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Breast milk and passive immunity during the COVID-19 pandemic

Edited by
Veronique Demers-Mathieu, Lars Bode and Johannes Van Goudoever

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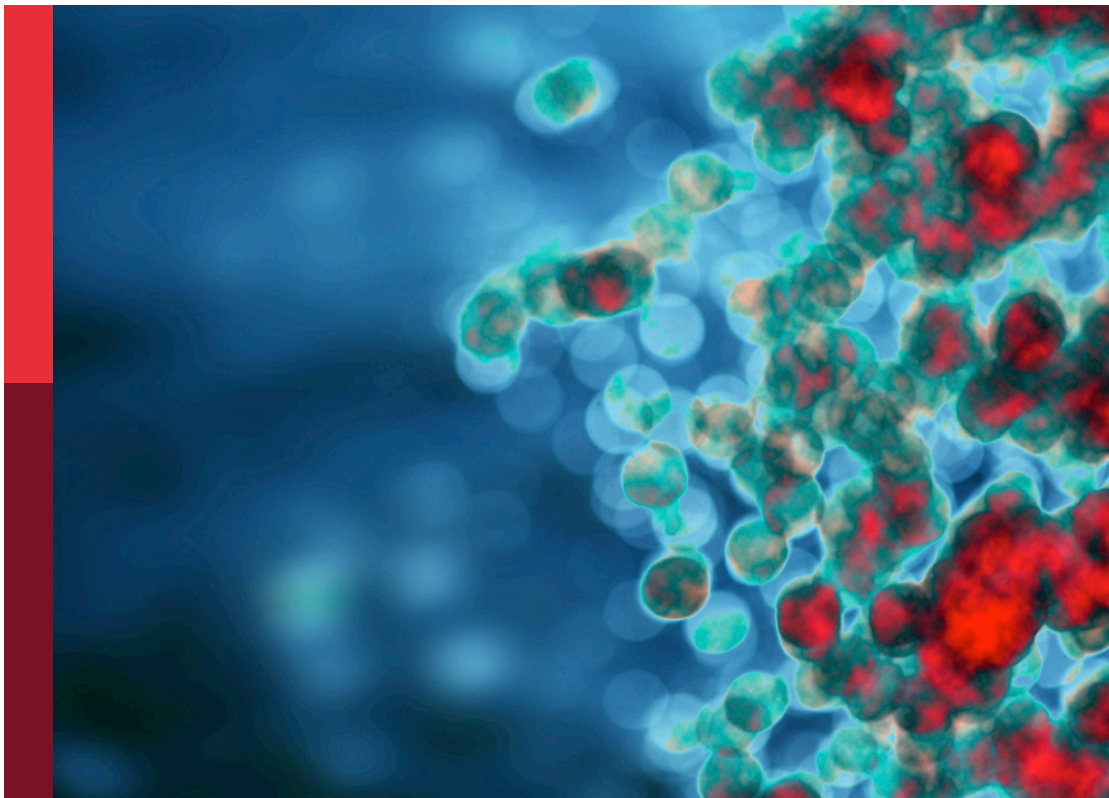

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Breast milk and passive immunity during the COVID-19 pandemic

Topic editors

Veronique Demers-Mathieu — Exagen, Inc., United States

Lars Bode — University of California, San Diego, United States

Johannes Van Goudoever — Amsterdam University Medical Center, Netherlands

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Willem Van Eden,
Utrecht University, Netherlands

*CORRESPONDENCE
Veronique Demers-Mathieu
✉ demersmv@gmail.com

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Editorial: Breast milk and passive immunity during the COVID-19 pandemic

Veronique Demers-Mathieu*

Department of Molecular Immunology, Exagen Inc., Vista, CA, United States

KEYWORDS

neutralizing antibody, mucosal immunity, breast milk antibodies, mRNA-based vaccine, lactating women, pregnant women, serum antibodies, immunogenicity

Editorial on the Research Topic
Breast milk and passive immunity during the COVID-19 pandemic

Neonates are born with an immature immune system, including a lack of IgG and secretory IgA (SIgA) production by plasma cells. Thus, newborns rely on the passive transfer of antibodies *via* the placenta (only IgG) and breast milk (80–90% SIgA/IgA, 5% IgG, and 10–15% IgM) to protect them against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection during the first 2 to 6 months of postnatal age. This editorial presents 10 contributing articles on the Research Topic "*Breast Milk and Passive Immunity during the COVID-19 Pandemic*." First, we describe the passive immunity from the placenta to the fetus after mRNA COVID-19 vaccination. Second, we evaluate the risk of transplacental transmission of SARS-CoV-2 to the fetus. Third, we elucidate that breast milk is not a vector of viral SARS-CoV-2 that can infect breastfed infants. Fourth, we discuss the maternal antibody response specific to SARS-CoV-2 after the two mRNA COVID-19 vaccine and the booster dose. Fifth, we report the safety of the mRNA COVID-19 vaccine and booster shot during breastfeeding. Lastly, we describe the relationship between maternal stress and antibody response in lactating mothers.

Passive immunity from the placenta to the fetus after mRNA COVID-19 vaccination

IgG is the only isotype passively transferred from the placenta to the fetus during pregnancy. Anti-SARS-CoV-2 IgG was present in cord blood and infants' blood from mothers vaccinated while pregnant, confirming the transfer of anti-SARS-CoV-2 IgG *via* the placenta to the fetal bloodstream (Hunagund et al.; Figure 1A). In contrast, serum anti-SARS-CoV-2 IgG was absent in infants born from mothers vaccinated after pregnancy, which could reduce their protection against COVID-19 infection.

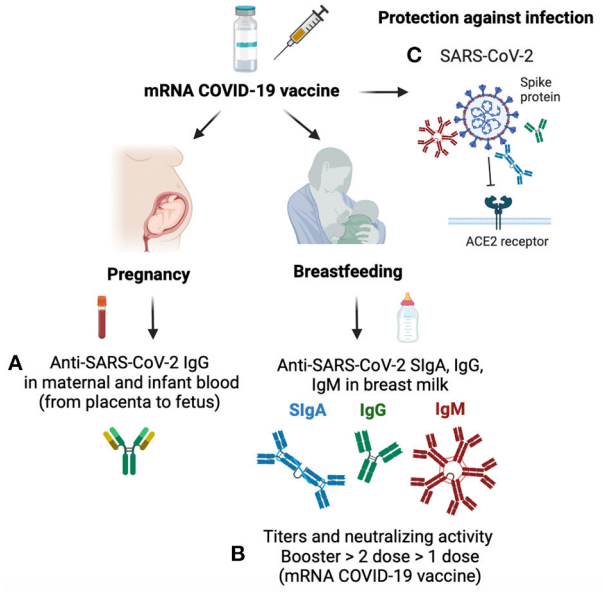

FIGURE 1
Mechanisms of antibody transfer during pregnancy and breastfeeding after mRNA COVID-19 vaccine. **(A)** Anti-SARS-CoV-2 IgG is transferred from the placenta to the fetus and is present in cord blood and infant blood from mothers vaccinated while pregnant. **(B)** Breast milk contains anti-SARS-CoV-2 IgG, IgA, and IgM after the first and second mRNA COVID-19 vaccines. Neutralizing activity and titer of anti-SARS-CoV-2 antibodies are higher after the booster (third dose) than after the second dose of the mRNA COVID-19 vaccine in breast milk, while only one dose has the lowest neutralizing capacity and antibody titers. **(C)** Anti-SARS-CoV-2 antibodies bind to the spike protein of SARS-CoV-2, which block the viral attachment to the angiotensin-converting enzyme (ACE) receptor and provide protection against COVID-19 infection in mothers and their breastfed infants.

Low risk of transplacental transmission of SARS-CoV-2 to the fetus

Most neonates born from COVID-19-infected mothers did not test positive for COVID-19, while few cases of newborns tested positive and presented early-onset symptoms (Rad et al.). Whether newborns with positive COVID-19 are due to the transplacental transmission of SARS-CoV-2 or infection after delivery is still not well-understood. The placenta provides a protective barrier to the fetus against maternal infections. However, vertical transmission of SARS-CoV-2 from the placenta to the fetus can happen when the virus-induce apoptosis and vascular damage in the placenta. SARS-CoV-2 can spread in maternal endothelium into fetal capillaries and then be aspirated through amniotic fluids. The presence of SARS-CoV-2 in blood samples from newborns was ~1%, suggesting the risk of transplacental transmission of COVID-19 to the fetus is low.

Breast milk is not a vector of viral SARS-CoV-2 that can infect breastfed infants

A few articles have reported the detection of low titers of viral SARS-CoV-2 RNA in breast milk samples. Lactating mothers and health professionals have been worried about the potential transfer of viral SARS-CoV-2 from breast milk to the infant. To confirm the safety of breastfeeding during maternal COVID-19 infection, breast milk was collected before and after washing the breast skin in lactating mothers with COVID-19 infection. Some breast skin swabs collected before washing the breast detected SARS-CoV-2 RNA in milk samples, while SARS-CoV-2 RNA was absent in all milk samples after washing the breast skin (Pace et al.). Breast skin contaminated with SARS-CoV-2 was associated with the presence of maternal caught and other family members in the household with COVID-19 infection. These findings explain why some breast milk samples in previous studies have detected positive results for SARS-CoV-2 RNA using RT-qPCR. Breast milk is likely not a potential source of viral RNA SARS-CoV-2 when mothers wash their breast skin before breastfeeding.

Maternal antibody response specific to SARS-CoV-2 during COVID-19 vaccine

After two mRNA COVID-19 vaccine doses, lactating mothers had detectable anti-SARS-CoV-2 IgG1, IgA, and IgM in serum samples, with an increase in all three isotypes after the second dose, especially IgG1 levels (Yeo et al.). After the second vaccine, all mothers had detectable anti-SARS-CoV-2 IgG1 and IgA in breast milk, whereas IgM was present in 87% of milk samples. Neutralizing antibodies increased after the second dose in serum and breast milk compared to the first dose (Figure 1B). The rapid increase of IgG after the second dose correlated with the specific B lymphocyte memory that prime a faster response with higher antibody levels. In contrast, IgA levels remained constant between the two first doses of the COVID-19 vaccine. Infants breastfed from vaccinated mothers did not have detectable neutralizing antibodies or vaccine mRNA in their serum.

The mRNA COVID-19 booster (total of three doses of mRNA vaccine) elevated antibody secretion in lactating mothers. The titers of IgG and IgA specific to SARS-CoV-2 were higher in breast milk after a third booster dose of mRNA COVID-19 than the peak after the first and second vaccines (Bender et al.; Figure 1B). The neutralizing capacity of breast milk antibodies produced during the third mRNA COVID-19 booster shot was higher than those with the second COVID-19 vaccine (pre-booster sample). Neutralizing activity of breast milk antibodies correlated with serum antibodies in mothers with the booster dose. These findings support the current guidance that all pregnant or lactating mothers should receive the mRNA COVID-19 booster dose to provide an optimal mucosal response to the mothers. Increased antibody secretion in vaccinated mothers during lactation could promote neonatal mucosal immunity *via* ingestion of breast milk IgA, IgG, and IgM. Anti-SARS-CoV-2 antibodies bind to the spike protein of SARS-CoV-2, which block the viral attachment to the angiotensin-converting enzyme (ACE) receptor and provide protection against COVID-19 infection (Figure 1C).

Like the mRNA vaccine, vector-based vaccines stimulated antibody response in lactating mothers. Paired longitudinal samples taken at 45 and 120 days after the second dose of COVID-19 vector-based vaccines showed that IgG levels waned over time in breast milk, while IgA levels remained stable in 100% of lactating women (Longueira et al.). A slight reduction of IgA titers in serum

relative to paired breast milk samples was detected 120 days after the second vector vaccine dose, suggesting a more sustained IgA production in mucosal secretion. In contrast, IgG levels in serum and breast milk (paired samples) decreased from 45 to 120 days after the second vaccine.

The mRNA COVID-19 vaccine is safe during breastfeeding

Some lactating mothers are hesitant to receive a COVID-19 vaccine due to the lack of knowledge on the immunogenicity of mRNA-based vaccines on nursing infants, as lactating mothers were excluded from initial clinical trials of mRNA vaccination. Paired blood and milk samples from lactating mothers and their infants were collected after the maternal mRNA vaccine (2 doses) to evaluate the immunogenicity of the mRNA molecule. Severe side effects were absent in infants breastfed from mothers vaccinated with mRNA COVID-19 (Golan et al.). Vaccine-related PEGylated protein concentrations did not increase in breast milk after COVID-19 vaccination. These results suggest that the mRNA-COVID-19 vaccine administered in lactating women did not lead to detectable immunogenicity in the infant's blood. In addition, low levels of intact mRNA vaccine were detected in maternal serum and breast milk samples, while infants' serum had no trace of mRNA molecule or serological evidence of infant sensitization (Yeo et al.). These findings confirm the safety of continuing breastfeeding during maternal mRNA COVID-19 vaccination.

A systemic review article demonstrated that lactating women receiving 2 doses of the mRNA COVID-19 vaccine are safe for them and their breastfed infants (Muyldermans et al.). Another mini-review article assessed that the safety and efficacy of the developed mRNA COVID-19 vaccines were comparable between pregnant, lactating, and non-pregnant women (Laguila Altoé et al.). The administration of mRNA COVID-19 vaccination in these groups promoted the production of neutralizing antibodies against SARS-CoV-2 in mothers and passive immunity in their infants.

Maternal stress and antibody response in lactating mothers

Antibody response is influenced by several factors related to maternal background and confounding factors, including psychological stress. Maternal stress could be elevated by stressful events, including the COVID-19 pandemic. Interestingly, the stress levels of lactating women were comparable between pre-pandemic and during the pandemic COVID-19 (Juncker et al.). However, maternal lifetime stressors were negatively correlated with breast milk IgA specific to SARS-CoV-2. Breastfed infants of mothers with high chronic stress levels might ingest lower breast milk SIgA/IgA.

Conclusion and perspective

This Research Topic in Frontiers in Nutritional Immunology provided new knowledge on the activation of antibody response during mRNA COVID-19 vaccine and booster dose in pregnant and lactating mothers and confirmed its safety for their infants. Additional investigations are required to identify the mechanisms of antibody protection when infants ingest breast milk with neutralizing antibodies against SARS-CoV-2. Finally, more studies are needed to reveal which maternal factors (including genetics, nutrition, preexisting immunity, and health conditions) enhance antibody responses during vaccination.

Author contributions

VD-M wrote the first draft of the manuscript, sections of the editorial article, revised, read, and approved the submitted version.

Conflict of interest

VD-M was employed by Exagen Inc.

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The Effects of COVID-19 on the Placenta During Pregnancy

*Habib Sadeghi Rad[1], Joan Röhl[1], Nataly Stylianou[1], Mark C. Allenby[2,3], Sajad Razavi Bazaz[4], Majid E. Warkiani[4], Fernando S. F. Guimaraes[5], Vicki L. Clifton[6] and Arutha Kulasinghe[1,5]**

[1] School of Biomedical Sciences, Queensland University of Technology, Brisbane, QLD, Australia, [2] School of Chemical Engineering, University of Queensland, St Lucia, QLD, Australia, [3] Centre for Biomedical Technologies, School of Mechanical, Medical and Process Engineering, Queensland University of Technology, Brisbane, QLD, Australia, [4] School of Biomedical Engineering, University of Technology Sydney, Sydney, NSW, Australia, [5] The University of Queensland Diamantina Institute (UQDI), Brisbane, QLD, Australia, [6] Mater Research Institute, University of Queensland, Brisbane, QLD, Australia

Coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a global pandemic. The virus primarily affects the lungs where it induces respiratory distress syndrome ranging from mild to acute, however, there is a growing body of evidence supporting its negative effects on other system organs that also carry the ACE2 receptor, such as the placenta. The majority of newborns delivered from SARS-CoV-2 positive mothers test negative following delivery, suggesting that there are protective mechanisms within the placenta. There appears to be a higher incidence of pregnancy-related complications in SARS-CoV-2 positive mothers, such as miscarriage, restricted fetal growth, or still-birth. In this review, we discuss the pathobiology of COVID-19 maternal infection and the potential adverse effects associated with viral infection, and the possibility of transplacental transmission.

Keywords: COVID-19, placenta, SARS-CoV-2, transplacental infection, pregnancy

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Veronique Demers-Mathieu, Medolac Laboratories, United States

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Pavithra Vijayakumar, University of Rochester, United States
Domenico Umberto De Rose, Bambino Gesù Children Hospital (IRCCS), Italy

***Correspondence:**
Arutha Kulasinghe
arutha.kulasinghe@uq.edu.au

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INTRODUCTION

The World Health Organization (WHO) declared a global pandemic of coronavirus disease 2019 (COVID-19) in March 2020, caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) (1). As of August 2021, the number of total cases surpassed 200 million and resulted in more than 4 million deaths. There is an ongoing effort to understand transmission, incidence, disease pathogenesis and the short- and long-term impacts following infection. In particular, the impact of SARS-CoV-2 infection on mothers and their babies (2). Evidence suggests that pregnant women with COVID-19 are more susceptible to severe disease with a higher risk of preterm birth (3–5), as well as higher risk of maternal and/or fetal death (6, 7). These findings are reminiscent of the dire outcomes from other similar respiratory viral infections, such as influenza A/H1N1 (8–11), severe acute respiratory syndrome (SARS) (12), and Middle East Respiratory Syndrome (MERS) (13, 14), where infected pregnant women are at increased risk of severe morbidity and mortality to both themselves and their infants (2). While most neonates born to SARS-CoV-2 positive mothers test negative and do not present with virus-induced disease, there have been some cases of newborns testing positive and presenting with early-onset symptoms (15). Whether this is due to the trans-placental transmission of SARS-CoV-2, or infection following delivery is still not well

understood (16–18). Examination of the placentas from SARS-CoV-2 positive mothers have mixed reports on viral positivity, and not all neonates born from mothers with a SARS-CoV-2 positive placenta test positive for the virus (19). This suggests that there is a protective mechanism/barrier within the placenta, where its success may rely on the presence or absence of certain receptors/pathways. Fortunately, SARS-CoV-2 positive neonates are yet to present with any congenital defects (20). In this review, we provide an overview of the literature of SARS-CoV-2 infection during pregnancy, as well as the pathobiology of the placenta which may protect the growing fetus.

IMMUNE SYSTEM ALTERATIONS DURING SARS-CoV-2 INFECTION

The immune system changes during pregnancy in such a way that it adapts to the growth of a semi-allogeneic fetus in the body of the mother, resulting in a distinct immune response to different infections during pregnancy (21–23). It has been well documented that in patients with COVID-19, particularly those with severe disease, have profound immune dysregulation (24). Studies have revealed an increase in blood leukocytes (leukocytosis), which was characterized by a decrease in lymphocytes (lymphopenia) and an increase in neutrophil-to-lymphocyte ratio (NLR) (25, 26). Using immunophenotyping analyses, researchers discovered that patients with severe COVID-19 had fewer natural killer (NK), CD3+, CD4+, and CD8+ T cells than those with the non-severe disease (27). NK cells were also found to be functionally exhausted during SARS-CoV-2 infection (28–30). Moreover, a reduction in circulating NK cell population has been reported during gestation (31). NK cells have key roles in the innate immune response by killing transformed cells, as consequence of viral infections or oncogenesis; NK cells are also major sources of pro-inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon gamma (IFN-γ), which can restore or activate the antiviral property of the myeloid compartment; thus, any decrease in these cell populations may alter the ability to clear viruses (32). Evidence has shown that lymphopenia and enhanced NLR can be further amplified by COVID-19 disease severity (33). Compared to patients with moderate COVID-19, individuals with severe disease had lower numbers of cytotoxic T lymphocytes (CTLs) (33). Studies investigating COVID-19 patients' lung tissue and bronchoalveolar lavage fluid (BALF) samples found T cell hyperactivation and/or upregulation of pro-apoptotic factors, including first apoptosis signal receptor (FAS), TNF-related apoptosis-inducing ligand (TRAIL), and caspase 3, as the main causes of T cell depletion (34–36). Alterations in CD4+ T cell population toward T helper-2 (Th)-2 phenotypes rather than Th1 phenotypes have been found during pregnancy, which contributes to the promotion of humoral immune responses over cellular immune responses (37). There is also a balance between regulatory T cell (Treg) and Th17 cells during pregnancy; with a shift towards Tregs to ensure fetal-maternal immune tolerance and to prevent fetal allograft rejection (38). In terms of innate immune cells, evidence suggests that, while absolute peripheral blood monocyte counts are not significantly different between patients with severe COVID-19 and those with moderate disease, the activation status of the monocyte/macrophage system is significantly altered (39). It was shown that monocyte/macrophage alterations caused by SARS-CoV-2 infection are similar to a condition known as familial hemophagocytic lymphohistiocytosis (HLH), a systemic inflammatory disorder involving cytokine production and cytopenia (40–42). HLH can be triggered either by abnormalities in genes regulating NK and cytotoxic CD8+ T cell degranulation or by conditions such as autoimmune disease, malignancy, and viral infection (40, 41). It was found that patients with H1N1 influenza who experienced the 'cytokine storm,' characterized by the extreme and excessive immune and inflammatory response (43), had mutations in genes associated with HLH (44). Many studies, however, do not support the link between HLH and COVID-19 (45–47). Wood et al., found that only three of 40 COVID-19 patients had Hscores >169, the cut-off used to identify HLH (47). Several studies have reported widespread infiltration of monocytes/macrophages in the lung tissue samples taken from COVID-19 patients (35, 48, 49). Single-cell studies revealed that monocyte-derived FCN1+ macrophages were the most abundant macrophage subset found in BALF samples from severe COVID-19 patients (35). Furthermore, it was discovered that peripheral monocyte trafficking and subsequent differentiation into macrophages in the lungs of COVID-19 patients contributes to pro-inflammatory responses and further activation of innate immune cells (49). Changes in the innate immune system during pregnancy, also, involve the pattern recognition receptors Toll-like receptors (TLRs), in particular TLR4 (50, 51). There are three different levels of TLR4 activation during pregnancy. First, TLR4 activation and the inflammatory response rise during the first trimester, allowing blastocyst implantation. Following that, a decrease in TLR4 activation happens during the second trimester in order to create an anti-inflammatory response for fetal growth. Eventually, TLR4 activation and the inflammatory response increase again in the third trimester to support labor and delivery (52). Infection with COVID-19 leads to pyroptosis of host cells and the release of danger associated molecular patterns (DAMPs) that can act as ligands for TLR molecules and trigger a greater inflammatory response (31). Studies are needed to determine whether such changes in the immune system result in higher susceptibility or are protective against COVID-19 during pregnancy (31).

Expression of ACE2 and TMPRSS2 in Placental and Fetal Cells

SARS-CoV-2 enters the body through the nasal passage and infects pulmonary cells by binding to the receptor angiotensin-converting enzyme 2 (ACE2) (31, 53–55). It has been found that ACE2 expresses in respiratory and intestinal track, placenta, ovaries, vagina, and uterus (56). Cell entry is further facilitated by viral spike (S) protein priming induced by trans-membrane serine protease 2 (TMPRSS2) (53–55). Cells co-expressing both ACE2 and TMPRSS2 have been found to have a higher

susceptibility to SARS-CoV-2 entry (57) (**Figure 1**). In addition, furin, trypsin, and cathepsins B and L have been reported to be capable of cleaving the spike glycoprotein binding at the S1/S2 site, allowing the virus to enter (53, 58, 59). ACE2 has been shown to be expressed by fetal kidney, ilium, and rectal cells from as early as 15 weeks, barely detectable at 15 weeks in the lungs with undetected expression thereafter, and undetectable in the cerebral ependymal, parenchymal and cardiac cells (60). It has been found that only a proportion of cells which are located in the fetal adrenal gland and the kidney co-expressed ACE2 and TMPRSS2. It was discovered that placental cytotrophoblasts and syncytiotrophoblasts (STBs) express ACE2 from 7 weeks onward, suggesting that SARS-CoV-2 could cross into the placenta at any gestational age (60). Investigation of ACE2 and TMPRSS2 co-expression in the developing embryo up to day 14 (from surplus IVF human embryos) has revealed the co-localization of these genes, raising concern to increased susceptibility to SARS-CoV-2 fetal infection in the early stages of embryonic development (61). To date, cohort studies of SARS-CoV-2 positive mothers with mild symptoms or asymptomatic, have reported no adverse effects to the mother or neonate regardless of the timing of the infection (i.e. first versus third trimester) (62, 63). However, women with severe SARS-CoV-2 infection that required critical care had higher odds of complications, particularly a higher incidence of iatrogenic pre-term delivery mostly due to fear of sudden maternal decompensation (64).

TRANSPLACENTAL VIRAL TRANSMISSION

The placenta offers a protective barrier that does not allow the fetus to become exposed to maternal infections (31). The human placenta primarily consists of a number of specific fetal-derived cells called trophoblasts, of which there are three main types. These include terminally differentiated multinuclear syncytiotrophoblast cells, which are in direct contact with the maternal blood and line the villus tree, progenitor villous cytotrophoblast cells, which underlie the syncytiotrophoblast, and invasive extravillous trophoblast (EVT) cells, which anchor the chorionic villi to the uterus and modify its vasculature (**Figure 2**) (31). Various potential causes may play a role in the vertical transmission of the virus from the mother to the fetus. These include direct damage to the villous tree with a break in the protective syncytiotrophoblast layer, which could be caused by virus-induced apoptosis and vascular damage in the placenta, spread through the virus-infected maternal endothelium to the extravillous trophoblast, trafficking of infected maternal immune cells throughout the syncytiotrophoblast, paracellular or transcellular transport (for example, immunoglobulin-mediated transcytosis) into fetal capillaries, transmission *via* swallowed or aspirated amniotic fluid (65, 66), as well as ascending infection from the vagina (**Figure 3**) (31). To define the possibility of vertical transmission of SARS-CoV-2 infection in different studies, a classification system has been proposed by a multidisciplinary team of the WHO (68). Given the timing of vertical transmission, *in utero*, intrapartum, and early postnatal period, four possibilities exist: confirmed, possible, unlikely, and indeterminate (68). Vertical transmission is considered "possible" if evidence suggests it but cannot confirm infection. However, if there is poor support of diagnosis, but vertical transmission cannot be completely ruled out, this is considered as "unlikely". The "indeterminate" possibility is when the tests required to define the classification have not been performed (68). Recent findings confirming the presence of SARS-CoV-2 mRNA or virions in syncytiotrophoblasts have strongly suggested transplacental infection caused by the SARS-CoV-2 (69, 70). Nonetheless, given that the presence of SARS-CoV-2 in the blood sample of COVID-19 patients is reported to be around

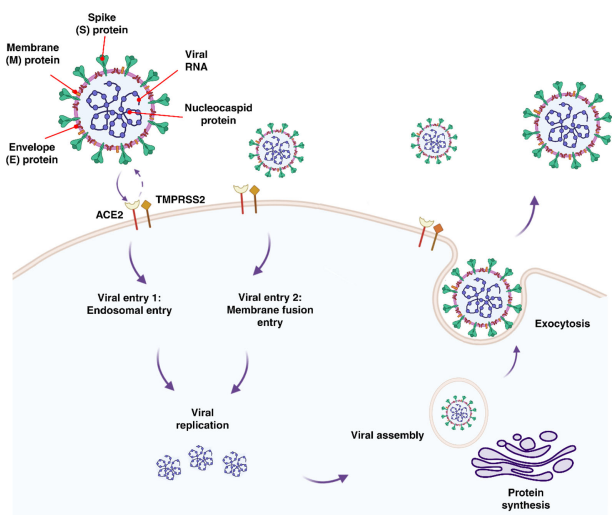

FIGURE 1 | Features, entry methods, and replication of SARS-CoV-2.

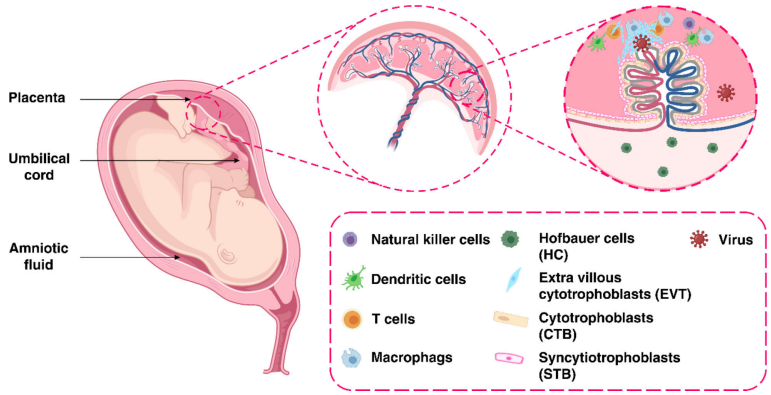

FIGURE 2 | The maternal-fetal interface.

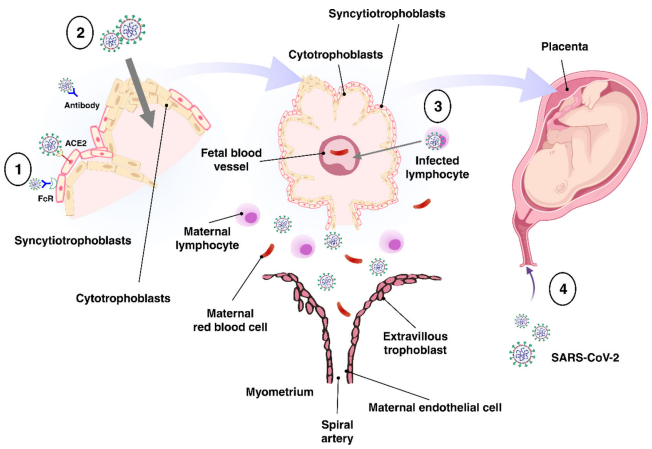

FIGURE 3 | Possible mechanisms of transplacental transmission. There are several potential mechanisms involved in the virus's vertical transmission from mother to fetus. (1) Infection caused by direct villous tree damage. (2) Infection through the maternal endothelium to the extravillous trophoblast. (3) Infection caused by maternal immune cell trafficking and transcellular transport. (4) Infection through the vagina. Adapted from (67).

1%, therefore the likelihood of SARS-CoV-2 being able to directly infect syncytiotrophoblasts is low (71). Another alternative way of transmitting SARS-CoV-2 infection to the neonate is through the vagina during childbirth (72, 73).

Whilst the possibility of transmitting SARS-CoV-2 from mother to fetus during pregnancy is suggested, the role of the placenta in infection with the virus has not yet been fully understood. However, evidence suggests that pathogens can overcome this barrier, infect the fetus, and even cause serious complications in newborns, such as microcephaly and ocular abnormalities (74). Such pathogens include Cytomegalovirus (CMV), herpes simplex virus (HSV), varicella-zoster virus, and Zika virus (ZIKV) (20, 75–77). It is currently unclear whether neonates who tested positive for SARS-CoV-2 have been infected with the virus from their mothers during pregnancy or have been infected during labor or after birth. (**Table 1**). Evidence based on infant antibody tests suggests vertical transmission of the virus may be possible. It was discovered that infants born to women infected with SARS-CoV-2 had higher immunoglobulin (Ig)G and IgM levels for SARS-CoV-2 (31, 89, 90). The presence of IgG in the fetus may indicate the transfer of this immunoglobulin from the mother to the fetus during pregnancy, but the presence of IgM indicates that the fetus has produced and secreted this immunoglobulin in response to viral infection because in contrast to IgG, IgM is unable to cross the placenta due to its higher molecular weight (89, 90).

BIOMARKERS OF SARS-CoV-2 INFECTION

Several studies have employed single cell RNA sequencing (scRNA-seq) to gain an understanding of the molecular features of SARS-CoV-2 infection (91–95). In a study by

TABLE 1 | Systematic review and meta-analysis studies on COVID-19 infection during pregnancy.

Publication name	Number of pregnant women with COVID-19	Findings	Conclusion
Vertical transmission of coronavirus disease 2019: a systematic review and meta-analysis (78)	NM	• SARS-CoV-2 RNA positivity was as follows • 0% (0/51) in amniotic fluid • 0% (0/17) in urine • 3.6% (1/28) in the cord blood • 7.7% (2/26) by placental sample analysis • 9.7% (3/31) by rectal or anal swab	• Vertical transmission of SARS-CoV-2 is possible but the likelihood of its occurrence is low • The rate of SARS-CoV-2 infection is almost similar to other pathogens causing congenital infections
Clinical outcomes of 201 neonates born to mothers with COVID-19: a systematic review (79)	223	• Fetal death was reported in two cases • Preterm birth was reported in 48 of 185 newborns • Birth asphyxia was reported in 1.8% of neonates • Respiratory distress syndrome was reported in 6.4% of neonates	• SARS-CoV-2 infection during pregnancy rarely affects fetal and neonatal mortality • SARS-CoV-2 infection during pregnancy can affect the fetal and neonatal morbidity
Maternal clinical characteristics and perinatal outcomes among pregnant women with coronavirus disease 2019. A systematic review (80)	322	• Premature birth was reported as the main adverse obstetric outcome in pregnant women • SARS-CoV-2 infection was not reported in samples, including breast milk, amniotic fluid, placenta or umbilical cord blood	• The study did not support the possibility of vertical transmission of SARS-CoV-2 in the third trimester
Clinical characteristics and outcomes of pregnant women with COVID-19 and the risk of vertical transmission: a systematic review (81)	230	• Premature birth was reported in 24.74% (24 out of 97) of newborns • SARS-CoV-2 infection was not reported in samples, including vaginal secretions, breast milk, amniotic fluid, placental blood, and placental tissues • 3.9% (5 out of 128) of newborns tested positive for SARS-CoV-2 RNA	• The main adverse event for newborn was premature delivery
Clinical characteristics and outcomes of pregnant women with COVID-19 and comparison with control patients: A systematic review and meta-analysis (82)	10,000	• Preterm birth was more common in pregnant women with COVID-19 than pregnant women without COVID-19 • The rate of vertical transmission was 5.3% • The rate of SARS-CoV-2 infection in neonates born to mothers with COVID-19 was 8%	• The higher likelihood of preterm birth in pregnant women with COVID-19 compared to pregnant women without COVID-19 may suggest a possible link between COVID-19 infection and pregnancy complications
Clinical Characteristics and Neonatal Outcomes of Pregnant Patients With COVID-19: A Systematic Review (83)	235	• SARS-CoV-2 infection was not reported in samples, including breast milk, amniotic fluid, and neonatal throat swab • Preeclampsia and premature delivery were reported as the major complications in pregnant women with COVID-19	• The study did not support the possibility of vertical transmission of SARS-CoV-2 infection, however it mentioned that the vertical transmission cannot be ignored
Pregnancy and Breastfeeding During COVID-19 Pandemic: A Systematic Review of Published Pregnancy Cases (84)	3,985	• Preterm birth was recorder in 23% of cases • SARS-CoV-2 infection was reported in samples, including amniotic fluid, breast milk, placenta, and cord blood, from pregnant women with COVID-19 • 61 newborns were found to be tested positive for SARS-CoV-2	• The study suggested that vertical transmission of SARS-CoV-2 is possible
COVID-19 (SARS-CoV-2) Infection in Pregnancy: A Systematic Review (85)	156	• Intrauterine/fetal distress and premature rupture of membranes were reported as the most common maternal/fetal complications	• The study suggested that COVID-19 infection may increase the risk of preterm birth and maternal death • The study did not support the possibility of vertical transmission of SARS-CoV-2 infection
Maternal and perinatal outcomes with COVID-19: A systematic review of 108 pregnancies (17)	108	• Maternal intensive care unit (ICU) admission was reported	• The study mentioned that the vertical transmission cannot be ruled out

(Continued)

TABLE 1 | Continued

Publication name	Number of pregnant women with COVID-19	Findings	Conclusion
		• One case of intrauterine fetal death and one case of neonatal case was reported	
COVID-19 in Pregnant Women and Neonates: A Systematic Review of the Literature with Quality Assessment of the Studies (86)	275	• Preterm birth was recorded in 28% of cases • 2 stillbirths were reported • 16 out of 248 neonates were tested positive for SARS-CoV-2 RNA, of which 9 of them were born to mothers with COVID-19 • SARS-CoV-2 infection was not reported in samples, including amniotic fluid, vaginal/cervical fluids, breast milk, and placental tissue	• The study mentioned that the vertical transmission is unlikely but it cannot be ruled out
Maternal Coronavirus Infections and Neonates Born to Mothers with SARS-CoV-2: A Systematic Review (87)	1457	• 64 cases of premature birth were reported • 16 cases of intrauterine fetal death or neonatal death were reported • 15 cases of maternal death were reported • 7 cases of miscarriage were reported • 19 cases of decreased fetal movements were reported • 5 cases of severe neonatal asphyxia were reported • 39 out of 1042 newborns were tested positive for SARS-CoV-2 infection • SARS-CoV-2 infection was reported in samples, including breast milk and placenta	• The study suggested that COVID-19 infection can be associated with maternal, fetal, and neonatal complications • The study mentioned that the vertical transmission cannot be ruled out
Vertical transmission of SARS CoV-2: a systematic review (88)	714	• 17 out of 606 neonates were tested positive for SARS-CoV-2 RNA • SARS-CoV-2 infection was reported in samples, including amniotic fluid, placenta and breast milk	• Possible vertical transmission of SARS-CoV-2 has been reported in some studies

NM, not mentioned; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; COVID-19, coronavirus disease 2019.

Lu et al., which compared ACE2 and TMPRSS2 gene expression between fetal, placental tissues and adult tissues, a small proportion of trophoblast cells, as well as various fetal organs such as the heart, kidney, stomach, and adrenal glands, had ACE2 expression. The study showed that only the kidney and adrenal gland expressed TMPRSS2 (96). Pique-Regi et al. discovered that very few cells during any of the three trimesters expressed both ACE2 and TMPRSS2. Using single-nuclear RNAseq (snRNA-seq), it has been shown that the placenta is unlikely to express ACE2 and TMPRSS2, and thus be infected by SARS-CoV-2 (59). Using scRNA-seq data, Ashary et al., identified only a small proportion of STB in the first trimester and EVT in the second trimester had ACE2 and TMPRSS2 expression. The $ACE2^{+}TMPRSS2^{+}$STBs were highly differentiated and expressed genes engaged in mitochondrial metabolism and glucose transport. In addition, the $ACE2^{+}TMPRSS2^{+}$EVTs were found to have endovascular trophoblast markers. The researchers found that these cells could be the targets of SARS-CoV-2 entry (97). Moreover, robust immune responses at the maternal-fetal interface of SARS-CoV-2-infected women was discovered (98). Researchers found overexpression of interferon-related genes, and increased activation of NK cells and T cells (98–100). Also, it was found that there was an association between SARS-CoV-2 infection and local immune responses at the maternal-fetal interface (98). in a study by Nagy et al, the impact of mutations in SARS-CoV-2 viral genes on clinical outcomes was explored. The study found that mutations in the nucleocapsid phosphoprotein-N, nonstructural proteins-4 (NSP4), NSP6, Open Reading Frame-3a (ORF3a), and ORF8 were associated with mild outcome, while mutations in NSP7 were linked to severe disease (101).

The identification of new biomarkers and prevention strategies requires the fundamental understanding and control of how SARS-CoV-2 spreads to the lungs and elicits a multi-organ inflammatory response. (**Table 2**). These infection processes rely on their location and spatial context: which cells in which tissue locations are most susceptible to infection (105), infected cell-to-uninfected cell associations, and biochemical factor release of different cell types in response to infection

TABLE 2 | Potential biomarkers of disease severity in COVID-19.

Analytes	Changes	Role	Ref
IL-1, IL-2, IL-6, TNF-a, G-CSF, GM-CSF, IFN-γ	Increase	Cytokine storm biomarker	(102)
CD3+, CD4+, CD8+, B cells, NK cells	Decrease	Clinical Hematological biomarker	(103)
CK, CK-MB, CRP, Ferritin, LDH, BUN, Creatinine, cTnI, AST, ALT, Total bilirubin	Increase	Clinical Biochemical biomarker	(104)

IL, interleukin; TNF-a, tumor necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; IFN-γ, interferon gamma; NK, natural killer; CK, creatine kinase; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; cTnI, cardiac troponin I; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

(106). These spatiotemporal relationships in the inflammatory cascade give rise to positive or negative prognoses, and their understanding can triage patients at greater or lesser risks of infection, of response to infection, and inform new therapeutics and treatment regimens (107). Spatial immunoprofiling is rapidly advancing due to several recent technologies: advanced instrumentation, molecular barcoding and immunolabelling, providing a much richer portrait of the immune landscape (108), and recent approaches in biostatics and theoretical biology are incorporating imaging data to deconstruct the relationships between cells and disease within their tissue context (109–111). Spatial resolved transcriptomics are changing the ways in which we interrogate complex tissues and were voted the 'Method of the Year 2020' by the journal Nature Methods (112). These technologies combine the benefits in advancements in microscopy and advanced imaging, with simultaneous read out of transcript and proteomic data, thereby alleviating the challenges associated with single cell or bulk profiling. The maintenance of spatial context is key in understanding the underlying cellular profiles, biology, specialization and tissue organization and has begun shedding light into consortia studies such as the Human Cell Atlas. A number of technologies currently exist for RNA applications: Nanostring GeoMX Digital Spatial Profiler (DSP), 10x Genomics Visium, MERFISH and proteomic: Nanostring GeoMX DSP, Akoya Biosciences CODEX, Imaging Mass spectrometry (IMC) (113). Recent application of these methodologies to COVID-19 autopsy tissue studies from lungs, kidney, liver and heart tissue has provided deep insights into cell types and genes implicated with severe COVID-19 disease severity (114).

