antibody assay targeting the RSV fusion protein in a pre-F stabilized conformation which permitted measurement of antibodies known to be an important target for RSV neutralizing antibodies[16]. Additionally, the use of recombinant palivizumab IgA allowed for accurate measurement with an IgA standard. Palivizumab IgA1 and IgA2 was used in a 3:2 ratio as found in human breast milk[27].

The results show a potential protective role against RSV ARI for breast milk pre-F IgG but not pre-F IgA antibodies. The difference in pre-F IgG between cases and controls is small, though statistically significant. The difference in protection across antibody isotypes is in accordance with studies specific to RSV and other pathogens such as HIV[28] and CMV[29]. Likewise, recombinant palivizumab-IgA offers less effective protection following intranasal administration than IgG in mice[23].

The relationship between breast milk pre-F IgG and time to infection was an exploratory analysis; the negative correlation merits further study in a larger population powered to look at this effect using more frequent sequential breast milk samples collected over time and a comparison to serologic assays. When looking at seasonality of breast milk pre-F antibodies in breast milk, IgA but not IgG decreased in the summer months possibly due to the shorter half life[32] and lack of exposure to RSV. Increases in breast milk preF IgG at 6 months postpartum may have reflected exposure and infection of the mothers. However, in our study we did not sample for respiratory viruses in asymptomatic or afebrile illnesses in women, therefore limiting our ability to detect these by molecular diagnosis.

There is consensus on the protective effect of breastfeeding on infant respiratory morbidity and mortality [33] with lower risk of RSV hospitalization and reduced disease severity when comparing breastfed to non-breastfed infants[34–36]. However, evidence for the mechanisms by which breast milk antibodies may enter the neonatal circulation is limited. Breast milk antibodies have been shown to reach the neonatal circulation in three children who were given antibody-rich human colostrum via a nasogastric tube[37]. After closure of the gut, uptake of IgG may occur via the FcRn receptor, which has been identified in the human intestine[38] and is involved in bidirectional transport across the enterocyte allowing for defense at the mucosal level[39]. Secretory IgA plays a role at the mucosal surface by neutralizing pathogens in the intestinal lumen in humans[39].

Boosting breast milk antibody via maternal vaccination may help protect infants from RSV disease. In a subunit RSV vaccine trial, increased IgA and IgG antibodies against RSV in breast milk were measured in vaccinated compared to non-vaccinated women[4]. Increased concentrations of breast milk antigen-specific antibodies have been measured following maternal vaccination against influenza, pertussis, RSV, and *Streptococcus pneumonia*[41]. Only one study examined the association between respiratory pathogen-specific antibodies and clinical outcomes in 57 infants[42]. In this study, maternal influenza vaccination and increased influenza-specific IgA in breast milk correlated with decreased episodes of infant respiratory illness, though IgG was not measured. Finally, there is evidence that high virus-specific IgA may interfere with vaccine response for rotavirus vaccination[43] which may be a consideration for RSV maternal vaccination.

The most important limitation of this study was that we did not measure pre-F antibodies in serum of all mothers of these infants or in cord blood. No blood was drawn from infants during this study so further study in infants was not possible. An alternative explanation for protection may be serum pre-F antibody titers in women and their infants. However, in a subset of 310 maternal infant pairs within the maternal vaccination cohort, neutralizing RSV antibody titers in cord blood were not shown to protect against RSV ARI[44]. For 44 maternal infant pairs that overlapped with the cohort in this study, we examined the correlation between breast milk pre-F IgG at 1 month postpartum and cord blood antibody titers and found a positive correlation between breast milk pre-F IgG antibodies and neutralizing antibody titers in cord blood (r(s)=0.29, p=0.05). We found no relationship between breast milk pre-F IgA and cord blood neutralizing antibody titers (r(s)=-0.07, p=0.6). In an exploratory analysis, we found no relationship between disease severity and breast milk pre-F IgG and IgA antibody titers from samples collected closest to the time of infection (data not shown). Furthermore, we found no relationship between breast milk pre-F antibody levels at the time points closest to infection and nasal swab PCR cycle threshold values (data not shown). Another limitation of this study is a facet of the study design. Although RSV ARI often occurs after 6 months of age[26,45], in this study we were limited to early RSV infection under 6 months of age by design. We did not measure antibody titers in the first month postpartum. The study population was small (n=174), though larger than almost all studies measuring antigen-specific antibodies against respiratory pathogens (n= 5 to 258) [41] and than any study measuring RSV antibodies in breast milk (n=57 to 130)[4,46,47]. These results should be replicated in a larger group in a different population with longer follow up time. An additional limitation was the choice to measure antibodies against pre-F but not exclude antibodies that bind epitopes present on postfusion F protein (sites II, IV).

