

Association of Human Milk Antibody Induction, Persistence, and Neutralizing Capacity With SARS-CoV-2 Infection vs mRNA Vaccination

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IMPORTANCE Long-term effect of parental COVID-19 infection vs vaccination on human milk antibody composition and functional activity remains unclear.

OBJECTIVE To compare temporal IgA and IgG response in human milk and microneutralization activity against SARS-CoV-2 between lactating parents with infection and vaccinated lactating parents out to 90 days after infection or vaccination.

DESIGN, SETTING, AND PARTICIPANTS Convenience sampling observational cohort (recruited July to December 2020) of lactating parents with infection with human milk samples collected at days 0 (within 14 days of diagnosis), 3, 7, 10, 28, and 90. The observational cohort included vaccinated lactating parents with human milk collected prevaccination, 18 days after the first dose, and 18 and 90 days after the second dose.

EXPOSURES COVID-19 infection diagnosed by polymerase chain reaction within 14 days of consent or receipt of messenger RNA (mRNA) COVID-19 vaccine (BNT162b2 or mRNA-1273).

MAIN OUTCOMES AND MEASURES Human milk anti-SARS-CoV-2 receptor-binding domain IgA and IgG and microneutralization activity against live SARS-CoV-2 virus.

RESULTS Of 77 individuals, 47 (61.0%) were in the infection group (mean [SD] age, 29.9 [4.4] years), and 30 (39.0%) were in the vaccinated group (mean [SD] age, 33.0 [3.4] years; $P = .002$). The mean (SD) age of infants in the infection and vaccinated group were 3.1 (2.2) months and 7.5 (5.2) months, respectively ($P < .001$). Infection was associated with a variable human milk IgA and IgG receptor-binding domain-specific antibody response over time that was classified into different temporal patterns: upward trend and level trend (33 of 45 participants [73%]) and low/no response (12 of 45 participants [27%]). Infection was associated with a robust and quick IgA response in human milk that was stable out to 90 days after diagnosis. Vaccination was associated with a more uniform IgG-dominant response with concentrations increasing after each vaccine dose and beginning to decline by 90 days after the second dose. Vaccination was associated with increased human milk IgA after the first dose only (mean [SD] increase, 31.5 [32.6] antibody units). Human milk collected after infection and vaccination exhibited microneutralization activity. Microneutralization activity increased throughout time in the vaccine group only (median [IQR], 2.2 [0] before vaccine vs 10 [4.0] after the first dose; $P = .003$) but was higher in the infection group (median [IQR], 20 [67] at day 28) vs the vaccination group after the first-dose human milk samples ($P = .002$). Both IgA and non-IgA (IgG-containing) fractions of human milk from both participants with infection and those who were vaccinated exhibited microneutralization activity against SARS-CoV-2.

CONCLUSIONS AND RELEVANCE In this cohort study of a convenience sample of lactating parents, the pattern of IgA and IgG antibodies in human milk differed between COVID-19 infection vs mRNA vaccination out to 90 days. While infection was associated with a highly variable IgA-dominant response and vaccination was associated with an IgG-dominant response, both were associated with having human milk that exhibited neutralization activity against live SARS-CoV-2 virus.

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JAMA Pediatr. doi:10.1001/jamapediatrics.2021.4897
Published online November 10, 2021.

The effect of COVID-19 on human milk composition remains poorly understood. Reassuringly, SARS-CoV-2 is rarely detectable in human milk samples, indicating human milk is unlikely a transmission risk to recipient infants.¹⁻⁴ Additionally, COVID-19 results in significant anti-SARS-CoV-2 antibody secretion into human milk. While current studies are small (15-22 individuals), individuals universally show a marked human milk IgA response with approximately 80% of individuals exhibiting IgA to SARS-CoV-2 receptor-binding domain (RBD) and/or spike proteins following infection.^{1,5-7} Anti-SARS-CoV-2 IgG and IgM are also detected in human milk postinfection but to a lesser extent, with one study showing that as many as 34% of lactating parents having no detectable anti-SARS-CoV-2 IgG or IgM in human milk.⁵ To our knowledge, the longest studies end at 3 months postinfection, limiting understanding of temporal trends of human milk antibody composition.⁷

To combat the COVID-19 pandemic, the US Food and Drug Administration granted emergency use authorization to the Pfizer-BioNTech (BNT162b2) and Moderna (mRNA-1273) vaccines in December 2020. Both vaccines use messenger RNA (mRNA) of the SARS-CoV-2 spike protein. The US Centers for Disease Control and Prevention recommended that pregnant and lactating parents receive these COVID-19 vaccines,⁸ despite not being included in initial vaccine trials. To date and to our knowledge, 5 peer-reviewed studies have investigated the human milk antibody response following mRNA vaccination with sample sizes between 5 and 84.⁹⁻¹³ These studies show that mRNA vaccination induces specific IgA, IgG, and IgM secretion into human milk⁹⁻¹³ and generally confirm that, in contrast to COVID-19 illness, human milk response to vaccination is IgG dominant,⁹ with IgA appearing approximately 2 weeks after the first dose. Neither IgA nor IgM antibodies increase after the second dose.¹² However, IgG antibody concentrations continue to rise after the second dose¹² and are more stable over time,^{10,11} with 97% of lactating parents exhibiting detectable human milk anti-SARS-CoV-2 IgG up to 6 weeks after the second dose.¹⁰ Longer-term studies are lacking.

