SARS-CoV-2 Antibodies in Breast Milk After Vaccination

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SARS-CoV-2 Antibodies in Breast Milk After Vaccination

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**Abbreviations:**

HM: human milk.

slgA: secretory immunoglobulin A

slgM: secretory immunoglobulin M

IgG: immunoglobulin G

RNA ribonucleic acid

**Article Summary.** The covid-19 vaccine in breastfeeding mothers yield passive antibody transfer and could be a protective measure against the disease in children of any age.

**What’s Known on This Subject:**

Recently, two studies have published the presence of antibodies against SARS cov 2 in human milk after breastfeeding mother vaccination.

**What This Study Adds:**

Mothers with different duration of breastfeeding were recruited. The interesting finding was the greater impact of vaccination on immunoglobulins in human milk with lactations greater than 23 months. This a lactation time specific effect on immunoglobulins, independent of other variables.
Contributors’ statement page:

- Dolores Sabina Romero Ramírez*, conceptualized, designed the study, coordinated, supervised data collection. She drafted the initial manuscript and revised the manuscript.
- María Magdalena Lara Pérez*, and Mercedes Carretero Pérez*, PharmG, MSc, MPH, drafted the initial manuscript, coordinated, supervised data collection and revised the manuscript.
- María Isis, Suárez Hernández and Ana María Fernández Vilar: conceptualized, designed the study and revised the manuscript.
- Saúl Martín Pulido Hospital, Lorena Pera Villacampa, Mónica Rivero Falero, Paloma González Carretero, Beatriz Reyes Millán designed the data collection instruments, collected data reviewed and revised the manuscript.
- Sabine Roper, traduction and revised critically manuscript.
- Miguel Ángel García Bello worked in statistic analysis, interpretation of data and revised the manuscript.

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.
Abstract

BACKGROUND AND OBJECTIVES: Passive and active immunity transfer through human milk (HM) constitutes a key element in the infant's developing immunity. Certain infectious diseases and vaccines have been described to induce changes in the immune components of HM.

METHODS: We conducted a prospective cohort single institution study from February 2 to April 4, 2021. Women who reported to be breastfeeding at the time of their COVID-19 vaccination were invited to participate. Blood and milk samples were collected on day 14 after their second dose of vaccine. IgG antibodies against nucleocapsid protein as well as IgG and IgM antibodies against the spike 1 protein receptor-binding domain (RBD) were analyzed in both serum and HM. The latter was tested for IgA, IgM, and IgG antibodies against SARS-CoV-2.

RESULTS: Most of the participants, ie, 94%, received the BNT162b2 mRNA COVID-19 vaccine and 6% the mRNA-1273 COVID-19 vaccine.

The mean serum concentration of IgG antibodies against the SARS-CoV-2 spike protein RBD in vaccinated individuals was 3379.6±1639.5 BAU/mL. All vaccinated study participants had IgG antibodies and 89% of them IgA antibodies against SARS-CoV-2 in their milk. IgA and IgG antibody concentrations in the milk of mothers who were breastfeeding ≥24 months were significantly higher than in mothers with breastfeeding periods <24 months (P <.001).

CONCLUSIONS: We found a clear association between COVID-19 vaccination and specific immunoglobulin concentrations in HM. This effect was more pronounced when lactation periods exceeded 23 months. The influence of the lactation period on immunoglobulins was specific and independent of other variables.

INTRODUCTION

Transfer of passive and active immunity through human milk (HM) is a key element in the infant protection against infections1. The mucosa is the point of entry for at least 90% of microorganisms; so the immunomodulatory capacity conferred by HM is important from the neonatal period on.1,2 Breastfed infants are better protected against different infectious diseases, like gastroenteritis, otitis media, urinary tract infection, neonatal sepsis, and necrotizing enterocolitis,3 as well as respiratory infections4 with a reduced frequency, duration, and risk of hospitalization than formula-fed infants.5-8
Protection through HM may go beyond cessation of breastfeeding, although not all the mechanisms are well known.9