Antibodies that target only antigenic site ø show high neutralizing activity[48] and may correlate even better with protection from RSV. The protective effect of breast milk pre-F specific antibodies against respiratory disease may have been underestimated because antibodies against all pre-F epitopes were measured which include less potent RSV-neutralizing or non-neutralizing antibodies. Finally, we did not measure antibodies against G protein, which have recently also been shown to display neutralizing activity and correlate with decreased disease severity in infants and young children[49] and should be assessed in future studies.

In conclusion, the current study provides evidence that pre-F IgG antibodies in breast milk may play a protective role against RSV-confirmed ARI in the first 6 months of life. Induction of pre-F IgG in breast milk may be a potential mechanism of protection of maternal RSV vaccine candidates.

Acknowledgments:

We would like to acknowledge Marijn Hazelbag and Christiana Naaktgeboren for assistance with the power analysis. We would also like to thank Marijn Hazelbag and Julia Drylewicz for critical review of statistical analysis and consulting on statistical methods. We would like to acknowledge Shari Cho for aiding with stability testing of antibodies in breast milk. Finally, we would like to acknowledge Margaret Green for provision of the de-identified samples for assay development.

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Table/ Figure Legends

Table 1. Baseline characteristics of children with and without RSV ARI in the first 6 months of life (cases and controls). Maternal and pediatric clinical and sociodemographic characteristics were compared between cases and controls. The Intergrowth-21 criteria[50] were used to calculate small for gestational age. Differences in mean/median of continuous variables were tested with the two-sided t-test or a non-parametric Mann-Whitney test when appropriate. Categorical variables were described with frequencies and percentages and compared between groups using χ^2 test. N/A: not applicable. *: Asterisk marks variables used to match controls to cases.

Table 2. Median antibody titers in breast milk of all 174 mothers, and cases and controls separately for all time points combined. Log_{10} prefusion F (pre-F) IgA, pre-F IgG, total IgA, total IgG concentrations are shown for all children, and cases and controls separately. Pre-F IgA and pre-F IgG antibodies are expressed in nanograms (ng)/ milliliter (mL). Total IgA and total IgG antibodies are measured in milligrams (mg)/ mL.