Only 1 study assessed potential neutralizing activity, finding the neutralizing activity of human milk against a nonreplicating SARS-CoV-2 pseudovirus 3 weeks after the second dose of mRNA vaccination was approximately half the activity detected after maternal COVID-19 infection.⁹ Postvaccination neutralization assays using live wild-type virus are lacking. This research gap particularly limits our understanding of the protective effect of human milk after vaccination as antibody levels alone may not be a good proxy for protection and live neutralization assays do not always match pseudo-neutralization assay results. For example, 94% of human milk samples from lactating parents who recovered from COVID-19 exhibited pseudo-neutralization activity, while only 18% exhibited neutralization activity against wild-type virus.⁷

Herein, we compare the temporal pattern and neutralizing activity of anti-SARS-CoV-2 antibodies in human milk following natural COVID-19 infection vs SARS-CoV-2 mRNA vaccination. We further assessed human milk ability to neutralize live wild-type virus from each group.

Key Points

Question How does human milk antibody composition and neutralization activity differ between lactating parents with COVID-19 infection vs those with COVID-19 messenger RNA vaccination?

Findings In this cohort study of a convenience sample of 47 lactating parents with infection and 30 lactating parents who were vaccinated, antibody response in milk after infection was IgA dominant and highly variable while vaccination was associated with a robust IgG response, which began to decline by 90 days after the second vaccine dose. Milk from both groups showed neutralization activity against live SARS-CoV-2 virus, which can be attributed to IgA and IgG SARS-CoV-2 antibodies.

Meaning COVID-19 infection and vaccination may result in significant antibodies in human milk that exhibit different temporal patterns, but both neutralize live SARS-CoV-2 virus.

Methods

All participants self-identified their race and ethnicity from the following categories: American Indian or Alaskan Native, Asian, Black or African American, Hawaiian or Pacific Islander, Hispanic, Non-Hispanic, White, more than 1, prefer not to answer, or other. All participants completed questionnaires at each sample collection documenting participant/infant COVID-19 symptoms, breast health, and infant feeding practices. All participants provided written online informed consent. This study was approved by the institutional review boards at the University of Rochester Medical Center and NYU Grossman School of Medicine.

Infection Cohort

This prospective study took place between July 2020 and April 2021. Eligibility included individuals 18 years or older currently lactating with an infant 6 months or younger with a COVID-19 diagnosis (from polymerase chain reaction test) within the previous 14 days. Participants were recruited nationally via word of mouth and social media. Participants collected human milk samples in their home on day 0 (date participants received collection materials) and thereafter on days 3, 7, 10, 28, and 90.

Vaccinated Cohort

This prospective study took place between December 2020 and May 2021. Participants were health care professionals who were receiving the first dose of either Pfizer-BioNTech (BNT162b2) or Moderna (mRNA-1273) vaccines between December 2020 and January 2021 and also lactating with an infant of any age. Previous COVID-19 diagnosis was an exclusion criterion. Lactating parents provided a human milk sample prior to receiving the first dose of vaccine, 18 days after the first dose, 18 days after the second dose, and 90 days after the second dose. Participants were recruited using flyers placed in the University of Rochester Medical Center.

Sample Collection

Participants collected all samples in the home. Collection kits were assembled aseptically by study personnel wearing masks

and gloves and were individually packaged to reduce potential contamination. Participants were instructed in clean techniques to obtain samples, including use of gloves and masks.

All participants were provided a sterile manual breast pump (Harmony; Medela) and reusable microwave sterilization pouch with instructions regarding how to sterilize pump parts between collections. Participants in the COVID-19 infection cohort were instructed to wash their hands and breast with warm soapy water for 20 seconds before sample collection. Participants refrained from nursing or expressing milk for at least 2 hours before sample collection. Participants expressed milk from 1 breast until the milk stopped flowing and mixed milk with a provided syringe and aliquoted up to 5 mL into provided tubes and stored the samples in the home in a freezer at -20°C . Participants in the vaccine cohort were allowed to use a personal electric breast pump for milk collection if preferred.

Frozen samples were assembled for return shipping by participants using provided materials. Participants packaged samples with several frozen ice packs and sent packages via overnight shipping to the University of Rochester Medical Center laboratory for analysis. Once received, samples were stored at -80°C until analysis.

SARS-CoV-2 mRNA and Antibodies in Human Milk

Total RNA was extracted from human milk and used as the input for a reverse-transcription quantitative polymerase chain reaction (real-time polymerase chain reaction) against SARS-CoV-2, previously validated for use with human milk.¹

Human milk samples were spun at 10 000 G for 10 minutes at 4°C , and skim milk was removed for antibody assessment. Concentrations of IgA and IgG reactive to SARS-CoV-2 RBD protein were assessed via indirect enzyme-linked immunosorbent assay. In brief, Nunc MaxiSorp 96-well plates (Thermo Fisher Scientific) were coated with $2\ \mu\text{g}/\text{mL}$ of recombinant SARS-CoV-2 RBD (Sino Biologicals) in 0.1 M of sodium carbonate with a pH level of 8.0 overnight at 4°C . Coated plates were blocked with immunoglobulin-free human serum albumin in phosphate-buffered saline containing 0.04% Tween-20 for 1 hour and washed with phosphate-buffered saline containing 0.04% Tween-20. Human milk samples were diluted 1:20 in human serum albumin/phosphate-buffered saline containing 0.04% Tween-20, then incubated on the plate for 2 hours at room temperature. Plates were washed and bound IgG and IgA were detected with horseradish peroxidase-conjugated anti-human IgG and anti-human IgA (goat polyclonal; Bethyl). Bound antibody was detected using 3,3',5,5'-tetramethylbenzidine substrate (Becton-Dickinson) and measuring absorbance at 480 nm after color development. Antibody concentration was standardized using a known high-titer serum sample for both IgA and IgG. The standard curve was a 1:5 dilution series of 1:100 dilution of the reference serum, and sample Ig concentration was back calculated from such standard curve set such that the 1:100 dilution of the standard serum corresponds with 100 antibody units (AU). Thus, AU reported here provide concentration estimates that are more easily clinically interpretable compared with dilution factors measured as titers. Criteria for classifying human milk immunoglobulin response to illness and assessment of IgA assay background are included in the eMethods in the Supplement.