HM includes many bioactive factors, such as secretory immunoglobulin A (sIgA), secretory immunoglobulin M (sIgM), immunoglobulin G (IgG), oligosaccharides, maternal glycoproteins, cytokines, nucleic acids, and leukocytes, which promote the baby's developing immunocompetence. Immunoglobulins are the most studied immunoprotective components in HM.10 Secretory IgA is the main isotype and is considered dominant in protecting the infant's mucosal surfaces without stimulating a substantial inflammatory response, ie, by intracellular neutralization, immune exclusion, and virus excretion.11,12 Second most abundant is pentameric sIgM, which activates the complement cascade and causes agglutination of recognized pathogens and innate immunological activities.9,11,13 IgG represents a lower proportion (2%) of immunoglobulins in HM, the implication of which is partly still unknown. It appears to be involved in immune surveillance in the intestinal lumen by binding to antigens, phagocytizing them, and transporting these antigen-IgG complexes to the lamina propria14 to activate B cells and thus affect the adaptive response of the infant.11,13 In in vitro models with human immunodeficiency virus (HIV), IgG is able to prevent infection at intestinal level.15,16

The impact of the cellular and biochemical composition of HM on infectious diseases in mothers and infants has been studied and described elsewhere.5,17 Changes in HM composition have also been observed following administration of certain vaccines during pregnancy or lactation.18-20
Since March 11, 2020, when the World Health Organization declared the global pandemic caused by SARS-CoV-2, the world has focused on studying this virus and preventing its spread. SARS-CoV-2 is a single-stranded ribonucleic acid (RNA), encapsulated virus, the infection of which can lead from an asymptomatic process to a severe, multi-systemic disease termed Covid-19. Children of all ages are susceptible to infection with this virus, and even those with mild or asymptomatic symptoms appear to be involved in disease transmission.\textsuperscript{21-23}

At the beginning of the pandemic, there were doubts about the safety of breastfeeding by SARS-CoV-2-infected mothers. Some authorities recommended against it. The current, global recommendation is to encourage breastfeeding, as no such route of transmission has been demonstrated, and its benefits outweigh the risks.\textsuperscript{24}

Studies on breast milk from mothers with COVID-19\textsuperscript{25} and on HM donors during the pandemic\textsuperscript{26} revealed the presence of anti-SARS-CoV-2 antibodies and their neutralizing capacity. This confers hope of protection for breastfed infants.

The first SARS-CoV-2 vaccines were given emergency use authorization by the FDA\textsuperscript{27,28} and appeared less than a year after virus sequencing. The initial exclusion of breastfeeding mothers and children in clinical trials reveals the need for studies to provide scientific information on these groups. We designed this study based on the hypothesis that vaccination against SARS-CoV-2 leads to antibody excretion into breast milk and passive antibody transfer to breastfed infants.
METHODS

Study Design, Population

In this study, we applied a prospective cohort design with a convenience sample of health care professionals who were breastfeeding their children at the time of vaccination against SARS-CoV-2. The exposed, vaccinated group consisted of either BNT162b2 mRNA- COVID-19 or mRNA-1273 COVID-19-vaccinated individuals. All mothers at the Hospital Universitario Nuestra Señora de Candelaria who reported breastfeeding and 8 breastfeeding mothers from other institutions were included. From February 2 to April 4, 2021, 102 vaccinated, potential study participants were invited for enrollment the day they were administered their second dose of vaccine. Four of them were excluded from final analyses (Fig 1) for COVID-19 symptoms at the time of vaccination, 1 for past SARS-CoV-2 infection, and 2 for presenting serum parameters also suggestive of past infection. Twenty four breastfeeding, non-vaccinated mothers without previous SARS-CoV-2 infection were recruited as a control group to determine the threshold for the presence of SARS-CoV-2-specific antibodies in HM. All participants gave their signed consent. Any type of breastfeeding at any infant age were accepted. Epidemiological variables and risk factors for severe COVID-19 disease in mothers and infants were collected (Table 1). Participants with HIV infection, diseases or treatment that cause immunosuppression, previous infections, or ongoing symptoms compatible with COVID-19 at the time of recruitment were excluded. Of the vaccinated study participants, 92 (94%) received the BNT162b2 mRNA COVID-19 vaccine and 6 (6%) the mRNA-1273 COVID-19 vaccine, with a mean time range between doses of 25 ±2 to 28±1 days.
Procedures

Maternal blood and milk sampling were scheduled on day 14 after the second dose of vaccine.