Once region- or cell-specific spatial information is derived from histology sections, statistical relationships between cells and tissues and mathematical predictions of their future behavior with or without treatment are often sought. There exist numerous tools to detect and segment single cell locations from this spatial information. While open-source *ImageJ*, developed in 1987, remains popular for microscopic image analysis (115–117), more recent software such as *CellProfiler*, *Icy*, *ilastik*, and *QuPath* provide user-friendly interfaces for the development of bioimage analysis macroscripts (118, 119). Once single-cell data can be derived, spatial relationships can be determined. The most common of which is intercellular clustering or associations, often calculated as cell density within concentric circles away from each cell's center and averaged across all imaged cells (120). For instance, to characterize the distribution of SARS-CoV-2 bodies from macrophages or monocytes or tissue structures to estimate inflammatory progression (121).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Taking into account the changing physiology and immune responses during gestation, pregnant women are more susceptible to developing severe COVID-19, which can lead to pregnancy-related complications. There is limited information for the association of COVID-19 and its direct complications to the growing fetus during pregnancy. These may include preterm birth, stillbirth, or long-term complications for the newborn (122). A study conducted on 827 pregnant women, who have been given the COVID-19 mRNA vaccine, found that the proportion of adverse pregnancy and neonatal outcomes were similar to incidence reported in similar studies conducted prior to the pandemic (123). Furthermore, vaccination of pregnant women has been shown to result in maternal IgG production 5 days after the first dose of vaccination, as well as the transplacental transfer of IgG 16 days after the first dose of vaccination (124). However, longitudinal follow-up is needed to monitor those who are vaccinated, especially during the first trimester, in order to be informed about maternal, pregnancy, and neonatal outcomes. Another important consideration with COVID-19 infection during pregnancy is that current diagnostic tests such as X-ray and CT scans cannot be performed in pregnant women due to potential risks to the growing fetus (125). These factors may therefore delay the diagnosis and treatment of pregnant women, particularly those with more severe symptoms.

These factors may therefore delay the diagnosis and treatment of pregnant women, particularly those with more severe symptoms. Screening tests may be helpful in this respect because of the possibility of transmitting the virus from the mother to the fetus. Understanding the disease progression and its relationship to manifestation severity is necessary to therapeutically intervene and reduce the associated morbidity.

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All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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COVID-19 mRNA Vaccination in Lactation: Assessment of Adverse Events and Vaccine Related Antibodies in Mother-Infant Dyads

*Yarden Golan[1,2], Mary Prahl[3,4], Arianna G. Cassidy[5], Caryl Gay[6], Alan H. B. Wu[7], Unurzul Jigmeddagva[8], Christine Y. Lin[5], Veronica J. Gonzalez[5], Emilia Basilio[5], Megan A. Chidboy[5], Lakshmi Warrier[9], Sirirak Buarpung[5], Lin Li[5], Amy P. Murtha[5], Ifeyinwa V. Asiodu[6], Nadav Ahituv[1,2], Valerie J. Flaherman[3] and Stephanie L. Gaw[5,8]**

[1] Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, San Francisco, CA, United States, [2] Institute for Human Genetics, University of California, San Francisco, San Francisco, CA, United States, [3] Department of Pediatrics, University of California, San Francisco, San Francisco, CA, United States, [4] Division of Pediatric Infectious Diseases and Global Health, University of California, San Francisco, San Francisco, CA, United States, [5] Division of Maternal-Fetal Medicine, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California San Francisco, San Francisco, CA, United States, [6] Department of Family Health Care Nursing, University of California, San Francisco, San Francisco, CA, United States, [7] Department of Laboratory Medicine, University of California, San Francisco, San Francisco, CA, United States, [8] Center for Reproductive Sciences, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California San Francisco, San Francisco, CA, United States, [9] Department of Medicine, University of California, San Francisco, San Francisco, CA, United States

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Veronique Demers-Mathieu, University of California, San Diego, United States

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***Correspondence:**
Stephanie L. Gaw
Stephanie.Gaw@ucsf.edu

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Background: Data regarding symptoms in the lactating mother-infant dyad and their immune response to COVID-19 mRNA vaccination during lactation are needed to inform vaccination guidelines.

Methods: From a prospective cohort of 50 lactating individuals who received mRNA-based vaccines for COVID-19 (mRNA-1273 and BNT162b2), blood and milk samples were collected prior to first vaccination dose, immediately prior to 2nd dose, and 4-10 weeks after 2nd dose. Symptoms in mother and infant were assessed by detailed questionnaires. Anti-SARS-CoV-2 antibody levels in blood and milk were measured by Pylon 3D automated immunoassay and ELISA. In addition, vaccine-related PEGylated proteins in milk were measured by ELISA. Blood samples were collected from a subset of infants whose mothers received the vaccine during lactation (4-15 weeks after mothers' 2nd dose).

Results: No severe maternal or infant adverse events were reported in this cohort. Two mothers and two infants were diagnosed with COVID-19 during the study period before achieving full immune response. PEGylated proteins were not found at significant levels in milk after vaccination. After vaccination, levels of anti-SARS-CoV-2 IgG and IgM significantly increased in maternal plasma and there was significant transfer of anti-SARS-CoV-2-Receptor Binding Domain (anti-RBD) IgA and IgG antibodies to milk. Milk IgA levels after the 2nd dose were negatively associated with infant age. Anti-SARS-CoV-2

IgG antibodies were not detected in the plasma of infants whose mothers were vaccinated during lactation.

Conclusions: COVID-19 mRNA vaccines generate robust immune responses in plasma and milk of lactating individuals without severe adverse events reported.

Keywords: COVID-19, SARS-CoV-2, lactation, antibodies, breastfeeding, human milk, mRNA vaccine, passive immunity

INTRODUCTION

An important benefit of human milk is the presence of IgA and IgG antibodies that provide passive immunity to the infant (1, 2). Anti-SARS-CoV-2 antibodies are present in milk from lactating women who were infected with SARS-CoV-2 (3, 4) or who received COVID-19 mRNA vaccines (5–14). Specifically, high titers of anti-SARS-CoV-2 IgG were reported after vaccination (6). In addition, IgG levels in milk were higher after vaccination compared to convalescent samples after SARS-CoV-2 infection (6, 14). The function of these antibodies in protection of infants against COVID-19 is not fully understood. In addition, it has recently been shown in a few studies that vaccines mRNA components are not present in milk samples after vaccination (15, 16), or were only detected in very low levels in some cases (14), providing reassurance that risks of exposure to the breastfed infant are minimal. Even though the infant receives passive immune protection from milk antibodies after vaccination, there is still significant hesitancy in the lactating population. Much of the concern is due to the lack of knowledge about the effect of mRNA-based vaccines on the nursing infant, as lactating mothers were excluded from initial clinical trials of mRNA vaccination (17). More studies that follow up on breastfeeding individuals and their infants after vaccination are needed to address concerns regarding the potential effects on infants, in order to prevent further delays in vaccination or early cessation of breastfeeding (18). In this study, we examined blood and milk samples from lactating mothers who received a COVID-19 mRNA vaccine, and their infants for the presence of anti-SARS-CoV2 antibodies and milk samples for the presence of PEGylated proteins which are part of the mRNA-based vaccines lipid nano-particles. In addition, we examine self-reported vaccine-related symptoms in order to address the gap of knowledge regarding vaccination efficacy and safety during lactation.

METHODS

Study Approval and Study Population

The institutional review board of the University of California San Francisco approved the study. Written, informed consent was obtained from all study volunteers in the COVID-19 Vaccine in Pregnancy and Lactation (COVIPAL) cohort study from December 2020 to June 2021. Eligible participants were actively lactating, planning to receive any COVID-19 vaccine, and willing to donate blood and/or milk samples.

Clinical Data Collection

Clinical data on vaccine side effects were collected through an online questionnaire that was sent to participants 21 days or more after each vaccine administration. Questionnaires were distributed using REDCap.

Sample Collection

Maternal blood and milk samples were collected at three time points: 1) up to 1 day before the 1st dose (pre-vaccine); 2) on the day of and prior to administration of the 2nd dose (after 1st dose); and between 4-10 weeks after the 2nd dose (after 2nd dose). In some cases, additional milk samples were collected up to 31 days before the 1st dose, 24 hours after each dose, and weekly for up to 4 weeks after the 2nd dose. Infant blood was collected by heel stick by trained study staff at 5-15 weeks after 2nd maternal vaccination.

Milk Processing

Fresh human milk samples were self-collected by participants into sterile containers at several time points before, during, and after vaccination. Milk samples were either collected immediately by the study staff or frozen by mothers in their home freezer as soon as possible after pumping. Samples were kept on ice during transport from home to the lab for processing. Milk was aliquoted and stored in -80°C until analyzed.

Measurement of SARS-CoV-2 Specific IgM and IgG in Plasma Samples

Whole blood was collected into tubes containing EDTA. Plasma was isolated from whole blood by centrifugation and immediately cryopreserved at -80°C until analysis. Anti-SARS-CoV-2 plasma IgM and IgG antibodies were measured using the Pylon 3D automated immunoassay system (19) (ET Healthcare, Palo Alto, CA). In brief, quartz glass probes pre-coated with either affinity-purified goat anti-human IgM (IgM capture) or Protein G (IgG capture) were dipped into diluted plasma samples, washed, and then dipped into the assay reagent containing both biotinylated, recombinant spike protein receptor binding domain (RBD) and nucleocapsid protein (NP). After washing, the probes were incubated with Cy®5-streptavidin (Cy5-SA) polysaccharide conjugate reagent, allowing for cyclic amplification of the fluorescence signal. The background-corrected signal of SARS-CoV-2 specific antibodies

was reported as relative fluorescent units (RFU). IgM and IgG measurements greater than 50 RFU were considered positive RFUs.

Measurement of IgA and IgG by ELISA Assay in Milk

After thawing, milk fat was separated by cold centrifugation (10,000g for 10 min, 4°C). Milk supernatant samples were diluted 1:2 in sample diluent buffer and were plated in duplicate on a 96-well plate containing S1 spike protein RBD (Ray-Biotech, GA, USA, IEQ-CoVS1RBD-IgG-1 and IEQ-CoVS1RBD-IgA-1). For monomeric IgA assays, samples were also plated in duplicate on a second 96-well plate coated with human albumin to account for non-specific binding. OD values for albumin were subtracted from the OD values for RBD. Each plate contained seven wells of serial dilutions (1:3) of a positive control from an inactivated serum sample which contains SARS-COV-2 S1 RBD protein human IgA antibody (provided with the kit) and one blank negative control. The mean absorbance of each sample was captured on an ELISA plate reader at 450 nm. Background values (blank negative control) were subtracted from the albumin and RBD plates. Standard controls were used to create a standard curve and determine the level of anti-RBD IgA and IgG in unit/ml.

Measurement of Polyethylene Glycol (PEGylated) proteins in human milk by ELISA. Milk supernatant was diluted 1:8 with the provided sample buffer and analyzed by PEGylated protein ELISA kit (Enzo, Farmingdale, NY, USA). Seven wells of each plate were loaded with serial dilutions (1:2) of mRNA-1273 or BNT162b2 to generate the standard curve for each vaccine (**Figure S1A**). The PEGylated Protein ELISA kit is a competitive assay specific to the backbone of PEG. Samples and controls were loaded on plates pre-coated with monoclonal antibody to PEG which binds in a competitive manner the PEG or PEGylated protein in the sample, or the PEG covalently linked to biotin which is mixed and incubated together with the tested samples. Due to the competitive assay, the amount of signal (OD) is inversely proportional to the concentration of PEG in the sample (**Figure S1A**). To ensure the ability of the kit to detect the vaccine PEGylated components in milk samples, mRNA-1273 or BNT162b2 vaccines were separately inoculated into human milk samples at three different concentrations (33µl/ml, 3.3µl/ml and 0.33µl/ml) and were analyzed separately (**Figure S1B**). Prism 9 (v 9.1.2) was used to interpolate PEGylated proteins concentration in the samples based on OD values, using a sigmoidal, four parameters logistic curve. Standard curve for mRNA-1273 or BNT162b2 were used to analyze participant milk samples based on the vaccine received. Of note, the assay measures all types of PEGylated proteins (if present in the sample), and not only the vaccine PEGylated proteins.

Statistics

All data analyses were conducted using Stata statistical software (v14, College Station, TX). Descriptive statistics included frequencies for categorical variables, and means, standard deviations, medians, and ranges for continuous variables. Group differences in categorical variables were analyzed using Fisher's exact test, and group differences in continuous variables were analyzed using Mann-Whiney U tests. McNemar tests were used to evaluate differences in symptom frequencies after each vaccine dose. Spearman correlation was used to assess the magnitude of associations between continuous variables. Non-parametric tests were used to accommodate non-normal distributions and small group sizes.

RESULTS

Participant Characteristics

During the study period, 50 participants answered all study questionnaires, provided blood and/or milk samples, had an infant up to 18 months old were included in this analysis. Two infants were diagnosed with COVID-19 during this study (**Table S1**, infant of participants 1 and 2). One mother reported that her infant had mild symptoms 1 week after the 2nd dose (not exclusively breastfed); this infant's vaccinated mother had a negative test at the time of the infant's positive PCR test. A second infant (exclusively breastfed) had positive plasma anti-SARS-CoV-2 IgG and IgA, despite the mother receiving the vaccine postpartum and reported no known prior COVID-19 infection. The mother's plasma was subsequently found to be positive to antibodies against SARS-CoV-2 nucleocapsid protein, indicating a likely natural asymptomatic SARS-CoV-2 infection (further details in **Table S1**). Two mothers were positive for COVID-19 and are presented in **Table S1** (participants 2 and 3)**;** they were excluded from further analysis of symptomatology. Cohort characteristics are presented in **Table 1**. Twenty-seven female participants [mean age 35.7 years (± 3.9)] received the BNT162b2 vaccine (Pfizer, 56%), and 21 received the mRNA-1237 (Moderna, 44%). The mean infant age at mother's 1st dose was 5 months (± 3.9). All mothers continued to feed their infants with milk at the time of the 2nd vaccination, and all except one continued up to the time of follow up sample collection (4-10 weeks after 2nd dose). There were no significant differences in maternal or infant characteristics by vaccine manufacturer.

Post Vaccination Symptoms

Self-reported symptoms after each vaccine dose are presented in **Table 2**. Fever, chills, headache, joint pain, muscle aches or body aches, and fatigue or tiredness were reported by significantly more participants after the 2nd dose than after the 1st dose (**Table 2**). All 21 participants (100%) who received the mRNA-1237 vaccine reported injection site symptoms, while only 21 (78%) of 27 BNT-162b2 recipients reported injection site symptoms (p=0.02) (**Table 2**). Two mothers reported slightly less milk production in the first 24-72 hours after vaccine doses (**Table 2**). With respect to infant symptoms, 12% of mothers reported at least one symptom after the 1st maternal vaccine dose (primarily gastrointestinal symptoms and sleep changes), and none reported an infant symptom after the 2nd dose

TABLE 1 | Sample characteristics overall and by vaccine manufacturer.

Sample Characteristics	Full Cohort (n = 48, 100%)	BNT162b2 (n = 27, 56%)	mRNA-1237 (n = 21, 44%)
Maternal characteristics			
Maternal age, years			
Median (min, max)	35 (27, 46)	35 (30, 45)	35 (27, 46)
Race/ethnicity, % (n)			
Asian	31% (15)	30% (8)	33% (7)
Black or African American	2% (1)	4% (1)	0% (0)
White/Caucasian	59% (28)	55% (15)	62% (13)
Other (Middle Eastern)	2% (1)	0% (0)	5% (1)
More than 1 race/ethnicity (White+Latina/Asian/Middle Eastern)	6% (3)	11% (3)	0% (0)
Highest level of education completed			
Some college	2% (1)	0% (0)	5% (1)
College graduate	17% (8)	19% (5)	14% (3)
Advanced degree	81% (39)	81% (22)	81% (17)
Work in health care?			
Yes, providing direct patient care	58% (28)	52% (14)	67% (14)
Yes, but not in direct patient care	19% (9)	15% (4)	24% (5)
No	23% (11)	33% (9)	9% (2)
Pre-Pregnancy Body Mass Index			
Median (min, max)	23.4 (19.1, 37.5)	23.4 (19.1, 35.9)	22.8 (19.6, 37.5)
Number of children			
1	40% (19)	41% (11)	38% (8)
2	46% (22)	41% (11)	52% (11)
3	12% (6)	15% (4)	10% (2)
4	2% (1)	3% (1)	0% (0)
Duration of most recent pregnancy, weeks			
Median (min, max)	39.0 (33.9, 41.1)	39.1 (33.9, 41.0)	39.0 (37.4, 41.1)
Infant characteristics			
Infant age at maternal 1st dose, months			
Median (min, max)	4.7 (0.1, 17.2)	4.8 (0.2, 15.2)	4.6 (0.1, 17.2)
Sex, % (n)			
Male	60% (29)	67% (18)	52% (11)
Female	40% (19)	33% (9)	48% (10)
Exclusively breastfeeding (and no solids)			
Yes	23% (11)	22% (6)	24% (5)
No	77% (37)	78% (21)	76% (16)
Days after vaccine that symptoms were assessed			
Dose 1			
Mean (SD)	78.7 (31.8)	78.3 (35.4)	79.2 (27.4)
Median (min, max)	81 (18, 154]	78 (18, 154)	86 (26, 117)
Dose 2			
Mean (SD)	59.6 (25.1)	62.6 (28.3)	55.8 (20.2)
Median (min, max)	58.5 (28, 133)	57 (29, 133)	60 (28, 89)

None of the characteristics above differed significantly by vaccine manufacturer.
Standard deviation (SD), minimum (min), maximum (max).

(**Table 3**). In summary, no severe adverse events (death, life threatening, hospitalization, disability) for mothers or nursing infants were reported in this cohort after vaccination, and reported symptoms resolved up to 72 hours after vaccination.

PEG Detection in Human Milk

Polyethylene glycol (PEG) is present in the lipid nanoparticles of the mRNA-based vaccines, and was reported to cause allergic reaction after vaccination in rare cases (20, 21). To address concerns about vaccine components passing to milk after vaccination, we performed ELISA assays to measure PEGylated proteins levels in milk after vaccination from 13 participants. PEGylated proteins were measured in milk samples collected before the vaccine, and at various time points post-vaccination (from 24 hours after 1st dose to 2 weeks after 2nd dose). Pre-vaccine PEGylated proteins concentration did not significantly differ from PEGylated proteins levels at any post-vaccine time point in either paired or unpaired comparisons (**Figure 1**).

Anti-SARS-CoV-2 Antibody Levels in Blood and Milk Samples After Vaccination

We analyzed blood and milk samples from lactating individuals for anti-SARS-CoV-2 antibodies to measure immune response after vaccination. Maternal blood anti-SARS-CoV-2 IgM and IgG antibodies increased significantly after the 1st dose **(Figure 2).** Anti-SARS-CoV-2 IgM levels were not significantly higher 4-10 weeks after the 2nd dose compared to samples collected after dose 1 (on the day of the 2nd dose) (**Figures 2A, B).** In contrast, anti-SARS-CoV-2 IgG levels increased significantly after the 2nd dose (P value <0.0001) when compared to samples collected

TABLE 2 | Symptoms after each vaccine dose.

Symptoms	Full Cohort:			After 1st dose			After 2nd dose		
	1st dose	2nd dose n = 48	p-value[a]	BNT162b2 n = 27	mRNA-1237 n = 21	p-value[b]	BNT162b2 n = 27	mRNA-1237 n = 21	p-value[b]
Injection site symptoms, % (n)									
Any injection site symptoms	88% (42)	88% (42)	>0.99	78% (21)	100% (21)	**0.02**	78% (21)	100% (21)	**.02**
Pain	88% (42)	85% (41)	0.71	78% (21)	100% (21)	**0.02**	78% (21)	95% (20)	0.12
Redness	4% (2)	10% (5)	0.08	0% (0)	10% (2)	0.19	4% (1)	19% (4)	0.15
Swelling	17% (8)	17% (8)	>0.99	7% (2)	29% (6)	0.12	11% (3)	24% (5)	0.27
Itching	4% (2)	4% (2)	>0.99	4% (1)	5% (1)	>.99	4% (1)	5% (1)	>.99
Rash around injection site	2% (1)	4% (2)	0.32	0% (0)	5% (1)	0.44	0% (0)	10% (2)	0.19
Generalized symptoms, % (n)									
Any general symptoms	48% (23)	92% (44)	**<0.001**	44% (12)	52% (11)	0.77	85% (23)	100% (21)	0.12
Fever	12% (6)	62% (30)	**<0.001**	19% (5)	5% (1)	0.21	52% (14)	76% (16)	0.13
Chills	8% (4)	48% (23)	**<0.001**	11% (3)	5% (1)	0.62	37% (10)	62% (13)	0.14
Headache	21% (10)	67% (32)	**<0.001**	11% (3)	33% (7)	0.08	56% (15)	81% (17)	0.07
Joint pain	8% (4)	31% (15)	**0.002**	7% (2)	10% (2)	>0.99	30% (8)	33% (7)	>0.99
Muscle/body aches	21% (10)	69% (33)	**<0.001**	30% (8)	10% (2)	0.15	59% (16)	81% (17)	0.13
Fatigue or tiredness	44% (21)	81% (39)	**<0.001**	41% (11)	48% (10)	0.77	67% (18)	100% (21)	**0.003**
Nausea	4% (2)	12% (6)	0.10	4% (1)	5% (1)	>0.99	7% (2)	19% (4)	0.38
Vomiting	0% (0)	0% (0)	—	0% (0)	0% (0)	—	0% (0)	0% (0)	—
Diarrhea	4% (2)	4% (2)	>0.99	4% (1)	5% (1)	>0.99	0% (0)	10% (2)	0.19
Abdominal pain	2% (1)	0% (0)	0.32	0% (0)	5% (1)	0.44	0% (0)	0% (0)	—
Rash not near injection site	0% (0)	0% (0)	—	0% (0)	0% (0)	—	0% (0)	0% (0)	—
Lump/swelling in breast (same side as injection)	0% (0)	2% (1)	0.32	0% (0)	0% (0)	—	4% (1)	0% (0)	>0.99
Lump/swelling in breast (opposite side as injection)	0% (0)	0% (0)	—	0% (0)	0% (0)	—	0% (0)	0% (0)	—
Mastitis	2% (1)	0% (0)	0.32	0% (0)	5% (1)	0.44	0% (0)	0% (0)	—
Decrease in milk supply	2% (1)	2% (1)	>0.99	0% (0)	5% (1)	0.44	0% (0)	5% (1)	0.44

[a]*McNemar's test.*
[b]*Fisher's Exact test. Statistically significant values (p<0.05) are indicated in bold.*

immediately prior to the 2nd dose (**Figures 2C, D**). There was no significant difference in blood antibody levels between participants who received the mRNA-1237 compared to the BNT-162b2 vaccine after dose 2 (determined by unpaired Mann-Whitney test).

We found significantly higher levels of IgA antibodies specific to SARS-CoV-2 RBD protein in human milk samples collected after the 1st dose of both BNT-162b2 and mRNA-1237 vaccines (**Figures 3A, B**). There was no significant increase in milk anti-RBD IgA after the 2nd vaccination as compared to after dose 1 (**Figures 3A, B**). Twelve individuals (25%, BNT-162b2 n=7; mRNA-1237 n=5) did not have detectable levels of anti-RBD IgA after either the 1st or 2nd dose (infants age at 1st dose range 1-11 months). Milk anti-RBD IgG levels increased after the 1st dose of vaccine and increased further after the 2nd dose (**Figures 3C, D**). There were no significant differences in milk anti-RBD IgG levels between women who received BNT-162b2 (**Figure 3C**) and mRNA-1237 (**Figure 3D**). These findings suggest that mRNA vaccine results in a robust immune response leading to increased anti SARS-CoV-2 antibody levels in blood, but also in milk during lactation.

TABLE 3 | Infant symptoms reported after maternal vaccination (write-in only).

INFANT SYMPTOMS	% (n)	Vaccine
After 1st vaccine dose		
None/no changes/blank	88% (42)	
"My baby seemed a little tired."	2% (1)	BNT162b2
"He started pooping a lot! And it was more sour smelling diarrhea like poops. I don't know if there is any correlation"	2% (1)	BNT162b2
"It could have been a fluke, but both my infant and I slept through the night for the first time the night after I received the 1st dose of the vaccine."	2% (1)	BNT162b2
"He had some diaper rash, but likely unrelated"	2% (1)	BNT162b2
"Rash on the face/worsening of baby acne"	2% (1)	mRNA-1237
"Disrupted sleep, waking at night when he usually doesn't. More fussy than normal."	2% (1)	mRNA-1237
After 2nd vaccine dose		
None/no changes/blank	100% (48)	

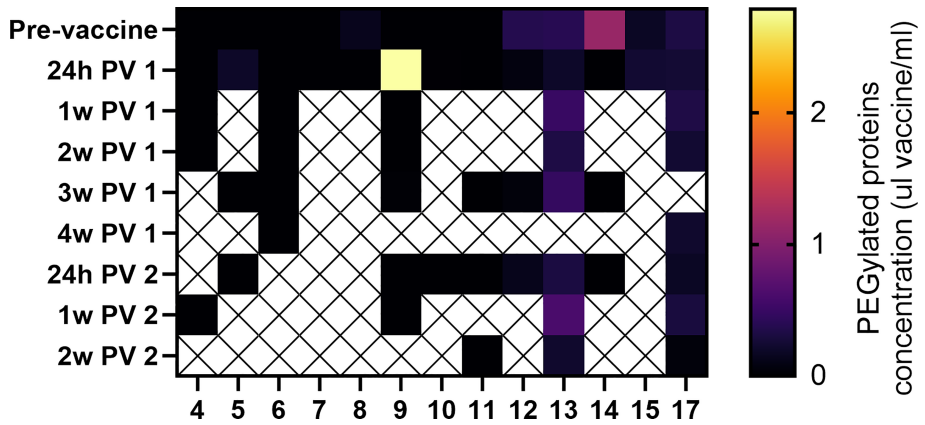

FIGURE 1 | Detection of vaccine PEG in human milk samples. PEGylated protein concentration in each sample were interpolated based on vaccine standard curves (**Figure S1**). No significant differences were observed between samples collected at any of the post vaccine (PV) time points and the pre-vaccine samples (paired and unpaired two-tailed t-tests). Y axes represent time of sample collection, as hours (h) or weeks (w) Post vaccine 1 (PV 1), or Post vaccine 2 (PV 2).

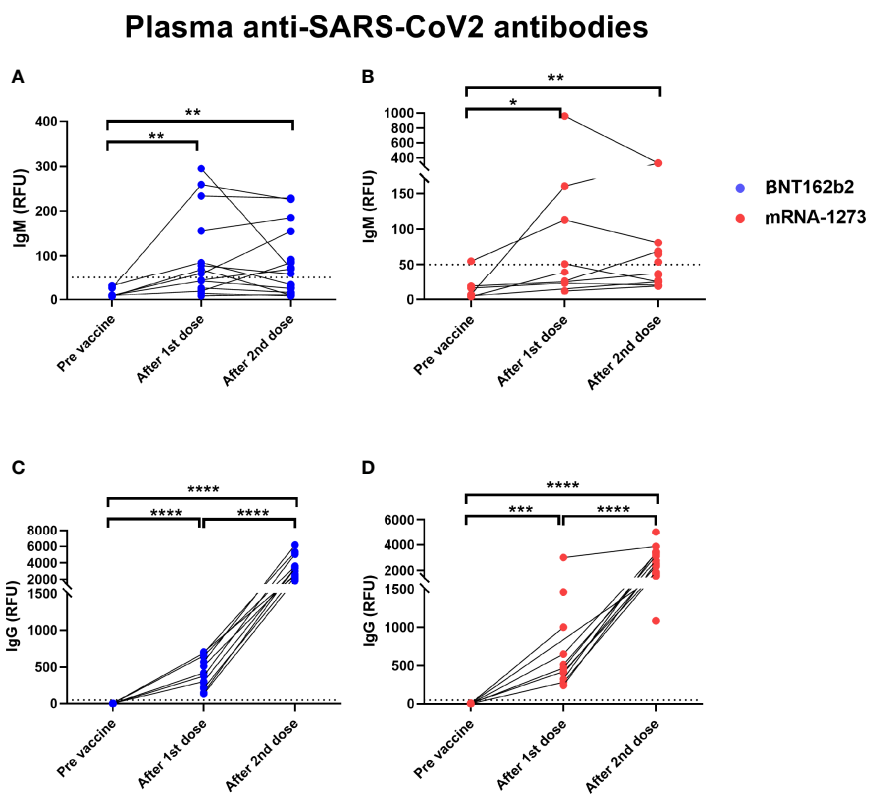

FIGURE 2 | Elevated levels of plasma anti-SARS-CoV2 antibodies in COVID-19 mRNA vaccinated lactating individuals. Anti-SARS-CoV2 IgM levels in plasma of lactating individuals receiving BNT-162b2 (n=19) **(A)** and mRNA-1273 (n=13) **(B)** COVID-19 vaccines (RFU- relative fluorescent units, dashed line represents positive cut-off >50 RFU). Anti-SARS-CoV2 IgG levels in plasma of lactating individuals receiving BNT-162b2 **(C)** and mRNA-1273 **(D)** COVID-19 vaccines. After 1st dose samples were collected on the day of the second vaccine, and after 2nd dose samples were collect 4-10 weeks post 2nd dose. Asterisks represent p-values: *= p-value <0.05, **= p-value <0.01, ***= <0.001, ****= <0.0001 as determined by unpaired Mann-Whitney test.

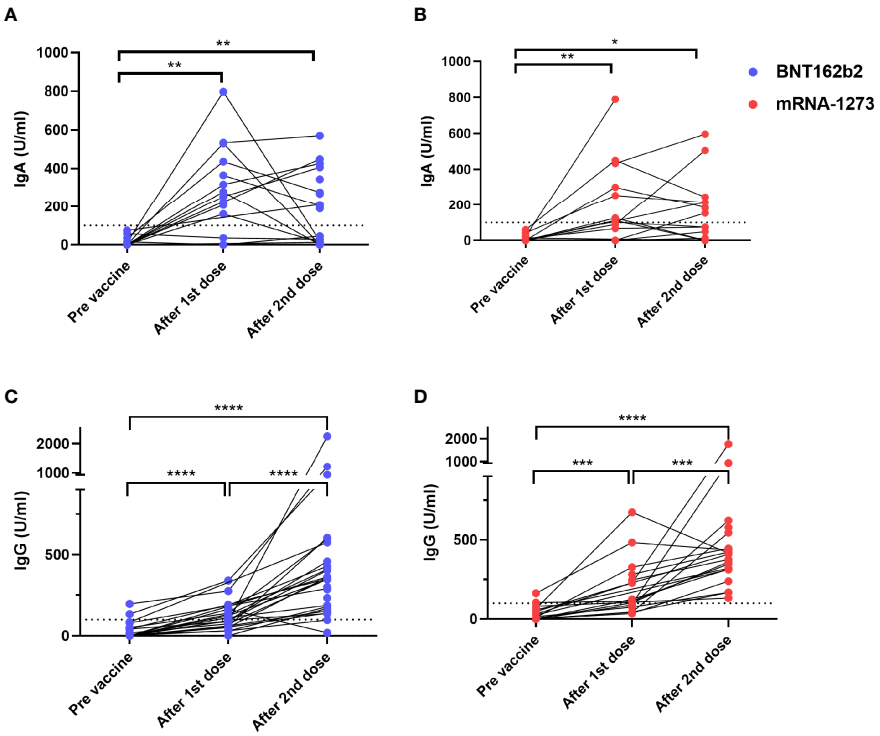

FIGURE 3 | Elevated levels of milk anti-SARS-CoV2 IgA antibodies in COVID-19 mRNA vaccinated lactating individuals. Milk samples from individuals receiving BNT-162b2 (n=27) **(A)** and mRNA-1273 (n=21) **(B)** COVID-19 vaccines were analyzed for anti-SARS-CoV2 IgA antibodies using ELISA at various time points as indicated on the X axis. After 1st dose samples were collected on the day of the second vaccine, and after 2nd dose samples were collect 4-10 weeks post 2nd dose. Milk anti-SARS-CoV2 IgG levels were measured using ELISA in milk samples from individuals receiving BNT-162b2 (n=27) **(C)** or mRNA-1273 (n=21) **(D)**. Asterisks represent p-values: *= p-value <0.05, **= p-value <0.01, ***= <0.001, ****= <0.0001 as determined by unpaired Mann-Whitney test. Dashed line represents positive cut-off >100 U/ml.

Correlations Between Antibody Levels, Participant Characteristics, and Symptoms

To better understand the differences in antibody responses between individuals in our cohort, we performed multiple correlation tests to determine whether IgG and IgA antibodies levels correlated with timing of sample collection after vaccination (range 4-10 weeks after 2nd dose), infant age at time of vaccination, or maternal BMI (**Table S2**). Milk IgA (but not IgG) levels measured after the second dose declined significantly as the infant age at time of vaccination increased (**Figures 4A, B).** There was no significant correlation between IgG and IgA levels and either the length of time after 2nd dose or maternal BMI (**Tables S2, S3**). The levels of IgG and IgA antibodies induced in milk were significantly correlated after 1st dose (**Figure 4C**), but not after the 2nd dose (**Figure 4D**). There was no correlation between the anti-SARS-CoV-2 IgG levels in blood and milk after 1st dose (**Figure 4E**), but there was a positive correlation between levels at 4-10 weeks after 2nd dose (**Figure 4F**).

Plasma Levels of Anti-SARS-CoV-2 IgG Are Not Detectable in Infants After Maternal Vaccination During Lactation

Although maternal IgG antibodies have been shown in multiple studies to transfer to the infant *in utero*, existing data suggests that milk-derived antibodies are not transferred to the infant blood circulation during breastfeeding (22, 23). To investigate whether maternal vaccination during lactation triggers infant immune responses, we analyzed infant blood samples from a subset of infants in our cohort (n=8). Blood samples were collected from these 8 infants (4 male, 4 female) at 68 days to 1 year of age (**Table S4**). Plasma was tested for the presence of anti-SARS-CoV-2 IgG and IgM and anti-RBD IgA. We evaluated infant blood samples collected at time frame of 4-10 weeks after 2nd dose as this time point corresponded to high anti-SARS-CoV-2 IgG levels in mothers' blood and milk (**Figures 2**, **3**). No antibodies were detected in the blood of nursing infants born to mothers who were vaccinated postpartum (**Figure 5**), despite high IgG levels in maternal blood and milk. In contrast, infants born to mothers who received both doses of vaccine during

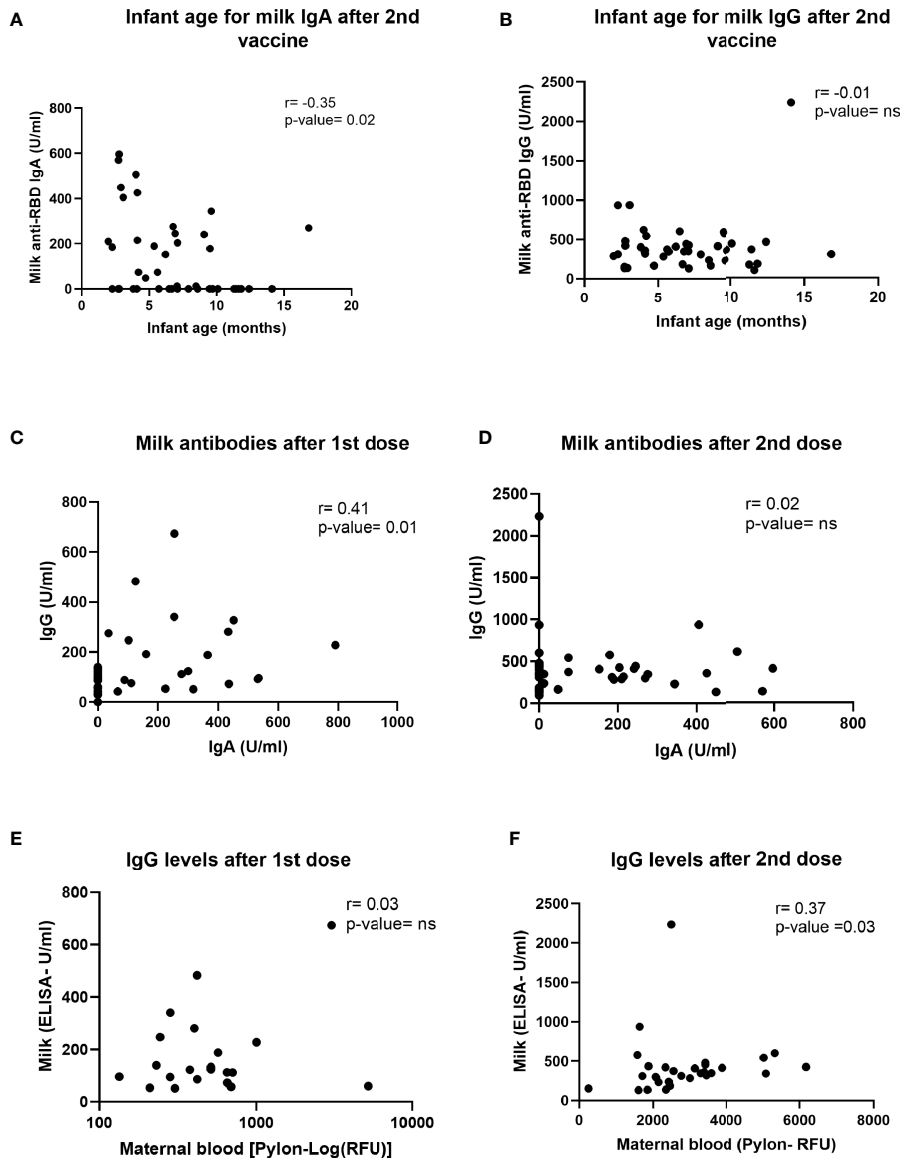

FIGURE 4 | Correlations between milk antibodies, blood antibodies and infant age. Two-tailed Spearman correlation was used to correlate milk IgA **(A)** and IgG **(B)** levels (Y axis) and infant age (X axis) 4-10 weeks after the 2nd dose administration (n=30). In addition, two-tailed Spearman correlation was used to correlate milk IgG (Y axis) and milk IgA levels (X axis) on the day of 2nd dose administration **(C)**, 21-28 days after 1st dose (n=35) and 4-10 weeks after 2nd dose **(D)**. We also tested correlation between milk (Y axis) and maternal plasma (X axis) IgG levels at day of 2nd dose **(E)** and 4-10 weeks after the 2nd dose administration **(F)** (n=30). Semi-partial correlations were used to assess relationships between variables while controlling for the effects of other relevant variables.

pregnancy had detectable plasma anti-SARS-CoV-2 IgG levels at birth (24) and at follow-up (data not showed). None of the follow-up infant blood samples had detectable levels of anti-RBD IgA antibodies. These results demonstrate that vaccination during lactation induces anti-SARS-CoV-2 antibodies in human milk but does not lead to detectable immunity in the infant, and does not provide additional transfer (or production) of anti-SARS-CoV-2 antibodies to the infant blood, in contrast to vaccination during pregnancy. Furthermore, maternal vaccination does not appear to stimulate an immune response in lactating infants, as expected.

CONCLUSION

Our study provides a detailed report on patient symptoms and antibody responses of the COVID-19 mRNA vaccines in lactating mothers. We found that the rates of reported symptoms were similar to the CDC report from the V-Safe registry (25) but higher than described in the clinical trials (26, 27), although we do not have a non-lactating comparison group. Comparing the mRNA vaccines made by current manufacturers, we found that lactating individuals may experience more vaccine-related side effects after mRNA-1237 compared to

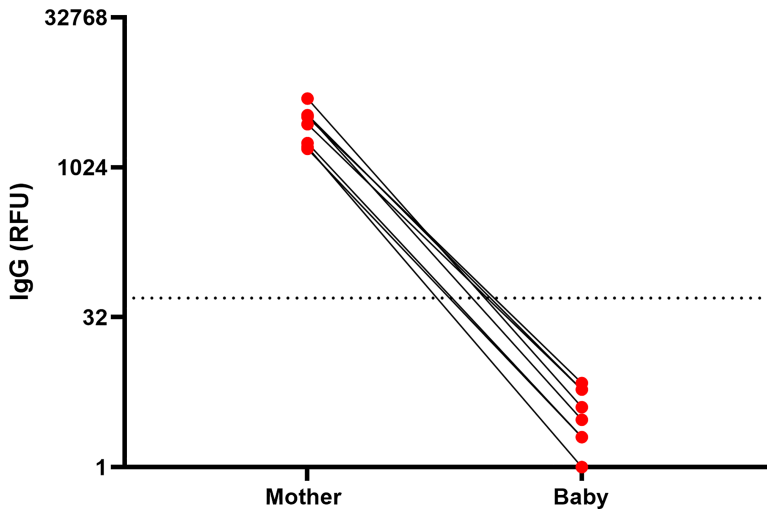

FIGURE 5 | Infants anti-SARS-CoV2 IgG levels after maternal vaccination during lactation. IgG levels were measured in blood samples of infants and mothers 61 days to 1 year postpartum, 61-129 days after 1st maternal vaccine administration. Maternal and infant samples were collected in the same week (except in one case in which the maternal sample was collected 18 days prior to the infant sample). (RFU- relative fluorescent units, dashed line represents positive cut-off >50 RFU). Sample characteristics and individual antibodies levels are presented in **Table S4**.