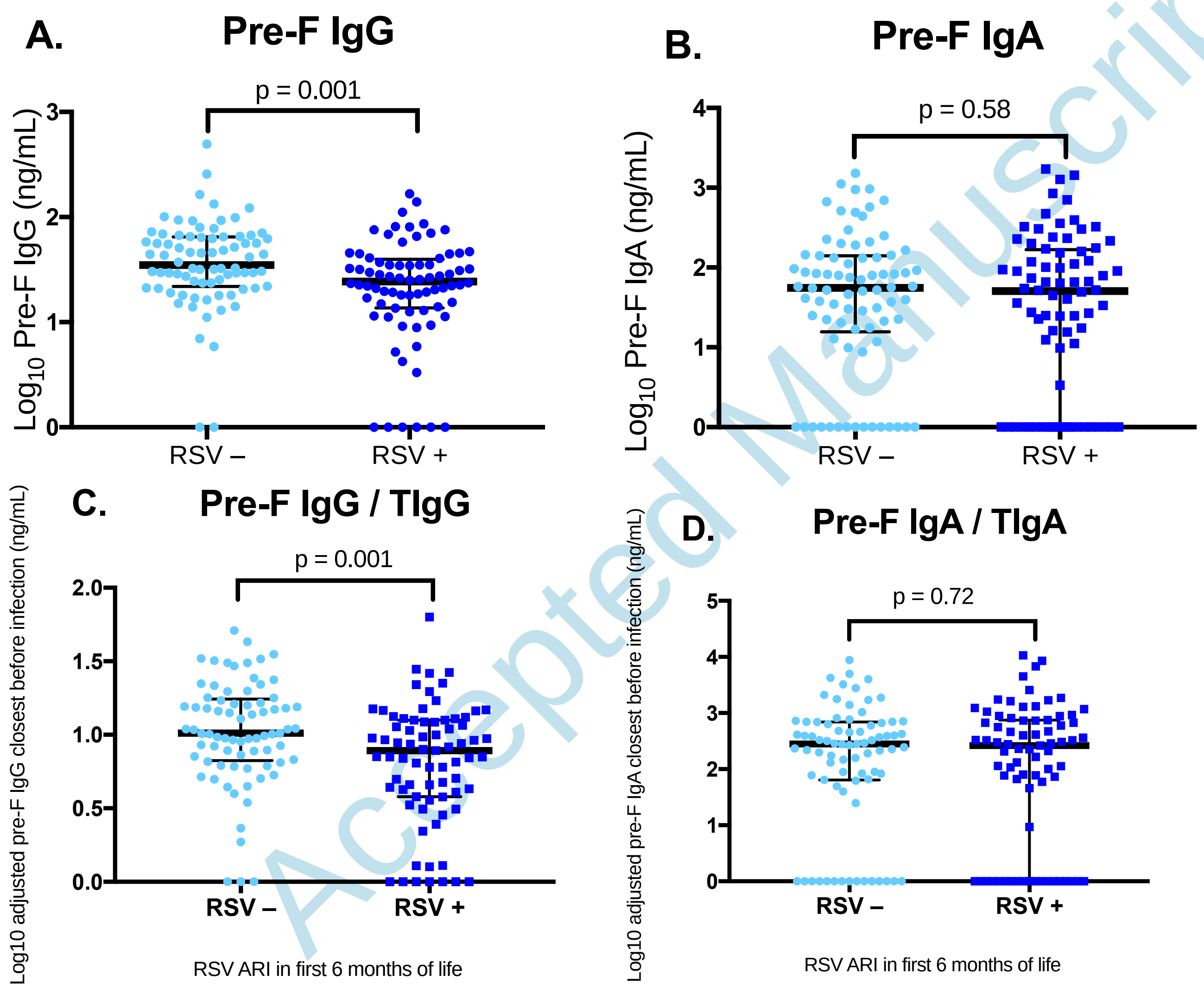
Figure 1. Pre-F antibody titers prior to time of infection in cases (RSV +) and matched controls (RSV –).

Mann-Whitney test was performed to compare medians of cases and controls. Pre-F antibody titer was compared for measurement prior to infection. For healthy controls antibody measurement at time of infection for age matched case was used. Ratio of pre-F IgA to total IgA was multiplied by $1x10^6$ in order to ensure values on the y-axis were greater than 0. Ratio of pre-F IgG and total IgG was multiplied by $1x10^4$ for the same reason. (A) Log_{10} pre-F IgG (B) Log_{10} pre-F IgA (C) Log_{10} pre-F IgG divided by log_{10} total IgG (D) Log_{10} pre-F IgA divided by log_{10} total IgA.

Figure 2. Mixed model analysis of pre-F antibody in cases and controls over time.