Human Milk IgA and non-IgA Fractions

As proof of concept to test the efficacy of IgA on neutralizing SARS-CoV-2, human milk samples were prepared into IgA and non-IgA fractions using peptide M agarose (Invivogen) using manufacturer's instruction. Details of the extraction are presented in the eMethods in the Supplement.

SARS-CoV-2 Neutralization

The neutralizing activity of human milk against SARS-CoV-2 was measured by microneutralization assay as previously described¹ in a subset of samples from participants with infection ($n = 10$) and who were vaccinated ($n = 20$) chosen randomly from those who exhibited a high antibody response in the first month following infection or first vaccine dose. Vero E6/TMPRSS2 cells were used (provided by Yoshihiro Kawaoka, National Institute of Infectious Diseases, Japan).¹⁴ Detailed assay procedures are provided in the eMethods in the Supplement.

Statistical Analyses and Reporting

Details of sample size determination are presented in the eMethods in the Supplement. For variables that are normally distributed, means (SDs) are reported; otherwise, medians (IQRs) are reported. Analyses were performed using R version 3.6.1 (R Foundation) and ggplot2 version 3.3.2. Antibody data between groups and time points were compared using paired and independent t test of log-transformed values. Comparisons of microneutralization titers between groups and time points was performed using permutation testing using functions `wilcox_test` (Wilcoxon rank sum test) and `wilcoxsign_test` (Wilcoxon signed rank test) from package `coins` for independent and paired data, respectively.¹⁵ Trendlines were generated using loess method in ggplot2 package. Two-sided P values were statistically significant at .05. Reporting of results follows the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines.¹⁶

Results

Cohort Characteristics

Characteristics of infection and vaccinated cohorts are presented in Table 1. Of 77 individuals, 47 (61.0%) were in the infection group, and 30 (39.0%) were in the vaccinated group. Eleven participants from the infection group were originally identified from a COVID-19 diagnosis resulting from standard screens at delivery, and 37 were identified via social media advertising. A flow diagram of eligibility screening and participant follow-up for the infection cohort is presented in eFigure 1 in the Supplement. The mean (SD) age of participants in the infection and vaccinated group was 29.9 (4.4) years and 33.0 (3.4) years, respectively ($P = .002$). The mean (SD) age of infants in the infection and vaccinated groups was 3.1 (2.2) months and 7.5 (5.2) months, respectively ($P < .001$). There was no difference in the racial distribution between groups, with 40 individuals in the infection group (85.1%; 95% CI, 72.3%-92.6%) and 29 individuals in the vaccinated group (96.7%; 95% CI, 83.3%-99.4%) identifying as White ($P = .39$). The infection group had a higher proportion of Hispanic individuals (12

Table 1. Cohort Characteristics

Characteristic	No. (%)		P value ^a
	Infection group (n = 47)	Vaccinated group (n = 30)	
Age, mean (SD) [range], y	29.9 (4.4) [20-38]	33.0 (3.4) [25-42]	.002 ^a
Race and ethnicity			
Race			
American Indian, Asian, or Black ^b	3 (6.3)	1 (3.3)	.39
White	40 (85.1)	29 (96.7)	
Other ^c	3 (6.4)	NA	
Ethnicity			
Hispanic	12 (25.5)	NA	<.001 ^a
Non-Hispanic	24 (51.1)	26 (86.7)	
Not reported	11 (23.4)	4 (13.3)	
BMI, mean (SD) [range] ^d	28.8 (6.4) [20.2-45.2]	27.1 (6.8) [19.9-45.1]	.27
Parity, mean (SD) [range]	2.2 (1.1) [1-5]	2.3 (1.3) [1-5]	.31
Gestational age at delivery, mean (SD) [range], wk ^e	39.0 (1.3) [36.0-41.5]	38.9 (1.8) [33.0-41.0]	.78
Birth weight, mean (SD) [range], g	3473 (439) [2735-4621]	3236 (540) [1644-4046]	.04 ^a
Infant sex			
Male	24 (51)	13 (43.3)	.51
Female	23 (49)	17 (56.7)	
Infant age, mean (SD) [range], mo	3.1 (2.2) [0.2-9.0]	7.5 (5.2) [0.5-23.4]	<.001 ^a
Breastfeeding exclusivity at day 0 or after dose 1, % human milk feeds ^f			
Mean (SD)	0.92 (0.18)	0.96 (0.15)	.32
Exclusively breastfeeding	36 (77)	27 (90)	
Vaccine			
BNT162b2 (Pfizer-BioNTech)	NA	12 (40)	NA
mRNA-1273 (Moderna)	NA	18 (60)	
Household member ever diagnosed with COVID-19	28 (59.6)	5 (16.7)	<.001 ^a
Infant			
Ever tested	17 (36)	13 (43)	.53
Ever diagnosed with COVID-19	9 (19)	2 (6.7)	

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); NA, not applicable.

^a P value for comparison between groups. Statistically significant P values are indicated.

^b These groups were combined to maintain anonymity of participants.

^c Other included self-report of Iranian American and unknown.

^d Data on 46 individuals from the infection group were reported.

^e Data on 45 individuals from the infection group were reported.

^f Breastfeeding exclusivity was calculated as percentage of total human milk/infant formula feeds that were human milk in a 24-hour period. Data on 46 individuals from the infection group were reported.

[25.5%] [95% CI, 15.3%-39.5%] vs 0%; $P < .001$), likely owing to a national recruitment pool and expanded potential eligibility in the infection group.