BNT-162b2 vaccine. These findings were in line with a report from another survey-based cohort study (28). Although in some studies, mRNA-1237 vaccine was shown to induce higher Spike and RBD-specific IgA titters in blood (8), in our cohort we found no significant differences in immune response between those vaccines.

Importantly, no severe side effects were reported in the infants of mothers vaccinated during breastfeeding. The reported symptoms (primarily gastrointestinal symptoms, rash, and sleep changes) were also reported in a larger cohort of vaccinated lactation mothers in a relatively similar low frequencies (28). However, both our study and the study by McLaurin-Jiang et al, are missing a non-vaccinated control group. The reported symptoms are common in lactating infants and might not be directly related to vaccine administration but to viral infection of other factors. For example, a mother in our study (**Table S1**), mother 2 and her baby were diagnosed with COVID-19 (based on serologic testing), and she reported that the infant was "Less active. Feverish" after the 2nd dose, but she didn't report the SARS-CoV-2 infection. Of note, we were able to confirm COVID-19 infection using anti-nucleocapsid antibody assay in this study; however, in other survey-based studies cases of infection cannot be ruled out, which may confound infant symptomatology reporting and assessments of associations with vaccination, rather than other mild viral infections. Further studies that compare symptoms in infants of vaccinated and non-vaccinated women are needed.

Our study found no significant increase in milk PEGylated protein concentrations at various time points after vaccine administration in a subset of samples analyzed in our cohort. We did observe one sample with higher concentration of PEGylated proteins 24 hours after 1st dose, compared to pre-vaccine sample (**Figure 1**, patient 9). This sample had PEGylated protein levels equivalent to 2.8µl/ml vaccine. However, we cannot confirm that the increased PEG in this single sample was from the COVID-19 vaccine, as PEG exposure may also be from other sources, such laxatives or ibuprofen. There was no increase in protein PEGylation concentration after 2nd dose in the same individual, and no unusual symptoms were reported in either the mother or her infant. These results demonstrate in a small cohort, that there is no significant increase in milk PEG levels after the first or second vaccination. Larger studies are needed to increase our understanding of the presence of PEG in human milk, and the biological relevance of these components after ingestion by the infant. Although expert consensus states there is minimal or no potential risk for the infant from maternal COVID-19 vaccination (29, 30), the minor symptoms that were reported (sleep changes and gastrointestinal symptoms) could be further investigated in future studies to determine if they are related to vaccination. Our findings also suggest that administration of maternal mRNA-based vaccine during lactation did not lead to a detectable immune response in the infant blood. These results further suggest that maternal vaccination during lactation cannot trigger infant immune responses to a degree that generates infant immunity.

We also demonstrate that COVID-19 mRNA vaccination induces significant increases in anti-SARS-CoV-2 IgM and IgG levels in lactating mothers' blood. Consistent with previous studies that showed IgM levels plateaued 28 days after COVID-19 infection (31), our results also demonstrated that 2nd dose did not induce significantly higher levels of IgM than was observed after 1st dose. In contrast, maternal blood IgG levels increased by 6-fold after the 2nd dose (compare to the levels after the 1st dose), highlighting the importance of the 2nd dose to boost the antibody response (27). We also observed a

similar pattern of increase in anti-RBD IgG levels in milk after the 2nd dose and positive correlation of blood and milk IgG levels. These findings stand in line with previous publications (32, 33) and strengthen our knowledge about the transport of milk IgG antibodies from the blood to the milk (34). In contrast, milk anti-RBD IgA levels measured 4-10 weeks after 2nd dose, were not significantly higher compared to their levels after the 1st dose. Spike-SIgA as well as IgA S2 titters in milk were previously reported to remain unaffected by 2nd vaccine dose (8) or to reach peak levels one week after the 2nd dose (5).

Twenty-five percent of women in our cohort had no detectable levels of anti-RBD IgA in their milk after vaccination. Similar findings were reported in other studies (5, 6), suggesting that production and transfer efficiency vary between individuals. Our analysis showed a weak but significant negative correlation between infant age and milk anti-RBD IgA levels, which might explain some of the variation in milk IgA levels observed between different individuals. Ten out of the 12 participants who had no detectable anti-RBD IgA, had infants older than 5.5 months at the time of sample collection. These findings are different from other publications that showed positive correlation of milk IgA levels (measured 2 weeks after 2nd dose) and baby age (32) or another study that didn't show correlation between these antibodies titters in milk and infant age (10 days after 2nd dose) (35). In our study IgA levels in milk were measured 4-10 weeks post 2nd dose and based on other publications we expect that milk IgA levels will be relatively lower at this stage in compared to 10-14 days after 2nd dose. These differences in the timing of measurements might explain the differences in our findings, compared to other studies (32, 35). Although we did not measure maternal blood IgA, other studies have shown that blood and milk IgA levels correlate when measured 7-10 days after the 2nd dose (13, 36). The relationship between infant age, breastfeeding exclusivity, milk IgA antibodies, and optimal timing of vaccination during lactation remains to be studied in detail.

Due to the lack of data about vaccination during pregnancy, many pregnant individuals were initially denied access to, declined, or were recommended to delay vaccination until after pregnancy. As such, many mothers have waited until after delivery to receive the vaccine. Although mothers vaccinated during lactation transferred antibodies to their infant through milk, which is an important component of mucosal immunity for the baby, there was no passive transfer of antibodies to the infant bloodstream (**Figure 5**), as occurs if the mother is vaccinated during pregnancy (8). Correlates of infant immune protection to COVID-19 are not yet well understood, however passive *in utero* transfer of IgG to the infant is important in the prevention of a number of infections including pertussis and influenza (37–39). Passively-transferred milk-derived IgA and IgG likely provide partial mucosal immune protection in infants, as breastfeeding is associated with lower risk of infections associated with mucosal defense, especially against respiratory infections (40–42). Two nursing infants in our cohort were infected with COVID-19 during the study (one a week post maternal 2nd dose, and the second one between 1st and 2nd maternal vaccine), indicating that at the time before full immune response is achieved in the vaccinated mother, typically 2-3 weeks after the 2nd dose milk antibodies cannot fully protect against SARS-CoV-2 infection (**Figure 2D**) and especially if the infant is not exclusively breastfed. Further studies are needed to determine the degree of protection conferred by IgA and IgG anti-SARS-CoV-2 antibodies that are present in milk. In addition, studies evaluating the additive benefit of both transplacentally-derived maternal IgG, as well as milk-derived IgA and IgG are needed to determine protection against COVID-19 in early infancy. Our findings underscore the importance of determining the optimal timing of vaccine administration to confer maximal protection against COVID-19 in infancy.

Strengths of our study include the prospective design and comprehensive symptom reporting by the vaccinated participants. We also report on longitudinal follow-up of infant immune responses, which has not been previously described. Furthermore, we included both BNT162b2 and mRNA-1273 vaccines and compared responses between the two vaccine manufacturers. Limitations include the small sample size, and that not all samples were able to be collected from all infant participants.

In summary, our study reports that no severe adverse events were noted in lactating individuals or their breastfeeding infants after COVID-19 mRNA vaccination. We demonstrated that human milk confers passive immunity to the infants, primarily through mucosal immunity in the gastrointestinal tract provided by IgA and IgG in milk. These results are important evidence to aid in counseling lactating individuals on the safety and efficacy of the COVID-19 mRNA vaccines, and the potential benefits to both the mother and infant.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The institutional review board of the University of California San Francisco. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YG, MP, VF, IA, and SG designed the study. AC and CL recruited participants. YG, MP, AC, UJ, AW, CL, VG, MC, LW, SB, LL, and EB conducted experiments and acquired data. YG, MP, CG, and SG analyzed data. YG, MP, AC, NA, and SG

wrote the manuscript. MP, AM, and SG provided funding. All authors assisted with editing the manuscript. NA, VF, and SG supervised the study. All authors contributed to the article and approved the submitted version.

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Milk From Women Diagnosed With COVID-19 Does Not Contain SARS-CoV-2 RNA but Has Persistent Levels of SARS-CoV-2-Specific IgA Antibodies

Ryan M. Pace[1†], Janet E. Williams[2†], Kirsi M. Järvinen[3], Courtney L. Meehan[4], Melanie A. Martin[5], Sylvia H. Ley[6], Celestina Barbosa-Leiker[7], Aline Andres[8,9], Laxmi Yeruva[8,9], Mandy B. Belfort[10], Beatrice Caffé[4], Alexandra D. Navarrete[11], Kimberly A. Lackey[1], Christina D. W. Pace[1], Alexandra C. Gogel[1], Bethaney D. Fehrenkamp[1], Miranda Klein[3], Bridget E. Young[3], Casey Rosen-Carole[3], Nichole Diaz[3], Stephanie L. Gaw[12], Valerie Flaherman[13], Mark A. McGuire[2], Michelle K. McGuire[1] and Antti E. Seppo[3*]*

[1] Margaret Ritchie School of Family and Consumer Sciences, University of Idaho, Moscow, ID, United States, [2] Department of Animal, Veterinary and Food Sciences, University of Idaho, Moscow, ID, United States, [3] Department of Pediatrics, Division of Allergy and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, NY, United States, [4] Department of Anthropology, Washington State University, Pullman, WA, United States, [5] Department of Anthropology, University of Washington, Seattle, WA, United States, [6] School of Public Health and Tropical Medicine, Tulane University, New Orleans, LA, United States, [7] College of Nursing, Washington State University Health Sciences Spokane, Spokane, WA, United States, [8] Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR, United States, [9] Arkansas Children's Nutrition Center, Little Rock, AR, United States, [10] Department of Pediatric Newborn Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, United States, [11] Elson S. Floyd College of Medicine, Washington State University, Spokane, WA, United States, [12] Department of Obstetrics, Gynecology & Reproductive Sciences, Division of Maternal-Fetal Medicine, University of California, San Francisco, San Francisco, CA, United States, [13] Department of Pediatrics, University of California, San Francisco, San Francisco, CA, United States

Background: Limited data are available regarding the balance of risks and benefits from human milk and/or breastfeeding during and following maternal infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Objective: To investigate whether SARS-CoV-2 can be detected in milk and on the breast after maternal coronavirus disease 2019 (COVID-19) diagnosis; and characterize concentrations of milk immunoglobulin (Ig) A specific to the SARS-CoV-2 spike glycoprotein receptor binding domain (RBD) during the 2 months after onset of symptoms or positive diagnostic test.

Methods: Using a longitudinal study design, we collected milk and breast skin swabs one to seven times from 64 lactating women with COVID-19 over a 2-month period, beginning as early as the week of diagnosis. Milk and breast swabs were analyzed for SARS-CoV-2 RNA, and milk was tested for anti-RBD IgA.

Results: SARS-CoV-2 was not detected in any milk sample or on 71% of breast swabs. Twenty-seven out of 29 (93%) breast swabs collected after breast washing tested

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***Correspondence:**
Antti E. Seppo
Antti_Seppo@URMC.Rochester.edu
Michelle K. McGuire
smcguire@uidaho.edu

[†]These authors have contributed equally to this work

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negative for SARS-CoV-2. Detection of SARS-CoV-2 on the breast was associated with maternal coughing and other household COVID-19. Most (75%; 95% CI, 70-79%; n=316) milk samples contained anti-RBD IgA, and concentrations increased (*P*=.02) during the first two weeks following onset of COVID-19 symptoms or positive test. Milk-borne anti-RBD IgA persisted for at least two months in 77% of women.

Conclusion: Milk produced by women with COVID-19 does not contain SARS-CoV-2 and is likely a lasting source of passive immunity *via* anti-RBD IgA. These results support recommendations encouraging lactating women to continue breastfeeding during and after COVID-19 illness.

Keywords: antibodies, breastfeeding, COVID-19, human milk, immunoglobulins, IgA, passive immunity, SARS-CoV-2

INTRODUCTION

Most studies examining milk produced by women with coronavirus disease 2019 (COVID-19) have demonstrated that it is an unlikely source of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) maternal-to-child transmission (1–4). Nonetheless, SARS-CoV-2 has been detected in a fraction of milk samples in some studies (5–7). The reasons for these disparate findings are unknown, but it is likely that sample collection and/or analysis methodology might play a role. Additionally, several studies have shown that milk antibody titers correlate with the milk's ability to neutralize SARS-CoV-2 infectivity (3, 8–11), thus likely offering immunological protection to the infant. Together, these findings support epidemiological evidence that breastfeeding while using appropriate hand and respiratory hygiene does not increase risk of infant SARS-CoV-2 infection (12, 13).

Individuals infected with SARS-CoV-2 typically develop a robust serum antibody response against the spike (S) glycoprotein within 2 weeks of illness onset. While early studies demonstrated that circulating levels waned by 2 months (14, 15), more recent research suggests more moderate declines with continued seropositivity at 6 to 8 months (16). Similarly, milk produced by women with COVID-19 contains substantial immunoglobulin A (IgA) targeting the S glycoprotein receptor binding domain (RBD) in the first month following infection (3, 9). However, little is known about the persistence of milk anti-SARS-CoV-2 IgA following maternal infection.

The presence of virus and anti-viral antibodies in milk contribute to the balance of risks and benefits that breastfeeding provides to infants of mothers with SARS-CoV-2 infection. The primary objective of this study was to use validated analytical methods and optimized longitudinal sampling to analyze milk produced after maternal COVID-19 diagnosis for the presence of SARS-CoV-2, as well as levels and duration of milk-borne anti-RBD IgA for up to 2 months after diagnosis. To further understand whether breast skin could be a possible source of viral RNA contamination in milk and/or represent a potential route of exposure to the infant, we also assessed the prevalence of SARS-CoV-2 on breast skin swabs collected before and after cleaning the breast.

MATERIAL AND METHODS

Experimental Design and Clinical Data/ Sample Collection

This multicenter study was carried out from April to December 2020 using a repeated-measures, longitudinal design. Maternal-infant dyads were recruited through participating institutions (University of Idaho; Washington State University; University of Rochester Medical Center; University of California, San Francisco; Brigham and Women's Hospital; University of Arkansas for Medical Sciences; Tulane University), and national social media advertising. To participate, women needed to be ≥18 years of age, lactating, have an infant less than 24 months old, and diagnosed with or tested for COVID-19 in the last seven days. No SARS-CoV-2 vaccine was available during the study period. Milk, breast skin swabs, and telephone surveys were ideally collected on three separate days during the first week post-diagnosis and again at 2-, 3-, 4-, and 8-weeks post-diagnosis. Participants self-collected milk and breast swab samples using provided collection kits, which were assembled aseptically by study personnel wearing masks and gloves. Mothers were instructed in clean techniques to obtain samples, including use of gloves and masks. Surveys included questions about COVID-19 testing results for all household members and maternal and infant COVID-19 symptoms. This multi-institutional study was reviewed and approved by the institutional review boards of the University of Idaho (20–056, 20–060), University of Rochester Medical Center (1507), University of California, San Francisco (20–30410), Brigham and Women's Hospital (2020P000804), University of Arkansas for Medical Sciences (260939), and Tulane University (2020–602). All participants gave written informed consent.

We previously reported (3) on 37 milk samples collected from 18 women in the first week post-diagnosis; of these women, 11 were recruited to provide additional longitudinal samples up to 2 months post-diagnosis for this study. In addition, we recruited 63 additional participants for this study (**Supplementary Figure 1**). No sample size calculation was performed due to logistical considerations and lack of preliminary data. Due to the nature of conducting this research during a pandemic and because test results were often not available soon after testing,

some women were recruited after being tested but prior to receiving the results for their COVID-19 tests. Because of this, women receiving a negative COVID-19 result after enrollment and were subsequently deemed ineligible to participate in the study and were not included in the total number of participants. Milk and swabs of the nipple/areola ("breast skin swabs") were collected as previously described (3). COVID-19 signs and symptoms (e.g., cough, fever, congestion, fatigue, malaise, difficulty breathing, chest pain, loss of smell and/or taste, and diarrhea) were recorded at study enrollment and at each sample collection. Milk samples collected prior to December 2019 from 5 healthy women located in the greater Rochester, NY area for general assay development were used as prepandemic control samples.

Laboratory Analysis

Total RNA was extracted from the first three milk samples collected from 47 women not previously reported on and extraction controls using the Quick-DNA/RNA Viral MagBead kit (Zymo Research, Irving, CA). Briefly, 200 µL of whole milk were mixed with 200 µL of 2X DNA/RNA Shield (Zymo Research) and incubated for 10 min prior to extraction following manufacturer's instructions. Total RNA was then used as the input for the CDC-designed SARS-CoV-2 reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay targeting two regions of the SARS-CoV-2 nucleocapsid gene, validated for use with human milk and replicated across two laboratories (University of Idaho and University of Rochester) as previously described (3). Per the CDC protocol, samples with Ct values <40 were considered positive. It is noteworthy that we did not reanalyze samples reported previously (3) for SARS-CoV-2 RNA because assay parameters had not changed. Total RNA was also extracted from swabs collected prior to the first three milk collections of 35 women not previously reported on and analyzed using the same RT-qPCR assay used for human milk with analysis only occurring at the University of Idaho. For breast swabs, swab heads were immersed in 400 µL 1X DNA/RNA Shield (Zymo Research), pulse vortexed for 20 seconds, incubated for 10 min, centrifuged at 500 x g for 1 min at 22°C, and then up to 400 µL of the liquid were used as input for RNA isolation. For extraction negative controls, 400 µL 1X DNA/RNA Shield were used as the input.

Concentrations of milk-borne anti-RBD IgA were determined in duplicate from delipidated milk using an enzyme-linked immunoassay (ELISA) as previously described (17) with the following modifications. Microtiter plates (Nunc Maxisorb, ThermoFisher Scientific) were coated with SARS-CoV-2 spike glycoprotein RBD (Sino Biological, Beijing, China) and blocked with 1% human serum albumin (HSA) (Millipore, Burlington, MA) in phosphate buffered saline containing 0.05% Tween-20 (PBS-T). Serum with known high anti-RBD IgA concentration (Ray Biotech, Peachtree Corners, GA) was used as a standard with dilution series ranging from 1:100 to 1:1,000,000 and milk samples were diluted 1:2 with 1% HSA and incubated in coated wells overnight at 4°C. After washing with PBS-T, bound antigen-specific antibodies were detected by incubating wells with horseradish peroxidase-conjugated polyclonal goat anti-human IgA antibody (Bethyl Laboratories, TX, USA), followed by washing with PBS-T and developing color with BD OptEIA reagent kit (Becton Dickinson). The color reaction was stopped after 10 min by adding 0.18 N sulfuric acid, and absorbance was read at 450 nm using a 96-well plate reader (BioTek, VT, USA). A standard curve was generated by fitting a 5PL equation to standard dilution series absorbances using plate reader software (Take5, BioTek, VT, USA), and sample concentrations were back calculated. Specific antibody concentrations are expressed based on standard serum (1 AU corresponds to the amount of specific IgA in 1:10,000 dilution of the standard serum). A positive cutoff threshold for positivity/antigen-specific binding was set as the sum of the mean and 2 times the standard deviation of RBD-specific IgA in prepandemic milk samples. SARS-CoV-2 RBD-specific IgA concentration for some of the samples were included in a previous study but were measured again in the present study for consistency. One participant's milk sample was not tested for IgA due to insufficient volume.

Statistical Analysis

R version 3.6.1 (18) and GraphPad Prism 9 were used for data analyses. The exact binomial test was used to calculate confidence intervals. The R package lmer (19) was used to perform univariate logistic regression to assess the relationships between the detection of viral RNA on breast skin swabs and the incidence of maternal respiratory symptoms or household COVID-19 symptoms/diagnosis with individual included as a random effect. Wilcoxon signed-rank test was used to assess the difference in anti-RBD IgA concentration between the first and second week of illness. Statistical significance was declared at $P<.05$.

RESULTS

Cohort Characteristics

Samples were collected from 64 women diagnosed with COVID-19 (**Supplementary Figures 1, 2**). Participant characteristics are presented in **Table 1**. Briefly, median age was 33 years (interquartile range [IQR], 30-36), and median time postpartum was 18 weeks (IQR, 2-32). Symptomatic and asymptomatic COVID-19 was reported in 83 (n=53) and 17% (n=11) of participants, respectively. Overall, relative to the day of diagnostic testing, we collected 78 milk samples from 40 women within the first week; 120 samples from 58 women between days 8 and 21; 89 samples from 47 women between days 22 and 56; and 29 samples from 29 women between days 57 and 106. In total, 316 milk samples were collected from 64 women. It is noteworthy that we have previously reported selected results from 37 milk samples produced by 17 of these women (3).

Evaluation of Milk and Breast Skin Swabs for the Presence of SARS-CoV-2 RNA

Here, we analyzed the first three milk samples (n=141) collected from 47 women and the first three breast skin swabs (n=99) collected prior to milk collection from 35 women not described in our previous report (3) for SARS-CoV-2 RNA (range, 0-37

TABLE 1 | Selected characteristics and behaviors of study participants.

Characteristic	No. (% or IQR)
Participants	64
Age,[a] median, y	33 (30-36)
Race	
Asian	1 (2)
Black	3 (5)
White	49 (77)
Other	8 (12)
Not reported	3 (5)
BMI,[b] median, kg/m^2	27 (23-31)
Parity,[a] median	2 (1-3)
Time postpartum,[a] median, wk	18 (2-32)
Breastfeeding status[c]	
Exclusively breastfeeding	19 (37)
Mixed feeding	33 (63)
COVID-19 symptoms	
Symptomatic	53 (83)
Asymptomatic	11 (17)
Infants tested for COVID-19[d]	20 (38)
Positive diagnosis	7 (35)

IQR, interquartile range; BMI, body mass index; COVID-19, coronavirus disease 2019. [a], Missing data from 1 participant; [b], Missing data from 4 participants; [c], Missing data from 12 participants; [d], Missing data from 11 participants; Percentages may not sum to 100 due to rounding.

days from diagnostic test). As with our prior report, all milk samples tested negative for SARS-CoV-2 in both laboratories. In contrast, while 71% (70/99; 95% CI, 61-79%) of the swabs collected before breast washing tested negative for SARS-CoV-2, 29% (29/99) generated Ct values that varied in degree of positivity (**Supplementary Table 1**). However, 27/29 (95% CI, 77-99%) of the companion swabs collected after breast washing tested negative for SARS-CoV-2. The two swabs that retained some degree of positivity after washing had a 70-80% reduction in the estimated viral load.

Using these swab data combined with our previously published swab data (3), we evaluated whether maternal respiratory symptoms of COVID-19 (cough, dyspnea, rhinorrhea/nasal congestion, and sneezing) or presence of household COVID-19 were related to the detection of SARS-CoV-2 RNA on the breast skin. Among the four maternal respiratory symptoms examined, only cough was related to the detection of viral RNA (odds ratio, OR, 4.78; 95% CI, 1.59-14.38; P<.01; 51% of swabs with cough versus 19% without cough) (**Table 2**). The presence of at least one other household member with COVID-19 was also associated with increased likelihood of detection of viral RNA on breast skin swabs (OR, 6.67; 95% CI, 1.79-24.92; P<.01; 53% of swabs with household COVID-19 versus 18% without household COVID-19).

Longitudinal Assessment of Milk Specific IgA

Assays for detecting IgA specific to the S glycoprotein RBD were conducted on 316 milk samples collected from all 64 women; 75% (95% CI, 70-79%) of these samples contained detectable anti-RBD IgA. The maximum concentration of anti-RBD IgA was two-fold higher in symptomatic women in comparison to asymptomatic women, although this difference was not statistically significant (P=.0610; symptomatic - 22.8 ± 27.1 AU, average ± standard deviation; asymptomatic - 11.2 ± 15.7 AU). Longitudinal analysis of samples collected from women who provided repeated samples for at least 2 months following onset of symptoms (n=24) or positive test (for asymptomatic women, n=2) demonstrated an increase (P=.02) in the concentration of anti-RBD IgA from the first to second week following onset of symptoms/positive test; 92% (24/26) of these women produced milk containing anti-RBD IgA by day 19 (**Figure 1A**). Of these 26 women, 77% (n=20) produced milk with anti-RBD IgA for 2 months or longer ("persistent IgA"), whereas 15% (n=4) produced milk without persistent levels of detectable anti-RBD IgA ("transient IgA"; **Figure 1B**). Of the two asymptomatic women with longitudinal data, one displayed persistent IgA while the other was IgA negative.

TABLE 2 | Association of respiratory signs/symptoms and viral RNA presence on the breast skin.

Sign/symptom	OR (95% CI)
Cough	4.78 (1.59-14.38)**
Dyspnea	0.91 (0.15-5.52)
Rhinorrhea/nasal congestion	2.94 (0.86-10.07)
Sneeze	0.22 (0.01-3.26)

***P<.01; n=116 breast skin swabs from 43 participants; OR, odds ratio; CI, confidence interval.*

DISCUSSION

Consistent with the preponderance of available evidence, we found no indication of SARS-CoV-2 in milk produced by women with COVID-19. Some breast skin swabs were found to contain detectable SARS-CoV-2 RNA, almost all of which were collected prior to washing the breast. Detection of SARS-CoV-2 RNA on the unwashed breast was related to the presence of maternal cough and others in the household with COVID-19.

Importantly, we detected a rapid, robust, and durable anti-RBD specific IgA response in the majority of mothers' milk. Our longitudinal study substantially extends prior knowledge by demonstrating: *(I)* the vast majority (92%) of women with COVID-19 have anti-RBD IgA in their milk; *(II)* concentrations of anti-RBD IgA increase during the first weeks following onset of symptoms or, if asymptomatic, following the day of diagnostic test; and *(III)* anti-RBD IgA is present in milk produced by most infected women for at least 2 months.

Our finding of a persistent antibody response in milk produced by women with COVID-19 is reassuring as it suggests passive immunity is likely conferred to recipient infants for at least 2 months after maternal infection. Passive immunity *via* milk is particularly important for breastfeeding children, including infants and neonates, as COVID-19 vaccines have yet to be approved for these populations. Further, our results showing a sustained anti-RBD antibody response in milk may have implications for the durability of vaccine-induced milk antibodies. Indeed, similar to SARS-CoV-2 infection and maternal vaccination against other respiratory pathogens (20, 21), recent data demonstrate that in the

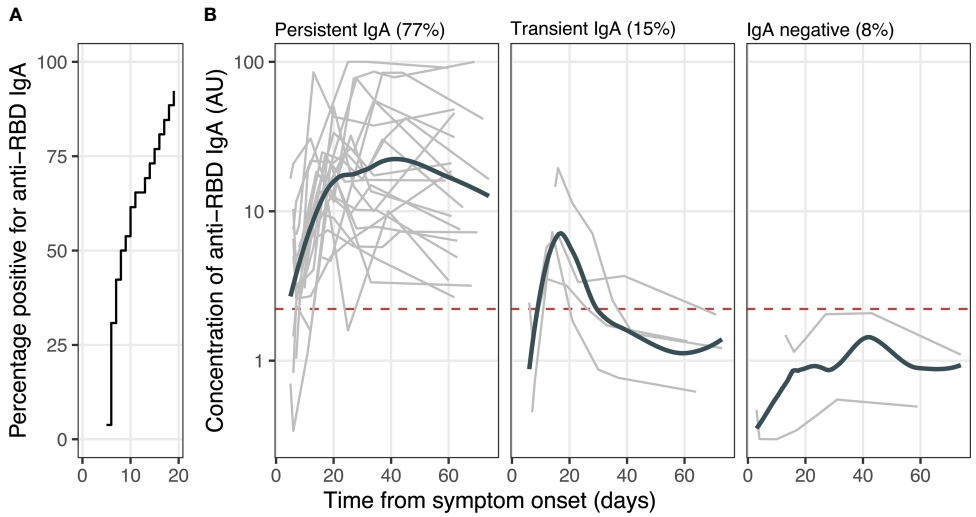

FIGURE 1 | Temporal dynamics of milk anti-RBD IgA. **(A)** Proportion of women with milk anti-RBD IgA. **(B)** Concentration of milk anti-RBD IgA. The gray lines represent individuals (*n*=26); bolded lines represent the group LOESS curves; and horizontal dashed red line denotes the cutoff for assay positivity/limit of antigen-specific binding.

days and weeks following maternal COVID-19 vaccination, a robust IgG-dominant milk antibody response is induced (22–25), although the longer-term durability of the milk-borne antibody response remains to be elucidated. Similarly, the mechanisms underlying the persistence or lack of specific antibodies in human milk are not well understood, but may be related to differences in the course of the infection, recurrent exposures and/or the migration of long-lived plasma cells from mucosal sites to the mammary gland (26–28)

Our previous (3) and current findings on the detection of SARS-CoV-2 RNA on the breast skin of a small number of participants prior to cleaning may provide an explanation as to why some milk samples in prior studies have yielded positive results for SARS-CoV-2 RNA *via* RT-qPCR. It is worth noting that only low titers of viral RNA were detected on the positive breast skin swab samples (**Supplementary Table 1**). Washing of the breast appeared effective in removing the RNA in almost all cases examined. Unfortunately, since the breast skin swabs utilized for viral RNA detection were inactivated prior to RT-qPCR, we were unable to examine whether the RNA represented viable virus or remnants of viral RNA and poses a potential risk of maternal-to-child transmission. However, it is notable that numerous studies examining neonatal outcomes during maternal SARS-CoV-2 infection have not found evidence of SARS-CoV-2 transmission *via* breastfeeding (12, 13, 29–33).

We recognize that this study was limited by self-reported COVID-19 diagnostic test results for most women, self-collection of samples, and lack of individuals with severe COVID-19 that required hospitalization, and an inability to assess functional immunity or virus neutralization. However, strengths of the study include a relatively large sample size, longitudinal sampling, collection methodology employing best practices for human milk research (34), and use of assays validated for human milk (3) to detect SARS-CoV-2 RNA and profile the dynamics of milk-specific IgA.

In summary, we found no evidence of SARS-CoV-2 in milk and documented the presence of anti-RBD IgA that persisted for at least 2 months in milk produced by most study participants. Beyond the health impacts of human milk as a source of nutrition, these data suggest that, on balance, human milk is not a source of SARS-CoV-2 transmission and may provide lasting passive immunity. Our findings also provide additional support for recommendations that lactating women with COVID-19 continue to breastfeed while they and others in the household take precautions, such as hand and respiratory hygiene, to prevent transmission *via* respiratory droplets (35).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This multi-institutional study was reviewed and approved by the institutional review boards of the University of Idaho (20-056, 20-060), University of Rochester Medical Center (1507), University of California, San Francisco (20-30410), Brigham and Women's Hospital (2020P000804), University of Arkansas for Medical Sciences (260939), and Tulane University (2020-602). All participants gave written informed consent.

AUTHOR CONTRIBUTIONS

Concept and design: RP, JW, KJ, CM, MMa, SL, CB-L, LY, AA, MB, SG, VF, MMc, MKM, and AS. Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: RP and JW. Critical revision of the manuscript for important intellectual content: All authors. Statistical analysis: RP, JW, and AS. Obtained funding: RP, JW, KJ, CM, MMa, SL, CB-L, LY, AA, MB, SG, VF, MMc, MKM, and AS. Administrative, technical, or material support: RP, JW, KJ, SG, VF, MMc, MKM, and AS. Supervision: RP, JW, KJ, CM, MMc, MKM, and AS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.801797/full#supplementary-material

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Neutralizing Activity and SARS-CoV-2 Vaccine mRNA Persistence in Serum and Breastmilk After BNT162b2 Vaccination in Lactating Women

Kee Thai Yeo[1,2,3†], Wan Ni Chia[4†], Chee Wah Tan[4†], Chengsi Ong[3,5], Joo Guan Yeo[2,3,6], Jinyan Zhang[4], Su Li Poh[2], Amanda Jin Mei Lim[2], Kirsten Hui Zhi Sim[1], Nursyuhadah Sutamam[2], Camillus Jian Hui Chua[2], Salvatore Albani[2,3‡], Lin-Fa Wang[4,7‡] and Mei Chien Chua[1,3,5,8‡]*

[1] Department of Neonatology, KK Women's & Children's Hospital, Singapore, Singapore, [2] Translational Immunology Institute, Singhealth Duke-NUS Academic Medical Centre, Singapore, Singapore, [3] Paediatrics Academic Clinical Programme, Duke-NUS Medical School, Singapore, Singapore, [4] Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore, Singapore, [5] KK Human Milk Bank, KK Women's & Children's Hospital, Singapore, Singapore, [6] Department of Paediatrics, KK Women's & Children's Hospital, Singapore, Singapore, [7] Singhealth Duke-NUS Global Health Institute, Singhealth Duke-NUS Academic Medical Centre, Singapore, Singapore, [8] Lee Kong Chian School of Medicine, Singapore, Singapore

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***Correspondence:**
Mei Chien Chua
Chua.Mei.Chien@singhealth.com.sg

†These authors share first authorship

‡These authors share senior authorship

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Background: There is limited information on the functional neutralizing capabilities of breastmilk SARS-CoV-2-specific antibodies and the potential adulteration of breastmilk with vaccine mRNA after SARS-CoV-2 mRNA vaccination.

Methods: We conducted a prospective cohort study of lactating healthcare workers who received the BNT162b2 vaccine and their infants. The presence of SARS-CoV-2 neutralizing antibodies, antibody isotypes (IgG, IgA, IgM) and intact mRNA in serum and breastmilk was evaluated at multiple time points using a surrogate neutralizing assay, ELISA, and PCR, over a 6 week period of the two-dose vaccination given 21 days apart.

Results: Thirty-five lactating mothers, median age 34 years (IQR 32-36), were included. All had detectable neutralizing antibodies in the serum immediately before dose 2, with significant increase in neutralizing antibody levels 7 days after this dose [median 168.4 IU/ml (IQR 100.7-288.5) compared to 2753.0 IU/ml (IQR 1627.0-4712.0), p <0.001]. Through the two vaccine doses, all mothers had detectable IgG1, IgA and IgM isotypes in their serum, with a notable increase in all three antibody isotypes after dose 2, especially IgG1 levels. Neutralizing antibodies were detected in majority of breastmilk samples a week after dose 2 [median 13.4 IU/ml (IQR 7.0-28.7)], with persistence of these antibodies up to 3 weeks after. Post the second vaccine dose, all (35/35, 100%) mothers had detectable breastmilk SARS-CoV-2 spike RBD-specific IgG1 and IgA antibody and 32/35 (88.6%) mothers with IgM. Transient, low intact vaccine mRNA levels was detected in 20/74 (27%) serum samples from 21 mothers, and 5/309 (2%) breastmilk samples from 4 mothers within 1 weeks of vaccine dose. Five infants, median age 8 months (IQR 7-16), were also recruited - none had detectable neutralizing antibodies or vaccine mRNA in their serum.

Conclusion: Majority of lactating mothers had detectable SARS-CoV-2 antibody isotypes and neutralizing antibodies in serum and breastmilk, especially after dose 2 of BNT162b2 vaccination. Transient, low levels of vaccine mRNA were detected in the serum of vaccinated mothers with occasional transfer to their breastmilk, but we did not detect evidence of infant sensitization. Importantly, the presence of breastmilk neutralising antibodies likely provides a foundation for passive immunisation of the breastmilk-fed infant.

Keywords: SARS-CoV-2 vaccine, mRNA vaccine, BNT162 vaccine, neutralizing antibodies, COVID-19, COVID-19 serological testing, breast feeding, breast milk expression

INTRODUCTION

Coronavirus disease 2019 (COVID-19) messenger RNA (mRNA) vaccines have been increasingly deployed in many countries as a means of controlling infectious spread and severity of the disease (1–4). Even so, the initial clinical trials evaluating these novel mRNA vaccines, encoding the spike protein of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), excluded breastfeeding and lactating women (4, 5). There is limited current data on the efficacy and safety of these SARS-CoV-2 vaccines in this group of mothers and their breastmilk-fed infants (6–8).

Emerging evidence from several cohort studies on lactating women have demonstrated the immunogenicity of the currently available mRNA vaccines (BNT162b2, mRNA-1273) among lactating women, with the induction of SARS-CoV-2-specific antibodies in the breastmilk post-vaccination (9–14). However, there is paucity of information on the functional neutralizing capabilities of SARS-CoV-2-specific antibodies in breastmilk and their dynamic and temporal relationship to serum levels after mRNA vaccination. Additionally, the potential adulteration of breastmilk with vaccine mRNA is currently unknown and raises safety concerns relating to the potential exposure of breastmilk-fed infant to the mRNA. Several international organizations including the World Health Organization (15) have recommended the continuation of breastmilk feeding following vaccination, while acknowledging the lack of safety data for mother and child.

To address these issues, we investigated the dynamics of SARS-CoV-2-specific immunoglobulin subtypes and their temporal relationship with SARS-CoV-2 neutralizing activity in the serum and breastmilk of lactating mothers through the 2-dose BNT162b2 mRNA vaccine, and the post-vaccination persistence of vaccine mRNA in the serum and breastmilk of these vaccinated mothers. We also examined serum of breastmilk-fed infants from vaccinated mothers to determine the presence of SARS-CoV-2 neutralizing antibodies and vaccine mRNA.

MATERIALS AND METHODS

Study Population

We evaluated the humoral responses of a cohort of healthcare workers who were lactating mothers working at a tertiary level women's and children's hospital in Singapore and had received the BNT162b2 COVID-19 vaccine (Pfizer/BioNTech) between 15 January and 31 May 2021. These front-line healthcare workers, were eligible if they consented to blood and breastmilk collection at specific timepoints after vaccination. All participants received both vaccine doses (30 μg/0.3 ml) 21 days apart. Breastmilk-fed infants from these lactating mothers were also recruited for the collection of a single serum sample with informed consent. At enrolment, maternal and infant demographic and clinical information were collected, including any significant symptoms after any of the two vaccine doses. The study was approved by the Singhealth Institutional Review Board and all participants provided written informed consent (CIRB Ref. No 2019/2906 & CIRB Ref. No. 2016/2791).

Biological Samples

Breastmilk samples (10mls each) were collected on day of vaccination (day 0) followed by days 1, 3, 7, 14, and 21 post-vaccination for both doses. Breastmilk sample on day 21 after dose 1 was collected before receipt of dose 2. All mothers were advised to express breastmilk into sterile containers and to immediately store them in their own freezers before transportation to the laboratory in coolers containing frozen cold packs. For the initial processing of these breastmilk samples, they were thawed and centrifuged twice at 2383g for 15minutes at 4°C. The fat layer was removed after each spin cycle and the resultant skim milk was transferred to a cryovial and frozen at -80°C until analysis.

Maternal serum samples were obtained at days 0 and 3 for dose 1 and days 0 and 7 for dose 2. Samples on day 0 of dose 2 was obtained before vaccine was administered. Infant serum samples were collected >3 weeks post maternal second dose. All serum samples (0.5ml to 1ml) were collected in serum separation blood collection tubes (Sarstedt AG & Co, Germany) and transported to the laboratory on the same day. These samples were centrifuged at 1300g for 10 minutes before serum was aliquoted into cryovials and stored at -80°C until analysis.

SARS-CoV-2 Surrogate Viral Neutralization Assay

We utilized a SARS-CoV-2 surrogate virus neutralization assay (cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit, GenScript Inc., USA) that detects the total immunodominant neutralizing antibodies targeting the viral spike protein receptor binding domain (RBD) in an isotype and species independent manner (16). This validated and commercialized test developed

with the D614 SARS-CoV-2 strain, measures the magnitude of antibody-mediated blockage of the interaction between angiotensin-converting enzyme 2 (ACE2) receptor protein and the RBD of SARS-CoV-2 which is required for viral entry into susceptible cells (17). Prior studies have documented that RBD-targeting neutralizing antibodies are immunodominant with SARS-CoV-2 infections (18, 19). The cPass kit results have shown 95.7% positive predictive agreement (95%CI 85.8-98.8%) and 97.8% negative predictive agreement (95%CI 92.5 – 99.4%) with 50% viral neutralization by plaque reduction neutralization tests (PRNT) in clinical studies (16, 20).

For serum samples, a final dilution of 1:20 was used according to manufacturer's recommendations. As the test was only validated on serum/plasma by the manufacturer, an optimization was performed and adapted for breastmilk samples. A final dilution of 1:5 was used as it gave no false positive background on pre-vaccinated breastmilk samples and allowed maximum volume of breastmilk to be tested. Apart from the sample amount used for testing, the rest of the assay was performed per manufacturer's instructions. The percent signal inhibition was calculated = [1-(Optical Density (OD) of sample/OD of negative control)]x100%. An inhibition signal of ≥30% was used as the cutoff for positive detection of SARS-CoV-2 neutralizing antibodies in all sample types (20).