Vaccines against COVID-19 introduce information from the spike glycoprotein receptor-binding domain (RBD) of SARS-CoV-2 and generate a humoral immune response with IgA, IgG, and IgM antibody production against its S1 subunit with its binding region for human cells, but do not generate antibodies against the SARS-CoV-2 nucleocapsid protein, which solely appear in infected patients and those who have had the disease. Individuals with serum IgG against the SARS-CoV-2 nucleocapsid protein (anti-SARS-CoV-2 N IgG-serum) were excluded from the study for previous SARS-CoV-2 infection.

The SARS-CoV-2 IgG Architect Abbott® assay was used for anti-SARS-CoV-2 N IgG detection. IgM antibodies against the spike protein of SARS-CoV-2 (anti-SARS-CoV-2 S1 IgM-serum) were assessed with the SARS-CoV-2 IgM Architect Abbott® assay. By default, data for both assays were expressed as qualitative “positive” or “negative” results, ie, the sample to positive ratio (S/P), given in detail in the Supplemental Information.

IgG antibodies against the receptor-binding spike domain S1 subunit (anti-SARS-CoV-2 RBD-S1 IgG-serum) were determined with the SARS-CoV-2 IgG II Quant Abbott® assay and results expressed as international standard units (unit of 1000 binding antibody units [BAU] per mL). According to the manufacturer, anti-SARS-CoV-2 RBD-S1 IgG concentrations of >560.90 BAU/mL correspond to a 95% probability
(95% CI: 78–99%) of neutralization capacity calculated by a plaque reduction equivalent to an inhibition of 50% of infection in cultured cells (ID50).

Blood extraction by venipuncture and milk collection were performed simultaneously. HM expression was carried out by the mothers in the hospital setting, usually in the morning, at least one hour after the last feeding, using an electric pump (SPECTRA S1®) and disposable extraction systems (Beldico®) equipped with 1 μm filters and non-return valves. The target amount for extraction, 20–30 mL, was collected in food standard polypropylene (PP) containers. Specific IgG (anti-SARS-CoV-2 RBD-S1 IgG-HM) and IgM antibodies (anti-SARS-CoV-2 S1 IgM-HM) in HM were determined with the same techniques used for blood serum. IgA (anti-SARS-CoV-2 S1 IgA-HM) was analyzed with the enzyme immunoassay anti-SARS-CoV-2 ELISA (IgA; Euroimmun®). Results are reported by calculating the ratio of the extinction of the control or patient sample over the extinction of the calibrator, S/P. Details are available in the Supplemental Information. The cut-off values in HM were calculated from the control milk samples as follows: mean + 2xSD. Thus, the cut-off was 0.12 BAU/mL for IgG and the S/P ratio for IgA 0.37. Following the manufacturer's instructions, an S/P ratio of 1 was used as cut-off for IgM in serum and HM.

The study was approved by the Institutional Review Board.

RESULTS

The clinical and demographic characteristics of the 98 vaccinated and the 24 control participants (Fig 1) are given in Table 1.
Immunogenicity

We detected anti-SARS-CoV-2 N IgG-serum antibodies in 2 vaccinated participants, who were, therefore, excluded for previous SARS-CoV-2 infection. Serum samples were obtained from 97 enrolled individuals on day 14±0.7 after the second dose of vaccine. The mean SARS-CoV-2 RBD-S1 IgG-serum antibody concentration in vaccinated participants was 3379.64±1639.46 BAU/mL (95% CI: 3049–3710). Neutralizing antibody titers, as defined by the manufacturer, were >560.9 BAU/mL in all vaccinated individuals. Two weeks post-vaccination, 22.5% of the samples (95% CI: 14.3–32.5) were positive for anti-SARS-CoV-2 S1 IgM-serum. We did not find a significant correlation between antibody levels in serum and maternal age or maternal body mass index (BMI). Serum of the control individuals was negative for anti-SARS-CoV-2 N and SARS-CoV-2 spike RBD IgM and IgG.

Antibodies in Breast Milk

The mean anti-SARS-CoV-2 RBD-S1 IgG level in the HM from the vaccinated participants was 12.19±11.74 BAU/mL (95% CI: 9.77–14.60; \( P < .001 \)) and, therefore, lower than in serum; but it was significantly higher than were the levels in the milk from the control group (0.02±0.05 BAU/mL [95% CI: 0.01 –0.05; \( P < .001 \)]). All vaccinated participants had anti-SARS-CoV-2 RBD-S1 IgG in their milk (95% CI: 96–100; Fig 2).