In the infection cohort, day 0 was a mean (SD) of 10.0 (8.3) days following the most recent reported positive polymerase chain reaction test result. Of 47 participants in the infection group, 42 (89%) experienced COVID-19 symptoms (eFigure 2 in the Supplement). The most commonly reported symptoms were loss of smell/taste (32 [66%]), headache (30 [64%]), and fatigue (29 [62%]).

All participants in the vaccinated cohort were recruited and consented within 14 days, based on earliest availability. Throughout this time period, the study team received approximately 300 inquiries per day. Among 30 vaccinated individuals, 12 (40%) received the Pfizer-BioNTech vaccine, whereas 18 (60%) received the Moderna vaccine.

SARS-CoV-2 mRNA and Antibodies in Human Milk Samples

SARS-CoV-2 mRNA was not detected in any human milk samples from either the infection or vaccinated cohort. Temporal changes in SARS-CoV-2 IgA and IgG within the group of participants with infection are presented in Figure 1. Antibody levels were compared with prevaccine levels in the vaccination cohort, assuming the majority of these participants had not had infection. Three patterns of antibody responses

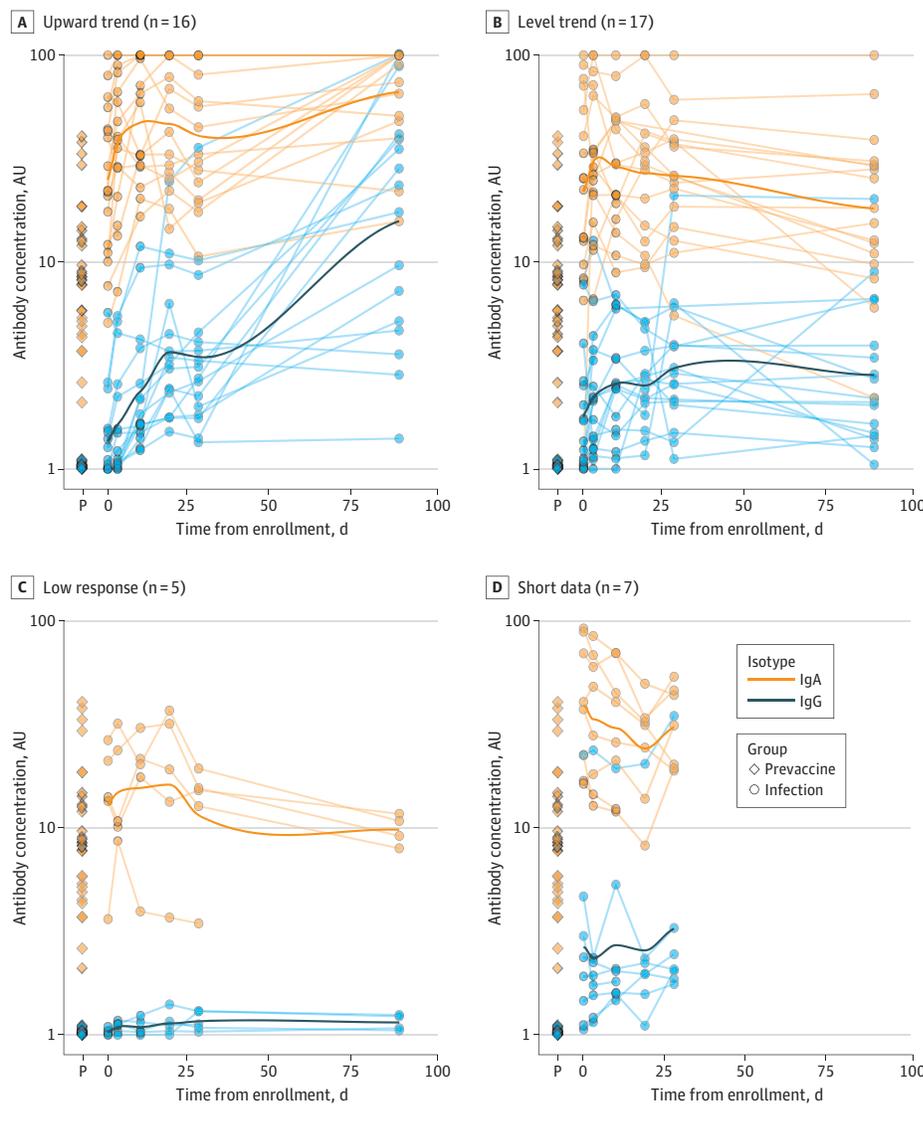
were identified: group A, initial IgA and IgG response with an upward trending levels up to 90 days (16 individuals [35.6%]); group B, initial IgA and IgG response with level trend up to 90 days (17 individuals [37.8%]); and group C, poor IgA and no IgG antibody response (5 individuals [11.1%]). A fourth group (group D; 7 [15.6%]) lacked long-term follow-up samples, and thus temporal patterns were not established. Table 2 provides the group means (SDs) of antibody concentrations at each point by temporal category of antibody response.

In groups A and B, IgA antibodies were already elevated on day 0 (mean [SD], 10.0 [8.3] days after diagnosis) of a mean (SD) of 34.2 (27.1) and 35.4 (31.3) AU in groups A and B, respectively. IgA response was large compared with pre-COVID-19 concentrations and lasted at least until 90 days after enrollment and was accompanied by a more modest IgG response (Figure 1).