Inhibition signal from individual samples from the cPass assay was converted to the World Health Organization (WHO) International Units (IU) based on previous calibration of this neutralization assay against the WHO International Standard (IS) for SARS-CoV-2 neutralization assays (21, 22), using a Excel-based conversion tool available online (https://github.com/Lelouchzhu/cPass-to-IU_Conversion). Based on recent studies using biological replicates from different international groups, cPass readings (% inhibition) were shown to be highly reproducible to International Units (IU)/ml of the WHO International Standard, with a pseudo R^2 at 0.978. cPass inhibition signal of 30% corresponds to a cut-off of 28 IU/ml for serum samples and 7 IU/ml for breastmilk samples based on the conversion to WHO International Standard and dilution factor for sample type.

Enzyme-Linked Immunosorbent Assay for Detection of SARS-CoV-2 Antibody Isotype

To determine the relative abundance of SARS-CoV-2 antibody isotypes and compare their temporal dynamics with neutralization activity, we performed semi-quantitative evaluations of the SARS-CoV-2 spike protein receptor binding domain (RBD)-specific antibody isotypes in serum and milk by enzyme-linked immunosorbent assay (ELISA). The 96-well plates were coated with 2μg/mL of SARS-CoV-2 RBD protein (RBD-His tag, expressed in HD293F cells from Genscript, USA) (100ng protein/well) diluted in bicarbonate buffer at 50μl/well in 4°C overnight. Plates were washed with wash buffer (0.05% Tween 20 in 1×DPBS) and blocked with 150μL of blocking buffer (BD OptEIA Assay Diluent, BD Pharmingen, USA). Block solution was discarded and plates were blotted dry. Serum diluted 1:100 in blocking buffer or neat breastmilk were added (50μl per well) and incubated for 2 hours at room temperature. Naïve human serum samples were added to each plate as negative controls.

This was followed by 5 more washes with wash buffer and incubated for 1-hour at room temperature with 50μl per well of 1μg/ml mouse anti-human IgG1 to IgG4 (Southern Biotech, USA), mouse anti-human IgM (GenScript Inc., USA) and goat anti-human IgA (Southern Biotech, USA). Plates were washed 5 times with wash buffer and underwent 1-hour incubation at room temperature with 50μl per well of 1:10000 anti-mouse IgG horseradish peroxidase (HRP) (Biolegend, USA) for detection of IgG1 to IgG4 and IgM isotypes, and 1:10000 anti-goat IgG-HRP (Biolegend, USA) for detection of IgA. Plates were washed 5 times with wash buffer and 50μl of TMB ELISA substrate (Life Technologies, USA) were added per well. Plate development was stopped by addition of 50μl KPL TMB Stop Solution (Sera Care, USA). Absorbance on the BioTek Cytation5 plate reader (Fisher Healthcare, USA) at 450nm and a reference background of 570nm was recorded. Corrected absorbance value was calculated (450nm-570nm). Corrected Optical Density at 450nm (OD_{450}) values for individual sample and isotypes was obtained by subtracting value of negative control of each isotype from each individual plate.

BNT162b2 mRNA Detection

RNA from breastmilk and serum samples was extracted using the E.Z.N.A Total RNA extraction kit (Omega Bio-tek Inc., USA) according to manufacturer's instructions. Briefly, samples were treated with lysis buffer and ethanol before binding of RNA to the HiBind RNA Mini Column. After several washes with wash buffer, RNA was eluted into nuclease-free water and immediately stored at -80°C before analysis. Synthesis of complementary DNA was performed using QuantiTect Reverse Transcription kit (Qiagen NV, Germany) and Real-time PCR was performed using SensiFAST SYBR No-ROX kit (Meridian Bioscience, USA). Primers were designed using the published BNT162b2 mRNA sequence (23). The following forward primer 5' TTCGCCCAAGTGAAGCAGAT 3' and reverse primer 5' CGGCCAGTGTCACTTTGTTG 3' with annealing temperature of 60°C was used. All samples were run in triplicates and repeated for result confirmation. Purified BNT162b2 mRNA was used as standard for quantification. Standard curves were generated for individual runs using spiked vaccine mRNA into non-vaccinated samples (see **Supplemental Figure 1**).

Statistical Analysis

Data generated are presented as median with interquartile range (IQR). Comparisons of median values of samples were performed using Mann Whitney U test. Correlation analysis was performed with Pearson correlation coefficients. Comparison of antibody isotype levels from pre-vaccination to the multiple post-vaccination timepoints were assessed using repeated measures mixed-effects model followed by *post hoc* Tukey's multiple comparisons test. Statistical significance was defined as $p<0.05$ and was two tailed. All statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, USA).

RESULTS

Study Population

We enrolled 35 lactating mothers who were frontline healthcare workers and received the two-dose BNT162b2 vaccine. Thirty-one women were recruited before their first dose and 4 were included just before their second dose. All participants completed the 2-dose course within 21 days. These mothers had a median age of 34 years (IQR 32-36), were predominantly of Chinese ethnicity (74%) and all had full-term deliveries (**Table 1**). The median age of their child and the length of lactation at the first vaccine dose was 7 months (IQR 5-14). All mothers were breastfeeding and/or feeding expressed breastmilk to their child. Five infants, with median age 8 months (IQR 7-16), were recruited into this study and provided serum samples. No participants were diagnosed with COVID-19 before or during the study period and none reported significant allergic symptoms with the vaccination.

Neutralizing Antibody Level

Serum of Vaccinated Mothers

To evaluate the neutralizing antibody responses from the 2-dose vaccination schedule in lactating women, a total of 21 mothers provided serum samples – 16 women provided 4 serum samples over 2 doses and 5 women provided 2 serum samples with the second dose. All women tested had detectable neutralizing antibody present in the serum just prior to the second dose (day 21) based on the 28 IU/ml cut-off (**Figure 1**). Median neutralizing antibody level at day 0-10 after the second dose (704.6 IU/ml, IQR 163.1-2784.0) was significantly higher than at day 0–3 after the first dose (6.4 IU/ml, IQR 3.4-8.9) (p<0.001). The median neutralizing antibody increased from dose 1 to dose 2 – with a rapid and significant increase in levels from day 0 to day 7-10 after vaccine dose 2 [median 168.4 IU/ml (IQR 100.7-288.5) vs 2753.0 IU/ml (IQR 1627.0-4712.0), p <0.001].

TABLE 1 | Clinical characteristics of the lactating mothers.

Characteristics	Total (n=35)
Median maternal age at first dose, years (IQR)	34 (32 – 36)
Maternal ethnicity, n (%)	
Chinese	26 (74)
Malay	5 (14)
Indian	1 (3)
Other	3 (9)
Median child gestation at birth, weeks (IQR)	39 (38 – 39)
Female child, (%)	22 (63)
Median age of child at maternal first dose, months (IQR)	7 (5 – 14)
Predominant mode of feeding, n (%)	
Breastfeeding	12 (34)
Expressed breast milk	17 (49)
Both breastfeeding + expressed breast milk	6 (17)
Estimated average volume of breastmilk per day, ml (range)	550 (190 – 1000)
Reported side effects after vaccine doses, n (%):	
Nil side effects	10 (29)
Myalgia	15 (43)
Fever	4 (11)
Rhinorrhea/Cough	3 (9)
Mastitis	1 (3)
Headache	1 (3)
Joint pain	1(3)

Breastmilk of Vaccinated Mothers

To determine the presence of neutralizing antibodies in breastmilk, 11 breastmilk samples per participant were collected from 31 women over the 2-dose vaccination and 6 samples per participant from 4 women with dose 2 of the vaccination schedule. There were minimal SARS-CoV-2 neutralizing antibodies present in the breastmilk (based on the 7 IU/ml cut-off) from day 0 of dose 1 up to day 3 post-dose 2 (**Figures 2A, B**). The neutralizing antibody levels increased significantly to a median of 13.4 (IQR 7.0-28.7) at 28 days (day 7 dose 2) from a median of 4.0 (IQR 2.7-4.9) at day 22-24 (p<0.001) (**Figure 2A**). Of those who provided breastmilk samples over 2 doses, only samples from 3 mothers did not have detectable neutralizing antibodies at any of the sampling timepoints up to 42 days [median 2.6 IU/ml (IQR 1.9-3.4)], in spite of these 3 mothers having detectable serum neutralizing antibodies.

Just prior to vaccine dose 2 (day 21), only 6/35(17.1%) mothers had detectable breastmilk neutralizing antibodies in spite of all mothers having detectable levels in their serum. After the second dose, there was moderate correlation between serum and breastmilk neutralizing antibody levels at day 7 dose 2 (r=0.39, p=0.08) (**Figure 3**). This was the timepoint with peak levels of breastmilk neutralizing antibodies detected for majority of mothers in this study.

Infant Serum

Five infants from the cohort of vaccinated mothers were recruited into the study and a single serum sample was collected from them. These samples were collected at a median of 48 days (IQR 44-57) after the second maternal vaccine dose. The age of these infants at the point of maternal vaccination ranged from 3 to 20 months. None had detectable neutralizing antibodies in their serum.

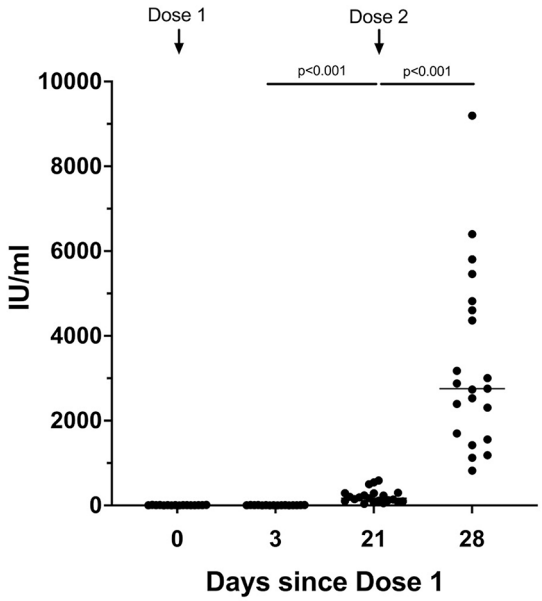
SARS-CoV-2 Spike RBD-Specific Antibody Isotypes

Serum of Vaccinated Mothers

Through the two vaccine doses, all mothers had detectable IgG, IgA and IgM isotypes in their serum with a notable progressive increase in all three antibody isotypes observed over the 4 study timepoints. There was significant boosting of the IgG1 levels 1 week after the second vaccine dose, with a 15-fold increase compared to day 21 after the first dose (median OD_{450} 0.02 (IQR 0.01-0.05) vs 0.35 (IQR0.33-0.40), P<0.0001) (**Figure 4A**). There was a lesser increment in the IgG3, IgA and IgM levels over the same timepoints (median OD_{450} 0.009 (IQR 0.008-0.014) vs 0.028 (IQR 0.02-0.04), p<0.0001), (median OD_{450} 0.05 (IQR 0.04-0.1) vs 0.22 (IQR 0.16-0.30), p<0.0001), and (median 0.04 (IQR 0.03-0.06) vs 0.07 (IQR 0.05-0.09), p<0.0001) (**Figures 4B–D**). IgG2 and IgG4 were not detected in the serum samples.

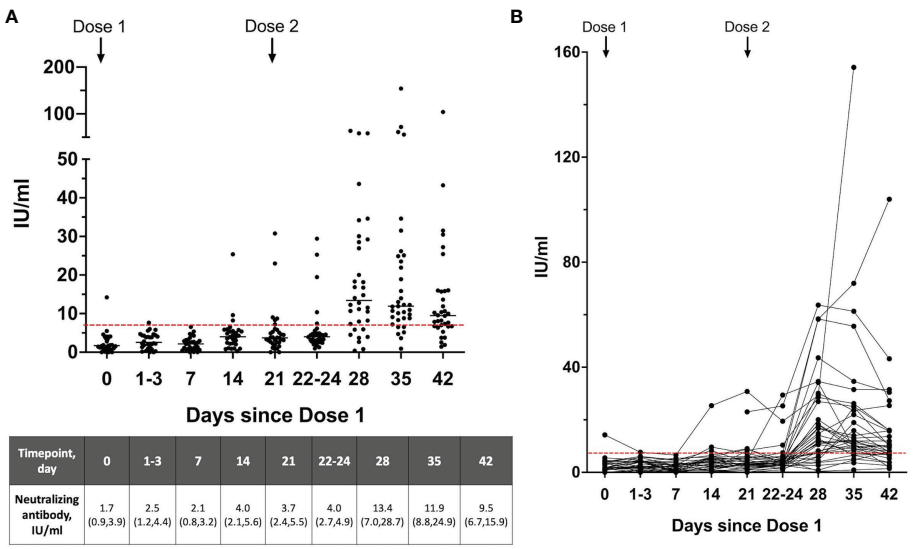
Breastmilk of Vaccinated Mothers

Up to day 21 of the first vaccine dose, SARS-CoV-2 spike RBD-specific IgG1 antibody were detected in the breastmilk of 23/31



Timepoint, day	0	3	21	28
Neutralizing antibody, IU/ml	6.9 (4.2,8.9)	5.2 (3.0,8.9)	168.4 (100.7,288.5)	2753 (1627,4712)

FIGURE 1 | Neutralizing antibody levels detected in serum of lactating women over 2 vaccine doses expressed as WHO SARS-CoV-2 International Standard (n=21). Serum samples on day 21 is taken prior to receipt of Dose 2. A level >28 IU/ml was used as the cutoff for positive detection of neutralizing antibodies. Neutralizing antibody levels are presented as median (interquartile range), IU/ml.



Timepoint, day	0	1-3	7	14	21	22-24	28	35	42
Neutralizing antibody, IU/ml	1.7 (0.9,3.9)	2.5 (1.2,4.4)	2.1 (0.8,3.2)	4.0 (2.1,5.6)	3.7 (2.4,5.5)	4.0 (2.7,4.9)	13.4 (7.0,28.7)	11.9 (8.8,24.9)	9.5 (6.7,15.9)

FIGURE 2 | **(A)** Neutralizing antibody levels and the **(B)** dynamics of neutralizing antibodies detected in the breastmilk over the 2-dose BNT162b2 mRNA vaccination expressed as WHO SARS-CoV-2 International Standard (n=35). Breastmilk samples on day 21 is taken prior to receipt of Dose 2. A level >7 IU/ml (dotted red line) was used as the cutoff for positive detection of neutralizing antibodies. Neutralizing antibody levels are shown as median (interquartile range), IU/ml.

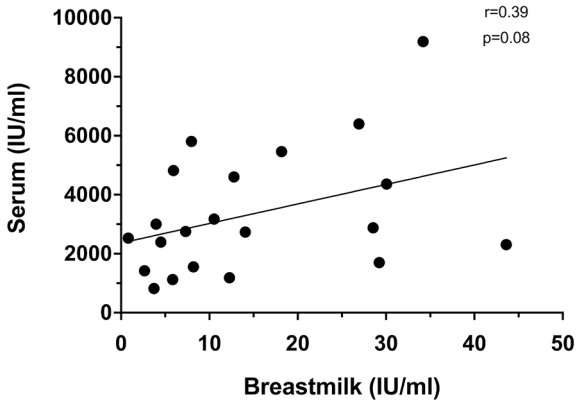


FIGURE 3 | Correlation between serum and breastmilk neutralizing at day 7 after dose 2 (n=21).

(74.2%) mothers; IgA in 31/31 (100%) and IgM in 26/31 (83.9%) mothers. Post the second vaccine dose, all (35/35, 100%) mothers had detectable SARS-CoV-2 spike RBD-specific IgG1 and IgA antibody and 32/35 (88.6%) mothers with IgM.

Breastmilk IgG1 rose significantly 7 days after the second vaccine dose with continued persistence and elevated levels 21 days after (Day of dose 2: median OD_{450} 0.001 (IQR 0-0.002); 7 days after dose 2: 0.08 (IQR 0.004-0.3); day 14 after dose 2: 0.11 (IQR 0.05,0.2); day 21 after dose 2: 0.06 (IQR 0.03-0.2) (**Figures 5A, D**). IgA and IgM in breastmilk (**Figures 5B, C**) increased gradually to peak levels at day 7 post dose 2 (peak median IgA OD_{450} 0.4 (IQR 0.3-0.7), peak median IgM 0.02 (IQR 0.01-0.07). Levels of these IgA and IgM antibodies subsequently decrease to pre-dose 2 levels after 3 weeks (**Figures 5E, F**). IgG2, IgG3 and IgG4 subclasses were not detected in breastmilk samples.

Infant Serum

None of the five infant serum analysed had detectable SARS-CoV-2 spike RBD-specific IgG, IgM and IgA antibodies.

BNT162b2 mRNA Detection in Serum and Breastmilk

Vaccine mRNA was detected in 20 serum samples from 15 mothers, out of 74 samples from 21 mothers tested. A total of 10/16 (63%) and 10/25 (40%) mothers had detectable vaccine mRNA at day 1-3 of dose 1 and day 7-10 of dose 2 respectively (**Figure 6A**). Five mothers had positive serum samples at both time points. The median vaccine mRNA amount (ng/100ml) were not different between the two timepoints – 16 (IQR 9-24) compared to 12 (IQR 9-18) (p=0.6) (**Supplemental Table 1**). None of the samples on days 0 and 21 post-dose 1 had detectable vaccine mRNA.

Five breastmilk samples from 4 mothers had detectable vaccine mRNA, out of 309 samples from 31 mothers tested (**Supplemental Table 2**). All positive samples were collected within 3 days of the vaccine doses - two samples from days 1 and 3 of dose 1 (**Figure 6B**) and another three from days 1 and 3 post dose 2. One mother had detectable vaccine mRNA in both breastmilk and serum samples. The median vaccine mRNA amount in both sample types were comparable: 14ng/100ml (IQR 8-23) in serum compared to 7ng/100ml (IQR 6-7) in breastmilk (p=0.2).

None of the serum samples from the five infants tested had detectable vaccine mRNA. Of the five, one infant was from a mother with detectable vaccine mRNA in the both breastmilk and serum and another three were from mothers with vaccine mRNA in the serum.

DISCUSSION

In this study, we described the dynamics of SARS-CoV-2-specific antibody isotype production and the associated inhibitory

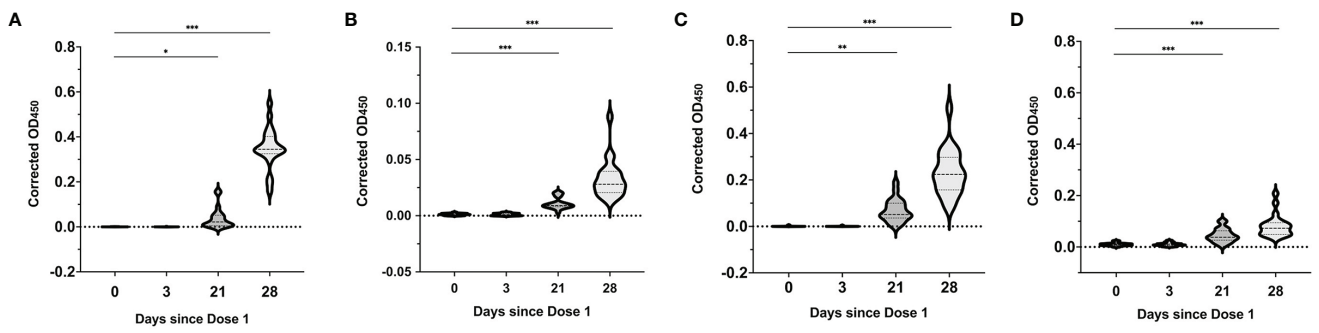


FIGURE 4 | SARS-CoV-2 spike RBD-specific antibody responses in serum over the 2-dose BNT162b2 mRNA vaccination. Median serum corrected OD_{450} values over the different time points for **(A)** SARS-CoV-2 RBD-specific IgG1, **(B)** IgG3, **(C)** IgA and **(D)** IgM isotypes. Violin plots with included boxplots showing kernel probability density of corrected OD_{450} with the dashed and dotted black lines representing the median and 25th/75th quartiles respectively. Comparisons of differences between time points were assessed using repeated measures mixed-effects model followed by *post hoc* Tukey's multiple comparisons test. The asterisk indicates P<0.05, double asterisk indicates P<0.001, the triple asterisk indicates P<0.0001.

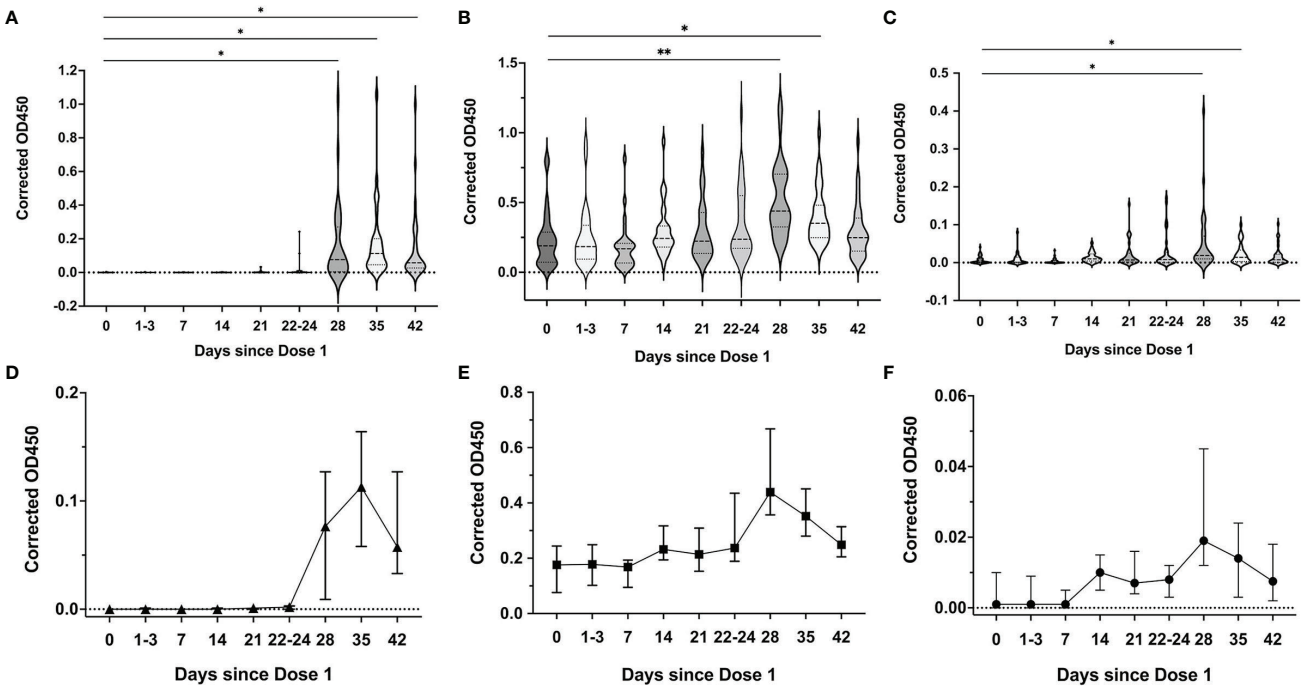


FIGURE 5 | SARS-CoV-2 spike RBD-specific antibody responses in breastmilk over the 2-dose BNT162b2 mRNA vaccination. Median breastmilk corrected OD_{450} values over the different time points for **(A)** SARS-CoV-2 RBD-specific IgG1, **(B)** IgA and **(C)** IgM isotypes. Violin plots with included boxplots showing kernel probability density of corrected OD_{450} with the dashed and dotted black lines representing the median and 25th/75th quartiles respectively. Comparisons of differences between time points were assessed using repeated measures mixed-effects model followed by *post hoc* Tukey's multiple comparisons test. Line graph depicting the dynamics of breastmilk (median with 95% CI as error bars) **(D)** IgG1, **(E)** IgA and **(F)** IgM isotypes over the time points. The asterisk indicates P<0.05, double asterisk indicates P<0.001.

activity in the serum and breastmilk of women vaccinated with the 2-dose BNT162b2 vaccine, over a 6-week period. As expected, there was detection of neutralizing antibodies and robust inhibitory responses in the serum just prior to and after the second vaccine dose, with all samples achieving significantly elevated neutralizing antibody levels by day 7 of this dose. The neutralization antibody levels and the associated SARS-CoV-2 RBD-specific antibody isotypes in the serum of these lactating women corresponds to previous reports of a rise in IgG, IgM and IgA specific to the spike and RBD segments of the SARS-CoV-2 virus after the first dose, and boosting of selected antibody isotypes by the second dose (10, 11). We similarly documented elevation in RBD-specific IgG1 and IgG3 levels in the serum of all mothers after the second vaccine dose. Recent studies have described the dominance of IgG1 and IgG3 response over IgG2 and IgG4 following SARS-CoV-2 mRNA vaccination and with natural infection (24–26). The lower IgM levels after the second vaccine dose may be related to enhanced class switching to IgG and IgA isotypes after this dose.

In the breastmilk, detection of neutralizing antibody and increase in inhibitory capability was notable 7 days after dose 2, corresponding with the peak levels of SARS-CoV-2 RBD-specific antibody isotypes, especially IgG1, and IgA levels at this point. These findings are concordant with previously reported increases in breastmilk SARS-CoV-2-specific IgA and IgG levels within a week after the second vaccine dose (10, 11, 27, 28). SARS-CoV-2 specific IgA in the breastmilk reportedly achieved maximal levels a week after the second dose, where SARS-CoV-2-specific IgG was only evident 1 week after the second dose (12, 14, 28–31). The persistence of neutralizing activity up to 3 weeks post dose 2 corresponds to the continued elevation in SARS-CoV-2 RBD-specific IgG1 but not IgA levels. In agreement with recent data, our study highlights the dominance of breastmilk SARS-CoV-2 IgG1 responses post-vaccination, as compared to higher IgA responses reported in natural infection (11, 30, 32, 33). The potential for therapeutic application of these antibodies for protection against COVID-19, especially neonatal infection, remains to be elucidated.

We noted one lactating mother who had detectable SARS-CoV-2-specific neutralizing antibodies in her breastmilk sample prior to and immediately after the first dose of BNT162b2 vaccination. There was no detectable neutralizing antibodies in her serum samples prior to and after the first vaccine dose (days 0 and 3), but with similar increases in the neutralizing antibodies after the second dose as the rest of the cohort. As this participant reported no significant COVID-19 exposure and the low community transmission of SARS-CoV-2 during the course of this study (34), this finding is likely due to cross-reactivity to antibodies from recent/past infection with non-SARS-CoV-2 coronavirus strains.

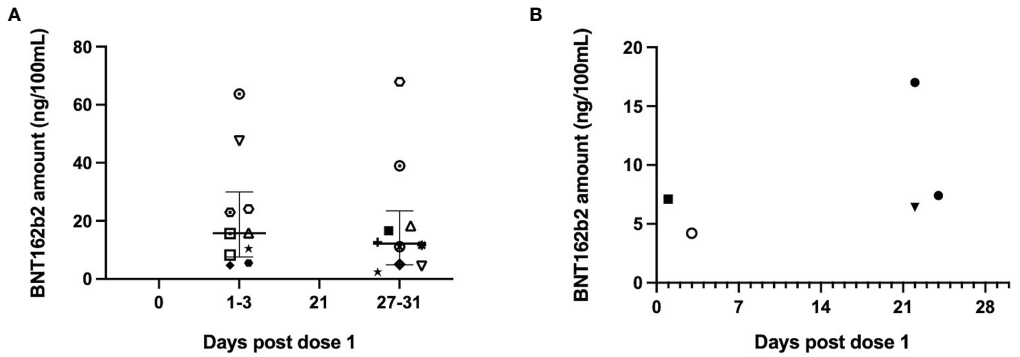


FIGURE 6 | The BNT162b2 mRNA amount detected in maternal serum and breastmilk across the different timepoints. Amount of BNT162b2 mRNA (ng/100ml) in **(A)** serum and **(B)** breastmilk across the different sampling timepoints. Only positive samples are plotted and specific shapes in the plots denote samples from individual mothers across both sample types types (median and 25^{th}/75^{th} quartiles plotted). Intact vaccine mRNA was detected in 20/74 serum samples tested from 21 mothers and 5/309 breastmilk samples tested from 31 mothers. Only 1 mother had detectable vaccine mRNA in serum and breastmilk samples (denoted by black filled square).

The neutralizing antibody levels in the serum and the breastmilk levels were poorly correlated prior to the second vaccine dose, with moderate positive correlation noted at a week after dose 2. This is likely a reflection of the boosting of antibody responses in the serum post the second dose and the enhanced excretion of antibodies into the breastmilk.

We also report the detection of low quantities of intact BNT162b2 mRNA in the serum and breastmilk samples – in 71% and 13% of the mothers investigated respectively. Several recent reports have inconsistently documented the presence of vaccine mRNA in the serum and breastmilk of women after BTN162b2 vaccination (13, 35, 36). The presence of intact vaccine mRNA in both sample types in our study highlights the stability and persistence of the vaccine mRNA nanoparticle within the bloodstream which may lead to infrequent transfer into breastmilk. The systemic spread of intramuscular delivery of lipid nanoparticle encapsulated-mRNA have been previously demonstrated in animal models (37). Importantly, our results were complemented by the lack of neutralizing antibodies and vaccine mRNA in the serum of breastmilk-fed infants from vaccinated mothers, suggesting the likely lack of significant exposure or sensitization of infant to the low levels of mRNA present in breastmilk. The concentration of mRNA detected in the serum and breastmilk are comparable, but the levels are still a small fraction of the vaccine dose given. The median concentration detected is 0.02% and 0.05% of the vaccine dose in 100ml of milk or serum respectively.

These results provide additional evidence on the immunogenicity of BNT162b2 mRNA vaccination among lactating women through the demonstration of robust SARS-CoV-2 neutralizing activity in the serum and breastmilk. Importantly, the presence of SARS-CoV-2-specific antibody isotypes and neutralizing antibodies in the breastmilk underscores the potential passive protection afforded to the breastmilk-fed infants. While we detected transient, low levels of intact vaccine mRNA in the serum and breastmilk, we did not detect any serological evidence of infant sensitization. These data provide additional support to the safety of current recommendations for continuing breastfeeding with maternal BTN162b2 mRNA vaccination.

The strengths of our study include the comprehensive, paired collection of serum and breastmilk samples at multiple collection timepoints over the 2-dose vaccine course which allowed us to investigate the presence and importantly, track the neutralizing ability of the antibodies present in both sample types. We utilized a rigorous detection method for the confirmation of the presence of low levels of intact vaccine mRNA in the serum and breastmilk. This study is limited by the convenience cohort of front-line healthcare workers and a small number of infants, which may limit generalizability to the general population. The positive detection of the vaccine mRNA in serum and breastmilk did not extend beyond 1 week after vaccination, although we were limited by the number of serum collection timepoints. Due to the self-collection and storage of breastmilk in the participants freezers prior to transport to the laboratory, this may have led to variation in sample quality which in turn could have led to degradation in the vaccine mRNA prior to detection (38).

Larger population-based study should be performed to confirm the persistence of intact vaccine mRNA in the serum and breastmilk of lactating women who received the SARS-CoV-2 vaccination and the possible sensitization of breastmilk-fed infants towards the intact mRNA. This will contribute additional evidence to support the safety of current and future recommendations for infant feeding with mRNA vaccination. Future studies should also further examine the SARS-CoV-2 inhibition activity of specific antibody isotypes present in breastmilk through mRNA vaccination, and the potential for infant protection against COVID-19 through the passive transfer of breastmilk-derived SARS-CoV-2-specific antibodies.

BNT162b2 mRNA vaccination was associated with the generation of robust SARS-CoV-2 neutralizing responses in the serum and breastmilk of lactating women. While we detected low levels of vaccine mRNA in the serum of vaccinated mothers and their breastmilk, we did not detect any serological evidence of

sensitization of the infant towards the mRNA, providing additional support to the safety of current recommendations for continuing breastfeeding with maternal BTN162b2 mRNA vaccination.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Singhealth Institutional Review Board (CIRB ref. no 2019/2906 & CIRB ref. no. 2016/2791). Written informed consent to participate in this study was provided by the participant or by the participant's parent/legal guardian for the infant.

AUTHOR CONTRIBUTIONS

Conceptualization: KY, WC, CT, JY, SA, L-FW, and MC. Recruitment of volunteers and laboratory samples collection: KY, CO, KS, and MC. Data acquisition, analysis and interpretation of data: KY, WC, CT, CO, JY, JZ, SP, AL, KS, NS, and CC. Drafting of the manuscript: KY, WC, CT, CO, and JY. Statistical analysis: KY and WC. Overall supervision: SA, L-FW, and MC. KY, WC, and CT had full access to all data in the study and take responsibility for the integrity and accuracy of the data presented. All authors critically revised the manuscript for intellectual content, approved the version of manuscript submitted and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.783975/full#supplementary-material

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Conflict of Interest: LW, WC, and CT are co-inventors on a patent for the surrogate virus neutralization assay commercialized by GenScript Biotechnology under the trade name cPass.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SYSTEMATIC REVIEW
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The Effects of COVID-19 Vaccination on Lactating Women: A Systematic Review of the Literature

Joke Muyldermans[1,2], Louise De Weerdt[3], Larissa De Brabandere[3], Kirsten Maertens[3] and Eline Tommelein[1]*

[1] Department of Pharmaceutical Sciences (FARM), Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel, Jette, Belgium, [2] Midwifery Education, Department of Health, University College Brussels, Jette, Belgium, [3] Centre for the Evaluation of Vaccination, Vaccine and Infectious Diseases Institute, University of Antwerp, Antwerp, Belgium

Objectives: The availability of new vaccines against COVID-19 urges for guidance about vaccination during lactation. We aimed to review the literature to get an insight into the effects of COVID-19 vaccination on lactating women.

Design: Systematic review.

Data Sources: We searched Ovid Embase Classic+Embase, PubMed and BioMed Central for articles published between December 1st 2020 and December 31st 2021.

Review Methods: The search strategy contained terms and combinations related to COVID-19 vaccination during lactation, including the MeSH terms "COVID-19", "COVID-19 Vaccines", "SARS-CoV-2", "Lactation", "Breast Feeding", "Pregnancy" and "Postpartum period". The database search was completed with a manual search of the reference lists of included articles. Data concerning country, study period, number of participants, type of applied vaccine, time points of sampling and outcome measures were collected from the selected manuscripts. The data are summarized and synthesized in a descriptive way.

Results: 30 manuscripts were included in this review. Data on safety of COVID-19 vaccination during lactation indicate no severe vaccine-related local and systemic reactions, both after first and second dose, neither in the mother nor the nursing child. No significant amount of vaccine components seems to appear in breast milk. Milk supply data after vaccination are inconclusive as there are no quantitative data available. Some women however observe a temporary increase or reduction in milk supply, without long-term effects. All prospective cohort studies demonstrated the presence of SARS-CoV-2-specific antibodies in breast milk of nursing mothers vaccinated against SARS-CoV-2. Nearly all studies were conducted with mRNA vaccines.

Conclusion: There is evidence that the administration of a COVID-19 vaccine is safe and poses no additional risk to the breastfeeding woman or the breastfed baby. After vaccination of the mother during the lactation period, antibodies appear in the milk, which could protect the infant against COVID-19. Professional associations and

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****Correspondence:***
Joke Muyldermans
Joke.Muyldermans@vub.be

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government health authorities should therefore recommend offering COVID-19 vaccines to breastfeeding women, as the potential benefits of maternal vaccination while breastfeeding outweigh the risks.

Keywords: COVID-19, obstetrics, immunology, breastfeeding, vaccination, lactation

HIGHLIGHTS

What is already known on this topic

- Healthcare professionals are at greater risk to get a COVID-19 infection
- It was recommended to prioritize healthcare professionals for vaccination with the new vaccines against COVID-19
- Many healthcare professionals are women and at fertile age, who are possibly breastfeeding
- Exclusively breastfeeding for six months and after that for two years in combination with complementary foods is recommended
- None of the COVID-19 vaccines currently authorized or in phase 3 have been trialed for women who are breastfeeding

What this study adds

- COVID-19 vaccines during breastfeeding pose no risk to the woman and infant
- The presence of antibodies in breast milk of nursing mothers after COVID-19 vaccination was demonstrated
- No significant amounts of COVID-19 vaccine components were found in breast milk after vaccination
- Some women report a temporary milk supply change after COVID-19 vaccination

INTRODUCTION

The COVID-19 outbreak was characterized as a pandemic in March 2020 by the World Health Organization (WHO) (1). SARS-CoV-2, the virus that causes COVID-19, appears with a variety of clinical manifestations. In most cases, the disease starts with influenza-like symptoms and evolves in some patients towards acute respiratory distress syndrome (ARDS) and pneumonia. However, other symptoms like, gastrointestinal, dermatological, neurological, cardiovascular, and renal manifestations have also been reported (2). Breastfeeding women can, similar to other populations, get infected by the SARS-CoV-2-virus.

In October 2020, the European Commission listed a number of key steps for effective vaccination strategies ensuring access to safe vaccines across Europe (3). By the end of 2020, a worldwide vaccination strategy deployed. In multiple countries, people in residential centers, along with health care providers (HCPs) were prioritized for vaccination. At the start of the vaccination strategy, mRNA-based, adenovector-based vaccines, inactivated whole virus and subunit vaccines were approved and used (4, 5).

The availability of new vaccines against COVID-19 and the recommendation to prioritize HCPs for vaccination, urged for guidance about vaccination during lactation as many of these HCPs are of fertile age. Initially, multiple governmental guidelines advised against vaccination during the lactation period, disregarding a breastfeeding woman's likelihood of developing a severe form of the COVID-19 after exposure to the virus. At a later stage, the vaccination campaign was extended to the general population, including women of fertile age. This led to discontent and a firm counter reaction in the scientific literature (5, 6). The Center for Disease Control and Prevention (CDC) (7), the European Medicines Agency (EMA) (8) and the Royal College of Obstetricians and Gynaecologists (RCOG) (9) have since reversed their stand and now advise to offer the vaccine to breastfeeding women (10). Although the currently marketed COVID-19 vaccines are non-replicating vaccines and therefore theoretically pose no risk (9, 11, 12), several institutions still mention that robust safety and immunogenicity data in this population of women is lacking.

Indeed, none of the COVID-19 vaccines currently authorized or in phase 3 have been trialed for women who are breastfeeding. Some of these vaccines are now being tested in an academic setting. As vaccination in the postpartum period and during lactation could however result in clinically relevant immunologic factors in breast milk that are protecting the child in early life, it is of importance that women have this information to decide whether to take the vaccine. Exclusively breastfeeding for the first six months, and after six months in combination with complementary foods, is recommended by the World Health Organization (13) and UNICEF (14), given the many physically and psychologically benefits of breastfeeding, for both mother and child. Risking a mother to stop breastfeeding earlier than intended because of vaccination, should be regarded as a threat to the health of both. On the other hand, refusing vaccination also entails risks for the mother, with an increased risk of infection and development of (severe) COVID-19.

We aim to review the literature to get an insight in the effects of vaccination with COVID-19 vaccines during the lactation period. This entails the safety of vaccination during lactation, the immune response in lactating women and the excretion of immunological factors in breast milk. Neonates rely on the transfer of immunity *via* the placenta and breast milk, since they are born with an immature immune system. The role of immunoglobulin G (IgG) transferred *via* the placenta is well established, but less is known about the transfer of antibodies and the mechanisms by which these antibodies provide protection to the neonate *via* breast milk (15). It is therefore plausible that the immune response triggered by vaccinating lactating women may be different from the general population.

The conclusions will contribute to the knowledge on COVID-19 vaccination and the results will benefit the population with respect to public health.

METHODS

Study Design and Searches

We searched Ovid Embase Classic+Embase, PubMed and BioMed Central for articles published between December 1st 2020 and December 31st 2021. The search strategy contained terms and combinations related to COVID-19 vaccination during lactation, including the MeSH terms "COVID-19", "COVID-19 Vaccines", "SARS-CoV-2", "Lactation", "Breast Feeding", "Pregnancy" and "Postpartum Period". The final literature search was performed on the 31st of December 2021. The database search was completed with a manual search of the reference lists of included articles (i.e. "snowballing"). The quality of reporting was supported by the use of the PRISMA guidelines (16). The detailed overview of the search strategy is provided in **Appendix 1**.

Eligibility Criteria and Study Selection

Manuscripts were eligible for inclusion if (1) study participants were women vaccinated with a COVID-19 vaccine during the lactation period and (2) the results reported on the safety of vaccination during lactation OR on the excretion of COVID-19 vaccine components in breast milk OR the excretion of immunological factors in breast milk after COVID-19 vaccination OR on the impact of COVID-19 vaccination on the breast milk production. Manuscripts published in English, French, Dutch, German or Spanish were included. Studies that focused on women with specific pathologies (e.g. transplant patients) were excluded. JM and ET independently screened titles, abstracts and full-texts of the retrieved manuscripts for eligibility. Each manuscript showing uncertainty regarding inclusion criteria was discussed with the other authors until consensus about inclusion.

Data Collection, Synthesis and Analysis

Data concerning country, study period, number of participants, type of applied vaccine, time points of sampling and outcome measures were collected from the selected manuscripts. The datasets were summarized and synthesized in a descriptive way.