We found a positive correlation (\( r = 0.36; 95\% \text{ CI}: 0.17–0.53; \ P < .001 \)) between anti-SARS-CoV-2 RBD-S1 IgG in serum and in HM, which was stronger with breastfeeding periods <24 months (\( r = 0.67; 95\% \text{ CI}: 0.52–0.78; \ P < .001 \)) than with ≥24 months (\( r = 0.32; 95\% \text{ CI}: 0.16–0.67; \ P = .19 \); Fig 3). However, the difference in the serum to milk
IgG correlations between the two time-frames (<24 months and ≥24 months) was not statistically significant ($P = .06$). Consequently, we cannot conclude that the serum to HM correlation of SARS-CoV-2 RBD-S1 IgG differed between the two time ranges.

We also found anti-SARS-CoV-2 S1 IgA in 89% of the HM samples (95% CI: 81–95). A strong positive correlation was observed between anti-SARS-CoV-2 S1 IgA-HM and anti-SARS-CoV-2 RBD-S1 IgG-HM ($r = 0.75; 95\% \text{ CI}: 0.65–0.83; P < .001$; Fig 3). We did not detect anti-SARS-CoV-2 S1 IgM in HM (95% CI: 2–5).

Furthermore, we did not find any maternal age- or BMI-related difference in HM immunoglobulins.

**Breastfeeding-Period Related Effects of the COVID-19 Vaccination**

With regard to the characteristics of the vaccinated study participants, we did not detect significant differences related to their breastfeeding periods (0–6, 6–12, 12–24, ≥24 months). We only observed differences related to the type of breastfeeding, ie, exclusive breastfeeding was more frequent in infants <6 months (supplemental Table 2).

When analyzing immunoglobulin levels by the mentioned subgroups (supplemental Table 3), we observed significant differences ($P < .001$) between breastfeeding periods of <24 months (group A) and ≥24 months (Group B) with higher anti-SARS-CoV-2 immunoglobulin levels in group B. The anti-SARS-CoV-2 S1 IgA-HM S/P ratio in group A was $1.35±1.17$ (95% CI: 1.1–1.6) and in group B $3.20±2.14$ (95% CI: 2.17–4.23). In group A, the anti-SARS-CoV-2 RBD-S1 IgG-HM was $9.16±7.22$ BAU/ml.
(95% CI: 7.50–10.84) and 24±17.57 BAU/ml (95% CI: 15.52–32.47) in group B (Fig 4).

Our group observed that both breastfeeding for ≥24 months and high anti-SARS-CoV-2 RBD-S1 IgG levels in serum predict high IgG levels in breast milk. In a linear and multiple regression model, these associations proved to be independent, ie, the effect of the HM-IgG concentrations during breastfeeding for ≥24 months is not associated with the mother's IgG levels in serum. Compared to a breastfeeding period of <24 months, lactation for ≥24 months led to an increase in the mean anti-SARS-CoV-2 RBD-S1 IgG in HM by 17.04 BAU/mL (95% CI: 12.07–22.15; \( P < .001 \)).

**DISCUSSION**

In our study, we found that all vaccinated mothers developed specific anti-SARS-CoV-2 RBD-S1 IgG antibodies in serum and in milk. This data points to a possible route of infant protection against the virus. The secretion of specific antibodies in naturally immunized mothers has been related to protection against enteric diseases, like Campylobacter, Vibrio cholerae, Salmonella typhimurium, norovirus, etc,\(^{30-33}\) as well as a decrease in respiratory infections.\(^{34,35}\)

Other authors have already described the presence of specific IgA and IgG in HM of SARS-CoV-2-infected mothers. It seems that the predominant response is reflected in an even higher IgA titer, which correlates with the neutralizing capacity demonstrated in HM.\(^ {25}\)
Moreover, certain vaccines have shown to induce changes in the protection-related composition of HM. In the randomized, clinical trial by Jarvis et al,\textsuperscript{18} mothers vaccinated against influenza in the third trimester of pregnancy had higher levels of antibodies against influenza A in their HM during the first 6 months postpartum than non-vaccinated mothers. This type of reaction was also seen in studies with other vaccines, such as the tetanus and pertussis (dTpa) and the anti-meningococcal vaccine.\textsuperscript{20,36,37} For that reason our group decided to assess potentially similar effects of SARS-Cov-2 vaccination.