Human Milk SARS-CoV-2 Antibodies in the Vaccinated Group

Temporal changes in SARS-CoV-2 IgA and IgG within the vaccinated cohort are presented in Figure 2. Compared with the infection group, vaccination was associated with a more uniform and elevated IgG response at 18 days after the first dose (mean [SD], 1.3 [1.3] AU prevaccination; mean [SD], 12.0 [17.8] AU at 18 days after the first dose; mean of differences, 11.2 [95% CI, 4.75-17.7]; $P = .001$), which further increased after the

Figure 1. Anti-SARS-CoV-2 IgA and IgG Antibody Levels in Human Milk Over Time After COVID-19 Infection



The IgA and IgG concentrations (antibody units [AU]/mL) of receptor-binding domain-reactive antibodies against SARS-CoV-2 are shown, and 4 patterns were identified: upward trending for IgA and IgG (16 [35.6%]) (A); level trend (17 [37.8%]) (B); poor antibody response (5 [11.1%]) (C); and long-term follow-up sample missing (7 [15.6%]) (D). Each dot presents the mean concentration of measurements in duplicate. The first sample was collected on day 0, which is the day of receiving collection kits (mean [SD], 10.0 [8.3] days after diagnosis by polymerase chain reaction); thereafter, samples were collected on days 3, 10, 19, 28, and 90. Shown in each panel is the antibody concentration of human milk collected prior to vaccination in the vaccine group.

booster shot (mean [SD], 44.2 [21.5] AU at 18 days after the second dose; mean difference to before vaccination, 43.0 AU [95% CI, 35.1-50.8 AU]; $P < .001$), compared with before the vaccination (mean [SD], 1.3 [1.3] AU). IgG responses decreased thereafter. At 90 days after the second dose, the IgG levels still had a higher mean than those observed during infection (mean [SD], 15.5 [28.2] AU at 90 days after infection; mean [SD], 29.4 [25.4] AU at 90 days after second dose of vaccination; $P = .048$). IgA response was uptrending at 18 days after the first dose; however, no further increase was seen after the second dose. With few exceptions, antibody levels somewhat decreased following the second dose, although they were still significantly higher compared with prevaccination levels (mean [SD], 12.0 [10.3] AU before vaccination; mean [SD], 30.3 [29.6] AU at 90 days after second dose; mean of differences, 18.1 AU [95% CI, 8.6-27.6]; $P < .001$). IgA antibody levels of participants after the first vaccine dose were comparable with those seen in participants with infection.

Human Milk SARS-CoV-2 Neutralizing Activity

Microneutralization titers from human milk of 10 participants with infection and 20 vaccinated participants are shown in Figure 3A. In the infection group, 5 of 10 samples had neutralizing antibodies already on day 0 (mean [SD], 10.0 [8.3] days after diagnosis). At days 28 and 90, microneutralization titer was significantly elevated above prevaccine titers (day 28: median [IQR], 20 [67.5]; $P < .001$ vs day 90: median [IQR], 10 [150]; $P < .001$ vs day 0: median [IQR] 2.2 [0]). Eight of 10 samples at day 28 and 9 of 9 samples at day 90 showed neutralizing activity. In the vaccinated group, 4 samples had neutralizing activity prevaccination, possibly owing to previous exposure to SARS-CoV-2. These 4 samples exhibit a wide range in both IgA and IgG antibody concentrations (shown in the yellow circles, Figure 3B). Human milk microneutralization titer increased from prevaccine (median [IQR], 2.2 [0]) to 18 days after the first dose (median [IQR], 5 [4.0]; $P = .01$) with 12 of 20 samples (60%) exhibiting neutralization activity. Human milk microneutraliza-

Table 2. Anti-SARS-CoV-2 Receptor-Binding Domain IgA and IgG Concentrations After Infection

Isotype	Temporal pattern ^a	Collection day											
		0		3		10		19		28		90	
		Mean (SD)	P value	Mean (SD)	P value	Mean (SD)	P value	Mean (SD)	P value	Mean (SD)	P value	Mean (SD)	P value
IgA ^b	A	34.2 (27.1)	<.001	48.2 (31.0)	<.001	56.6 (33.2)	<.001	57.0 (34.4)	<.001	51.4 (33.9)	<.001	75.2 (30.7)	<.001
	B	35.4 (31.3)	.01	40.5 (30.8)	<.001	35.8 (24.9)	<.001	35.6 (29.3)	<.001	32.5 (23.1)	<.001	26.0 (24.3)	.01
	C	15.7 (8.6)	.34	17.0 (10.2)	.12	18.7 (9.5)	.19	21.0 (13.5)	.22	13.2 (6.0)	.47	9.9 (1.7)	.54
	D	47.9 (31.2)	<.001	41.8 (27.3)	<.001	37.0 (23.4)	.001	27.7 (13.8)	.004	33.3 (14.5)	<.001	NA	NA
IgG ^b	A	1.6 (1.2)	.03	2.0 (1.6)	.01	3.1 (3.1)	<.001	5.2 (5.8)	<.001	5.6 (8.4)	<.001	31.9 (35.0)	<.001
	B	2.4 (2.2)	.004	2.9 (2.9)	<.001	3.1 (2.0)	<.001	2.8 (1.3)	<.001	4.2 (4.7)	<.001	4.1 (4.7)	<.001
	C	1.0 (0.0)	.52	1.1 (0.1)	.1	1.1 (0.1)	.21	1.1 (0.2)	.17	1.2 (0.1)	.06	1.2 (0.1)	.09
	D	4.8 (7.3)	.03	4.5 (7.8)	<.05	4.4 (6.2)	.02	4.5 (7.0)	.04	6.9 (12.3)	.03	NA	NA