RESULTS

Included Studies

In Total, 2,373 manuscripts were identified. We screened 2,163 titles and 60 abstracts for eligibility. We screened the full text of 26 manuscripts and excluded 7. Eleven manuscripts were added *via* manual search of the references (**Figure 1**). We included manuscripts reporting on women that were vaccinated with the messenger RNA (mRNA) mRNA-1273 (Moderna) and vaccines BNT162b2 (Pfizer–BioNTech), the adenoviral vector vaccines ChAdOx1 nCoV-19 (Oxford - AstraZeneca) and JNJ-78436735 (Johnson & Johnson) and the inactivated whole-virus SARS-CoV-2 vaccine by Sinovac Biotech Ltd.

Of the 30 included references, 1 study reports on the excretion of COVID-19 components in breast milk (17), 20 studies report on the excretion of antibodies in breast milk (18–36), 1 study report on the excretion of other immunological factors (37) and 1 study reports on the impact of vaccination on breast milk production (38). Two studies report on both side effects and the excretion of COVID-19 antibodies (39, 40), 1 study reports on the excretion of COVID-19 antibodies and the excretion of vaccine components (41), 3 studies report on side effects and the impact of vaccination on breast milk production (42–44) and 1 study reports on side effects, the impact of vaccination on breast milk production and on breast milk production (45).

Side-Effects in the Mother After COVID-19 Vaccination During Lactation

Six publications report on side-effects of COVID-19 vaccination during lactation in a total of 7,241 women, of which 4,509 vaccinated with the BNT162b2 vaccine, 2,669 with the mRNA-1273 vaccine and 23 with the JNJ-78436735 vaccine (39, 42–45).

In a first study (42), data from 17,525 women vaccinated with a COVID-19 vaccine were included of which 6,815 were lactating women. The other women were either pregnant women (n = 7,809) or women from fertile age planning to get pregnant at the moment of the first vaccine dose (n = 2,901). Of the vaccinated lactating women, 4,156 received the BNT162b2 vaccine, 2,596 received the mRNA-1273 vaccine, 23 received the JNJ-78436735 vaccine and from 40 women it was not known which vaccine they received. The most common adverse reactions in all participating women after a first vaccine dose were pain at the injection site (16,019/17,525; 91.4%) and fatigue (5,489/17,525; 31.3%). Fatigue was more reported after the second dose (10,399/17,525; 69.2%). No difference in the rate of adverse events by vaccine-type was reported across all groups (42).

Another study (43) (n = 180, of which 128 lactating women received the BNT162b2 vaccine and 52 the mRNA-1273) reported similar proportions of general side-effects for both the BNT162b2 (89.4%) and mRNA-1273 (98.1%) vaccine after the first dose in lactating women. The most common reactions in lactating women after a first dose were pain at the injection site (105/126; 86.8% BNT162b; 50/52, 96.2% mRNA-1273) fatigue (31/126; 26.3% BNT162b2; 12/52; 23.1% mRNA-1273) and headache (28/126; 23.7% BNT162b;13/52; 25.0% mRNA-1273). Following the second dose, lactating women receiving the mRNA-1273 vaccine reported significantly more side-effects like chills (36/52 vs. 55/123, 75.0% vs.47.8%), muscle/body aches (41/52 vs. 71/123, 83.7% vs.61.7%), fever (23/52 vs. 28/123, 46.9% vs. 24.3%) and vomiting (4/32 vs. 1/123, 8.5% vs. 0.9%) ($p<0.05$) in comparison with women receiving the BNT162b2 vaccine. Local symptoms including redness at injection site (15/52 vs. 3/123, 31.9% vs. 2.6%), swelling at injection site (14/52 vs. 8/123, 29.8% vs. 6.1%) and itching at injection site (8/52 vs. 5/123, 17.4% vs. 4.4%) were more common after the second dose with the

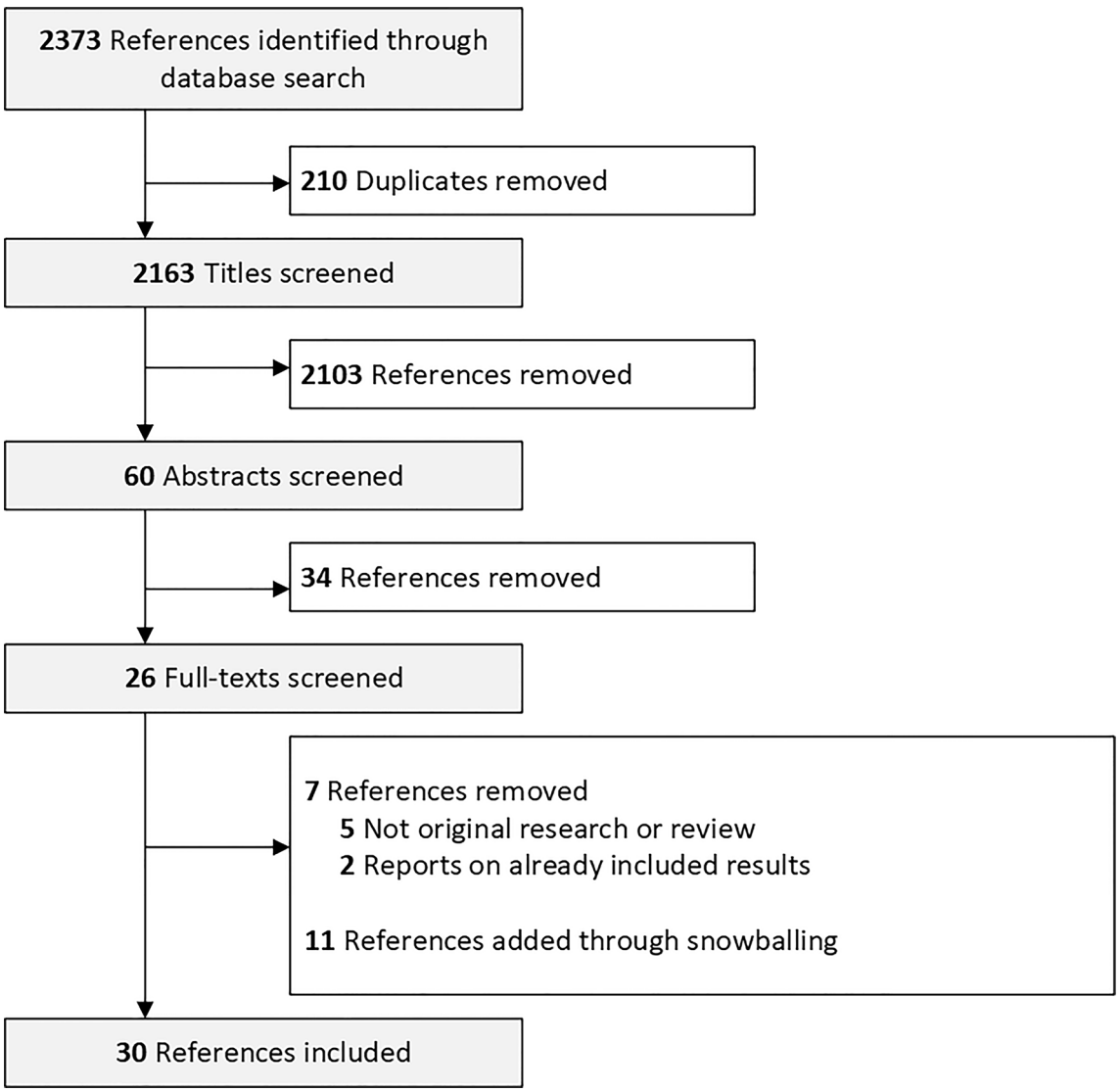

FIGURE 1 | Flow diagram of literature search and manuscript selection.

mRNA-1273 vaccine ($p<0.05$) then after the BNT162b2 vaccine (43).

In a third study (45), (n = 48), fever, chills, muscle or body aches, fatigue and/or tiredness and joint pain were significantly less reported by lactating women after the first dose than after the second dose. All 21 participants (100%) who received the mRNA-1237 vaccine reported symptoms at injection, while only 21 (78%) of 27 BNT162b2 participants reported symptoms at injection site ($p=0.02$). During the study, two infants were diagnosed with COVID-19. One week after the second dose, mild symptoms by one infant were reported, while the infant's PCR test was positive, the mother who was vaccinated had a negative test. A positive plasma anti-SARS-CoV-2 IgG (immunoglobulin G) and IgA (immunoglobulin A) was found in another infant, despite the mother reported no known prior SARS-CoV-2 infection and receiving the vaccine postpartum. A likely natural asymptomatic COVID-19 infection could be indicated, since antibodies against SARS-CoV-2 nucleocapsid protein were found in the mother's plasma (45).

A fourth study (39) (n = 84) reported similar adverse reactions after the first and second dose in lactating women. Forty-seven women (55.9%) reported one or more vaccine-related side effect after the first dose of which 40 local pain at injection site (47.6%), and 8 fatigue (9.5%). After the second dose, 52 women (61.9%) reported side-effects, of which 34 local pain at injection site (40.5%), 28 fatigue (33.3%) and 10 fever (11.9%) (39). In the final included study (44), local reactions at the injection site (redness, pain or swelling) were reported by 57 of 88 women (64.8%), headache, muscle pain or joint pain was reported by 52/88 (59.1%) and fatigue by 54/88 (61.4%). Five out

of 88 (5.7%) lactating women reported neck or axillary lymph node swelling after their second dose of the BNT162b2 vaccine. Mastitis was reported by three (3.4%) women and breast engorgement which resolved after 24 hours was reported by one woman (44).

In a fifth study (40) (n = 26) one or more side effects were reported by 57% of the participants after the first dose. After the second dose, one or more side effects were reported by 81% of the participants. After the first dose, the most reported side effects were local pain or swelling (6/26, 28.6%) and muscles aches (5/26, 23.8%). After the second dose fatigue (7/26, 33.3%), local pain or swelling (6/26, 28.6%), fever (5/26, 23.8%) and headache (5/26, 23.8%) were the most frequently reported side effects (40).

Overall, we can conclude that side-effects in lactating mothers after COVID-19 vaccination are similar to other individuals like pregnant women and women who are planning to get pregnant (42). The most common side-effects shown in these studies were fatigue, fever, headache, chills, muscle pain and pain at injection site. These side-effects are mild and similar to side-effects described in the general population (46). Studies showed increased reactions following the second dose of BNT162b2 and mRNA-1237 vaccines compared to the first dose (39, 42, 43, 45).

Side-Effects in the Infant After COVID-19 Vaccination During Lactation

Three studies evaluated side-effects in the infant after a COVID-19 vaccination to the mother, including a total of 7,043 infants (4,311 of whom the mother was vaccinated with the BNT162b2 vaccine, 2,669 with the mRNA-1273 vaccine, 23 with the JNJ-78436735 vaccine, 40 of which the vaccine type was not specified) (42, 43, 45).

In the first study (n = 6,815) 208 breastfeeding mothers reported to have concerns about the infant after the first dose (3.0%) and concerns were reported by 267 breastfeeding mothers after the second dose (4.4%) (42). In another study (n = 180) the most common side-effects seen in the nursing children were similar for both mRNA vaccines following the first dose (poor sleep: 4/129, 3.4% BNT162b2; 3/53, 5.9% mRNA-1273 and irritability: 2/129, 1.7% BNT162b2; 2/53, 3.9% mRNA-1273) and second dose (poor sleep: 7.8% BNT162b2; 8.3% mRNA-1273 and irritability: 12/126, 10.3% BNT162b2; 5/53, 10.4% mRNA-1273). The only side-effect showing a significant difference between the mRNA-1273 and BNT162b2 vaccine after the second dose was drowsiness after the second vaccine dose. Mothers who received the mRNA-1273 vaccine reported significantly more drowsiness in the infant after the second dose in comparison to mothers who received the BNT162b2 vaccine (3/53 vs. 0/129; 6.4% vs. 0.0%; p=0.02) (43). In another study with 48 participants (27 BNT162b2 and 21 mRNA-1273), 12% (6/48) of mothers reported to have seen at least one symptom after the first vaccine in their infants. These included gastrointestinal (2/48; 4.0%; BNT162b2), sleep changes (3/48; 6.0%; 2 BNT162b2, 1 mRNA-1273) and rash/baby acne (1/48; 2.0%; mRNA-1273). No mothers reported an infant symptom after the second vaccine dose (45).

Excretion of COVID-19-Vaccine Components in Breast Milk

Two manuscripts report on the excretion of COVID-19 vaccine particles in breast milk, including a total of 16 women (15 vaccinated with the BNT162b2 vaccine and 1 with the mRNA-1273 vaccine) (17, 41).

The first study included 6 women receiving a COVID-19 vaccine of which 5 women were vaccinated with the BNT162b2 vaccine, and 1 with the mRNA-1273 vaccine. When testing breast milk samples, no mRNA was found in their milk 4-48 hours post-vaccination (17). The second study included 10 women receiving the BNT162b2 vaccine of which milk samples were analyzed at four timepoints (pre-vaccination, 1-3 days after the first dose, 7-days after the first dose and 3-7 days after the second dose). Across all timepoints, a minimal transfer of BNT162b2 mRNA in human milk was found. Very low levels of vaccine mRNA were only found on rare occasions (4/40) and this within the first week after the first dose or second dose. Detectable levels of vaccine mRNA were not shown in 90% (36/40 samples) of the samples. The highest concentration of BNT162b2 mRNA found was 2 ng/mL. In the worst-case scenario, this would mean 0.67% of the given vaccine dose being transferred to the infant in 100 mL of breast milk (41).

mRNA vaccines contain a part of the genetic code of the SARS-Cov-2 virus, more specifically that of the SARS-Cov-2 S spike or "S" protein. They are encapsulated in very small specialized particles consisting of fats, cholesterol and polyethylene glycol. The small amount of polyethylene glycol-2000 in the BNT162b2 vaccine was also not found in breast milk (45).

Excretion of Antibodies in Breast Milk

The eventual list of included manuscripts covering the excretion of antibodies in breast milk is presented in **Tables 1**, **2**. It covers an overview of the number of participants, applied vaccines, timepoints of breast milk collection and IgG and IgA responses in breast milk for every study. Our final sample comprised of 18 published (18, 19, 21–33, 39, 40) manuscripts reporting on 716 women receiving a COVID-19 vaccine during the lactation period and 6 preprints (20, 35, 36, 41, 45, 48) on 151 women receiving a COVID-19 vaccine during the lactation period. In total, 665 women were vaccinated with the BNT162b2 vaccine, 125 with the mRNA-1273, 13 with the JNJ-78436735, 44 with the ChAdOx1 nCoV-19 and 20 with the Sinovac Biotech Ltd. The studies were performed in 9 different countries (i.e. USA, Israel, Spain, Brazil, Italy, Poland, Portugal, Singapore and The Netherlands).

The presence of antibodies in breast milk (mainly IgA and IgG) after vaccination against SARS-CoV-2 during lactation, have been demonstrated by several prospective cohort studies. Nearly all studies were conducted with mRNA vaccines (See **Tables 1**, **2**). Only one study included participants vaccinated with the JNJ-78436735 vaccine (48), one study included participants vaccinated with the Sinovac Biotech Ltd. Vaccine (22) and two studies included participants vaccinated with the ChAdOx1-S vaccine (19, 32).

TABLE 1 | Overview of vaccination studies in breastfeeding women with data on antibody secretion in breast milk.

Study	No. of participants	Applied vaccine(s)	Time points	IgA response in BM	IgG response in BM
Baird et al. (21) *(USA)*	6	BNT162b2 (n = 3) mRNA-1273 (n = 3)	Before vaccination (BV) + day 1, 4, 7, 11, 14 after 1st dose + 1 day before 2nd dose + day 1, 4, 7, 11 and 14 after 2nd dose	Elevated levels of IgA beginning at day 7 after 1st dose. Prior to the 2nd dose, levels of IgA decreased. Levels of IgA increased sharply after 2nd dose.	Elevated levels of IgG after 1st dose, beginning at day 7, with an IgG dominant response. The level of IgG decreased prior to 2nd dose. IgG levels sharply increased after 2nd dose.
Calil et al. (22) *(Brazil)*	20	Sinovac Biotech Ltd. (inactivated whole-virus SARS-CoV-2 vaccine)	BV + Weekly after 2nd dose for 3 weeks + until 4 months after 1st dose (n=10)	After the 1st dose, mean levels of IgA increased in the first two weeks. At week 5 and 6, significantly higher mean values were obtained compared to week 1, 2, 3 and 4. At week 7, specific IgA antibody levels above the seroconversion were found in milk samples of 10 mothers. IgA levels were above the seroconversion in milk samples 4 months after the 1st dose (n=10).	Not Applicable
Charepe et al. (23) *(Portugal))*	14	BNT162b2	1-3 weeks after 1st dose + 1-3 weeks after 2nd dose	IgA was detected in breast milk after vaccine administration. In 35.7% (5/14) of milk samples, IgA was present after the 1st dose. IgA was present in 21.4% (3/14) after the 2nd dose.	IgG was detected in breast milk was detected after vaccine administration. After the 1st dose, IgG was present in 7.1% (1/14). IgG presence increased to 42.9% (6/14) after the 2nd dose.
Collier et al. (24) (*USA)*	16	mRNA-1273 (n = 5) BNT162b2 (n = 11)	Close to each vaccine dose and between 2-8 weeks after 2nd dose	The median IgA titer was 25 after vaccination.	The median IgG titer was 97 after vaccination.
Esteve-Palau et al. (25) (*Spain)*	33	BNT162b2	Around 2 weeks after 1st dose (T1) + 2 (T2) and 4 weeks (T3) after 2nd dose	Not Applicable	Median IgG levels for breast milk were found at each time point: 1 (0-2.9) AU/mL for T1, 78 (33.7-128) AU/mL for T2, and 50.4 (24.3-104) AU/mL for T3.
Gray et al. (26) *(USA)*	31	mRNA-1273 (n = 15) BNT162b2 (n = 16)	BV (T1) + Day of 2nd dose (T2) + between 2-6 weeks after 2nd dose (T3)	in milk samples after mRNA-1273 vaccination, higher S- and RBD-specific IgA responses were found compared to the BNT162b2 vaccine. There was no significant rise in IgA after either dose.	from T1 to T3 IgG rose significantly (3.44-3.50; p=0.002), but not from T1 to T2 (3.44-3.45, p=0.7).
Guida et al. (27) *(Italy)*	10	BNT162b2	20 days after 1st dose (T1) (before 2nd dose) + 7 days after 2nd dose (T2)	Not Applicable	Anti-SARS-CoV-2 S antibodies were detected in two (40%) milk samples with a low concentration (1.2 +/- 0.3 U/mL) at T1. In all milk samples anti-SARS-CoV-2 S antibodies were detected at T2 (41.5 +/- 47.5 U/mL).
Jakuszko et al. (28) *(Poland)*	28	BNT162b2	Day 8 and 21 after 1st dose (day 21 prior to 2nd dose) + Day 29, 43 after 2nd dose	No differences in the absolute values were observed on day 8. On day 29 after the 2nd dose, the highest concentrations of IgA were observed, with a decrease on day 43.	In the absolute values, there were no differences observed on day 8. In 14/28 (50%) positive IgG samples were observed on day 22 and in all women on days 29 and 43. On day 29 after the 2nd dose the highest concentrations of IgG were observed. A decrease was seen on day 43.
Juncker et al. (40) *(The Netherlands)*	26	BNT162b2 (6 one dose, 20 2 doses)	BV + Day 3, 5, 7, 9, 11, 13 and 15-17 after 1st dose + before 2nd dose + Day 3, 5, 7, 9, 11, 13 and 15-17 after 2nd dose	After vaccination, a higher inter-individual variability in IgA was observed. IgA started rising 5 to 7 days after 1st dose, with an increase of 12% per day. On day 15 a three-fold increase was seen, compared to baseline. From day 15 after 1st dose and just before 2nd dose, IgA levels decreased by 43%. IgA levels stabilized at 50% of peak level. At 2nd dose peak level was 1.3 times higher compared to peak level 7 days after 1st dose. After the 2nd dose IgA gradually declined, decreasing by 33% until the end of sample collection 35 days after 1st dose IgA increased by 2.4 times.	Not Applicable

(Continued)

TABLE 1 | Continued

Study	No. of participants	Applied vaccine(s)	Time points	IgA response in BM	IgG response in BM
Kelly et al. (18) *(USA)*	5	BNT162b2	BV + Day of 1st dose + weekly following until between 40-90 days after 1st dose	In all samples, IgA levels were elevated compared to pre-vaccine baseline. Two weeks after the 1st dose, IgA remained sustained. Following the 2nd dose gradual decline in IgA over time was seen.	In all samples, IgG levels were elevated relative compared to pre-vaccine baseline. Starting at 20 days after 1st dose, IgG remained sustained at an elevation through final milk sample.
Lechosa-Muñiz et al. (19) *(Spain)*	110	BNT162b2 (n = 70) mRNA-1273 (n = 20) ChAdOx1-S (n = 20) (only one dose was administrated from ChAdOx1-S)	30 days after 2nd dose for BNT162b2 and mRNA-1273 30 days after 1st dose for ChAdOx1-S	According to the type of vaccine, the mean IgA titers observed were different. Mothers who receveid the BNT162b2 vaccine had a mean of 0.11 (AU), for mRNA-1273 the mean was 0.10 (AU) and for ChAdOx1-S (one dose) the mean was 0.04 (AU). Comparing mean of IgA in mother milk from mothers vaccinated with BNT162b2 vs. ChAdOx1-S, there were significant differences found.	According to the type of vaccine, the mean IgG titers observed were different. Mothers who received the BNT162b2 vaccine had a mean of 0.41 (AU), for mRNA-1273 the mean was 0.45 (AU) and for ChAdOx1-S (one dose) the mean was 0.09 (AU). Comparing mean of IgG in mother milk from mothers vaccinated with BNT162b2 or mRNA-1273 vs. ChAdOx1-S (Sidakmethod), there were significant differences found. No differences in mean IgG could be found between those mothers vaccinated with BNT162b2 vs. mRNA-1273.
Low et al. (29) *(Singapore)*	14	BNT162b2	BV (T1) + 1-3 days after 1st dose (T2) + 7-10 days after 1st dose (T3) + 3-7 days after 2nd dose (T4) + 4-6 weeks after 2nd dose	A strong IgA response at 3-7 days after the 2nd dose (T4) was induced by vaccination. From T4 mother milk samples showed medians of 827 pM of anti-spike and 282 pM of anti-RBD IgA, a significantly higher level compared to the concentrations from earlier time points ($p < 0.001$). A reduction was observed 4-6 weeks after 2nd dose in the anti-spike (median: 499 pM) and the anti-RBD (median: 0 pM) IgA response.	At 3-7 days after the 2nd dose (T4) the median concentrations of anti-spike and anti-RBD IgG were 392 and 188 pM (picomolar). In all mother milk samples an increase in IgG was observed at T4. At 4-6 weeks after the 2nd dose (T5) the IgG levels remained high, with median concentrations of 657 pM anti-spike IgG and 184 pM anti-RBD IgG. Compared to the IgG concentration the levels at after the 2nd dose (T4, T5) were significantly higher compared to the concentration before vaccination ($p < 0.001$).
Nir et al. (30) *(Israël)*	64	BNT162b2	During postpartum hospitalization, mean time interval between 2nd dose and delivery was 21.7 (+/- 11.0)	Not Applicable	SARS-CoV-2 IgG was found in all breast milk samples.
Perl et al. (39) *(Israël)*	84	BNT162b2	BV + Weekly for 6 weeks beginning 2 weeks after 1st dose	Mean levels of IgA increased rapidly. At 2 weeks after the 1st dose, mean levels of IgA were significantly elevated, compared to mean levels before vaccination (2.05 ratio; $p < 0.001$). An increase from 61.8% positive tested samples to 86.1% 1 week after the 2nd dose. Until the last sample, mean levels remained elevated. At 6 weeks, 65.7% of samples tested positive.	The first 3 weeks after vaccination IgG remained low. An increase was seen at week 4 (20.5 U/mL; $p = 0.004$). At that point 91.7% of samples tested positive, even more increasing to 97% at weeks 5 and 6.
Romero Ramirez et al. (31) *(Spain)*	98	BNT162b2 (n = 92) mRNA-1273 (n = 6)	14 days after 2nd dose	IgA was found in 89% of the samples (95% CI: 81–95).	Anti- SARS-CoV-2 RBD-S1 IgG in all milk samples of vaccinated mothers. The mean IgG level was 12.19 ± 11.74 BAUs per mL (95% CI: 9.77–14.60; p <.001). The mean IgG levels were significantly higher than the levels from the control group (no vaccination, no previous infection) (0.02 ± 0.05 BAUs per mL [95% CI: 0.01–0.05; p<0.001]).
Schwartz et al. (47) *(Israël)*	61	BNT162b2	Time of sample collection according to vaccination was not mentioned in the article	In 15% of mother milk samples IgA was detected in secretory form. A median of 0.4 S/Co (IQR, 0.3e 0.7) was found.	A median igG concentration of 6.3 S/Co (IQR, 5.1e 7.4). was found in all mother milk samples.

(Continued)

TABLE 1 | Continued

Study	No. of participants	Applied vaccine(s)	Time points	IgA response in BM	IgG response in BM
Selma-Royo et al. (32) (*Spain*)	75	BNT162b2 (n = 30) mRNA-1273 (n =21) ChAdOx1-S (n = 24) (only one dose was administrated from ChAdOx1-S)	14 days after 1st dose, 14 days after 2nd dose of mRNA vaccines.	IgA had a strong reactivity after the 2nd dose. After the 1st dose, IgA levels were higher in mRNA-1273 vaccinated women compared to ChAdOx1-S vaccinated women (p<0.0001) and BNT162b2 vaccinated women (p=0.002). No differences were found between the two mRNA-based vaccines after the 2nd dose. After the 2nd dose, IgA levels did not further increase.	IgG had a strong reactivity after the 2nd dose. After the 1st dose, higher levels of IgG were induced by the BNT162b2 vaccine and the mRNA-1273 vaccine, compared to the ChAdOx1-S vaccine. The maximum effect with the mRNA-based vaccines was induced 2 weeks after the 2nd dose. A higher percentage of samples from mRNA-based vaccines remained positive compared to ChAdOx1-S 2 weeks after the 1st dose (p<0.0001). After the 1st dose, a higher increment of IgG was shown in mother milk samples from mothers receiving a mRNA vaccine, compared to the ChAdOx1-S vaccine (p<0.0001). IgG levels reached higher levels after the 2nd dose, compared to the 1st dose.
Valcarce et al. (33) (*USA*)	21	BNT162b2 (n = 14) mRNA-1273 (n = 7)	BV (T1) + 16–30 days after 1st dose (T2) + 7–10 days after 2nd dose (T3)	IgA statistically significantly increased between samples before vaccination (T1) to 16-30 days after the 1st dose (T2) (p < 0.0007) and from T1 to 7-10 days after the 2nd dose (T3) (p < 0.0001). A positive result for SARS-CoV-2 IgA was found in 85% after full vaccination based on the established cutoff value.	All samples were positive for SARS-CoV-2 IgG by 7-10 days after the 2nd dose based on the established cut off value.

BNT162b2, Pfizer-BioNTech vaccine; mRNA-1273, Moderna vaccine; ChAdOx1-S, Oxford – AstraZeneca vaccine; JNJ-78436735, Johnson & Johnson vaccine; AB, antibody; BV, before vaccination; IgA, immunoglobulin A; IgG, immunoglobulin G; No., number; RBD, receptor-binding domain; pM, picomolar; BM, breast milk.

In total, 17 published and 6 preprint studies researched the presence of IgA in breast milk after COVID-19 vaccination (18–24, 26, 28, 29, 31–33, 35, 36, 39, 40, 45, 47, 48). In general, the included studies show particularly an increase of antibody titers in breast milk after the second dose and that this is highly correlate with the levels present in the mother's blood. An increase of anti-SARS-CoV-2 specific IgA was found in most studies one week after the first dose. In the different studies, the interval between the first and second dose of mRNA vaccines was between 21 and 35 days, however, the participants of the two studies using the adenovector-based vaccines ChAdOx1 nCoV-1, did not receive a second dose. After the intermediate timepoint between the first and second vaccine dose, a decrease of IgA was reported towards the second dose. In most studies, the highest concentrations were observed one week after the second dose (18, 20–23, 28, 29, 32, 33, 36, 39, 40, 45). Three studies compared IgA between the mRNA-based vaccines (BNT162b2, n=123, and mRNA-1273, n=45) and the adenovector-based vaccines ChAdOx1 nCoV-19 (n=44) or JNJ-78436735 (n=13) (19, 32, 48). A first study found a significant difference in the mean IgA titers in breast milk of mothers vaccinated with BNT162b2 vs. ChAdOx1 nCoV-19 (p=0.02). For mothers who received the mRNA-1273 vaccine, the BNT162b2 vaccine and the ChAdOx1 nCoV-19 vaccine (one dose) respectively, the mean antibody titers observed in milk were 0.10 (± SD 0.07), 0.11 (± SD 0.12) and 0.04 (± SD 0.07) (AU, Arbitrary Units) (19). Another study found that IgA levels were higher after the first dose mRNA-1273 vaccinated women compared to ChAdOx1 nCoV-19 (one dose) (p<0.0001) and BNT162b2 (p=0.002). After the second dose, no differences were observed between the mRNA-based vaccines. After the notification of severe episodes of immune thrombotic thrombocytopenia after vaccination with ChAdOx1 nCoV-19, participants did not receive a second dose. Therefore there is no information on antibody responses in breast milk available after the second dose of ChAdOx1 nCoV-19 (32). A third study compared the mRNA-based vaccines with the JNJ-78436735 vaccine. Positive levels of Spike-specific IgA, exhibiting a mean endpoint titer of 15, was found in 23% of the JNJ-78436735 recipient milk samples. Comparing to the mRNA-1273 vaccine group, this was significantly lower (p=0.025) (48).

In total, the presence of IgG in breast milk after vaccination against SARS-CoV-2 in the lactation period was researched in 17 published and 4 preprint studies (18, 19, 21, 23–26, 28–33, 35, 36, 39, 47, 48). In some milk samples, an increase of anti-SARS-CoV-2 specific IgG was found one week after the first dose and increasing towards 2 weeks after the first dose. After the second dose, also an increase of IgG antibodies was seen (18, 21, 23, 25–29, 32, 33, 36, 39). Three studies compared ant-SARS CoV-2 RDB-S1 IgG between the mRNA-based vaccines (mRNA-1273 and BNT162b2) and the adenovector-based vaccines ChAdOx1 nCoV-19 or JNJ-78436735 (19, 32, 48). In a first study, according to the type of vaccine, the mean IgG titers were different, being 0.41 (± SD 0.10) for mothers who received BNT162b2, 0.45 ((± SD 0.08) for mRNA-1273, and 0.09 (± SD 0.08) (AU) ChAdOx1-S (one dose).

Comparing mean of IgG of lactating women vaccinated with mRNA-1273 or BNT162b2 vs. ChAdOx1-S significant

TABLE 2 | Overview of vaccination studies in breastfeeding women with data on antibody secretion in breast milk, published as preprint.

Study	Number of participants	Applied vaccine(s)	Time points	IgA response in BM	IgG response in BM
Fox et al. *(USA)*	10	BNT162b2 (n = 6) mRNA-1273 (n = 4)	BV + 14 days after 2nd dose	In 6 out of 10 undiluted post-vaccination Spike specific IgA were found.	All post-vaccination samples contained Spike-specific IgG.
Fox et al. *(USA)*	50	BNT162b2 (n = 23) mRNA-1273 (n = 14) JNJ-78436735 (n = 13)	1 week BV + 14 days after 2nd dose for BNT162b2 and mRNA-1273/28 days after 1st dose of JNJ-78436735	After vaccination, 71% of mRNA-1273 (mean endpoint titer of 19) and 52% of BNT162b2 (mean end point titer of 22). Of JNJ-78436735 mother milk samples 23% (endpoint titer of 15). The endpoint titer of JNJ-78436735 IgA was significantly lower than that of the mRNA-1273 vaccine group ($p = 0.025$).	After vaccination, 100% of mRNA-1273 (mean endpoint titer of 120) and 87% of BNT162b2 (mean endpoint titer of 180) mother milk samples contained levels of Spike-specific IgG. There was no significant difference in the mean IgG titers of both mRNA vaccine groups. both mRNA vaccine groups exhibited significantly higher specific milk IgG compared to sample of the JNJ-78436735 vaccine group. Only 38% of JNJ-78436735 samples contained levels of specific IgG (mean endpoint titer = 10; $p < 0.0001$).
Friedman et al. *(Israel)*	10	BNT162b2	Day 7 (T1) and day 14 (T2) after the 1st dose + day 7 (T3) and day 14 (T4) after 2nd dose	At 14 days after the 1st dose, a first significant increase in antibody titers was seen. This upward trend peaked at 7 days after the 2nd dose. A slight decrease was seen in titers 14 days after the 2nd dose. IgA in mother milk exhibited a potential neutralization capacity in all mothers.	At 14 days after the 1st dose, a first significant increase in antibody titers was seen. This upward trend peaked 7 days after the 2nd dose. A slight decrease was seen in titers 14 days after the 2nd dose. Anti-spike IgG in mother milk exhibited a potential neutralization capacity in all mothers.
Golan et al. *(USA)*	48	BNT162b2 (n = 27) mRNA-1273 (n = 21)	BV (T1) + day of 2nd dose (T2) + between 4-10 weeks after 2nd dose (T3)	Twelve individuals (BNT162b2 n=7; mRNA-1237 n=5) did not have detectable levels of anti-RBD IgA at T1 and T2. There were significantly higher levels of IgA antibodies specific to SARS-CoV-2 RBD protein found in mother milk samples after the 1st dose (T2). Compared to anti-RBD IgA at T2, there was no significant increase 4-10 weeks after the 2nd dose.	Not Applicable
Golan et al. *(USA)*	23	BNT162b2 (n = 14) mRNA-1273 (n = 9)	BV (T1) + day of 2nd dose (T2) + 4 weeks after 2nd dose (T3)	After the 1st dose significantly higher levels of IgA AB specific to SARS-CoV-2 RBD protein in mother milk samples were found. At the day of 2nd dose 17 out of 19 samples were positive for anti- SARS-CoV-2 IgA AB. Four weeks after the 2nd dose 13 out of 15 samples were positive for anti-SARS-CoV-2 RBD IgA. A variation in anti-SARS-CoV-2 RBD IgA AB levels was found in samples collected from 0 to 64 days after 1st doses. Four weeks after the 2nd dose IgA levels largely remained stable.	Not Applicable
Low et al. *(Singapore)*	10	BNT162b2	BV (T1) + 1-3 days after 1st dose (T2) + 7-10 days after 1st dose (T3) + 3-7 days after 2nd dose (T4)	At 3-7 days after the 2nd dose, the sharpest rise of IgA antibody production was found, with a median 374 pM. In mother milk sample of one mother, IgA was not detected in one mother 3-7 days after the 2nd dose.	At 3-7 days after the 2nd dose, the sharpest rise of IgG antibody production was found, with a median of 1110 pM.

BNT162b2, Pfizer-BioNTech vaccine; mRNA-1273, Moderna vaccine; ChAdOx1-S, Oxford – AstraZeneca vaccine; JNJ-78436735, Johnson & Johnson vaccine; AB, antibody; BV, before vaccination; IgA, immunoglobulin A; IgG, immunoglobulin G; No., number; RBD, receptor-binding domain; pM, picomolar; BM, breast milk.

differences were found (p=0.01), but there were no differences found between those mothers vaccinated with mRNA-1273 vs. BNT162b2 (19). In another study, higher levels of IgG were induced by the mRNA-1273 vaccine and BNT162b2 vaccine compared to the ChAdOx1-S vaccine after the first dose. Compared to the ChAdOx1-S vaccine 2 weeks after the first dose, a higher percentage of samples from mRNA-based vaccines remained positive for anti-SARS-CoV-2 IgG (p<0.0001) (32). In a third study, positive levels of Spike-specific IgG were found in 100% of mRNA-1273 and 87% of BNT162b2 post-vaccine milk samples. Of these mRNA vaccine groups, there were no significant differences in mean IgG titers. Both groups, of the mRNA-1273 vaccine and BNT162b2 vaccine, exhibited significantly higher specific milk IgG compared to milk samples from mothers vaccinated with the JNJ-78436735 vaccine. Only 38% of JNJ-78436735 samples contained positive levels of specific IgG (mean endpoint titer = 10; p < 0.0001) (48).

In addition, the presence of binding neutralizing antibodies was revealed in two studies evaluating the immunogenicity of mRNA-based vaccines (24, 36). These data may indicate that breast milk has the potential to add to infant protection by passively transferred antibodies through breast milk.

Excretion of Other Immunological Factors in Breast Milk

Only one manuscript reported on the excretion of other immunological factors than antibodies. The study included 14 women, all receiving the BNT162b2 vaccine. The study showed that vaccination is not only able to increase the amount of antibodies in breast milk, but also induces spike-reactive CD4+ T cells in breast milk, especially after the second dose of the BNT162b2 vaccine. These spike-reactive CD4+ T cells may have a protective function in the upper respiratory tract of infants (37).

Impact of Vaccination on Breast Milk Production

Five manuscripts report on the impact of vaccination on breast milk production and included a total of 11,586 lactating women receiving a COVID-19 vaccine (38, 42, 43, 45). These studies report on 4,399 women vaccinated with the BNT162b2 vaccine, 2,669 with the mRNA-1273, 23 with the JNJ-78436735 and for 40 the vaccine type was not specified (42, 43, 45). One of these studies vaccinated 4,445 women with either the mRNA-1273 or the BNT162b2 vaccine. This was not specified (38).

In a first study (n = 180), a temporary reduction in breast milk supply was reported by some women after vaccination with a COVID-19 mRNA vaccine (71% BNT162b2, 29% mRNA-1273). A decrease in milk production after the BNT162b2 vaccine was reported by 7.3% (9/126) and 8.0% (9/123) women after the first and second dose, respectively. The percentage of women reporting a decrease in milk production after the mRNA-1273 vaccine was 11.5% (6/52) after the first dose and 23.4% (11/52) after the second dose. The difference between the BNT162b2 vaccine and the mRNA-1273 was statistically significant (p<0.05). Milk supply returned to normal within three days in all cases. In contrast, an increase in milk supply was reported by some women. More production was reported after the first dose by 3.3% (4/126) of mothers who received the BNT162b2 vaccine, but was not reported by mothers receiving a first dose of the mRNA-1273 vaccine. After the second dose, 3.6% (4/123) of mothers who received the BNT162b2 vaccine reported an increase in milk supply, and 6.4% (3/52) of mothers who received the mRNA-1273 vaccine. Finally, a milk color change to blue-green color was reported by 3 mothers after vaccine administration (2/126, 8.0%, BNT162b2; 1/52, 7.1%, mRNA-1273) after the administration of the first dose and by 2 mothers (1/123, 4.0%, BNT162b2; 1/52, 6.2%, mRNA-1273) after the second dose (43).

In a second study, 4,455 breastfeeding mothers who received either the BNT162b2 vaccine or the mRNA-1273 vaccine filled in an online survey. An increase in milk supply was reported by 3.9% of mothers and a decrease was reported by 6.0% of mothers (38). In a third study of 6,815 lactating women, 339 participants reported a decreased milk supply no longer than 24 hours after the first dose (5.0%) and 434 participants after the second dose (7.2%) (42). In another, relatively small study with 48 mothers, 2 mothers reported a slight decrease in milk production in the first 24-72 hours after the first and second dose (45).

An interruption of breastfeeding after the first dose by 155 of 6,815 participants (2.3%) and 130 of 6,056 individuals after the second dose (2.2%) was reported by Kachikis et al. (2021). Whether the breastfeeding interruption was a deliberate choice of the mother, imposed upon from the health care provider or a consequence of decreased production was not specified (42).

One study looked at milk production after receiving the BNT162b2 vaccine (n = 88). A change in milk supply was reported by one woman, increase or decrease was not specified. One woman reported a transient bluish-green color of her breast milk after her first vaccine dose. This was not reported after her second dose (44).

DISCUSSION

Main Findings of the Review

We reviewed the literature on the safety of COVID-19 vaccination during lactation in women and neonates. Subsequently, we summarized the effects of COVID-19 vaccination during lactation on the excretion of COVID-19 vaccine components in breast milk, the excretion of immunologic factors in breast milk and on the production of breast milk. In general, currently available data point towards a reassuring safety profile of COVID-19 vaccination during lactation with comparable side-effects in lactating compared to pregnant, non-pregnant and non-lactating women of childbearing age. While vaccine components are barely or not detectable in breast milk, most studies report the presence of anti-SARS-CoV-2 IgA and IgG in breast milk at several timepoints post vaccination, up to 8 weeks after the second vaccine dose. Additionally, a large proportion of the antibodies in breast milk exhibits neutralizing capacity against the virus offering potential additional protection to the nursing infant. The impact on milk supply appears to remain very limited.

Combining the Reported Study Outcomes

Since neonates are born with an immature immune system, they rely on the transfer of antibodies *via* the placenta and breast milk for their protection during the first vulnerable months and years of life. The mechanisms by which the antibodies in breast milk provide protection to the neonate still remain unclear (15). At the moment of publication, worldwide, 47 out of 224 countries recommend COVID-19 vaccination for some or all lactating women whereas 77 countries state that lactating women can receive, may receive or can choose to receive the vaccine. In 8 countries, only certain groups of lactating people (e.g. health care professionals, women with underlying conditions) can or may choose to receive the vaccine. One country state that lactating women should not receive the vaccine, with certain exceptions and 23 countries do not recommend COVID-19 vaccination for lactating women. For 68 countries, there is currently no information on their policy regarding COVID-19 vaccination during lactation (10).