In our study, we found a direct link between COVID-19 vaccination and specific immunoglobulins in HM. All the analyzed HM samples contained specific IgG antibodies and 89\% of them specific IgA antibodies. These findings are in line with other recent studies on vaccinated mothers.\textsuperscript{38,39} In breast milk, the predominant response to vaccination was observed for IgG, which the mentioned authors attribute to parenteral administration.\textsuperscript{39} Our group observed a higher percentage of mothers with anti-SARS-CoV-2 RBD-S1 IgG in their milk, although we cannot compare quantification outcomes, as a semi-quantitative technique was used for anti-SARS-CoV-2 S1 IgA evaluation. What we did find was a strong, positive correlation between anti-SARS-CoV-2 RBD-S1 IgG concentrations and the IgA S/P ratio in breast milk. We believe that this finding is more likely to be related to the immune response to the vaccine antigens\textsuperscript{40} rather than the route of administration. Brady et al reported an IgA-IgG response and neutralizing capacity of milk from breastfeeding mothers who had been administered live-attenuated (intranasal) versus inactivated (intramuscular) influenza vaccine,\textsuperscript{19} although the response was stronger upon parenteral administration.
The authors conclude that the entry through the mucosa is not enough to elicit a high IgA response to this vaccine.

In our study, all mothers developed IgG antibodies against SARS-CoV-2 RBD-S1 in serum with concentration that, according to the manufacturer, predict a potentially neutralizing capacity. Moreover, we found a highly significant correlation between the antibody levels in serum and those in HM. Hence, serum antibody concentrations seem to predict the appearance of antibodies in HM. Moreover, serum antibody levels strongly correlated with those in HM in lactations of <24 months, although this statement should be interpreted with caution as the correlation between lactation periods of <24 months and ≥24 months did not result significant (P=0.06). The association between serum and HM antibody levels in lactation periods of ≥24 months was less pronounced than in shorter periods, which may suggest some intervening mechanism, eg, a local antibody production in the breast itself, a point that should be explored in-depth. Our finding that breastfeeding periods ≥24 months had a stronger influence on vaccine-induced immunoglobulin concentrations in HM than shorter ones and that the effect of the lactation period on immunoglobulins was specific and independent of other variables may encourage further study as well.

No anti-SARS-CoV-2 RBD-S1 IgM antibodies were detected in 88% of the samples taken 14 days after the second dose of vaccine. IgM antibodies have not yet been well studied in this context. This primary response may be transient and short-termed. More data is needed to clarify this point.
The composition of HM, including its immunological components, is dynamic and changes throughout lactation.\textsuperscript{13,41} The changes detected by our group suggest mechanisms that adapt to the immune development of the baby by which mothers initially protect the infant through abundant sIgA and sIgM in their transitional milk and subsequently contribute to the development of the baby's adaptive immunity with IgG antibodies in their mature milk.\textsuperscript{13,14,42} Although our finding of higher immunoglobulin levels in HM samples where breastfeeding was prolonged for over 23 months was unexpected, a positive correlation between the length of breastfeeding (between >6 months and 2 years of age) and the immunoglobulin concentration in milk, regardless of the mother's age or BMI, had been described previously.\textsuperscript{43,44}

In addition, increased concentrations of proteins,\textsuperscript{41,45} immunoglobulins, lactoferrin, and lysozyme have been described in lactation periods of over 18 months and during the involution of the breast near weaning. This phenomenon may be particularly beneficial when there is a need for augmented local protection against infections and may favor that sick infants can return to the breast during this period.\textsuperscript{46}

A limitation of this study was that we do not have access to biosafety level 3 (BSL3) facilities and, therefore, have not been able to perform \textit{in vitro} plaque reduction neutralization tests (PRNT), the gold standard for determining SARS-CoV-2 antibody deactivating effectiveness. However, ELISA receptor-binding domain-based assays have been described as a valid alternative to assess the neutralization capacity of said antibodies in HM.\textsuperscript{47,48}
Another possible limitation of this study is the difference in breastfeeding periods between control and vaccinated participants. Only two mothers in the control group had extended lactation periods of over 2 years. We do not consider this difference to be clinically relevant or interfere with the results of our study. The women in the control group had not suffered the disease and therefore did not present anti-SARS-CoV-2 antibodies neither in serum nor in milk, regardless of their breastfeeding period. In our sample, overall antibody levels were rather homogeneous in the control group, and the two mothers who extended breastfeeding to ≥24 months did not display high antibody levels in their milk.