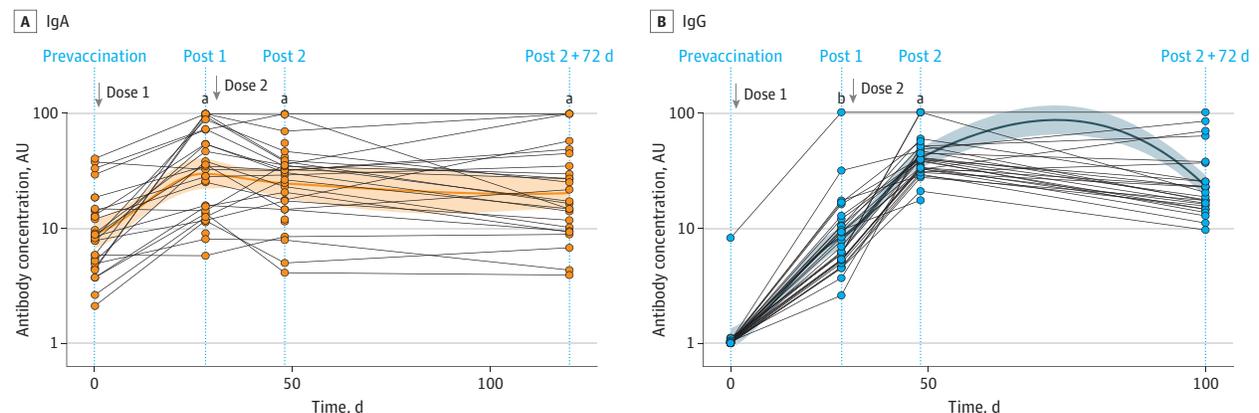
Abbreviation: NA, not applicable.

^a Temporal pattern of response was classified into 3 categories of antibody response: group A, initial IgA and IgG response with an upward trending levels up to 90 days (16 [35.6%]); group B, initial IgA and IgG response with level trend up to 90 days (17 [37.8%]); and group C, poor IgA and no IgG antibody

response (5 [11.1%]). A fourth group (group D; 7 [15.6%]) lacked long-term follow-up samples, and thus temporal patterns were not established.

^b Concentrations are expressed as mean (SD) in antibody units followed by a P value that is calculated using unpaired t test in comparison with corresponding isotype anti-receptor-binding domain levels in prevaccinated healthy controls.

Figure 2. Anti-SARS-CoV-2 IgA and IgG Antibody Levels in Human Milk Over Time After SARS-CoV2 Messenger RNA Vaccination



The IgA and IgG concentrations (antibody units [AU]/mL) of receptor-binding domain-reactive antibodies against SARS-CoV-2 are shown. Each dot presents the mean concentration of measurements in duplicate. The first sample was collected prevaccination and then 18 days after first vaccination (post 1; mean [SD], 18.1 [1.7] days after the first dose), 18 days after second vaccination (post 2; mean [SD], 18.6 [2.8] days after the second dose), and 90 days after second vaccination (post 2 + 72 d; mean [SD], 83.2 [17.3] days after the second dose).

For the statistical comparison, paired t test for each time point compared with concentrations prevaccine were conducted. P values are shown on the top of the graph.

^a P < .001.

^b P = .001.

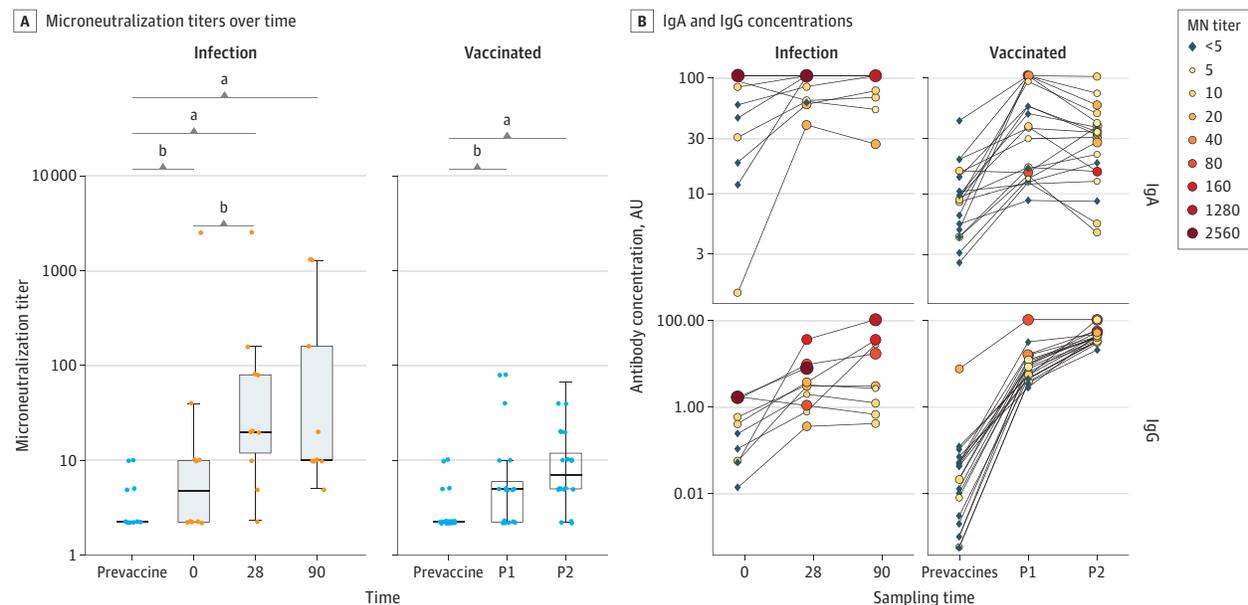
tion titer remained elevated at 18 days after the second dose (median [IQR], 7.5 [7.5]; P < .001) with 17 of 20 samples (85%) exhibiting microneutralization activity. Microneutralization titer did not differ between 18 days after the first dose and 18 days after the second dose. Microneutralization titer of the vaccine group at 18 days after first dose was significantly lower than microneutralization titer of the infection group at day 28 (P < .001, Wilcoxon rank sum test). In Figure 3B, neutralization titers are plotted twice: once together with IgA and once with IgG levels. There was no consistent association between human milk IgA or IgG concentrations and neutralizing activity at the various points. Figure 4 shows the neutralizing titers of both IgA and non-IgA human milk fractions from 3 participants with infection and

3 vaccinated participants. In these proof-of-concept experiments, both the IgA and non-IgA fraction showed similar neutralizing capacity with 2 vaccinated samples showing a higher microneutralization titer with non-IgA (ie, IgG containing) fraction. No significant differences were observed in human milk IgA or IgG levels or microneutralization titers between participants vaccinated with Moderna vs Pfizer vaccines.