Despite the fact that the strategy of vaccinating lactating women with COVID-19 vaccines is frequently recommended on a global level, none of the COVID-19 vaccines currently authorized or in phase 3 have been trialed for women who are breastfeeding. Since COVID-19 is a new disease, it is important to consider whether vaccination during lactation is safe and effective. The safety of administrating inactivated vaccines to lactating women was already shown for other vaccine-preventable infectious diseases (49) and also sIgA and IgG after postpartum vaccination against pertussis and influenza are proven to be secreted into breast milk (15).

Three studies, all using mRNA-based vaccines, focused on the safety and side-effects of COVID-19-vaccination in lactating women (42, 43, 45). Among all participants, the most common described side-effects were pain at the injection site, fatigue, chills, headache, muscle/body aches, fever and vomiting. These side-effects are similar to the ones seen in the general population (46).

The excretion of COVID-19 vaccine components in breast milk was investigated in two studies (17, 41). Although these studies were small, it is reassuring that no or very low concentrations of mRNA were detected (17, 41). If low concentrations of mRNA reach the breastfed infant through breast milk, it is very likely that there will be no uptake by the gastrointestinal system. The small amount of polyethylene glycol-2000 (PEG-2000) in the BNT162b2 vaccine is not found in breast milk. This is important to know, since PEG-2000 can cause anaphylaxis in very rare cases (45). No studies were performed on the excretion of COVID-19-vaccine components in breast milk after vaccination with any other type of COVID-19 vaccine.

Multiple studies showed that sIgA and IgG against the SARS-CoV-2 spike protein are present in breast milk after COVID-19 mRNA vaccination (20, 21, 26, 37, 50, 51). These studies show that the antibody titers (sIgA and IgG) in breast milk mainly increase after a second dose and that they are strongly correlated with the antibody levels present in the mother's blood. In most published studies, antibody levels are measured relatively shortly after vaccination, i.e. within 2 to 8 weeks after the second dose. At the time of publication, most studies focused on measuring antibody levels until 2 to 8 weeks after the second dose. Long-term results on antibody levels after vaccination are not published yet.

Nearly all studies were conducted with mRNA vaccines. Only one study was conducted with the JNJ-78436735 vaccine (48), one study was conducted with the Sinovac Biotech Ltd. vaccine (22) and two studies were conducted with the ChAdOx1-S vaccine (19, 32).

Five studies looked at the effect of COVID-19 vaccination on milk production (38, 42–45). Only a few women reported a temporary reduction in breast milk supply. Milk supply returned to normal within one to three days. Some women reported an increase in milk supply.

Strengths and Limitations of the Review

To the best of our knowledge, this is the most complete and extensive review on the effects of COVID-19 vaccines when administered to lactating women. The insights of this review are important for policy makers that can adapt guidelines and inform women on whether to take the vaccine or not during lactation as vaccination during lactation could result in clinically relevant immunological factors in breast milk and therefore offer additional protection to the infant.

Despite specific selection criteria of the studies included in this review, differences in vaccine schedules, sample collection timepoints, sample processing and data monitoring complicated head-to-head comparisons between studies and performing a meta-analysis was not possible. This also means that at this point, it is not possible to compare the different vaccine platforms used in lactating women. Also, since COVID-19 vaccination is a rapidly evolving field, pre-prints or publications might have been missed when published between submission and publication of the manuscript.

The review did not include studies on COVID-19 vaccination of pregnant women and the effect of antibodies in their breast milk.

Recommendations for Future Practice and Research

Side-effects for both mother and infant were researched in only four studies. Sample sizes were however large enough to provide a first indication of safety. However, the effect of vaccination on the milk production was studied based on (retrospective) reporting by the mother, which could have led to a recall bias. Quantitative research into the effect on milk supply could give more insights and could be used for further recommendations about vaccination during lactation. Additionally, it is essential that research on specific breastfeeding related side-effects is performed. This includes for example breast engorgement or the development of mastitis. These are currently often not taken into account. It would also be of interest to know whether changing the vaccine administration place (i.e. leg instead of arm) would lead to less breastfeeding related side-effects.

At this point, most studies researched the excretion of antibodies after COVID-19 vaccination with an mRNA-based vaccine. The two studies that used the adenovector-based vaccine ChadOx1-S were stopped after administration of the first dose.

At the moment, in most countries, adenovector-based vaccines are no longer used in women of childbearing age, but there are other vaccines developed with this platform for other diseases, such as Ebola. The information on the effect of adenovector-based vaccines in lactating women could be interesting to extrapolate to other vaccines in development that have the potential to be used in women of fertile age. Research on the safety, side-effects and excretion of antibodies after a full vaccine schedule including booster vaccination is therefore adamant. Worldwide the booster vaccination (a third vaccination after mRNA-based vaccines or the ChadOx1-S vaccine or a second vaccination after the JNJ-78436735 vaccine), is being recommended (52, 53). At the moment there are no published studies on the effects of COVID-19 booster vaccination in lactating women. Finally, a qualitative analysis of the subclass, glycosylation profile and functional properties of vaccine-induced antibodies would be of interest to enlighten the immunology during breastfeeding. This could be completed along more research into cellular and humoral immune responses during lactation. Lastly, one of the main questions are whether these immunological factors excreted into breast milk also have protective effects for the infant.

Additionally, as the WHO and UNICEF recommend 6 months exclusive breastfeeding and afterwards until the age of 2 years in combination with complementary foods (13, 14), more knowledge on the effects of vaccination on the long term is indispensable. Most studies focused on breast milk analysis up to 2 or 8 weeks after the second dose. Therefore, there is a need for follow-up studies taking samples with longer time intervals and longer follow-up.

Safety of vaccination during lactating period need to be assessed at early stages of product development. In order to achieve this, vaccine manufacturers and regulators must work closely with specialists in lactation, infectious diseases and public health experts in order to improve maternal and infant health and to build confidence in vaccines. Breastfeeding women therefore need to be included in clinical trials and the need for appropriate safety data is critical.

CONCLUSION

There is no evidence that the administration of a COVID-19 vaccine poses an additional risk to the breastfeeding woman or the breastfed infant. Data on safety of vaccines against SARS-CoV-2 virus indicate no severe vaccine-related local and systemic reactions, both after first and second dose. Milk supply data after vaccination indicate that some women report a temporary reduction in milk supply, without a long-term effect and milk supply returned to normal within a few days whereas other women reported a stimulation of breast milk production. All prospective cohort studies have demonstrated the presence of antibodies (mainly sIgA and IgG) in breast milk of nursing mothers vaccinated against SARS-CoV-2. Nearly all studies were conducted with mRNA vaccines. These studies mainly showed that the antibody titers in breast milk mainly increase after the second dose and are associated with the levels present in the mother's blood.

After vaccination of the mother, antibodies appear in the milk, which could better protect the infant against COVID-19. Professional associations and government health authorities should recommend offering COVID-19 vaccines to breastfeeding women, as the potential benefits of maternal vaccination while breastfeeding outweigh the theoretical risks.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JM and ET conducted the review. LW, LB, and KM read the review and gave new input and comments. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: All authors have completed the ICMIJE uniform disclosure form at www.icmje.org/disclosure-of-interest/. ET received a fee for personal consulting for expertise considering smoking cessation counselling in the community pharmacy from Johnson and Johnson.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDIX 1: DETAILED SEARCH STRATEGY OF THE SYSTEMATIC REVIEW

A. Define text words & synonyms for the text words

1. (COVID-19* or SARS-CoV-2* or corona disease* or COVID*).af.
2. (breastfeeding* or lactation* or breast milk*).af.
3. (postpartum* or postnatal period* or puerperium*).af.
4. (pregnancy*).af.

B. Perform test searches – I

5. 1 AND 2
6. 1 AND 3
7. 1 AND 4
8. Limit to following languages: German, Dutch, English, French & Spanish
9. 5, 6, 7 AND 8 AND 2020/12/01 to 2021/12.31.date

C. Identify "controlled vocabulary" (keywords) used for the indexing of databases (MeSH)

10. "COVID-19 Vaccines" or "COVID-19" or "SARS-CoV-2" (Mesh)
11. "Lactation" or "Breast Feeding" (Mesh)
12. "Postpartum Period" (Mesh)
13. "Pregnancy" (Mesh)

D. Perform test searches – II

14. 10 AND 11
15. 10 AND 12
16. 10 AND 13
17. Limit to following languages: German, Dutch, English, French & Spanish
18. 14, 15 AND 16 AND 2020/12/01 to 2021/12/31.date

E. Remove duplicates

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BRIEF RESEARCH REPORT
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Coronavirus Disease 2019 Vaccine Booster Effects Are Seen in Human Milk Antibody Response

Jeffrey M. Bender[1,2], *Yesun Lee*[2], *Wesley A. Cheng*[2], *Carolyn J. Marentes Ruiz*[2] *and Pia S. Pannaraj*[1,2]*

[1] Department of Pediatrics, Pediatric Infectious Diseases, Keck School of Medicine, University of Southern California, Los Angeles, CA, United States, [2] Pediatric Immunization Advancement Laboratory, Children's Hospital Los Angeles, Los Angeles, CA, United States

Infants remain at high risk for severe coronavirus disease 2019 (COVID-19). Human milk contains high levels of protective SARS CoV-2 specific antibodies post-infection and primary vaccine series, but levels decline over time. We hypothesized that the COVID-19 booster vaccine augment antibody production and the protection afforded to human milk-fed infants. We prospectively enrolled pregnant or lactating mothers planning to receive COVID-19 vaccination. We measured human milk IgG, IgA, and IgM antibodies targeting the SARS CoV-2 receptor binding domain within the spike protein and human milk neutralization activity against SARS CoV-2 in 10 lactating mothers from pre-COVID-19 primary series vaccine to post-booster dose. Human milk SARS CoV-2 specific IgG increased significantly from pre- to post-booster levels (median OD 0.33 vs. 2.02, $P = 0.002$). The IgG levels post-booster were even higher than the peak level after the primary series (2.02 vs. 0.95, $P = 0.03$). The increase in SARS CoV-2 specific IgA levels was not significant (0.10 vs. 0.33, $P = 0.23$). There was a strong correlation between paired maternal blood and milk IgG and IgA levels (IgG rho 0.52, $P < 0.001$, IgA rho 0.31, $P = 0.05$). Post-booster neutralizing activity was elevated compared to pre-booster levels (66% vs. 12% inhibition, $P = 0.002$). COVID-19 vaccine booster elicits SARS CoV-2 specific antibodies in human milk at higher levels compared to the initial primary series. This finding suggests that three doses of COVID-19 mRNA vaccination leads to improved mucosal response in human milk and reinforces current guidance recommending all pregnant or lactating mothers receive full COVID-19 vaccine courses with a booster dose.

Keywords: breastmilk, breastfeeding, serology, IgA, pregnancy, infant, COVID-19, immunization

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***Correspondence:**
Pia S. Pannaraj
ppannaraj@chla.usc.edu

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INTRODUCTION

As with many other viral illnesses in infants, breastfeeding remains one of the most important ways by which families may protect their newborn children from infection with severe acute respiratory syndrome coronavirus 2 (SARS CoV-2) (1). Epidemiologic surveys initially showed a disproportionate low impact of associated coronavirus disease 2019 (COVID-19) on infants and young children (2–5). The reason for this is likely multifactorial. Maternal transfer of protective SARS CoV-2 specific antibodies is thought to be a major contributor to this natural protection. Numerous studies have demonstrated that SARS CoV-2 infection of mothers during pregnancy leads to transplacental transfer of neutralizing IgG and production of IgA in breastmilk (6–9).

Unfortunately, this relative protection is not complete. Infants with COVID-19 have now been shown to be at risk for increased morbidity and mortality and hospitalization rates have increased worldwide (10–14).

Now as the pandemic continues, efforts to vaccinate pregnant and breastfeeding mothers against SARS CoV-2 are critical to further protect them and their infants. As in natural infection, vaccines appear to provide some level of transferred antibodies from mother to infant both through transplacental transport and breastmilk (6, 15–22). Most USA studies thus far have focused on the standard 2 dose series of the messenger ribonucleic acid (mRNA) COVID-19 vaccines (BNT162b2, Pfizer-BioNTech; mRNA-1273, Moderna) or single dose of the adenovirus based COVID-19 vaccine (Ad26.COV2.S, Janssen/Johnson and Johnson). In November 2021, the Centers for Disease Control and Prevention (CDC) recommended that all adults over the age of 18 receive a booster dose of mRNA COVID-19 vaccines 6 months after completing their primary vaccine regimen (1). We hypothesized that this booster COVID-19 vaccine dose would lead to further antibody production and augment the protection afforded to human milk fed infants. Through this brief report, we demonstrate the subsequent maternal antibody response after booster mRNA COVID-19 vaccine dose in breastfeeding mothers.

METHODS

Study Design/Participants

We prospectively consented and enrolled pregnant or lactating mothers who planned to receive COVID-19 vaccination. Demographic data including pre-existing conditions and prior infection with SARS CoV-2 was obtained at time of enrollment. Participants with known prior infection with SARS CoV-2 were excluded and confirmed in participating subjects by evaluating pre-vaccine blood and breastmilk for antibody response. This study was reviewed and approved by the institutional review board at Children's Hospital Los Angeles.

Sample Collection

We collected blood and human milk samples at the following time points: pre-vaccination; 1-, 3-, 6-, and 9-months post-initial vaccine dose, and 1-month post-booster vaccine dose. The pre-booster sample collection was defined as the last timepoint prior to the booster vaccination at 6 or 9 months. Individuals received booster doses following emergency use authorization; this occurred between 6 and 9 months for the individuals in our study. Human milk was collected at each time point until the participant stopped lactating. Post booster milk samples were collected between 26-38 days after booster dose. Human milk samples were self-collected at home just prior to each visit in sterile containers or collection bags using electrical or manual pumps. Milk samples were then stored at−80 degrees Celsius (°C) until antibody testing was performed. Blood samples (3-4mL) were drawn during each visit in red top tubes. Blood samples were transported to the laboratory within 2 h of collection where serum was extracted from coagulated blood *via* centrifugation and stored overnight at−20°C for next day serology testing.

SARS CoV-2 Specific Serology Testing in Human Milk

Measurement of human milk IgG, IgA, and IgM antibodies targeting the SARS CoV-2 receptor binding domain (RBD) within the spike protein was performed using a modified enzyme-linked immunosorbent assay (ELISA) technique (6, 23). In brief, to remove cells and fat, thawed human milk samples were centrifuged at 1,000 G for 10 min twice. The separated supernatant was then diluted to 1:10 and placed in high binding 96-well plates that were previously coated with recombinant SARS CoV-2 RBD protein. These plates were then incubated for 2 h at room temperature. Plates were then washed with PBS-1% Tween20 (PBS-T). Next, diluted (1:3000) secondary enzyme labeled antibodies for IgG, IgA, and IgM (Rockland) were added and incubated for 1 h. We then added 100 uL of O-phenylenediamine dihydrochloric marker substrate (Sigma-Aldrich) to each well and incubated for 20 min prior to quenching with 50 uL of 3 molar hydrochloric acid. Optical density values were measured at 490 nm (OD_{490}). We established positive cutoff values based on the mean plus three standard deviations of 20 archived negative control human milk samples collected before 2020 (IgG 0.20, IgA 0.21, and IgM 0.14). The assays were performed in duplicate.

SARS CoV-2 Specific Serology Testing in Blood

Serum IgG and IgA antibodies targeting the SARS CoV-2 RBD were also measured using a previously described technique (23). All samples were analyzed on the same plate for each isotype assay. We further tested the level of IgG against SARS CoV-2 nucleocapsid protein (GenScript) in pre- and post-booster blood samples of each participant to determine if any SARS CoV-2 infection occurred in the subjects before or during the study.

Neutralizing Antibody

We measured human milk neutralization activity against SARS CoV-2 using a surrogate virus neutralization assay (sVNT, GenScript). This assay has been previously shown to correlate with the SARS CoV-2 90% plaque reduction neutralization test titer assay (24). We modified this assay as described previously to work with human milk (6). In brief, human milk was mixed with an equal volume of horseradish peroxidase conjugated to recombinant SARS CoV-2 RBD protein and incubated at 37°C for 30 min. Next, 100 uL of each mixture was then added to microtiter plate wells coated with angiotensin-converting enzyme-2 and incubated at 37°C for 15 min. We then added 100 uL of indicator solution (3,3′,5,5′-tetramethylbenzidine) to each well. The plates were then incubated in the dark at 22–25°C for 15 min. Lastly, we added 50 uL of the stop solution and immediately measured the light absorbance at 450 nm. A simple percent inhibition was calculated using negative control values. Using the mean percent inhibition plus three standard deviations of 20 archived negative control human milk samples collected

before 2020, we established a cutoff value for neutralization at ≥25% inhibition. The assay was performed in duplicate.

Data Analysis

Statistical analysis was performed using R Studio v4.0.3 (R Studio). Standard descriptive statistics of median, range, and percent positive based on established cutoffs for each assay at all time points was calculated. Non-parametric variables were then analyzed using Wilcoxon matched-pairs signed-rank tests. Spearman correlation coefficient was used to calculate correlations between serum and human milk values. All tests were designed to be 2-tailed with $P < 0.05$ considered significant.

RESULTS

Participants

We identified 10 lactating mothers who we followed and obtained blood and human milk samples pre-vaccine all the way through third booster dose of the COVID vaccine between December 2020 and January 2022 (**Table 1**). The average age of participating mothers was 35.1 years (range 30.9-42.8). There were few comorbid conditions identified in this cohort with the most common being allergies and obesity. Three were pregnant at time of enrollment and initial vaccination and thus do not have pre-vaccine milk samples. The average gestational age at time of delivery was 38 weeks. Seven of the 10 infants born were female. All mothers reported exclusive breastfeeding at enrollment with the age appropriate introduction of solids as the infants developed. Nine mothers received a full three dose vaccine series with BNT162b2 (Pfizer-BioNTech). One mother was given a single dose Ad26.COV2.S (Janssen/Johnson and Johnson) boosted with a single dose of mRNA-1273 (Moderna). The Ad26.COV2.S individual was included in the primary analysis of pre- vs. post-booster comparisons; she was excluded from the post-primary series analysis to minimize heterogeneity.

TABLE 1 | Participant characteristics of 10 lactating mothers receiving the primary series and booster vaccination against COVID-19.

Characteristics ($n = 10$)	*N* (%)
Age, years [mean(range)]	35.1 (30.9–42.8)
Race	
Asian	2 (20)
White	8 (80)
Ethnicity	
Non-hispanic	10 (100)
Highest level of education	
College, Bachelor's degree	4 (40)
Post-graduate degree	6 (60)
Co-morbid condition	
Allergies	4 (40)
Cancer (past)	1 (10)
Gestational diabetes with last pregnancy, resolved	1 (10)
Other endocrine	1 (10)
Obese (BMI > 30)	3 (30)
Pregnant at enrollment	3 (30)
Gestational age at delivery, weeks [mean, (range)]	38.6 (37.0–39.9)
Infant gender, female	7 (70)
Exclusive breastfeeding at enrollment	10 (100)

SARS CoV-2 Specific Antibodies Increased After Booster Dose in Human Milk

We evaluated the SARS CoV-2 RBD specific antibodies in a total of 48 human milk samples from the 10 lactating mothers (**Figures 1A–C**, **Supplementary Table 1**). Pre-COVID vaccine SARS CoV-2 specific IgG, IgA, and IgM levels were all low, suggesting that all participants were immunologically naïve to SARS-CoV-2. Two mothers enrolled at time of first vaccine and one was vaccinated during pregnancy; thus, these three subjects did not provide pre-COVID vaccine milk samples. After the primary vaccine series, SARS CoV-2 specific antibodies increased, peaked at 1 month, and then waned over time. After the booster, human milk SARS CoV-2 specific IgG levels were shown to increase from pre-booster levels (median OD_{490}: pre-booster 0.33, post-booster 2.02, $P = 0.002$). The post-booster IgG levels were higher than the initial post primary vaccine series peak (post-primary 0.95, post-booster 2.02, $P = 0.03$). SARS-CoV-2 specific IgA levels showed non-significant increases post-booster compared to pre-booster (pre-booster 0.10, post-booster 0.33, $P = 0.23$) and post-primary vaccine series (post-primary 0.23, post- booster 0.33, $P = 0.09$). IgM levels demonstrated little change over the study period.

COVID Vaccine Booster Led to an Increase in Neutralizing Activity in Human Milk

As seen with SARS CoV-2 specific antibody levels, the percent inhibition peaked in human milk 1 month after initial COVID vaccine and waned with time approaching the booster vaccine dose (**Figure 1D**, **Supplementary Table 1**). Post-booster neutralizing activity increased compared to pre-booster levels (median pre-booster 12%, post-booster 66%, $P = 0.002$). The booster vaccine dose led to a non-significant increased neutralizing effect over the peak 1-month post-primary vaccine peak (post-primary 41%, post- booster 66%, $P = 0.09$). All 10 samples collected post-booster demonstrated neutralization with >25% inhibition.

SARS CoV-2 Specific Antibodies in Human Milk Correlated With Paired Blood Samples

In comparing blood SARS CoV-2 RBD specific antibodies to those found in human milk collected at the same time point, we found a moderate correlation (IgG correlation coefficient ρ 0.52, $P < 0.001$; IgA ρ 0.31, $P = 0.05$; **Figure 2**). As blood levels rose with initial vaccine, both IgG and IgA were similarly elevated. That pattern persisted during the subsequent waning of antibody and post-booster spike. No participants tested positive for COVID-19 during the study period. Furthermore,

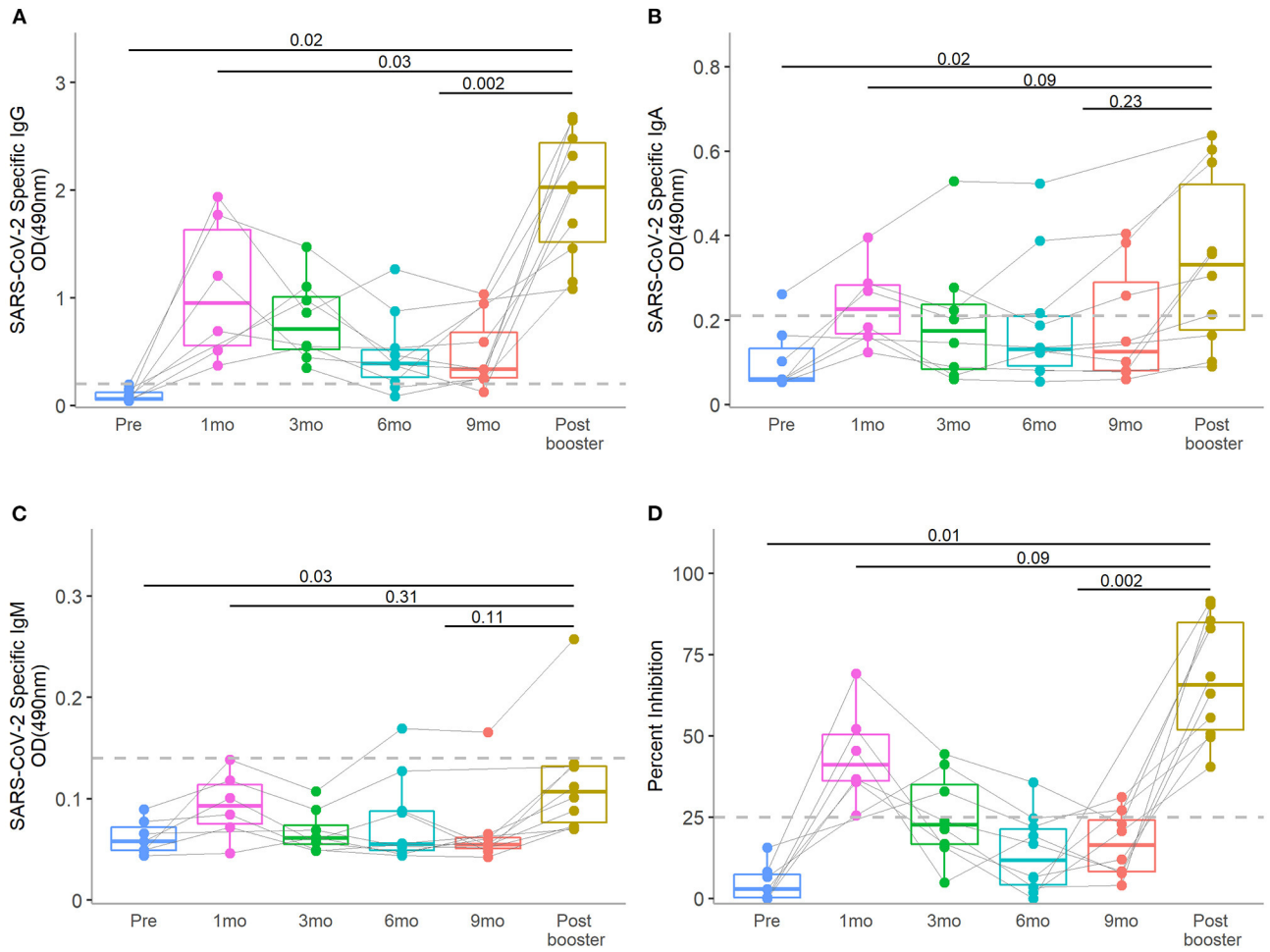

FIGURE 1 | Human milk SARS-CoV-2-specific IgG, IgA, and IgM of antibody levels and neutralizing activity at pre-vaccination: 1-, 3-, 6-, and 9-months post-primary initial vaccine and 1-month post-booster vaccine dose. The median level of SARS-CoV-2-specific IgG, IgA, and IgM and neutralization activity at the 1-month post-booster time point was compared to the peak post-primary vaccination and pre-booster time points in human milk **(A–D)**, respectively]. Dotted lines in y-axis indicate the positive cut-off OD_{490} values of 0.20, 0.21, and 0.13 for IgG, IgA, IgM, respectively. Dotted line in y-axis of **(D)** indicate the positive cut-off of 25% neutralizing activity. Wilcoxon matched pairs signed rank tests were used for statistical analysis. Error bars indicate 95% confidence intervals.

we confirmed that all pre- and post-booster blood samples were IgG negative against SARS CoV-2 nucleocapsid protein. Therefore, we ensured that all responses observed were due to vaccination.

DISCUSSION

Through this study, we described that COVID-19 vaccine booster elicits human milk antibody response. SARS CoV-2 specific antibodies (IgG and IgA) increase in human milk after booster dose of mRNA COVID-19 vaccine to levels even higher than the peak after the initial vaccine series. Human milk antibodies boosted with COVID-19 vaccines were further found to have increased neutralizing activity compared to the waning pre-booster activity. The responses correlated with the paired serological blood samples from the lactating mothers. All these findings reinforce current guidance recommending all pregnant or lactating mothers receive full COVID-19 vaccine courses with a booster dose.

Most respiratory infections disproportionately affect infants with a developing immune system (25). Although initial data suggested that infants and children were less likely to acquire SARS-CoV-2 (3–5), increasing rates of pediatric SARS-CoV-2 infections and hospitalizations have been observed worldwide (12–14). Maternal vaccination during pregnancy leads to transplacental antibody transfer (6–9). Breastfeeding may be another important strategy to protect infants. We and others have demonstrated that SARS-CoV-2 specific antibody in human milk following maternal vaccination with the primary series was followed by a slow wane in antibody levels as is seen in natural infection (6, 15–22). Through this study, we observe a clear increase in human milk IgG, IgA, and neutralizing antibody following COVID-19 booster vaccination. To our surprise, the booster dose induced antibody levels

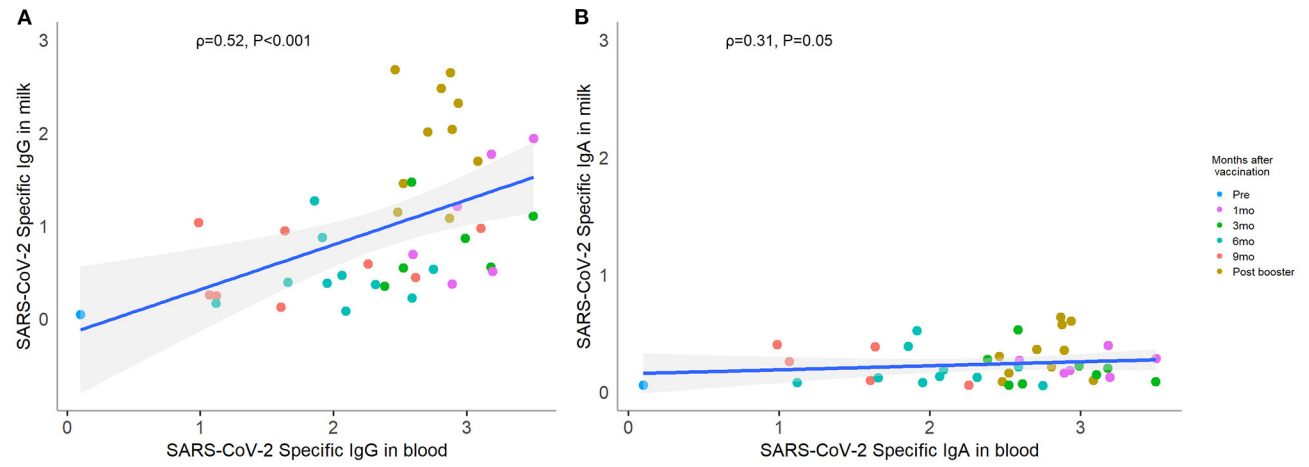

FIGURE 2 | Correlation between paired human milk and blood SARS-CoV-2-specific antibody. Forty-one paired human milk and blood samples collected at the same time point were included in the correlation analysis. Each color point represents each visit. The level of SARS-CoV-2-specific IgG **(A)** and IgA **(B)** in breastmilk showed positive correlations with the same isotypes in blood. Correlations were computed using Spearman correlation coefficient labeled ρ.

even greater than levels generated by the initial vaccination series. This suggests that three doses of mRNA vaccination may provide the optimal mucosal response. Larger studies on how antibody production in lactating mothers may be augmented with multiple doses of COVID-19 vaccine are needed moving forward.

The ability of human milk antibodies to neutralize potential pathogens provides a barrier of protection for infants as they develop. Secretory IgA and IgG can neutralize viruses at the mucosal surface before infection of epithelial cells occurs (26, 27). Previous studies looking at antenatal influenza vaccine in pregnant mothers have demonstrated subsequent production of influenza specific neutralizing antibodies in human milk (28). Neutralizing antibodies induced by COVID-19 vaccines appear to be the key correlate of protection from COVID-19 in animal and human studies (29, 30). Previous studies have demonstrated this neutralization property of human milk SARS-CoV-2 specific antibodies post-primary vaccine series (6, 15, 18, 21). Through this study, we further observe an increase in the ability of these human milk antibodies to neutralize SARS CoV-2 after the COVID vaccine booster dose. Neutralization appeared to be stronger than the peak seen after initial vaccine series, but the difference was not statistically significant likely due to our small numbers. This emphasizes the importance of not only vaccinating lactating mothers to protect their infants but making sure they receive their booster dose as well.

This study has some limitations. The most important limitation is that this is a real-world observational cohort with a small sample size. Though relatively small, this was a unique opportunity to examine 10 COVID naïve mothers who continued to breastfeed through initial and booster doses of vaccine. Despite the small size, we were able to provide statistically significant evidence of antibody response post-COVID-19 booster vaccine. We observed a higher SARS-CoV-2 specific IgG level post-booster compared with the peak post-primary series level. A larger sample size is needed to determine if differences in IgA and neutralizing activity would reach significance. Second, we acknowledge heterogeneity in the study participants. Some received the primary series while pregnant while others were post-partum. The study primarily included mothers vaccinated using the BNT162b2, Pfizer-BioNTech mRNA COVID vaccine. One mother received the Ad26.COV2.S, Janssen/Johnson and Johnson vaccine boosted by a dose of the mRNA-1273, Moderna vaccine. As more vaccine platforms become available, variability around human milk antibody production and protection will need to be evaluated. Furthermore, changing SARS CoV-2 variants and variant-specific boosters may require re-evaluation. Third, despite showing the neutralizing effect of the stimulated antibody after COVID booster vaccine dose, epidemiological studies will be needed to demonstrate protection in infants. A recent study showed that maternal vaccination with mRNA COVID-19 vaccine during pregnancy is effective against COVID-19 hospitalization among infants <6 months of age (31). How much protection was from transplacental transfer vs. from breastfeeding is not clear.

With the ongoing global pandemic, the CDC has strongly recommended all pregnant and lactating mothers to receive a full course of COVID vaccinations including a booster dose (1). Unfortunately, this remains an at-risk population, and vaccine hesitancy has hampered efforts to immunize and protect these mothers and their infants. Our data provides additional evidence to support maternal COVID-19 booster vaccination, as milk-delivered antibodies could offer breastfed infants additional protection against COVID-19.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board at Children's Hospital Los Angeles. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JB, YL, and PP provided conceptualization and study design. WC and CM recruited and enrolled patients. YL conducted experiments and performed data analysis. JB drafted the initial manuscript draft. JB, YL, and PP critically reviewed and revised the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.898849/full#supplementary-material

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Vaccine Protection Through Placenta and Breastfeeding: The Unmet Topic in COVID-19 Pandemic

Ariane Laguila Altoé[1,2], *Anna Paula Marques Mambriz*[3], *Daniela Maira Cardozo*[4], *Joana Maira Valentini Zacarias*[1,3], *Jeane Eliete Laguila Visentainer*[1,3] and *Larissa Danielle Bahls-Pinto*[1*]

[1] Department of Basic Health Science, Laboratory of Immunogenetics, State University of Maringa, Maringa, Brazil, [2] Department of Medicine, State University of Maringa, Maringa, Brazil, [3] Department of Clinical Analysis and Biomedicine, Postgraduate Program in Biosciences and Physiopathology, State University of Maringa, Maringa, Brazil, [4] Clinical Hospital, State University of Campinas, Campinas, Campinas, Brazil

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***Correspondence:**
Larissa Danielle Bahls-Pinto
ldbpinto2@uem.br

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The coronavirus disease 2019 (COVID-19) pandemic has turned pregnant women's healthcare into a worldwide public health challenge. Although initial data did not demonstrate pregnancy as a more susceptible period to severe outcomes of acute severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) infection, there are an increasing number of reports showing that not only pregnant women might be at significantly higher risk than non-pregnant women by COVID-19 but also the fetus. These findings may be related to adaptive changes that occur during pregnancy, such as the reduction in the residual respiratory capacity, the decrease in viral immune responses, and the increased risk for thromboembolic events. Additionally, despite the SARS-CoV-2 vertical transmission evidence being uncommon, maternal illness severity might reflect serious perinatal and neonatal outcomes. Thus, protecting the maternal–fetal dyad against COVID-19 is critical. Even though pregnant women initially were excluded from vaccine trials, several studies have provided safety and efficacy of the overall vaccine COVID-19 platforms. Vaccination during pregnancy becomes a priority and can generate benefits for both the mother and newborn: maternal neutralizing antibodies are transmitted through the placenta and breastfeeding. Moreover, regarding passive immunization, human milk contains other bioactive molecules and cells able to modulate the newborn's immune response, which can be amplified after the vaccine. Nonetheless, many issues remain to be elucidated, considering the magnitude of the protective immunity transferred, the duration of the induced immunity, and the optimal interval for pregnant immunization. In this review, we assessed these unmet topics supported by literature evidence regarding the vaccine's immunogenicity, pregnancy immune heterogeneity, and the unique human milk antiviral features.

Keywords: COVID-19, SARS-CoV-2, vaccine, human milk, passive immunization

INTRODUCTION

In December 2019, a virus called severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) was identified in China (1). This new respiratory disease was named coronavirus disease 2019 (COVID-19) by the WHO, and in March 2020, it was declared a pandemic (2). The case fatality rate (CFR) of COVID-19 was estimated at 2.3% (3, 4), which is reflected in more than 475 million cases and 6.1 million deaths registered worldwide to date (5). This remarkable ability to spread is explained by the high viral transmissibility added to characteristics such as long incubation period, infectivity capacity before the beginning of symptoms, and a large number of asymptomatic cases and/or mild diseases (6). In fact, it is estimated that approximately 55%–60% of infected individuals present some symptom, and the majority of them (81%) develop mild disease (fever, cough, fatigue, dyspnea, myalgia, headache, and diarrhea); of the other infected individuals, 14% evolve to severe disease, and 5% develop the critical disease, frequently needing to stay in an intensive care unit (ICU) (3, 5). However, these statistics may be different in some risk groups such as frontline healthcare professionals; elderly people; patients with heart, pulmonary, or neurologic diseases; patients with diabetes mellitus, obesity, or immunosuppression; and pregnant/postpartum women (6).

Concerning pregnancy and lactation, although the initial studies involving pregnant women were not conclusive (7), a series of severe complications in pregnant women and their newborns have been associated with SARS-CoV-2 infection (8). This outbreak was expected as previous coronavirus pandemic diseases such as SARS and Middle East respiratory syndrome (MERS) had already presented similar risks for mother and child (5, 7, 9, 10). Thus, although early studies have shown that pregnant women have milder symptoms than non-pregnant women in SARS-CoV-2 infection (5, 11–13) and a lower incidence of gestational and neonatal complications (5, 14–16), growing evidence suggests that pregnant women diagnosed with COVID-19 are at increased risk for ICU admission and need for invasive ventilation/extracorporeal membrane oxygenation (ECMO), higher morbidity and mortality, and higher odds of maternal–fetal complications (such as preterm birth and miscarriage), thrombosis, intrauterine fetal growth, intrauterine transmission, congenital anomalies, and neurologic abnormalities) when compared to those without COVID-19 (8, 14–20).

In this mini-review, we summarize the last information about COVID-19 vaccines in use by pregnant women, with an emphasis on its immunogenicity in this particular group and on the transmission of the acquired immunity to the fetus and the newborns (**Figure 1**).

PECULIARITY OF IMMUNE SYSTEM DURING PREGNANCY

Pregnant women are usually considered at high risk for infectious diseases; it happens due to physiologic, cardiopulmonary, and immunologic changes in their bodies during pregnancy (3, 21). In

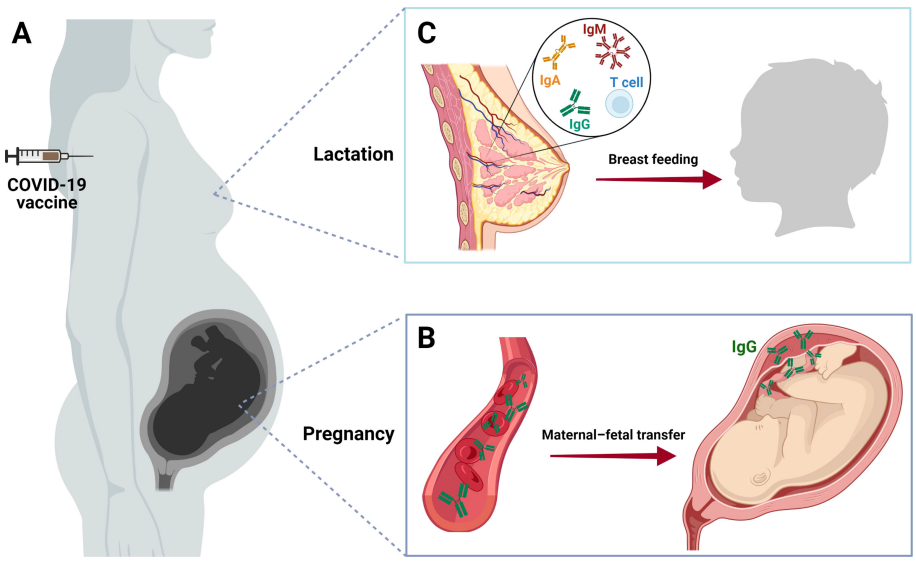

FIGURE 1 | COVID-19 vaccine in pregnancy and lactation. **(A)** Two pathways of maternal–fetal protection against SARS-CoV-2 after COVID-19 vaccination. **(B)** After receiving the COVID-19 vaccine, pregnant women start to develop antibodies against the virus (IgG). Thus, immunized women are able to transmit anti-SARS-CoV-2 IgG molecules from their blood to the fetus. This process occurs passively through the placenta, and it is confirmed by the presence of these antibodies in cord blood or the newborn serum after birth. **(C)** Passive immunization of the newborn also happens through breastfeeding, which can be demonstrated by the presence of anti-SARS-CoV-specific IgA, IgM, IgG, and T cells in breast milk. These findings reinforce the importance of pregnant and lactating women to complete the vaccination schedule, protecting themselves and their infants from the severe manifestations of COVID-19. Created with BioRender.com.

this period, the diaphragm is pushed to a higher position as the uterus expands; this can create an obstacle for the lungs to expand, additionally the upper respiratory tract swells, and the oxygen demand increases. Thus, the intolerance to hypoxemia makes pregnant women more likely to develop respiratory disease complications, including COVID-19 (22, 23). In this period, marked by significant hormonal changes, a shift in the balance between T helper 1 (Th1)-mediated and T helper 2 (Th2)-mediated immunity can be observed: a decrease in Th1 response leads to a dominant Th2 humoral immune response, which results in a lower secretion of proinflammatory cytokines, such as interleukin-2 (IL-2), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) and an increase in anti-inflammatory cytokines (IL-4, IL-10, and IL-13), respectively (24, 25). Indeed, a decrease in NK cells and plasmacytoid dendritic cells (which compromises the production of type 1 IFN) and a decrease in phagocytic activity were observed. This scenario creates an immune tolerance for the fetus but increases the susceptibility of pregnant women to SARS-CoV-2 infection (26).