CONCLUSIONS

In conclusion, BNT162b2 and mRNA-1273 COVID-19 vaccines generate immunity in vaccinated mothers and are associated with vaccine-specific immunoglobulin concentrations in HM. This effect persists in breastfeeding periods of over 2 years. Immunity from breastfeeding and its possible impact on infant protection from SARS-CoV-2 infection is a hope for breastfeeding girls and boys, for whom the prospect of vaccination in this pandemic is still a long way off.

There are only few studies on HM composition in breastfeeding periods of >2 years, and its immunological benefit is often underestimated. The stronger effect of COVID-19 vaccination on HM immunoglobulins in lactation periods >2 years suggests a need to increase support and health policies that encourage such long breastfeeding periods in times of a pandemic. More studies are needed on how long these antibodies last in HM and on their implication in protecting the breastfeeding population over time.
Acknowledged

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The authors also thank each and every one of the Medical Laboratory scientists and technicians who performed the laboratory procedure and the team of vaccinators for their help in locating nursing mothers at the time of vaccination against SARS-CoV-2.

The authors also thank all the families who participated in this study.

References


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<tr>
<th>Characteristic</th>
<th>Control group (N = 24)</th>
<th>Vaccinated group (N = 98)</th>
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<td>36 (33.2–38.7)</td>
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<td><strong>Mother’s risk factors for severe COVID-19, n (%)</strong></td>
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<tr>
<td>Liver disease</td>
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<td>.36</td>
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<td><strong>Child’s risk factors for severe COVID-19, n (%)</strong></td>
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IQR, interquartile range. \(^a\) Body mass index at the time of screening = weight (kg)/height (m\(^2\)). \(^b\) Cancer, chemotherapy, immunomodulators, radiotherapy, immunosuppressants, or corticosteroids (eg, >20 mg/day of prednisone or equivalent) for more than 14 days in the last 6 months, or immunoglobulins in the last 3 months. CPAP (continuous positive airway pressure).
Figure 1 Participant enrollment.
Figure 2 Boxplot of the immunoglobulins anti-SARS-CoV-2-S1 IgA-HM, anti-SARS-CoV-2 S1 IgM-HM, and anti-SARS-CoV-2 RBD-S1 IgG-HM in human milk (HM) of the vaccinated and control study participants. The gray, dotted line stands for the positive cut-off, calculated as the mean OD+2SD of the control milk samples for each immunoglobulin. Red dots represent the participants with a lactation period of ≥24 months.
Figure 3 Scatterplots representing the positive correlation between (A) anti-SARS-CoV-2 RBD-S1 IgG-serum and anti-SARS-CoV-2 RBD-S1 IgG-HM both expressed in BAU/mL, and (B) anti-SARS-CoV-2 RBD-S1 IgG-HM and anti-SARS-CoV-2-S1 IgA-HM expressed as S/P ratio. Red dots represent the participants with a lactation period of ≥24 months. Pearson correlation coefficients (r) were determined when $P < .05$. 
Figure 4. Boxplot of human milk immunoglobulins anti-SARS-CoV-2 S1 IgA-HM, anti-SARS-CoV-2 S1 IgM-HM, and anti-SARS-CoV-2 S1 IgG-HM (all anti-SARS-CoV-2 spike protein RBD) in participants grouped according to their lactation period (0–5 months, 6–11 months, 12–23 months, and ≥24 months).
"SARS-CoV-2 Antibodies in Breast Milk After Vaccination"

98 professionals who reported that they were breastfeeding at the time of COVID-19 vaccination were included.

100% of vaccinated participants reached serum titers antibodies related to neutralizing capacity.

"The impact of vaccination on specific immunoglobulins in HM was greater on lactations greater than 23 months"

Ig A-2 and Ig G specific SARS-CoV in milk in those mothers who were breastfeeding > 23 months were significantly higher.

Supplemental Figure 5 Graphical abstract.

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SUPPLEMENTAL LABORATORY PROCEDURE:

Procedures

The blood sample was collected by direct venipuncture in a coagulation activator serum tube. They were centrifuged at 3,500 rpm for 10 minutes.

The serum was frozen at -20°C to subsequently analyze the IgM against the RBD antigen receptor of the S1 protein (SARS-CoV-2 Spike RBD IgM).