Discussion

We demonstrate different kinetics and concentrations of anti-SARS-CoV-2 antibodies in human milk between lactating par-

Figure 3. Anti-SARS-CoV-2 IgA and IgG RBD Antibody Levels in Human Milk Compared With Microneutralization Activity



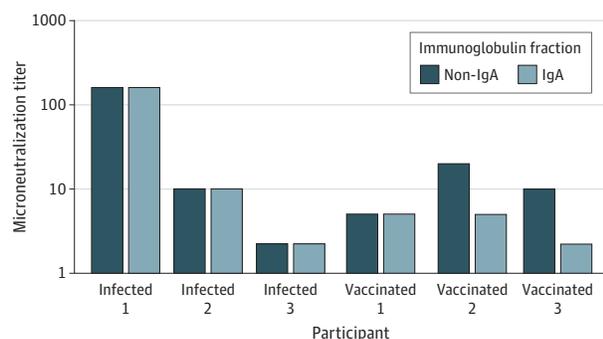
A, Microneutralization (MN) titers of participants with infection (n = 10) and vaccinated participants (n = 20) over time. In the infection group, day 0 indicates the first sample collection (mean [SD], 10.0 [8.3] days after diagnosis). B, MN titer plotted against both human milk IgA concentrations (top panel) and IgG concentrations (bottom panel) in the infection (left panel) and vaccinated (right panel) groups. AU indicates antibody unit; prevaccine, MN

titer from the prevaccination samples in the vaccine group; P1, 18 days after the first dose; P2, 18 days after the second dose.

^a $P < .001$.

^b $P < .05$.

Figure 4. Anti-SARS-CoV-2 Microneutralization Activity in the IgA vs Non-IgA Fractions of Human Milk



Shown is the microneutralization titer of human milk samples from 3 randomly selected participants with infection and vaccinated participants. Human milk was collected at day 28 in participants with infection and 18 days after the second dose in vaccinated participants. IgA fraction from each sample was isolated yielding an IgA-containing fraction and a non-IgA (IgG-containing) fraction.

ents with infection and vaccinated lactating parents. Infection resulted in a relatively universal rapid and long-lasting IgA response compared with more variable IgG response. In contrast, vaccination results in an initially lower human milk IgA response that increased after the first vaccine dose and then decreased after the second dose. Conversely, human milk IgG response to vaccination was uniformly larger and more stable compared with lactating parents with infection. Human milk showed

neutralizing activity in both infection and vaccinated groups with slightly higher activity in the infection group. Neutralizing activity can be likely attributed to both IgA and IgG.

COVID-19 infection was associated with a rapid IgA response in human milk, elevated above the vaccine group's prevaccine levels by 10 days after diagnosis and lasting at least 90 days. Human milk IgG response to infection was highly variable. In the majority of the participants, IgG response was not detected on day 0 but did increase over time. While 73% of participants' human milk demonstrated an upward or level trend in IgA and IgG antibodies out past 90 days postinfection, 11% of human milk showed a low/no response to infection, demonstrating significant interindividual variation. Collectively, we conclude that the first antibody upregulated in human milk due to COVID-19 infection is IgA; this response is long-lasting in most individuals (89%). This IgA-dominant human milk response to infection confirms other preliminary work.^{5,7}

Compared with infection, mRNA vaccination was associated with a larger IgG response after the first dose with further increases after the second dose; however, IgG responses decreased thereafter. Previous studies of anti-SARS-CoV-2 spike IgG demonstrate sustained high concentrations in human milk post-vaccine but end follow-up at 6 weeks after the first dose,¹⁰ likely missing the decrease over time captured in our longer follow-up. Still, at 90 days after the second dose, the IgG levels were higher than those generated as a result of infection, which corroborates other non-peer-reviewed reports comparing human milk IgG from vaccinated lactating parents vs lactating parents with infection.¹⁷ Human milk IgA levels after the first dose of vac-

ination were comparable with human milk from parents with infection; however, this response was not long-lasting and deteriorated over time in the majority of individuals, despite receipt of the second dose. This contrasts with others' data on human milk spike protein IgA postvaccination, which shows increases following each dose and may indicate antigen-specific responses.¹³ Interestingly, the mean IgA (but not IgG) levels were already positive prevaccination. This may indicate polyreactivity of IgA responses in human milk or, less likely, cross-reactivity with other seasonal coronaviruses.^{1,6} Any such cross-reactivity reflected in our detection of anti-RBD IgA would artificially inflate the concentrations detected but may represent clinically relevant IgA concentrations that are capable of binding SARS-CoV-2 RBD.

To our knowledge, this study has the longest follow-up of human milk postvaccine compared with previously published studies. In addition, the human milk sampling was well controlled and analogous in the infection and vaccinated cohort, allowing for accurate description of differences in the temporal human milk response between groups. The differential antibody response between infection and vaccination is somewhat anticipated as mucosal exposure would be expected to elicit an IgA response, whereas systemic vaccination would preferentially induce an IgG response.