Despite this period of women's life being marked mainly by an immunotolerant profile, actually pregnancy involves a triphasic immune modulation, characterized by an alternation between proinflammatory, anti-inflammatory, and a second proinflammatory state, in that order, over the three trimesters (22). Thus, women in the first and third trimester of pregnancy have a proinflammatory profile, and for this reason, when infected with SARS-CoV-2, they are more likely to develop the cytokine storm, leading to bad maternal and fetal prognoses (21, 23).

PREGNANT COVID-19 VACCINATION: STATE OF THE ART

Fortunately, different kinds of COVID-19 vaccines are now available to the global population, and the evolution of epidemiological data has shown that they are essential for the control of SARS-CoV-2 spread and, especially, for the decrease of COVID-19 morbidity–mortality worldwide (27, 28). All the COVID-19 vaccines approved for use in the population are allowed during pregnancy if the benefits outweigh the possible risks (15, 16, 29, 30). Initially, two anti-COVID-19 vaccines, which use mRNA technology, were authorized: Pfizer/BioNTech (Germany and USA) and Moderna (USA) (31). The first one is administered in 2 doses, with 3 weeks of interval between them, and the second one also involves 2 doses, but with 4 weeks of interval. Both vaccines have about the same effectiveness, approximately 94.1% to 95% (32). Other than that, mRNA technology was also approved for three viral vector vaccines: Oxford-AstraZeneca (UK and Sweden), Sputnik (Russia), and Janssen (Belgium) (13, 32). The recommended administration is 2 doses for AstraZeneca, with an interval of <6 or >12 weeks between first and second doses (effectiveness from 55.1% to 81.3%); Sputnik uses 2 doses administered 3 weeks apart (effectiveness of 91.6%); Janssen was proposed as a single-dose vaccine (effectiveness of 66% against moderate to severe to critical COVID-19 and 76.7% to 85.4% against critical disease) (32). A sixth approved vaccine called Sinovac-CoronaVac (China, and lately produced by Instituto Butantan in Brazil) uses inactivated SARS-CoV-2 virus antigen and is administered in 2 doses (2–4 weeks apart between then; effectiveness of 83.7% against moderate disease to 100% against severe disease) (32, 33).

None of the approved COVID-19 vaccines contain a replicant virus; thus, they cannot cause the disease. Studies with animals did not demonstrate dangerous effects related to Pfizer, Moderna, AstraZeneca, Sputnik, and Janssen vaccines in pregnancy (13). In general, the side effects of vaccination are similar in pregnant and other groups, with non-specific side effects due to activation of the immune system being the most worrying (34). Although rare, some immune-mediated complications were already described, such as myocarditis/pericarditis after immunization with mRNA vaccines and Guillain–Barré syndrome and thrombotic events after viral vector vaccines (35–37). It is important to note the rare cases of post-COVID-19 vaccine thrombosis with thrombocytopenia syndrome (TTS) occur by a mechanism distinct from thromboembolic events that usually happen during pregnancy and post-childbirth (38–40). Moreover, according to a systematic review and meta-analysis recently published, there are no class-wide effects of adenovirus-based vaccines on thrombocytopenia or coagulopathy in pregnancy or the general population (41). Thus, after several investigations, authorities determined that adenovirus vector vaccines could be used by pregnant women, and the TTS occurrence probability is similar to that in the general population (42). The decision about the better choice between the abovementioned vaccine platforms should be discussed between the health professional and the pregnant/lactating woman, considering the effectiveness, security, and other parameters (43, 44). Still, there are few published data on the COVID-19 vaccine in pregnant women, mainly because they are not usually included in vaccine clinical trials due to safety and responsibility concerns (45); nevertheless, several studies support its safety and effectiveness (46, 47).

HUMORAL IMMUNE RESPONSE POST-COVID-19 VACCINE IN PREGNANT WOMEN

Prospective cohorts revealed that anti-SARS-CoV-2 humoral and cellular responses are similar between immunized pregnant and non-pregnant women and more robust when compared to infected and unvaccinated individuals (43, 48–50); this proves that vaccination gives higher immunity than natural infection by SARS-CoV-2 (51). A study performed by Collier et al. showed that after receiving COVID-19 mRNA vaccine, both pregnant and non-pregnant women had their titers of IgG and IgA against the receptor-binding domain (RBD) from spike protein of SARS-CoV-2, and the titers of the pseudovirus neutralizing antibody (NT50) similarly increased (52). Another study that compared anti-SARS-CoV-2 IgG levels

between mRNA COVID-19 vaccinated pregnant women and SARS-CoV-2 diagnosed pregnant women found that while vaccination increased levels of anti-S1 and anti-RBD IgG antibodies, the infection was associated with higher levels of anti-S2 and IgG neutralizing antibodies (46).

Additionally, Golan et al. demonstrated that serum levels of anti-SARS-CoV-2 IgM and IgG antibodies were significantly higher after the first dose of the mRNA vaccine; furthermore, the second dose significantly increased anti-SARS-CoV-2 IgG serum levels, but not anti-SARS-CoV IgM serum levels, characterizing a secondary immune response against the virus (53). These findings are in agreement with those found by Leik et al., who showed that the anti-spike IgG and anti-RBD IgG titers increased after the first dose of the vaccine but were much higher when pregnant women received the second dose (46). These results highlight the importance of the second dose to the development of higher titers of protective antibodies in pregnant women.

Another important topic related to vaccination is the durability of conferred protection. In this regard, studies demonstrated that approximately 5 to 6 months after taking the second dose of the SARS-CoV-2 vaccine, its effectiveness naturally starts to decrease (54–57). Thus, in order to recover the immune response against the virus, a booster dose has been recommended for some high-risk groups, including pregnant women (58). A recent study demonstrated that women who received the third dose in the last trimester of pregnancy presented higher levels of anti-spike IgG in maternal and cord blood (59), which suggests that women with a complete vaccination schedule (two initial doses followed by a booster dose) transmit a higher concentration of antibodies to the infant than those who only received the first and second doses. These findings indicate the benefits of early COVID-19 immunization protocol in pregnant women, which are sustained by results that demonstrate that COVID-19 vaccination during early pregnancy is not associated with an increased risk of fetal structural anomalies (60).

Maternal–Fetal Anti-SARS-CoV-2 Antibody Transmission

The transmission of humoral immunity from mother to fetus or newborn throughout the placenta or human milk is well established. As explored below and summarized in **Table 1**, studies that investigated if it also occurs for anti-SARS-CoV-2 antibodies found that pregnant women who had COVID-19 active infection or received the COVID-19 vaccine developed anti-SARS-CoV-2 IgM, IgG, and IgA, and these antibodies were transferred to the fetus *via* placental transport or breastfeeding (48, 51, 53, 70–73).

Regarding the transplacental route of infant's passive immunization, Leik et al. reviewed studies that demonstrated anti-spike IgG, anti-RBD IgG, and neutralizing IgG in blood samples of newborns of vaccinated women; furthermore, these antibody levels were higher among those whose mothers had received two doses of vaccine (46). Additionally, a recent paper showed that after a third dose, the levels of neutralizing antibodies against SARS-CoV-2 were higher in both mother blood and cord blood, strengthening the importance of a boost dose to increase humoral immune transfer to the newborn (59). Importantly, it was demonstrated that the majority of maternal IgG is transferred to the fetus in the last 4 weeks of gestation (70, 74). This information is crucial to better determine the administration period for this specific public, to ensure the protection of the newborns from possible infections. Thus, the seroprotection during the beginning of the infant's life can be enhanced by a booster dose of the COVID-19 vaccine at the beginning of the third trimester of pregnancy, once the magnitude of the maternofetal transfer is increased in this period.

There are many studies showing the presence of neutralizing anti-SARS-CoV-2 IgA, IgM, and IgG antibodies in breast milk of vaccinated women and women previously infected by COVID-19 (48, 51, 53, 65–67). An interesting study developed by Gray et al. addressed the magnitude of generated immunity post-vaccine (Pfizer or Moderna) in lactating women, which showed an increased level of virus-specific IgG after the vaccination and a high antibody level transferred to the neonate through breastfeeding, although the levels of IgA did not increase in breast milk, as expected, after the boost. In this context, these researchers concluded that IgG titers dominate in the breast milk of women who received the COVID-19 vaccine, whereas IgA titers dominate in the breast milk of women with previous SARS-CoV-2 infection (48). These results are in consonance with a prospective cohort study in Spain, which also found specific anti-SARS-CoV-2 IgG antibodies in breast milk after Pfizer vaccination (with levels even higher after the second dose) (65), but contrasts with a study by Valcarce et al., who demonstrated that after mRNA COVID-19 vaccination (Pfizer or Moderna), there was a predominance of SARS-CoV-2 IgA in human milk when compared to SARS-CoV-2 IgG levels (68).

To evaluate the duration of vaccine immunity, Perl et al. performed a cohort study including 84 lactating women and analyzed a total of 504 samples of breast milk collected before administration of the Pfizer vaccine and then, once a week—starting 2 weeks after the administration of the first dose—for 6 weeks. They found elevated levels of anti-SARS-CoV specific IgA during the follow-up: 61.8% of antibody positivity in the breast milk samples 2 weeks after the first dose; more than 85% positivity of these antibodies after week 4 (1 week after administration of the second dose of vaccine) and about 65.7% positivity at week 6 (67). Additionally, this same study analyzed anti-SARS-CoV IgG in the samples after vaccination and observed that the antibody levels remained low during the first 3 weeks, started to increase at week 4 (91.7% of samples testing positive for anti-SARS-CoV-2 IgG), and reached the peak at weeks 5 and 6 (97% of positivity). Therefore, considering the vigorous secretion of SARS-CoV-2-specific IgA and IgG in breast milk for at last 6 weeks after mRNA vaccination, these authors suggested that vaccination of lactating women offered protective effects against COVID-19 in the newborn (67). Similar findings were found by a recent Brazilian study that evaluated the presence of anti-SARS-CoV-2 IgA antibodies in human milk samples of women who received the CoronaVac vaccine, with the two doses of the vaccine administered 4 weeks apart. It was observed that the levels of anti-SARS-CoV-2 IgA

TABLE 1 | Anti-SARS-CoV-2 antibodies production and maternal–fetal transfer after COVID-19 vaccination.

References	Producer	Type of vaccine	N pregnant	N lactating	Antibodies researched	Main findings in serum	Main findings in breast milk	Main findings in umbilical cord
(48)	Pfizer-BioNTech	BNT162b2 mRNA	41	16	IgM, IgA, and IgG anti-spike, RBD, S1, and S2	1. Increase in all antibodies at the first and second doses 2. Significant increase in IgG at the third dose 3. Dominant IgG antibody response	1. Increase in all antibodies in the first and second doses and significant increase in IgG in the third dose 2. Increased transfer of IgG1 RBD at the third dose 3. IgG transfer *via* breast milk	1. Anti-spike IgG and RBD found in the cord 2. Transfer of IgG *via* the placenta
	Moderna-NIH	mRNA-1273	43	15	IgM, IgA, and IgG anti-spike, RBD, S1, and S2	1. Increase in all antibodies at the first and second doses 2. Significant increase in IgG at the third dose 3. Dominant IgG antibody response 4. More robust anti-spike and anti-RBD IgA response	1. Increase in all antibodies in the first and second doses and significant increase in IgG in the third dose 2. Increased transfer of IgG1 RBD at the third dose 3. IgG transfer *via* breast milk	1. Anti-spike IgG and RBD found in the cord 2. Transfer of IgG *via* the placenta
(61)	CoronaVac®	Inactivated Virus Antigen	1	0	Total Neutralizing Antibodies to SARS-CoV-2	Positive reaction for neutralizing antibodies in NB serum, 24 h after birth	NA	NA
(62)	Pfizer-BioNTech	BNT162b2 mRNA	0	14	IgM, IgA, and IgG anti-spike	Maternal IgG and IgM increased after second dose	IgG and IgA present in approximately 40% of samples	NA
(53)	Pfizer-BioNTech	BNT162b2 mRNA	0	19	IgA and IgG anti-RBD	1. High maternal IgG levels after second dose 2. IgG detection in babies whose mother was vaccinated during pregnancy but not after delivery	1. Higher levels of IgA 2. Increase in IgG after second dose	NA
	Moderna-NIH	mRNA-1273	0	13	IgA and IgG anti-RBD	1. High maternal IgG levels after second dose 2. IgG detection in babies whose mother was vaccinated during pregnancy but not after delivery	1. Higher levels of IgA 2. Increase in IgG after second dose	NA
(63)	Pfizer-BioNTech	BNT162b2 mRNA	0	70	IgA and IgG anti-RBD	Detection of IgG and IgA in the mother's serum	Detection of IgG and IgA	NA
	Moderna-NIH	mRNA-1273	0	20	IgA and IgG anti-RBD	Detection of IgG and IgA in the mother's serum	Detection of IgG and IgA	NA
	AstraZeneca	Replication-deficient simian adenovirus vector ChAdOx1-S	0	20	IgA and IgG anti-RBD	Lower detection of IgG and IgA in maternal serum	Lower detection of IgG and IgA	NA
(64)	Pfizer-BioNTech	BNT162b2 mRNA	0	21	Anti-spike SIgA, IgA, IgG, and IgM; spike T cells	Detection of IgA, IgG, and IgM	1. Detection of IgA, IgG, and IgM 2. Immune transfer to breast milk occurs through spike SIgA, IgG, and T cells	NA
	Moderna-NIH	mRNA-1273	0	2	Anti-RBD IgG, IgA, and IgM; spike T cells	Detection of IgA, IgG, and IgM.	1. Detection of IgA, IgG, and IgM 2. Immune transfer to breast milk occurs through spike SIgA, IgG, and T cells	NA
(65)	Pfizer-BioNTech	BNT162b2 mRNA	0	84	Anti-spike IgA and IgG	NA	Detection of IgA and IgG	NA
(66)	Pfizer-BioNTech	BNT162b2 mRNA	0	33	Anti-spike IgG	Detection of IgG	Detection of IgG	NA
(67)	CoronaVac®	Inactivated Virus Antigen	0	20	Anti-spike IgA	NA	Detection of IgA	NA
(68)	Pfizer-BioNTech	BNT162b2 mRNA	0	14	Anti-spike IgA and IgG	Detection of IgA and IgG	Detection of IgA and IgG	
	Moderna-NIH	mRNA-1273	0	7	Anti-spike IgA and IgG	Detection of IgA and IgG	Detection of IgA and IgG	NA
(69)	Pfizer-BioNTech	BNT162b2 mRNA	Not cited	25	Anti-spike IgM, IgG and IgA	Detection of IgA and IgG	1. Detection of IgA, IgG, and IgM 2. The SARS-CoV-2 antibodies induced by mRNA vaccines persist for at least 6 months	NA
	Moderna-NIH	mRNA-1273	Not cited	2	Anti-spike IgM, IgG and IgA	Detection of IgA and IgG	1. Detection of IgA, IgG, and IgM 2. The SARS-CoV-2 antibodies induced by mRNA vaccines persist for at least 6 months	NA

N, number of individuals; NA, not analyzed; NB, newborn.

started to increase in the first 2 weeks after the first dose of the vaccine, and they were significantly higher 5–6 weeks after vaccination (66).

Another study, developed by Perez et al., analyzed human milk samples from 27 women collected three times at 1, 3, and 6 months after they received the BNT162b2 (Pfizer) vaccine (25 of 27 women) or mRNA-1273 (Moderna) vaccine (2 of 27 women) (69). Concerning IgM antibodies, 7 of 24 (29.1%) women were positive after 1 month, 6 of 27 (22.2%) were positive after 3 months, and after 6 months post-vaccination, these antibodies were not detectable in breast milk. IgG antibodies were positive in breast milk samples from 24 of 24 (100%) lactating women in the first month, 25 of 27 (92.6%) in the third month, and 9 of 12 (75.0%) in the sixth month. On the other hand, 12 of 24 (50%) lactating mothers were positive for SARS-CoV-2-specific IgA 1 month after vaccination, 7 of 27 (25.9%) were positive at 3 months, and at 6 months' IgA levels were not detected at significant levels above the baseline. The authors also evaluated the neutralizing activity of the cited antibodies and found that 20 of 24 (83.3%) breast milk samples showed neutralizing capacity at 1 month; 19 of 27 (70.4%) had neutralization activity at 3 months; only 3 of 12 (25.0%) maintained this neutralizing activity by month 6. In other words, they concluded that COVID-19 mRNA vaccination induced the production of SARS-CoV-2-specific antibodies for at least 6 months after vaccination, and neutralizing antibodies persisted for at least 3 months (69).

There is a lack of research that evaluates the efficiency of anti-SARS-CoV-2-specific IgM, IgG, and IgA transfer from breast milk to the infant's serum (62, 75). A study by Yeo et al. analyzed the serum of 5 infants (age 3 to 20 months) of vaccinated women that were breastfeeding; a single serum sample was collected at a median of 48 days after their mothers received the second dose of BNT162b2 vaccine, and the researchers observed that there were no neutralizing antibodies detected in their serum (76). These results were also observed by Golan et al. in a study that did not identify anti-SARS-CoV-2 IgG antibodies in the plasma of infants whose mothers were vaccinated with mRNA-based vaccines for COVID-19 (mRNA-1273 and BNT162b2) during lactation (53). Additionally, a longitudinal cohort study by Schwartz et al. detected SARS-CoV-2 IgG in the oral mucosa of 3 of 5 (60%) breastfed infants of lactating women who were vaccinated against COVID-19 with the BNT162b2 messenger RNA vaccine but also did not find these antibodies in the infants' serum (77). Therefore, further studies are needed to better understand these points.

CELLULAR IMMUNE RESPONSE POST-COVID-19 VACCINE IN PREGNANT WOMEN

Besides humoral response, it is known that cellular immune response mediated by T cells is crucial for the combat of SARS-CoV-2 infection: while $CD4^+$ T cells are important to develop specific antibodies against the virus, $CD8^+$ T cells have a role in the identification and destruction of infected cells (62). A study that evaluated the participation of cellular immunity in the lactation of women vaccinated against COVID-19 revealed that after vaccination with COVID-19 mRNA, non-pregnant, pregnant, and lactating women had an increase in anti-SARS-CoV-2 $CD4^+$ and $CD8^+$ T cell counts, and this immune response was more robust to vaccine than to natural infection (52). In this context, another study with pregnant women who received the Pfizer vaccine showed that although the concentration of their antibodies against the virus decreased after several months of vaccination, their memory $CD4^+$ and $CD8^+$ cells continued to express proinflammatory cytokines (such as IFN-γ, TNF-α, and IL-2), which indicates that vaccination in pregnant women, as in other individuals, provides long-term protection against SARS-CoV-2 (78).

Maternal Immune Cells in Human Milk and Cellular Immunity Transmission

The development of the newborn immune system starts *in utero* and is highly boosted by passive immunization through breastfeeding (79). Faced with an immature adaptive immune system that has not had the time to build up the necessary repertoires of cell clones and memory to permit the neonatal defense, the newborn takes into account immune cells and other defense components coming from breast milk. Of these components, we can highlight the high amounts of antibodies (mainly IgA), cytokines and other proteins (primarily lactoferrin) transferred from mother to child, and components of maternal cellular immunity such as macrophages, polymorphonuclear neutrophils, and lymphocytes (composed by approximately 83% of T cells and 4%–6% of B cells) (80–83). Breast milk lymphocytes are very abundant at delivery, decline over the first month postpartum to a steady state, and persist for up to 2 years (84–87). This was confirmed by a flow cytometry study that identified and quantified the $CD45^+$ leukocyte populations in human breast milk and found cells like myeloid precursors, neutrophils, immature granulocytes, $CD16^+$ and $CD16^-$ monocytes, non-cytotoxic T cells, cytotoxic T and NK cells, eosinophils, basophils, B-cell precursors, and B cells (87). It is already known that the leukocytes are able to survive in the environment of the child's digestive tract; reach the blood, lymph nodes, spleen, and other tissues/organs; and phagocytize and fight against pathogens (81, 82). Therefore, the properties offered by this group of cells provide active immunity to the infants, besides stimulating the achievement of their own immunocompetence (83).

Additionally, evidence shows that there are a few differences between breast milk and blood leukocytes: breast milk T cells and macrophages have more motility than those in blood, and colostrum lymphocytes have effector functions that can be transferred through breast milk and benefit the infant to respond against threats (82). Therefore, the properties offered by this group of cells provide active immunity to the infants, besides stimulating the achievement of their own immunocompetence (83).

The presence of memory T cells in human milk suggests that these cells were transferred from the mother to the infant to provide a rapid response against specific pathogens until their immune system becomes fully operative. In fact, studies demonstrated transferred breast milk memory $CD4^+$ and $CD8^+$ T cells in infants' Peyer's patches, spleen, and bone marrow; it is known that T lymphocytes in the intestine of neonates are recent thymic emigrants (progenitors of mature naive T lymphocytes) (80, 83); therefore, these specific memory T cells probably originated from their mothers through lactation. Sabbaj et al. analyzed a group of virus-infected lactating women and demonstrated that cytomegalovirus (CMV), influenza virus, Epstein–Barr virus (EBV), and HIV-specific $CD8^+$ T cells were found in the breast milk. This suggests that an effector memory phenotype of $CD8^+$ T cells is passed through breastfeeding to the newborns (88). Although studies about memory T cells against SARS-CoV-2 in human milk are scarce, Armistead et al. observed that the lactating breast contains a distinct T-cell population that can be modulated by maternal vaccination with potential implications for infant passive protection. These researchers have identified SARS-CoV-2 spike-specific T cells in mRNA vaccinated in lactating women (89). Another study conducted by Gonçalves et al. involving lactating women who received mRNA vaccination found a combination of spike-reactive T cells and anti-SARS-CoV-2 secreted IgA in their milk, which shows that immune transfer to the infant could linger even after weaning, especially because of long-lived memory T cells transferred (64). Such evidence points to the great importance of maternal vaccination, especially for the SARS-CoV-2 virus, as a cellular immunization strategy for the newborn through lactation.

OTHER BIOACTIVE COMPOUNDS IN HUMAN MILK

Human milk has a list of maternal immunomodulatory, antiviral, and anti-inflammatory elements that helps in the development of the newborn's immune response (79). Recent research has shown that the risk of severe viral respiratory infections in infants is negatively associated with the duration of breastfeeding (90). Therefore, a crucial role in human milk is played by other components in addition to IgA, such as oligosaccharides, proteins (such as lactoferrin), lipids, and pro- and anti-inflammatory factors (TNF-α, interleukin-1 [IL-1], interleukin-10 [IL-10], prostaglandins E2 [PGE2], etc.) (91–93).

There is a lack of studies on the antivirals' effects of breast milk against SARS-CoV-2, but some authors suggest that newborns can be protected from COVID-19 by milk proteins like lactoferrin, casein, and immunoglobulins, which have antiviral effects (94). It has already been reported that lactoferrin enhances NK cell activity, promotes neutrophil aggregation and adhesion, and blocks the SARS-CoV from entering host cells during the infection (93); it is likely that these findings could also be applied to SARS-CoV-2. Indeed, these breast milk bioactive molecules can have their immune response amplified after women's vaccination, as has already been evidenced in the immunization against human rotavirus (95).

CONCLUSION

The peculiarities of the immune system during pregnancy are one of the reasons pregnant women are included in a higher risk group for respiratory infections. With the emergence of the COVID-19 pandemic and the uncertainties around it, the concerns around pregnant women increased, mainly due to the possibility of maternal–fetal transmission of the virus. In this review, we assessed that the safety and efficacy of the developed COVID-19 vaccines did not differ between pregnant, lactating, and non-pregnant women. Furthermore, besides reducing the risks of post-COVID-19 complications, the benefits of vaccinating these groups are not restricted to them, since the production of neutralizing antibodies against SARS-CoV-2 by the mother can be transmitted to the fetus. Several studies showed that immunized women can transmit anti-SARS-CoV-2 IgG through the placenta, as has been confirmed by the presence of these antibodies in cord blood or the newborn serum after birth. Additionally, the seroprotection during the beginning of the infant's life can be boosted by early third-trimester vaccination of their mothers, seeing that the magnitude of the maternal–fetal transfer is increased in this period. The passive immunization of the newborn also happens through breastfeeding; studies demonstrated the presence of anti-SARS-CoV-2 specific IgA, IgM, IgG, and T cells in the breast milk some weeks after a mother's vaccination. These findings add even more benefits to breastfeeding, which naturally confers protection to infants due to the immunomodulatory, antiviral, and anti-inflammatory molecules that compose the human milk. Therefore, more studies involving pregnant and lactating women are needed to better characterize the vaccine immunogenicity among these populations. These results may help to create public health policies and to optimize the vaccine schedule, considering the durability of post-vaccine immunity, to ensure maternal–fetal protection against COVID-19.

AUTHOR CONTRIBUTIONS

ALA, APMM, and DMC performed an extensive review of literature about issues contemplated in the manuscript. ALA wrote the manuscript. JMVC, JELV, LDB-P and DMC reviewed the intellectual content and also helped to draft the manuscipt. JMVZ, JELV, and LDB-P conceived the proposal of the mini-review. All authors approved the final manuscript.

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Maternal Stress and Human Milk Antibodies During the COVID-19 Pandemic

Hannah G. Juncker[1,2], Eliza J. M. Ruhé[1], Aniko Korosi[2], Johannes B. van Goudoever[1], Marit J. van Gils[3] and Britt J. van Keulen[1]*

[1] Department of Pediatrics, Emma Children's Hospital, Amsterdam Reproduction & Development Research Institute, Amsterdam UMC, Vrije Universiteit, University of Amsterdam, Amsterdam, Netherlands, [2] Swammerdam Institute for Life Sciences - Center for Neuroscience, University of Amsterdam, Amsterdam, Netherlands, [3] Department of Medical Microbiology and Infection Prevention, Amsterdam Infection and Immunity Institute, Amsterdam UMC, University of Amsterdam, Amsterdam, Netherlands

Importance: *SARS-CoV-2*-specific antibodies in human milk might protect the breastfed infant against COVID-19. One of the factors that may influence human milk antibodies is psychological stress, which is suggested to be increased in lactating women during the COVID-19 pandemic.

Objective: To determine whether psychological stress is increased in lactating women during the COVID-19 pandemic, and if maternal stress is associated with the level of *SARS-CoV-2*-specific antibodies in human milk.

Design: Population-based prospective cohort study.

Setting: Data collection took place in the Netherlands between October 2020 and February 2021.

Participants: Lactating women living in the Netherlands were eligible to participate in this study. In total, 2310 women were included.

Exposures: Stress exposure during the COVID-19 pandemic was determined using the Perceived Stress Scale (PSS) questionnaire and maternal lifetime stress was determined by the Life Stressor Checklist – revised (LSC-r) questionnaire.

Main Outcome(s) and Measure(s): Stress experience during the COVID-19 pandemic was compared with a pre-pandemic cohort. *SARS-CoV-2*-specific antibodies in human milk were measured using an Enzyme-Linked Immunosorbent Assay (ELISA) with the Spike protein of *SARS-CoV-2*. The association between maternal stress and human milk antibodies was determined using a multiple regression model.

Results: The PSS score of lactating mothers was not increased during the pandemic compared to the PSS score in the prepandemic cohort. Six hundred ninety-one participants had *SARS-CoV-2*-specific antibodies and were included in the regression models to assess the association between maternal stress and human milk antibodies. No association was found between PSS scores and human milk antibodies. In contrast, the LSC-r score was negatively associated with *SARS-CoV-2*-specific IgA in human milk ($\beta = 0.98$, 95% CI: 0.96–0.997, $p = 0.03$).

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Talat Kilic,
Inönü University, Turkey

***Correspondence:**
Johannes B. van Goudoever
h.vangoudoever@amsterdamumc.nl

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Conclusions and Relevance: Our results suggest that lactating women in the Netherlands did not experience higher stress levels during the COVID-19 pandemic. Breastfed infants of mothers with high chronic stress levels receive lower amounts of antibodies through human milk, which possibly makes them more vulnerable to respiratory infections. This emphasizes the importance of psychological wellbeing during lactation.

Keywords: SARS-CoV-2, stress, COVID-19, lactation, passive immunity, breast milk

INTRODUCTION

COVID-19 usually has a mild course in children; however, young infants are more susceptible to severe disease development, which could be due to an immature immune system (1). Human milk provides additional immunological protection for these infants as it contains multiple immunological components. Human milk antibodies are suggested to play an important role in the protection against respiratory infections (2–5). Antibodies against the *severe acute respiratory syndrome coronavirus* 2 (*SARS-CoV-2*) have been found in human milk after maternal infection and vaccination (6–12). It is very likely that these antibodies play a critical role in protecting the infant against COVID-19. Indeed, breastfeeding in *SARS-CoV-2* positive mothers, protects their infants from developing symptoms of COVID-19 (13). Moreover, although *SARS-CoV-2* RNA has been detected in human milk, replication competent *SARS-CoV-2* has not been isolated and transmission of the virus to the infant through human milk has not been reported (14–18).

Human milk antibody titers are influenced by many different factors, including maternal psychological stress (19–21). However, there is still controversy on the effect of maternal stress on the secretion of immunoglobulin A (IgA), the most abundant antibody in human milk (19, 22–25). Most studies point toward the view that perceived stress reduces IgA in human milk (19). It is important to elucidate this relationship, as it is plausible to assume that maternal stress might be increased during the COVID-19 pandemic. Indeed, several studies have highlighted concerns about the mental health of postpartum women in the COVID-19 pandemic, showing an increase in depressive symptoms, anxiety and maternal distress (26–29). The mental state and overall functioning of the mothers may have suffered from the lockdown measures due to limited access to support systems, changes in hospital policies including unaccompanied pregnancy checkups, mother-infant separation policies, and the stress that comes from their overall concerns about exposure to COVID-19 (29).

The aim of this study is to investigate maternal psychological stress during the COVID-19 pandemic and its potential impact on *SARS-CoV-2*-specific antibodies in human milk. We hypothesize that maternal psychological stress is higher during the pandemic and that perceived stress levels are negatively associated with IgA against *SARS-CoV-2* in human milk.

METHODOLOGY

Study Design and Population

The COVID MILK – POWER MILK study is a prospective cohort study, which included lactating women between October 12th and February 23th in the Netherlands who did not yet receive a *SARS-CoV-2* vaccine. Participants were recruited via (social) media and could sign themselves up by sending an e-mail. Ethical approval was obtained from the Medical Ethics Committee of the Amsterdam UMC, location VUmc. Written informed consent was obtained from all participants.

Study Procedures and Sample Collection

To determine *SARS-CoV-2* antibodies, a human milk and blood sample were collected during a study visit. In the morning of the appointment, participants were instructed to empty one breast completely before the first feeding moment, either manually or with an electric breast pump, mix the milk and subsequently store 20 ml in the refrigerator until collection by the researcher. During the study visit, 5 ml of blood was collected. At the study site, serum and milk samples were stored at −80°C up until analysis. After the study visit, participants received a questionnaire, which included two validated test tools to examine the level of stress experienced by the participants.

Perceived Stress Scale (PSS)

To investigate stress during the COVID-19 pandemic and its influence on maternal antibodies, the PSS questionnaire was used. The PSS is a validated 14-item questionnaire developed by Cohen et al. (30, 31). The questionnaire aims to determine how stressful one experiences certain situations (30, 31). For each question the respondent is asked to indicate how many times they felt a certain way since the outbreak of COVID-19. Each question is scored on a 5-point Likert Scale ranging from 0–4 (0 = never; 1 = almost never; 2 = sometimes; 3 = fairly often; 4 = very often).

Life Stressor Checklist – Revised (LSC-r)

To investigate the influence of maternal lifetime stressors on human milk antibody levels, the LSC-r questionnaire was used. The LSC-r evaluates the maternal lifetime history of stress. The validated checklist is a 30-item scale to identify the exposure to traumatic events or other stressful life events (32). For this research, we used the questions that form a comparative baseline for lifetime traumatic stress. We combined two scoring methods of the questionnaire for this study. This approach combines a score for high magnitude stressors (criteria A stressors) and

a score for low magnitude stressors (other life stressors) (32) resulting in an overall life stressor score ranging from 0–13, with the highest score representing the highest level of lifetime history of stress (32).

Determination of SARS-CoV-2 Antibody Titers in Human Milk and Serum

Before analysis, the collected human milk and serum samples were stored at the Amsterdam UMC, location VUmc, at −80°C. To assess the *SARS-CoV-2*-specific IgA antibodies in human milk and IgG antibodies in serum, an enzyme-linked immunosorbent assay (ELISA) with the *SARS-CoV-2* spike protein was used as described previously (33). In brief, whole human milk and serum samples were diluted 1:10 or 1:100, respectively, in 1% casein PBS (Thermo Scientific) and IgA or IgG were detected using horseradish peroxidase (HRP)-labeled goat anti-human IgA (Biolegend) or HRP-labeled goat anti-human IgG (Jackson, Immunoresearch), respectively, which were validated using monoclonal antibodies. A relative operating characteristic (ROC) curve analysis was performed to determine the cut-off value for both milk and serum samples using pre-pandemic negative samples and polymerase chain reaction proven positive samples. The human milk samples were considered positive at an optical density (OD) 450 nm cut-off value of 0.502, and the serum samples at an OD450 nm value of 0.452. With these cut-off values, the sensitivity was 67.9% (95% confidence interval (CI): 61.0–74.1%) for IgA antibodies in human milk with a specificity of 99.0% (95% CI: 94.7–100.0%) and for serum IgG antibodies the sensitivity was 95.9 (95% CI: 92.9–97.6%) with a specificity of 99.1 (95% CI: 94.9–100%). For cross-comparison, negative and positive controls were included in each run.

Statistical Analysis

The obtained data is registered in the Clinical Data Management System "Castor Electronic Data Capture (EDC)." In order to perform the statistical analysis, the data was transferred into IBM Statistical Package for Social Sciences Statistics (SPSS) for Windows version 26. Characteristics were described in descriptive statistics including frequencies, mean values with standard deviations (SD) or median with interquartile ranges (IQR). Participants with missing data for stress measures or antibody levels were excluded from further analyses.

We compared PSS scores in our cohort with a recent study conducted in the United States before the outbreak of COVID-19 (34). This pre-pandemic cohort consisted of 151 lactating mothers between 18 and 40 years old who filled out the PSS questionnaire at weeks 1 and 2 postpartum, as well as at 1-, 2-, 3-, and 6-months postpartum. This pre-pandemic cohort was comparable with our cohort in baseline characteristics including age, BMI and history of depression. Unpaired *t*-tests were performed to compare PSS scores between this pre-pandemic cohort and our cohort for each month postpartum.

To investigate the influence of maternal stress on human milk antibodies, lactating mothers who tested positive for *SARS-CoV-2*-specific antibodies in serum or human milk were included. IgA values were log-transformed before analyses. Due to a non-linear relation between PSS and IgA levels, participants were divided in three groups: low stress (PSS 0-14.99), moderate stress (15.00–21.99) and high stress (22.00–56.00) based on the 33.3–66.6 percentiles. Pearson Chi square tests, one-way ANOVA and Kruskal Wallis tests were used to assess differences in characteristics between PSS subgroups based on the distribution.

To examine the association between PSS and LSC-r scores and maternal antibodies, multiple regression analyses were performed. The PSS regression model was adjusted for factors that differed between the PSS groups. In literature, age of the mother, BMI of the mother, parity, lactation stage and sex of the child have shown to influence antibody levels in human milk (21, 23, 35, 36). Those variables were added to the LSC-r regression model when they influenced the model with >10%. To correct for the logarithmic transformation, the following formulas were used to accurately interpret the regression coefficients: $\beta = e^{\beta}$ and 95.0% confidence interval$= e^{(\beta \pm 1.96\ x\ standard\ error)}$. For the statistical analysis, the hypothesis was tested two-tailed and a *p*-value of < 0.05 was considered statistically relevant. GraphPad Prism for Windows (version 8.2.1.) was used to illustrate the data distributions.

RESULTS

Stress Levels of Lactating Mothers During the COVID-19 Pandemic

Baseline Characteristics

In total, 2310 mothers participated in the study, of whom 2,163 (94%) filled out the characteristics questionnaire (**Table 1**). The participants were on average 33.2 (SD ± 3.9) years of age and were breastfeeding their child for 38.0 (25.0–59.0) weeks.

Postpartum PSS and LSC-r Scores

The PSS questionnaire was completed by 2,162 participants (94%). These women had a mean PSS score of 19.56 (SD ± 7.97). The PSS scores increased over the first postpartum year [$r = 0.09$, 95% CI: 0.12–0.40, $p < 0.001$, $N = 1{,}619$ (two-tailed)] (**Figure 1**). We compared the PSS scores of the women in our cohort to PSS scores in a pre-COVID-19 cohort of lactating women. The mean PSS score in this pre-pandemic cohort of 151 lactating women up to 6 months postpartum was 18.69 (SD ± 0.47) (34). The women up to 6 months postpartum in our cohort had a mean PSS score of 18.41 (SD ± 7.64) ($N = 494$), which did not differ from the pre-pandemic cohort at any time postpartum (mean difference: −0.27, 95% CI: −0.95–0.40, $p = 0.43$) (**Figure 2**). The LSC-r questionnaire was completed by 2162 participants (94%) and they scored a median of 1.00 (IQR: 0.0–3.0).

Maternal Stress and *SARS-CoV-2*-Specific Antibodies in Human Milk

Baseline Characteristics

Of the total study population, 691 participants tested positive for *SARS-CoV-2*-specific IgA in human milk or IgG in serum. These participants were categorized into subgroups based on their PSS scores: low ($N = 182$), moderate ($N = 245$) and high ($N = 219$) PSS groups. The per subgroup characteristics are depicted in **Table 1**. Women with high PSS scores had more mental illnesses ($p < 0.0001$), were breastfeeding for a longer time period ($p =$

TABLE 1 | Participants characteristics based on perceived stress scores (PSS) in participants with an ELISA confirmed SARS-CoV- 2 infection in serum or human milk.

Maternal characteristics	Total (*N* = 2,310)	*SARS-CoV-2* positive (*N* = 691)[a]	Perceived Stress Scale groups			*p*-value
			Low (*N* = 182)[a]	Moderate (*N* = 245)[a]	High (*N* = 219)[a]	
Age mother – years* (± SD)	33.1 (±3.8) (*N* = 2,223)	33.2 (± 3.9) (*N* = 662)	33.5 (± 3.9) (*N* = 175)	33.0 (± 3.8) (*N* = 240)	33.3 (± 4.1) (*N* = 208)	0.46
Body Mass Index** (IQR)	23.3 (21.3–26.0) (*N* = 2,226)	23.3 (21.4–25.9) (*N* = 646)	23.1 (21.4–25.5) (*N* = 182)	23.0 (21.1–25.8) (*N* = 245)	23.6 (21.–26.2) (*N* = 219)	0.16
Chronic illness No. (%)	306/2,224 (13.8)	81/646 (12.5)	18/182 (10.0)	35/245 (14.3)	28/219 (12.8)	0.41
Autoimmune disease No. (%)	72/2,262 (3.2)	15/646 (2.3)	2/182 (1.1)	7/245 (2.9)	6/219 (2.7)	0.46
Psychological disease No. (%)	408/2,221 (18.4)	106/645 (16.4)	20/182 (11.0)	28/245 (11.4)	58/218 (26.6)	0.0001
Smoking No. (%)	42/2,196 (1.9)	12/636 (1.9)	0/179 (0)	7/242 (2.9)	5/215 (2.3)	0.048
Alcohol consumption No. (%)	1,014/2,196 (46.2)	338/636 (53.1)	96/179 (53.6)	136/242 (56.2)	106/215 (49.3)	0.33
LSC-r score** (IQR)	1.0 (0.0–3.0) (*N* = 2,226)	1.0 (0.0-3.0)	1.0 (0.0–2.0) (*N* = 182)	1.0 (0.0–3.0) (*N* = 245)	2.0 (1.0–4.0) (*N* = 219)	0.0001
Education level						0.02
- Primary and lower secondary No. (%)	31/2,263 (1.4)	7/673 (1.0)	3/182 (1.6)	3/245 (1.2)	1/219 (0.4)	
- Upper secondary No. (%)	338/2,263 (14.9)	114/673 (16.9)	22/182 (12.1)	37/245 (15.1)	54/219 (24.6)	
- Bachelor equivalent No. (%)	1,008/2,263 (44.5)	291/673 (43.2)	82/182 (45.1)	99/245 (40.4)	97/219 (44.3)	
- Master and Doctoral equivalent No. (%)	842/2,263 (37.2)	245/673 (36.4)	73/182 (40.1)	103/245 (42.0)	65/219 (29.7)	
Infant characteristics						
Age child – weeks** (IQR)	34.0 (24.0–50.0) (*N* = 2,122)	38.0 (25.0–59.0)	37.0 (26.0–56.3) (*N* = 174)	35.0 (23.3–55.0) (*N* = 234)	42.0 (28.0–66.0) (*N* = 210)	0.005
GA at delivery – weeks** (IQR)	40.0 (39.0-40.9) (*N* = 2,164)	40.1 (39.0-40.9)	40.0 (39.0-40.9) (*N* = 176)	40.2 (39.3-41.0) (*N* = 240)	40.0 (39.0–40.9) (*N* = 211)	0.52
Birth Weight – grams* (± SD)	3,566 (±517) (*N* = 2,160)	3,582 (± 517) (*N* = 637)	3,579 (± 510) (*N* = 175)	3,605 (± 517) (*N* = 240)	3,562 (± 532) (*N* = 208)	0.72
Primipara No. (%)	865/2,185 (39.6)	244/635 (38.4)	76/179 (42.5)	92/242 (38.0)	76/214 (35.5)	0.37
Sexe- Boy No. (%)	1,071/2,233 (45.5)	318/635 (50.0)	83/179 (46.4)	122/242 (50.4)	113/214 (52.8)	0.45
Delivery						
Vaginal delivery No. (%)	1,835/2,236 (82.1)	532/635 (78.1)	147/179 (82.1)	204/242 (84.3)	181/214 (84.6)	0.79
Instrumental delivery No. (%)	129/2,236 (5.8)	37/635 (5.8)	14/179 (7.8)	13/242 (5.4)	10/214 (4.7)	0.38
Caesarian section No. (%)	269/2,236 (12.0)	84/635 (13.2)	25/179 (13.9)	34/242 (14.0)	25/214 (11.7)	0.72

*Data are given as number/the total of participants who answered the specific question (%), mean (± Standard Deviation) and median (interquartile range: 25th percentile- 75th percentile). Data given as mean and median are given with the total amount of participants who filled out the specific question (N=). * = Data given as mean. ** = Data given as median. LSC-r score, life stressor checklist – revised calculated score; Education is classified according to the International Standard Classification of Education; GA, Gestational Age.*

[a]Participants with SARS-CoV-2-specific antibodies who filled out the PSS questionnaire were divided into PSS subgroups (low, moderate and high). The p-value represents whether there is a significant difference between the low, moderate and high perceived stress groups.