The milk extraction was carried out in the hospital setting, preferably in the morning, at least one hour after the last feeding, by means of mechanical extraction (SPECTRA S1®) and disposable extraction systems, Beldico® with 1 μm filter and non-return valve. The objective volume of the extraction was 20-30 mL collected in food standard Polypropylene (PP) pot. The samples were immediately sent to the laboratory and after centrifugation at 3,500 rpm for 15 minutes, the supernatant was removed with a Pasteur pipette, repeating the same procedure twice. Once skimmed, they were frozen at -20°C for later analysis.

The presence of antibodies in serum was analyzed using appropriate assay kits according to the manufacturers' instructions.

The cut-off values for HM testing were calculated from the control milk samples as follows: mean + 2xSD. Thus, the cut off was 0.12 BAU/mL and for IgG and 0.37 S/P ratio for IgA, respectively. For IgM assessment in serum and HM, 1 S/P ratio was used as cut off, following the manufacturer's instruction.

Laboratory procedures

Anti-SARS-CoV-2 N IgG: The SARS-CoV-2 IgG Architect Abbott® assay is a chemiluminescent microparticle immunoassay (CMIA) used for the qualitative detection of IgM antibodies to SARS-CoV-2 in human serum and plasma.
The assays results are reported as a numerical Index value (ratio of the chemiluminescent signal between the samples and a calibrator) that give a qualitative result of “Positive” or “Negative”.

The SARS-CoV-2 IgG detect immunoglobulin class G (IgG) antibodies to the nucleocapsid protein of SARS-CoV-2 from patients with signs and symptoms of infection who are suspected of corona coronavirus virus disease (COVID-19) or in serum of subjects that may have been infected by SARS-CoV-2. The presence of antibodies was analyzed using appropriate assay kits according to the manufacturers’ instructions on the automated Abbott ARCHITECT i2000SR instrument.

**Anti-SARS-CoV-2 S1 IgM:** It’s CMIA used for the qualitative detection of IgM antibodies against the SARS virus- CoV-2 (SARS-CoV-2 IgM Architect Abbott®).

The presence of antibodies was analyzed using appropriate assay kits according to the manufacturers’ instructions on the automated Abbott ARCHITECT i2000SR instrument.

Results are reported as an Index (ratio of the chemiluminescent signal between the samples and a calibrator), with values >1.4 indicating a positive result.

IgM antibodies to SARS-CoV-2 are generally detectable in blood several days after initial infection, although the duration of time antibodies are present post-infection is not well characterized. Individuals may have detectable virus present for several weeks following seroconversion. SARS-CoV-2 antibody negative samples collected 15 days or more post symptom onset should be reflexed to a test that detects and reports SARS-CoV-2 IgG.

**Anti-SARS-CoV-2 RBD-S1 IgG:** The SARS-CoV-2 IgG II Quant assay is a chemiluminescent microparticle immunoassay (CMIA) used for the qualitative and quantitative determination of IgG antibodies to SARS-CoV-2 in human serum and plasma on the Alinity and ARCHITECT i Systems. The
SARS-CoV-2 IgG II Quant assay is to be used as an aid in the diagnosis of SARS-CoV-2 infection in conjunction with clinical presentation and other laboratory tests. The assay is also to be used as an aid in evaluating immune status of individuals with quantitative measurement of IgG antibodies against the spike receptor-binding domain (RBD) of SARS-CoV-2.

The spike glycoprotein (S-protein), has a pivotal role in viral pathogenesis, mediating binding to target cells through the interaction between its receptor-binding domain (RBD) and the human angiotensin converting enzyme 2 (ACE2) receptor. The S-protein has been found to be highly immunogenic, and the RBD is possibly considered the main target in the effort to elicit potent neutralizing antibodies.