Importantly, whether a dominant IgA or IgG response, both infection and vaccination generated human milk with neutralizing activity. Among other benefits, human milk provides protection against morbidities including respiratory and diarrheal illnesses, owing to specific and non-specific immune factors including antibodies.¹⁸⁻²⁰ Studies on human milk antibodies to respiratory pathogens have focused on associations between maternal vaccination during pregnancy and human milk IgG and IgA levels to pertussis, pneumococcus, influenza, and meningococcus,^{21,22} but few studies assess function/clinical benefit from these antibodies. A Bangladeshi study reported significantly higher vaccine-specific, viral-neutralizing IgA levels to influenza A/New Caledonia in human milk over time among vaccinated lactating parents.²³ Additionally, infants exclusively breastfed by parents vaccinated against influenza during pregnancy had significantly fewer respiratory febrile illnesses in the first 6 months.²³ We recently showed that unlike IgG antibodies, influenza virus hemagglutinin-specific IgA antibody levels and patterns in human milk were mostly discordant compared with serum, providing specific, passive protection to the infant.²⁴

In a previous study of lactating parents with COVID-19 infection or who are suspected of having infection, 79% of human milk exhibited SARS-CoV-2 antibodies, and 94% of human milk samples were capable of neutralizing a pseudovirus of SARS-CoV-2.⁷ However, only 18% of human milk was capable of neutralizing wild-type SARS-CoV-2,⁷ emphasizing the necessity to test neutralization capacity with wild-type virus. Indeed, our results suggest that 80% of samples exhibit neutralization activity 28 days postinfection and 100% at 90 days postinfection. These data are the first to demonstrate this neutralization activity in human milk postvaccination, to our knowledge. A study of human milk 3

weeks after the second dose of mRNA vaccine ($n = 16$) showed that human milk had approximately half of the neutralization activity against a SARS-CoV-2 pseudovirus compared with human milk from lactating parents after infection ($n = 5$), but most human milk postvaccination exhibited some pseudo-neutralization activity.⁹ Our findings corroborate these initial data with wild-type virus assays, showing that 60% of human milk samples exhibit neutralization activity after the first dose and 85% after the second dose. Together, this supports the likelihood of human milk providing infant protection proceeding lactating parental SARS-CoV-2 infection or immunization.

Limitations

This study contains sources of potential bias. For example, most participants in the infection group were identified via social media marketing. This preselects for participants who have the fiscal and time resources to partake in social media. This group also had different exclusion criteria on infant age (≤ 6 months) compared with the vaccinated group (no upper limit on infant age). Additionally, the vaccinated group demographics were narrow, given that the vaccine was only available to health care professionals at the time of recruitment. This explains the older age and higher education level of the vaccinated group and introduces bias to the study. It is a limitation that only mRNA vaccinations were included as alternative vaccines were not yet available in the US. Recent non-peer-reviewed data suggest that mRNA vaccine may result in enhanced human milk IgG response compared with adenovirus-vectored vaccines.¹⁷ While our study presents the longest published follow-up of human milk postvaccination to our knowledge, larger and longer-term studies are needed including parents breastfeeding older children. Owing to its importance in infectivity, we focus on anti-SARS-CoV-2 antigen (RBD) while antibodies to other known antigens of the spike protein and nucleocapsid are detectable in human milk.^{4,5,13} We use the samples taken prior to receiving the first dose of vaccine from the vaccine cohort as a baseline for comparison with the immunoglobulin concentrations detected in the infection cohort. While both assays detect specific IgA and IgG anti-RBD antibodies, the inherent background of the IgA assay is greater than that of the IgG assay.

Conclusions

In this cohort study of lactating parents, SARS-CoV-2 mRNA vaccination was found to be associated with a robust IgG-predominant response in human milk that began to decline by 90 days after the second vaccine dose. This is in contrast to antibody response to infection, which was variable and IgA dominant with 73% of participants showing an upward or level trend in human milk antibodies out past 90 days after infection. Both illness and vaccination resulted in human milk with neutralization activity against live wild-type SARS-CoV-2. Our data suggest that both IgA and IgG contribute to the neutralizing capacity, implying clinical benefit to infants receiving human milk from parents with COVID-19 infection or who are vaccinated.

ARTICLE INFORMATION

Accepted for Publication: October 5, 2021.

Published Online: November 10, 2021.
doi:10.1001/jamapediatrics.2021.4897

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Obtained funding: Young, Ferri-Huerta, Järvinen.

Administrative, technical, or material support: Seppo, Diaz, Rosen-Carole, Nowak-Wegrzyn, Cruz Vasquez, Ferri-Huerta, Nguyen-Contant, Fitzgerald, Topham, Järvinen.

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Conflict of Interest Disclosures: Dr Young reported grants from the National Institute of Diabetes and Digestive and Kidney Diseases and the Gerber Foundation outside the submitted work during the conduct of this study. Dr Seppo reported grants from the National Institute of Allergy and Infectious Diseases (NIAID) during the conduct of the study. Dr Rosen-Carole reported grants from the National Institutes of Health during the conduct of the study. Dr Nowak-Wegrzyn reported grants from the NIAID during the conduct of the study. Dr Järvinen reported grants from the NIAID during the conduct of the study and grants from Janssen outside the submitted work. No other disclosures were reported.

Funding/Support: This work was supported by the National Institute of Allergy and Infectious Diseases

(grant U01 AI131344-04S1) and in-kind support from Medela.

Role of the Funder/Sponsor: Neither funder had a role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Additional Contributions: We thank Kaili Widrick, MS, Katherine Murphy, Miranda Klein, MPH, and Elizabeth Catlin, CNM, for their technical assistance. All individuals are employees of the University of Rochester and were not compensated outside of their standard salary. We are very grateful to the families that graciously donated their time and milk to this study.

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