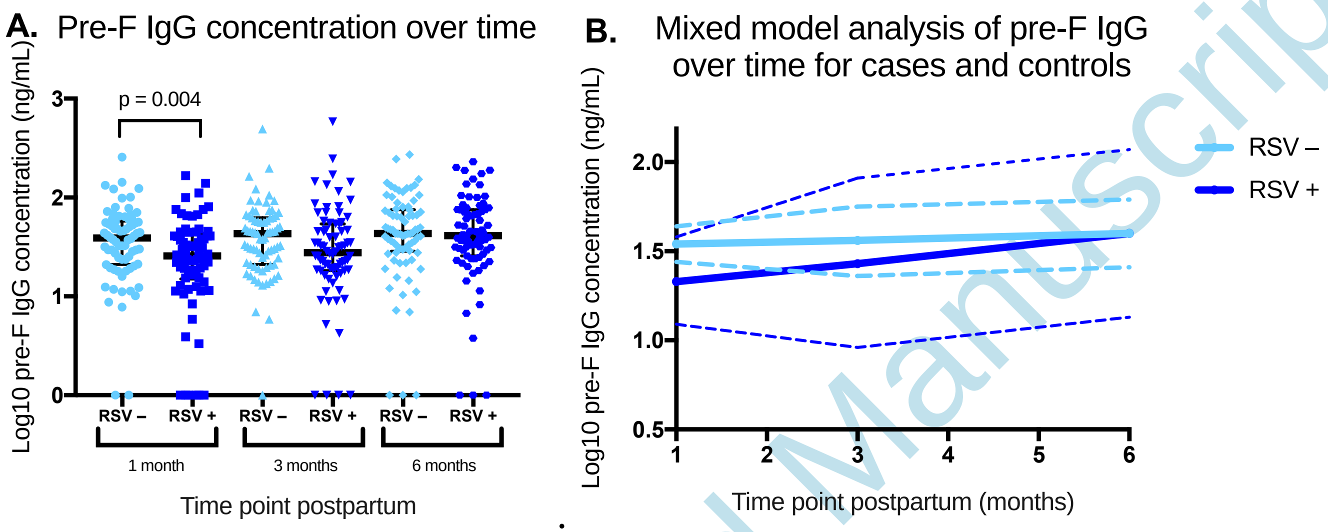
A linear mixed model analysis was used to examine the effect of RSV on pre-F antibodies at different time points. (A) Log_{10} pre-F IgG antibody concentration at 1, 3 and 6 months postpartum for cases (RSV +, dark blue) and controls (RSV –, light blue), with medians indicated in black. (B) Linear mixed model analysis for log_{10} pre-F IgG in cases and controls. Solid line is the mean and dashed line indicates the 95% confidence interval (CI). (C) Log_{10} pre-F IgA antibody concentration at 1, 3 and 6 months postpartum for cases and controls, medians in black. (D) Linear mixed model analysis for log_{10} pre-F IgA in cases and controls. Solid line is the mean and dashed line indicates the 95%CI.

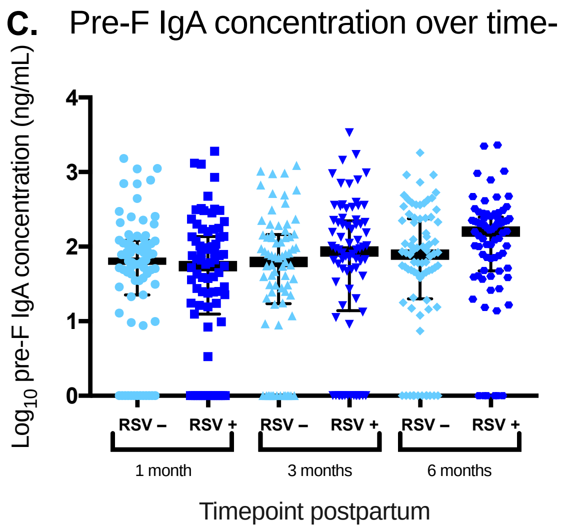
FIG 1.



A.
Pre-F IgG
p = 0.001
Log10 Pre-F IgG (ng/mL)
3
2
1
0
RSV –
RSV +
B.
Pre-F IgA
p = 0.58
Log10 Pre-F IgA (ng/mL)
4
3
2
1
0
RSV –
RSV +
C.
Pre-F IgG / TIgG
p = 0.001
Log10 adjusted pre-F IgG closest before infection (ng/mL)
2.0
1.5
1.0
0.5
0.0
RSV –
RSV +
RSV ARI in first 6 months of life
D.
Pre-F IgA / TIgA
p = 0.72
Log10 adjusted pre-F IgA closest before infection (ng/mL)
5
4
3
2
1
0
RSV –
RSV +
RSV ARI in first 6 months of life

FIG 2


A. Pre-F IgG concentration over time
Log10 pre-F IgG concentration (ng/mL)
p = 0.004
3
2
1
0
RSV –
RSV +
RSV –
RSV +
RSV –
RSV +
1 month
3 months
6 months
Time point postpartum
B. Mixed model analysis of pre-F IgG over time for cases and controls
Log10 pre-F IgG concentration (ng/mL)
2.0
1.5
1.0
0.5
1
2
3
4
5
6
Time point postpartum (months)
RSV –
RSV +