**Anti-SARS-CoV-2 S1 IgA:** Anti-SARS-CoV-2 ELISA (IgA) Euroimmun® is enzyme immunoassay (ELISA) provides semiquantitative in vitro determination of human antibodies of the immunoglobulin class IgA against SARS-CoV-2 in serum. The microplate wells are coated with recombinant structural protein (S1 domain) of SARS-CoV-2. The presence of antibodies in serum was analyzed using appropriate assay kits according to the manufacturers’ instructions on the DS2® system, an automated microplate technology. Results can be evaluated semiquantitatively by calculating a ratio of the extinction of the control or patient sample over the extinction of the calibrator.
Table 2 Characteristics of vaccinated participants according to duration of breastfeeding

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>[0,23] b months (N=76)</th>
<th>(23,50) b months (N=24)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age-yr</td>
<td>36 (33-38)</td>
<td>37.5 (35-40)</td>
<td>.38</td>
</tr>
<tr>
<td>Body-mass index c</td>
<td>23.3 (20.8-25.7)</td>
<td>22.2 (19.9-26.8)</td>
<td>.95</td>
</tr>
<tr>
<td>Infant feeding modality (%)</td>
<td></td>
<td></td>
<td>.23</td>
</tr>
<tr>
<td>Exclusive breastfeeding</td>
<td>28 (37)</td>
<td>0 (0)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Partial breastfeeding</td>
<td>5 (6)</td>
<td>0 (0)</td>
<td>.33</td>
</tr>
<tr>
<td>Breastfeeding and complementary feeding</td>
<td>43 (57)</td>
<td>24 (100)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Child's sex (%)</td>
<td></td>
<td></td>
<td>.99</td>
</tr>
<tr>
<td>Male</td>
<td>35 (46)</td>
<td>11 (46)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>41 (54)</td>
<td>13 (54)</td>
<td></td>
</tr>
<tr>
<td>Childbirth (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age</td>
<td>39.9 ± 1.1</td>
<td>39.3 ± 1.8</td>
<td>.16</td>
</tr>
<tr>
<td>Birth weight*</td>
<td>3255 (2991-3486)</td>
<td>3250 (3043-3625)</td>
<td>.61</td>
</tr>
</tbody>
</table>

aMedian and IQR (interquartile range). b2 groups were divided according to the duration of lactation of 0-23 months, ≥24 months. cThe body-mass index is the weight in kilograms divided by the square of the height in meters. This calculation was based on the weight and height measured at the time of screening.
### Table 3 Immunoglobuline of vaccinated participants according to duration of breastfeeding

<table>
<thead>
<tr>
<th></th>
<th>[0,23] (N=76)</th>
<th>(23,50] (N=24)</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-SARS-CoV-2 RBD-S1 IgG-HM</strong></td>
<td>8(4.74-11.19)</td>
<td>18.06(12.13-25.36)</td>
<td><em>P</em> &lt; .001 Wilcoxon</td>
</tr>
<tr>
<td><strong>Anti-SARS-CoV-2 S1 IgM-HM</strong></td>
<td>0.02(0.02-0.03)</td>
<td>0.03 (0.02-0.04)</td>
<td><em>P</em> = .01 Wilcoxon</td>
</tr>
<tr>
<td><strong>Anti-SARS-CoV-2 S1 IgA-HM</strong></td>
<td>1.05(0.56-1.69)</td>
<td>2.67(1.71-5.89)</td>
<td><em>P</em> &lt; .001 Wilcoxon</td>
</tr>
</tbody>
</table>

*Median and IQR (interquartile range). b2 groups were divided according to the duration of lactation of 0-23 months, ≥24 months. cThe body-mass index is the weight in kilograms divided by the square of the height in meters. This calculation was based on the weight and height measured at the time of screening. The IgA e IgM value is expressed in a ratio of the extinction of participant vaccinated over the extinction of the calibrator. The Ig G is expressed in BAU/mL.*
Table 4. Immunoglobulin of vaccinated participants and control participants

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>vaccinated participants</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-SARS-CoV-2 RBD-S1 IgG-Serum</strong></td>
<td>0.41±0.37</td>
<td>3379.64±1639.46</td>
<td>P &lt; .001, t.test</td>
</tr>
<tr>
<td><strong>Anti-SARS-CoV-2 RBD-S1 IgG-HM</strong></td>
<td>0.02±0.05</td>
<td>12.19±11.74</td>
<td>&lt;0.001, t.test</td>
</tr>
<tr>
<td><strong>Anti-SARS-CoV-2 S1 IgM-HM</strong></td>
<td>0.01±0.00</td>
<td>0.04±0.08</td>
<td>0.003, t.test</td>
</tr>
<tr>
<td><strong>Anti-SARS-CoV-2 S1 IgA-HM</strong></td>
<td>0.21±0.08</td>
<td>1.73±1.59</td>
<td>&lt;0.001, t.test</td>
</tr>
</tbody>
</table>