C. Pre-F IgA concentration over time-
Log10 pre-F IgA concentration (ng/mL)
4
3
2
1
0
RSV –
RSV +
RSV –
RSV +
RSV –
RSV +
1 month
3 months
6 months
Timepoint postpartum

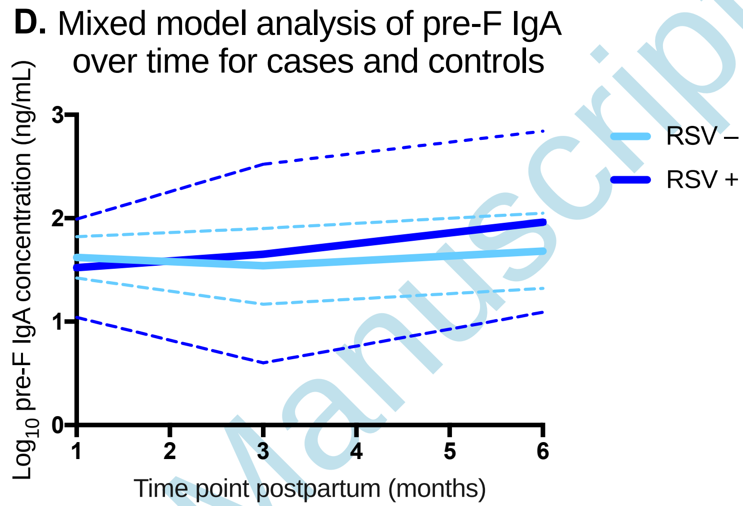

D. Mixed model analysis of pre-F IgA over time for cases and controls
Log10 pre-F IgA concentration (ng/mL)
3
2
1
0
1
2
3
4
5
6
Time point postpartum (months)
RSV –
RSV +

Table 1. Maternal and pediatric clinical characteristics of cases and controls

Characteristic	Cases (n=87)	Controls (n=87)	p-value
Maternal			
Median age (IQR)	22 (19 – 27)	22 (20 – 26)	0.64
Mean body mass index (SD)	21.0 (2.5)	20.7 (2.9)	0.55
Literacy, n/N (%)	47/82 (57.3)	47/81 (58.0)	0.93
Nulliparous, n/N (%)	31/87 (35.6)	35/87 (40.2)	0.53
Exclusive breastfeeding, n/N (%)	57/86 (66.3)	49/87 (56.3)	0.21
Household smoking, n/N (%)	3/82 (3.7)	4/81 (4.9)	0.72
Influenza vaccination*, n/N (%)	40/87 (46.0)	40/87 (46.0)	0.99
# respiratory episodes during pregnancy, n/N (%)	5/87 (5.8)	6/87 (6.9)	0.76
# respiratory episodes after delivery, n/N (%)	6/87 (6.9)	4/87 (4.6)	0.52
Pediatric			
Mean age at RSV illness, months (SD)	3.1 (1.5)	N/A	N/A
Mean birth weight, grams (SD)	2767 (401)	2802 (488)	0.63
Low birth weight, n/N (%)	15/75 (20.0)	20/74 (27.0)	0.31

Median gestational age (IQR)	40 (39– 41)	40 (39– 41)	0.33
Small for gestational age, n/N (%)	35/75 (46.7)	30/74 (40.5)	0.45
Preterm*, n/N (%)	6/87 (6.9)	6/87 (6.9)	0.99
Female sex, n/N (%)	40/87 (46.0)	38/87 (43.7)	0.76

Table 2. Antibody measured in breast milk at all time points combined, log-adjusted data

Breast milk antibody measured	**All** (n=454) (Log_{10} ng/mL)		**Cases** (n=227) (Log_{10} ng/mL)		**Controls** (n=227) (Log_{10} ng/mL)	
	Median	IQR	Median	IQR	Median	IQR
Prefusion IgA (n=450)	1.9	1.3 – 2.3	2.0	1.4 – 2.3	1.8	1.3 – 2.2
Prefusion IgG (n=449)	1.6	1.3– 1.8	1.5	1.3– 1.7	1.6	1.4 – 1.8
Total IgA (n=452)	5.3	5.2 – 5.4	5.3	5.2 – 5.4	5.3	5.2 – 5.4
Total IgG (n=447)	4.5	4.4 – 4.7	4.5	4.4 – 4.7	4.6	4.4 – 4.7