0.005), smoked more often ($p = 0.048$), and scored higher on the LSC-r questionnaire ($p < 0.0001$).

PSS Scores and Maternal SARS-CoV-2-Specific Antibodies

To compare maternal *SARS-CoV-2*-specific antibodies between the PSS groups, a multiple regression was performed. No differences were observed in *SARS-CoV-2*-specific antibody levels in human milk between the PSS groups in both the unadjusted and adjusted model (**Table 2**, **Figure 3**).

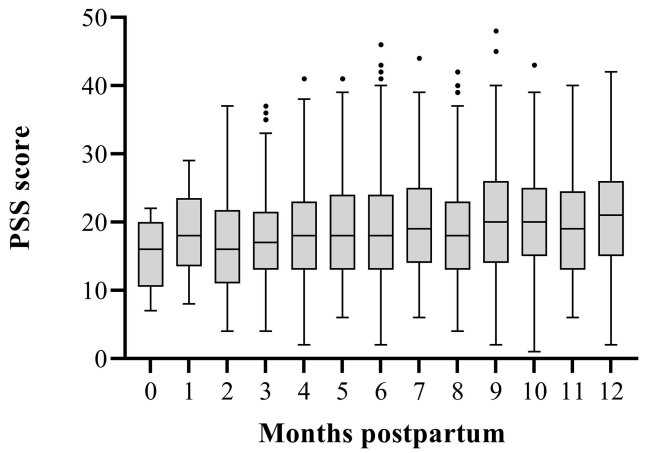

FIGURE 1 | Perceived Stress Scale (PSS) scores up to 12 months postpartum. This figure shows the increase in PSS scores over the first postpartum year. The box represents the interquartile range with median PSS scores. Whiskers present the data range (Q1/Q3 +/−1.5IQR). · = outlier.

LSC-r Scores and Maternal SARS-CoV-2-Specific Antibodies

To investigate the relationship between LSC-r scores and maternal *SARS-CoV-2*-specific antibodies, a multiple regression was performed. After adjustment for covariates, the LSC-r score was negatively associated with IgA in human milk (B = 0.98, 95% CI: 0.96–0.997, $p = 0.03$) (**Table 3**, **Figure 4**).

DISCUSSION

In contrast to our hypothesis, the results of this study suggest that lactating women did not experience higher levels of stress during the COVID-19 pandemic compared to lactating women before the pandemic. Interestingly, maternal lifetime stressors, but not current perceived stress, were negatively associated with human milk antibodies against *SARS-CoV-2*.

Several studies assessed stress levels in lactating women during the COVID-19 pandemic, of which the majority showed that stress and anxiety levels were increased, while some studies showed similar stress levels during and before the pandemic

TABLE 2 | The association between PSS scores and *SARS-CoV-2*-specific antibodies in human milk.

PSS score subgroups	Unadjusted model		Adjusted model	
	β (95% CI)	*P*-value	β (95% CI)	*P*-value
Low - Moderate	1.04 (0.96–1.13)	0.34	1.06 (0.97–1.15)	0.23
Low - High	1.04 (0.96–1.13)	0.35	1.07 (0.98–1.17)	0.15
Moderate - High	1.00 (0.92–1.08)	0.98	1.01 (0.93–1.10)	0.76

PSS, Perceived Stress Scale.
The regression model was adjusted for LSC-r scores, psychological disease, smoking, the age of the child and education level.

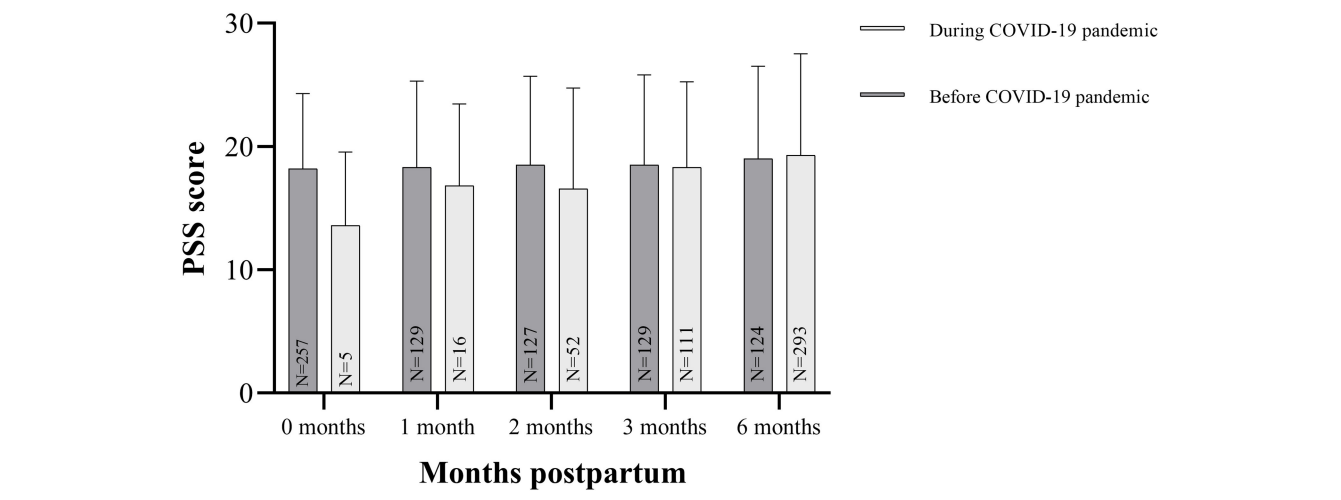

FIGURE 2 | Perceived Stress Scale (PSS) scores during and before the COVID-19 pandemic. In this figure, the PSS scores are displayed as mean (SD) of the specific postpartum group up to six months postpartum. Mean PSS scores of a U.S. cohort before the COVID-19 pandemic are obtained in Paul et al. (34). There were no differences between our study cohort and the pre-pandemic cohort.

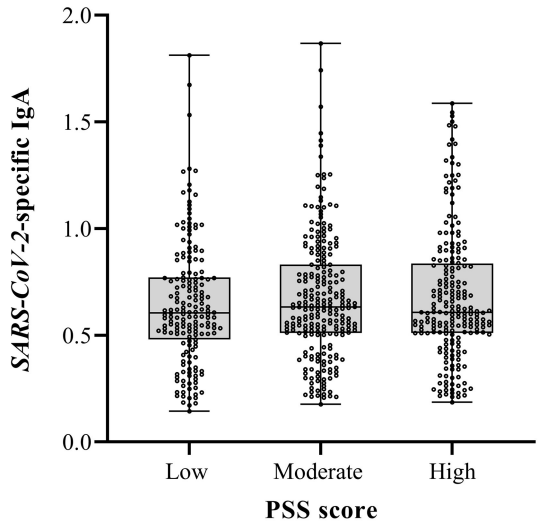

FIGURE 3 | Perceived Stress Scale (PSS) scores and *SARS-CoV-2* specific Immunoglobuline A (IgA) in human milk. The boxes represent the interquartile range with median *SARS-CoV-2* specific IgA in human milk for the different PSS groups. Whiskers present the data range (Q1/Q3 +/−1.5IQR). The dots indicate the individual measurements. No differences in SARS-CoV-2 specific human milk IgA were found between groups.

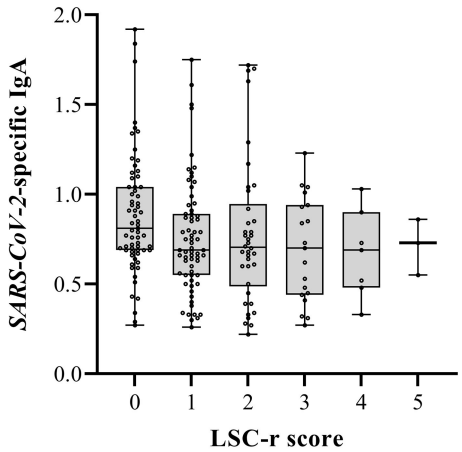

FIGURE 4 | Life Stressor Checklist-revised (LSC-r) scores and *SARS-CoV-2* specific Immunoglobuline A (IgA) in human milk. The boxes represent the interquartile range with median *SARS-CoV-2* specific IgA in human milk for the different LSC-r scores. Whiskers present the data range (Q1/Q3 +/−1.5IQR). The dots indicate the individual measurements. Multiple lineair regression models were used to determine the association between *SARS-CoV-2*-specific IgA in human milk and the LSC-r scores (adjusted *p*-value 0.03).

TABLE 3 | The association between LSC-r scores and *SARS-CoV-2*-specific antibodies in human milk.

	Unadjusted model		Adjusted model	
	β (95% CI)	***P*-value**	**β (95% CI)**	***P*-value**
LSC-r score	0.98 (0.97–1.00)	0.08	0.98 (0.96–0.997)	0.03

LSC-r, Life Stressor Checklist – revised.
We included age child, sex infant, parity, BMI, age mother to test for potential covariates. The age of the child was considered a confounder and was adjusted for in our model.

(26, 28, 37–43). The studies that found higher stress levels were carried out at the onset of the pandemic. It could be suggested that the lack of knowledge of the effects of COVID-19 in lactating women and infants at the very beginning of the pandemic caused stress. Considering that our study was conducted 7 months into the pandemic, this could entail that the women who participated in this study had potentially already adapted to the situation and that stress levels were normal again. Moreover, it could be suggested that lactating women did not experience increased stress levels during the pandemic due to other factors, such as a reduction in social and work obligations. For example, working from home results in reduced travel time and spending more time with family (44, 45).

Previous literature on the relationship between stress and human milk antibodies is controversial. Either positive, negative and no associations between maternal stress, anxiety or depression and human milk IgA have been reported (19, 22–25, 46). The before mentioned studies were hampered by their relatively small samples sizes (n = 50–119) and differed in type and timing of stress measurement, set up and human milk collection, hampering comparability between studies. Most of the before mentioned literature showed a negative association between maternal stress and human milk antibodies (19, 23, 25, 47). In our study, perceived stress among postpartum women showed no relation with *SARS-CoV-2*-specific antibodies in human milk. However, an inverse association between lifetime stressors and human milk antibodies was observed, also after correcting for possible confounders. This suggests that chronic stress levels may have more pronounced consequences for the maternal immune system compared to current stress levels. Indeed, former research states that chronic stress diminishes the immune response (22, 48–51).

Our study is strengthened by the large sample size, making it possible to identify and adjust for confounding factors. Human milk samples were collected in a standardized way, to minimize collection bias. Moreover, both acute as well as chronic stress was measured. Finally, the study questionnaire was completed by 94% of our study population, which minimizes missing data and improves the reliability and generalizability of our study results. A limitation of our study is that the stress levels were self-reported via questionnaires and that no biological stress measures were included. Moreover, our cohort consisted mostly of highly educated women. It might be that this is not entirely representative for perceived stress levels of all lactating women. In addition, to compare stress levels during the pandemic with pre-pandemic stress levels, our cohort was compared to a pre-pandemic cohort from the United States. Preferably, pre- and during pandemic stress levels should be measured in the same cohort. Finally, *SARS-CoV-2*-specific antibodies may depend on several other factors, including time after infection and severity

of symptoms. However, as our sample size is relatively large, we expect that the influence of these factors on our results is minimal.

At this point, we can only speculate what the stress-related changes in human milk antibodies mean for the protection of the breastfed infant. However, as infants drink this milk multiple times a day for a long period, it can be suggested that the protection will be affected. Large sample-sized, population-based studies are needed to address the actual effect of decreased human milk antibody levels on the protection of the breastfed infant from infections. Moreover, future studies should consider adding biological indicators of stress, for example human milk or hair cortisol concentrations, to assess stress levels in lactating women. Lastly, it would be valuable to measure total immunoglobulins and/or other immunological components in human milk to be able to investigate the effects of stress on the total immunological properties of human milk.

CONCLUSION

The results of this study demonstrated that lactating women in the Netherlands did not experience higher perceived stress levels seven months into the COVID-19 pandemic compared to stress levels of lactating women prior to the COVID-19 pandemic. Moreover, lifetime stress was associated with reduced *SARS-CoV-2*-specific antibodies in human milk, while current perceived stress was not. Our findings emphasize the importance of psychological well-being of lactating women and the need to identify and guide (expecting) mothers with high chronic stress levels.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by METc VUmc. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HJ and BK had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: HJ, ER, AK, JG, and BK. Acquisition, analysis, or interpretation of data: HJ, ER, MG, AK, JG, and BK. Drafting of the manuscript: HJ, ER, and BK. Critical manuscript revision for intellectual content: MG, AK, JG, and BK. Statistical analysis: HJ, ER, and BK. Obtained funding: MG, JG, and BK. Administrative, technical, or material support: HJ, ER, MG, JG, and BK. Supervision: JG and BK. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: JG was the founder and director of the Dutch National Human Milk Bank and a member of the National Health Council and has been a member of the National Breastfeeding Council from March 2010 to March 2020.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of Vaccination Against Influenza, Pertussis, and COVID-19 on Human Milk Antibodies: Current Evidence and Implications for Health Equity

Soumya Hunagund[1†], *Yarden Golan*[2,3†], *Ifeyinwa V. Asiodu*[4], *Mary Prahl*[5,6] *and Stephanie L. Gaw*[1,7*]

[1] Division of Maternal-Fetal Medicine, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco, San Francisco, CA, United States, [2] Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, San Francisco, CA, United States, [3] Institute for Human Genetics, University of California, San Francisco, San Francisco, CA, United States, [4] Department of Family Health Care Nursing, University of California, San Francisco, San Francisco, CA, United States, [5] Department of Pediatrics, University of California, San Francisco, San Francisco, CA, United States, [6] Division of Pediatric Infectious Diseases and Global Health, University of California, San Francisco, San Francisco, CA, United States, [7] Center for Reproductive Sciences, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco, San Francisco, CA, United States

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***Correspondence:**
Stephanie L. Gaw
Stephanie.Gaw@ucsf.edu

[†]These authors have contributed equally to this work

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Human milk contains three antibody classes that confer mucosal immunity to the breastfed infant: secretory IgA (SIgA), secretory IgM (SIgM), and IgG. Influenza and pertussis vaccines administered during pregnancy induce pathogen specific SIgA and IgG responses in human milk that have been shown to protect the breastfed infant from these respiratory illnesses. In addition, mRNA vaccines against the SARS-CoV-2 virus administered during pregnancy and lactation induce anti-SARS-CoV-2 IgG and IgA responses in human milk. This review summarizes the immunologic benefits of influenza, pertussis, and COVID-19 vaccines conferred by human milk. Additionally, future research direction in human milk immunity and public health needs to improve lactational support are discussed.

Keywords: human milk, COVID - 19, influenza, pertussis, vaccination, infant health, immunization

INTRODUCTION

Human milk has been shown to have numerous benefits for infants (1–5) as well as for breastfeeding mothers which experience short-term and long-term health benefits (5–8). The World Health Organization recommends exclusive breastfeeding for the first six months after birth, and up to two years with the introduction of complementary foods (9). Unfortunately, due to systemic and structural barriers such as racism, lack of workplace accommodations, and inequitable access to human milk feeding resources, breastfeeding disparities and inequities remain (10–12). In general, breastfeeding initiation and duration rates are higher among Asian and White mothers and lower among Black and Indigenous mothers in the U.S (13).

Vaccination during pregnancy and lactation not only has immune protection for the mother, but also provides immunologic benefits for their child through the transfer of immune factors *in utero* and through human milk. Pregnant women and those who have recently given birth may face increased vulnerability to infections and severe illness (14, 15). Thus, vaccines serve as a critical component of preventative healthcare for pregnant and lactating women and an important public health intervention (16, 17). However, inequities and disparities also extend to vaccinations. Presently, in the U.S., children, adolescents, and adults who are uninsured, living in rural communities, have lower levels of income, and identify as a person of color, experience lower rates of recommended vaccination (18–20). Given the benefits and significance of human milk, lactation, and vaccines across the life course, the barriers need to be addressed to make certain that all mothers and infants, especially those most marginalized, have access to critical resources and supports during the perinatal period.

In this review, we discuss the barriers that need to be addressed to improve equity, and summarize the literature regarding humoral immunity in the human milk after influenza and pertussis vaccinations, as well as the latest data on human milk immunity conferred by the mRNA-based COVID-19 vaccines.

ANTIBODIES IN HUMAN MILK

For the first few months of life, the infant's immune system is immature and they therefore rely on maternal passive immunity for protection and to distinguish pathogenic from commensal bacteria (21). During pregnancy, specific maternal IgG antibodies are transferred from the mother through the placenta to the fetal bloodstream to provide systemic immunity that confers protection for the first few months of infancy. Maternally-derived antibodies gradually decrease during the first year of life while the infant builds protective immune responses through vaccination and early life pathogen exposure (22). After birth, lactating mothers continue to transfer milk-derived antibodies to their newborn which provide passive mucosal immunity. Human milk contains protective immunologic components including immune cells, cytokines, glycoproteins (e.g. lactoferrin), human milk oligosaccharides, and antibodies such as maternal secretory IgA (SIgA), secretory IgM (SIgM), and IgG (21, 23, 24). In humans, mucosal barriers close shortly after birth, and therefore human milk antibodies are prevented from passing into the bloodstream due to decreasing permeability of the gut. As a result, milk antibodies predominately provide mucosal immunity (25, 26).

Serum IgA is a monomer, whereas mucosal IgA is a dimer. The IgA dimers in the mammary gland bind to polymeric immunoglobulin receptor (pIgR) on the basolateral surface of the epithelial cells and travel across the cell to the apical surface (27). There, the external domain of the pIgR bound to the dimeric mucosal IgA is cleaved, and the remaining compound is secreted into the human milk as SIgA (26). SIgA provides first line protection along mucosal surfaces including the respiratory and digestive tracts (27). It has also been shown to be protective against various diarrheal diseases as infants consuming human milk with higher SIgA levels were more likely to be asymptomatic for these diseases (24, 28–31). Pentameric secretory IgM usually produces the primary antibody response to an antigen and activates the complement cascade upon antigen binding. SIgM is delivered to human milk through the same mechanism as SIgA. IgG is the least prominent antibody in human milk. Monomeric IgG from maternal blood is delivered in human milk through binding of the neonatal Fc receptor (FcRn) on epithelial cells in the mammary gland (32–35). Human milk-derived maternal IgG binds intraluminal pathogens in the infant's gut and helps protect against enteric infections (36, 37). Milk antibodies main functions are summarized in **Table 1**.

ANTIBODY COMPOSITION IN HUMAN MILK POST-INFLUENZA AND PERTUSSIS VACCINATION

Influenza Vaccination

Influenza (flu) viruses are RNA viruses (38), that can cause severe illness, particularly in pregnant people who are at high risk for infectious complications leading to hospitalization (39). Influenza vaccines are updated annually to optimize protection against circulating influenza viruses that are predicted to be the most common in the upcoming year (40). Currently in the U.S., inactivated virus quadrivalent vaccines are recommended for pregnant individuals, which protect against four different types of flu viruses. There is year-to-year variability in vaccine efficacy, due to a number of factors including antigenic mismatch, pre-existing immunity, and the limited ability to predict the dominant viruses each year. However, the efficiency of the flu

TABLE 1 | Function and location of human milk antibodies.

Antibodies	Structure	Location	Function
SIgA	Dimer	Mucosal sites including respiratory and digestive tracts	-Intracellular neutralization (forming complexes with the viral proteins) -Virus excretion through transcytosis of immune complexes to the intestine lumen. -Immune exclusion to prevent pathogens penetration.
SIgM	Pentamer	Mucosal sites including respiratory and digestive tracts	-Intracellular neutralization -Ability to activate complement
IgG	Monomer	Primarily in blood	-Pathogenic neutralization including viruses and bacteria

vaccines typically ranges between 50-70% in pregnancy but may be less for other populations (41, 42).

Influenza vaccination during pregnancy leads to a 40% decreased risk of influenza-related hospitalization in pregnant women (43), as well as a significant increase in maternal and infant serum influenza IgG levels (44, 45). In addition, numerous studies have shown a decrease in the incidence of influenza in infants born to vaccinated mothers up to 6 months post-delivery (45–48). This protection is mostly attributed to transplacentally-derived IgG antibodies which are transferred during pregnancy, and wanes in the infants typically three to six months after delivery (44, 45, 49–51). However, in breastfed infants, human milk-derived antibodies may also provide additional layer of influenza protection in the infant during the breastfeeding period. A recent study evaluated longitudinal levels of anti-influenza IgA in human milk, samples were collected from lactating individuals after administering the trivalent inactivated influenza vaccine or a 23-valent pneumococcal polysaccharide vaccine (control) to pregnant women in the third trimester (52). Human milk anti-influenza IgA levels in milk were maintained at a significantly higher level in those who received the influenza vaccine for at least 6 months after delivery compared to controls (52). In addition to IgA, anti-influenza IgM and IgG are also present in milk but at lower levels (53). Human milk also contains varying levels of immune cells including innate cells, memory T cells, and plasma B cells (54–58), but limited data exists on the antigen-specificity of these cells and response to infection or vaccination. A prior study demonstrated influenza-specific CD8 T cells in human milk (57, 59). However, the degree of protection conferred by milk immune cells remains unknown. Breastfeeding exclusivity is associated with lower rate of infant febrile respiratory illness (52, 60) compared to non-exclusively breastfed infant. Additional studies are needed to understand the various factors in milk that confer this protection to infants.

Pertussis Vaccination

Pertussis (also known as whooping cough) is a childhood respiratory illness caused by the bacterium *Bortadella pertussis*. Pertussis (PT) booster immunization during the late second or third trimester of pregnancy is an important public health strategy to reduce the morbidity and mortality from whooping cough in neonates. Since 2010, the pertussis vaccine has been recommended for all pregnant people between 27- and 36-weeks of gestation in order to provide protective antibodies to the fetus for protection against pertussis, in the critical early months of the infant's life when they are most at risk for serious disease (61, 62). In the U.S., this is typically administered through the combined Tdap vaccine, which also provides protection against tetanus, diphtheria, in addition to pertussis (63).

Studies have demonstrated that after maternal vaccination high levels of anti-PT IgG is present in newborn blood due to transplacental transfer from mother to infant (64). After delivery, pertussis-specific IgA as well as IgG are present in colostrum and mature human milk and are detected for at least 8 weeks postpartum after maternal vaccination during pregnancy (65, 66).

The effectiveness of maternal vaccination in infant protection against PT infection at the first months of life ranges from 88 to 93% (67–70). Further, infants with pertussis whose mothers received the TdaP vaccine had lower risks of hospitalization, ICU admission, and shorter hospital stays compared to mothers who were not vaccinated (71). In summary, vaccination with Tdap during or shortly after pregnancy greatly increases the level of anti-PT antibodies in human milk (64–66, 72, 73) and may contribute to the protection provided to the infant against pertussis infection.

IMMUNE RESPONSES IN HUMAN MILK FOLLOWING COVID-19 VACCINATION

BNT162b2 (BioNTech and Pfizer) and mRNA-1273 (Moderna) are mRNA-based vaccines approved by the Food and Drug Administration (FDA) to use against COVID-19 (74, 75). In addition, two vector-based vaccines AZD1222 (Oxford/AstraZeneca) and Ad26.COV2.S (Johnson & Johnson/Janssen) are widely used worldwide (76–80). However, due to the timing of vaccine approval, there is currently limited data on vector-based vaccines in pregnancy and lactation, and for purposes of this review we will focus on mRNA vaccines. BNT162b2 and mRNA-1273 vaccines contain the mRNA sequence of the SARS-CoV-2 Spike protein, coated by a lipid-nanoparticle envelope. Upon administration, the lipid nanoparticles are absorbed by cells, and the mRNA sequence is released into the cytoplasm, where it is translated into Spike protein that is presented on the cell surface of vaccinated cells. This Spike protein is recognized by immune cells to generate a robust and specific immune response against the Spike protein (81, 82). These vaccines have been found to be highly efficient in prevention of severe COVID-19 disease (83, 84) and to be safe for administration during pregnancy and lactation (85–95).

For mothers that were vaccinated while pregnant, their infants had detectable levels of anti-SARS-CoV-2 IgG antibodies in cord blood and in infant follow up blood samples, demonstrating transfer of these IgG antibodies *via* the placenta to the fetal bloodstream (96–98). Similar to influenza and pertussis vaccination during pregnancy (44, 46), SARS-CoV-2 vaccination during pregnancy reduced the risk of infant hospitalization for COVID-19 up to 4-6 months of age by 30-70% (99, 100). In contrast, infants born to mothers vaccinated after pregnancy did not have anti-SARS-CoV-2 IgG in their blood (25, 101). However, COVID-19 vaccination during pregnancy and lactation both elicited transfer of anti-SARS-CoV-2 antibodies to human milk (25, 96, 97, 102–105).

Since SARS-CoV-2 is a novel pathogen, the implementation of COVID-19 vaccines has provided a unique opportunity to understand primary immune responses in human milk to a novel antigen in lactating people. We have summarized multiple studies that have evaluated mRNA vaccination during lactation and human milk antibodies (**Table 2**). Most studies have found an initial increase of milk IgG 14-21 days after the first dose of vaccine, with further robust increased levels peaking at 7 days

TABLE 2 | Summary of various studies evaluating vaccination during lactation and human milk antibodies with regards to the mRNA-1273 and BNT162b2 vaccines including sample size of lactating women, timepoints measured, and mean infant age.

Author	Vaccines	Measured anti-bodies (Ab) in human milk	Sample size (lactating women)	Timepoints	Mean infant age	Findings overall	Findings on milk IgG	Findings on milk IgA
Kelly et al. (102)	BNT162b2	Anti-spike IgG and IgA Ab levels	5	1. Pre-vaccine 2. 10-19 days post vaccination 3. 20-29 days post vaccination 4. 30-39 days post vaccination 5. >40 days post vaccination	9.8 months	-Both IgG and IgA levels were increased post vaccination	-Anti-spike IgG remained significantly increased 20 days post dose 1 to >40 days compared to pre-vaccine levels	-Anti-spike specific IgA were significantly increased 2 weeks post dose 1 to >40 days compared to pre-vaccine levels, although a decreasing level of mean IgA was observed at >40 days post dose 1
Perl et al. (103)	BNT162b2	Anti-spike IgG and IgA Ab levels	84	Pre-vaccine and weekly samples up to 6 weeks after first dose.	10.32 months	-Both IgG and IgA levels remained elevated in human milk 6 weeks post vaccination	-Mean anti-COVID specific IgG levels were low until week 3, and dramatically increased at week 4 and remained elevated at weeks 5 and 6	-Mean anti-COVID specific IgA levels increased significantly at 2 weeks post-first dose, decreased before the 2nd dose, and increased sharply 1 week post-second dose at week 4. -IgA levels remained elevated throughout the rest of the time points although steadily decreased.
Rosenberg-Friedman et al. (104)	BNT162b2	Anti-spike and RBD IgG and IgA Ab levels compared with a pre-pandemic control population	10 healthcare workers	1. 7 days post-first dose 2. 14 days post-first dose 3. 7 days post-second dose 4. 14 days post-second dose	5.13 months	-IgG: IgA ratios were calculated and suggested that IgA was the greatest at all time points, although the ratio increased significantly at 7 and 14 days post second dose, suggesting an increase in IgG over time post second dose. IgG and IgA levels increased at each time point and stopped increasing on 14 days post-second dose. - IgA production rate decreased 14 days post-second dose. IgG peaked at 14 days post-second dose whereas IgA showed a small decline at 14 days post-second dose.	-Anti-spike IgG at 7 days after first dose did not increase significantly compared to the controls, although increased significantly on day 14. Levels peaked on 7 days post second dose. -Anti-RBD IgG had a similar trend as above	-Anti-spike IgA increase significantly compared to controls 14 days after first dose. Levels peaked 7 days after second dose. -Anti-RBD IgA had a significant increase 7 days post second dose compared to controls.
Gray et al. (96)	mRNA-1273 and BNT162b2	Anti-spike and RBD IgG, IgA, and IgM Ab levels	31	1. Before first dose 2. After 1st dose: day of and before the 2nd dose 3. 2-6 weeks post-second dose	7.3 months (median)	-A significant increase of COVID specific IgG, IgA, and IgM was measured after first and after second dose compared to baseline.	-Increase in IgG was measured after second dose suggesting the boost facilitated an increase in transfer of IgG to human milk.	-IgA transfer in human milk did not increase after second dose compared to IgA levels after first dose.
Young et al. (105)	mRNA-1273 and BNT162b2	Anti-RBD IgG and IgA Ab levels	30	1. pre-vaccine 2. 18 days post-first dose	7.5 months	-Both IgG and IgA levels were increased post vaccination	-Large increase in IgG 18 days post-first dose and an additional increase	-IgA levels increased at 18 days post-first dose, and didn't

(Continued)

TABLE 2 | Continued

Author	Vaccines	Measured antibodies (Ab) in human milk	Sample size (lactating women)	Timepoints	Mean infant age	Findings overall	Findings on milk IgG	Findings on milk IgA
				3. 18 days post-second dose 4. 90 days post-second dose			18 days after the second dose. It was followed by a decline at 90 days post-second dose.	further increase post-second dose
Golan et al. (25)	mRNA-1273 and BNT162b2	Anti-RBD IgG and IgA Ab levels in human milk and IgG and IgM in serum	50	1. Pre-vaccine 2. After first dose: day of and before the second dose 3. 4-10 weeks after the second dose	4.7 months (median)	-Both IgG and IgA levels were increased post vaccination -IgG levels were positively correlated between blood and milk between 4-10 weeks after the second dose	-IgG levels increased after the first dose and had a greater increase after the second dose.	- IgA levels significantly increase after the first dose, with no further increase 4-10 weeks after second dose.
Lechosa-Muñiz et al. (79)	BNT162b2, mRNA-1273 and ChAdOx1-S	Anti-RBD IgG and IgA Ab levels in human milk and serum	110	30 days after the second dose of the vaccine (or after first dose for ChAdOx1-S)	15.9 months	Significantly higher levels of IgG and IgA were found after mRNA-based vaccine vs. ChAdOx1-S.		
Selma-Royo et al. (80)	BNT162b2, mRNA-1273 and ChAdOx1	Anti-RBD IgG and IgA Ab levels in human milk	86	pre-vaccination, 1 week, 2 weeks, and 3–4 weeks post the 1st dose of vaccine; 1 week, 2 weeks, and 3–4 weeks post 2nd dose.	11-14.3 months	-Significant increase in IgA and IgG in milk with higher levels after second dose. -Antibody levels depend on vaccine type.	-IgG levels increased after the first dose with greater increase after the second dose.	- IgA levels after vaccination were lower compared to milk from COVID-19-infected women.

after the second dose and remaining elevated for at least 6 weeks (78, 96, 102–105). In most lactating people, 4-10 weeks after the second dose, anti-SARS-CoV-2 IgG levels in milk were still significantly higher compared to their levels before vaccination (25, 105). Additionally, IgA levels generally peak at 14-18 days after the first dose, increase slightly for one week after the second dose, but decrease thereafter (96, 102–105). In contrast to the significant increases in IgG levels after the second dose, studies have shown that IgA levels in milk do not rise further when measured > 18 days after the second dose (25, 101, 105).

Studies on the association between blood and milk levels after SARS-CoV-2 vaccination during lactation have found a positive correlation between serum and human milk SARS-CoV-2 IgG levels measured at 4-10 weeks after second dose (25, 106). Interestingly, one study measured milk IgG and IgA in pregnant women who were vaccinated for both SARS-CoV-2 and TdaP during pregnancy and found similar levels between anti-Spike (SARS-CoV-2) antibodies and anti-tetanus toxoid (TT) antibodies (104). These findings further strengthen our knowledge about the mechanism and absolute level of transferred IgG antibodies from the serum to human milk *via* FcR transfer in the mammary gland (107).

Mothers who were infected with COVID-19 during pregnancy or lactation had a universally rapid anti-SARS-CoV-2 IgA secretion in human milk, lasting >90 days after diagnosis. In contrast, vaccination during pregnancy or lactation results in a robust anti-SARS-CoV-2 IgG secretion to milk with a less dominant IgA response (97, 105, 107–109). Though antibody functional responses may be similar after SARS-CoV-2 vaccination vs infection, as was demonstrated by comparable levels of neutralizing antibodies (97, 105). These findings suggest that exposure through natural infection leads to increased secretion of mucosal related IgA antibodies in mucosal organs, such as the mammary gland, which may be a distinct immune response than what is generated after mRNA-based vaccines. In animal models, additional intranasal vaccination induces mucosal boost immunity in addition to the systemic immunity that is induced after mRNA-based vaccines (110). Approaches

boosting mucosal immunity may be useful to increase secretion of antibodies to human milk, however further research is needed in this area.

Similar to other vaccinations and infections, there is limited data on the presence SARS-CoV-2 antigen-specific human milk immune cells on infant protection against disease (111). Using animal models, it was shown that cells from milk can survive the digestive tract and can traffic into infant organs (112, 113). Interestingly two recent studies have demonstrated the presence of SARS-CoV-2 specific Spike-reactive T cells in human milk after vaccination (111, 114). However, it is unknown if human milk cells provide immune protection to the respiratory tract or gastrointestinal tract of human infants or if they are taken up in the infant gut into systemic circulation. The role of these antigen-specific immune cells in human milk in regard to infant protection requires further study.

Protection of Infants

Further studies are needed to evaluate the protective effects of breastfeeding and milk SARS-CoV-2 antibodies against COVID-19 infection in infants. Exclusively breastfed infants usually consume human milk every 1-3 hours, providing them with frequent doses of milk antibodies. Upon weaning, milk antibodies decay rapidly in the infant, and this mode of passive immunity ends. Neonates and infants with COVID-19 often present with gastrointestinal symptoms (115). However, there is limited information to date on whether SARS-CoV-2 achieves gastrointestinal viral invasion or whether SARS-CoV-2 causes bystander mucosal inflammation that contributes to these symptoms (116–118). Interestingly, anti-SARS-CoV-2 IgA and IgG have been detected in one-third of 24 infant stool samples after maternal vaccination (119). Further studies are needed to determine the impact of local mucosal protection by human milk derived SARS-CoV-2 antibodies in the infant gut.

COVID-19 Vaccines Safety During Lactation

COVID-19 vaccination for lactating women is recommended by the Centers for Disease Control and Prevention (CDC) to reduce the risk of complications from COVID-19, and the World Health Organization (WHO) recommends continuing of breastfeeding after vaccination (120, 121). Maternal vaccination during lactation protects the mother from severe COVID-19 disease and as discussed above may also protect the infant. A large survey-based study including over 10,000 lactating individuals found minimal disruption of lactation after vaccination (around 2% of the individuals), with 6% of individuals reporting decrease in milk supply (122). Reduction in milk supply was reported in 5-7% of the women, which was more common after the second dose. Most symptoms resolved within 24-72 hours after vaccination (25, 122, 123). Symptoms in the breastfed infant in the short term after maternal vaccination were reported in 2-7% the cases, with sleepiness and fussiness being the most common symptom (25, 122, 123). Other symptoms such as fever and gastrointestinal symptoms were reported in 1-2% of the infants (25, 122, 123). Few studies examined transfer of vaccine particles to human milk after vaccination (101, 124) and found minimal transfer of vaccine mRNA to human milk in less than 2% of the samples (out of 309 samples examined). In addition, a single study measured polyethylene glycol (PEG) which is present in the lipid nanoparticles of the mRNA-based vaccines in milk and found no significant increase in PEG in milk after vaccination (25). It is not clear whether the infant symptoms reported are specifically related to vaccine particle transfer, and further research is needed in this area. There is a lack of clinical trials that carefully examine infant side effects after vaccination in this vulnerable population of lactating dyads. Future trials should include these populations and outcomes. However, based on the data collected so far in multiple prospective studies, the benefits of vaccination outweigh the risk for mother and her infant.

COVID-19, Lactation, and Equity Issues

Despite the known maternal and infant health benefits of breastfeeding and vaccination, significant inequities persist among the most vulnerable groups that are presented with unique challenges to lactation support and vaccine access. There are a lack of studies examining barriers to breastfeeding during the COVID-19 pandemic. Access to commercial tele-lactation companies offering online lactation support is limited especially for those who have lost their jobs and may not be able to afford lactation or internet services (117). It is essential to provide resources to the communities and populations purposively marginalized. For instance, in certain parts of large cities with previous inequitable health care access, such as the South Side of Chicago, the COVID-19 pandemic has exacerbated the reduction of open hospitals (118). Hospitals are typically the primary source of breastfeeding education and in communities with already low rates of breastfeeding. Barriers of marginalized populations are being aggravated rather than reduced during the pandemic. There is also inadequate funding to lactation services in institutions and agencies. Addressing systemic and structural barriers and increasing funding to lower resourced communities can begin to reduce health care disparities by providing essential services, such as open hospitals and consistent breastfeeding education so that families understand the short- and long-term importance of vaccination and breastfeeding.

DISCUSSION

Future Directions

The studies presented here have demonstrated the benefits of influenza, pertussis, and SARS-CoV-2 vaccination for pregnant and lactating individuals and the presence of anti-pathogens antibodies in human milk following vaccination. Further epidemiological studies are needed to determine the level of disease protection to infants against COVID-19 provided by maternal vaccination through human milk. Additionally, the quantity of human milk required to be ingested to confer a protective effect in an infant is unknown. To address this question, detailed study of infant feeding patterns is needed to distinguish between various quantities and patterns of human

milk consumption. Current studies usually compare only exclusively breastfed to nonexclusively breastfed infants as a group. In addition, studies that measure the durability of milk antibodies in infant mucosal surfaces, such as the oropharynx, are necessary to better understand the protection of milk antibodies against pathogens that are transmitted *via* these organs.

Longitudinal studies to evaluate the persistence of human milk antibodies after vaccination, and the effect of a third and fourth mRNA-based vaccine doses on human milk are needed. Similarly, long-term follow up on infants of COVID-19 vaccinated mothers is needed as the pandemic evolves to provide more data on protection of these infants with continued breastfeeding.

In summary, while the immunologic benefits of breastfeeding have long been promoted, there is still much to learn regarding the dynamics of immune responses during lactation. More work is needed to understand the precise mechanisms of immune protection seen in breastfed infants. However, the potential benefits of breastfeeding and human milk are nullified if there is not equitable access and support for lactation, particularly in vulnerable communities. Research and financial support for qualitative studies and community-engaged programs are needed to improve advocacy for education and resources in lactating communities of color.

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EDITED BY
Veronique Demers-Mathieu,
Exagen, Inc., United States

REVIEWED BY
Antti E Seppo,
University of Rochester, United States
Larissa Bahls-Pinto,
State University of Maringá, Brazil

*CORRESPONDENCE
Andrea V. Gamarnik
agamarnik@leloir.org.ar
Diego S. Ojeda
dojeda@leloir.org.ar

†These authors have contributed equally to this work

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SARS-CoV-2-Specific IgG and IgA response in maternal blood and breastmilk of vaccinated naïve and convalescent lactating participants

Yesica Longueira[1†], Diego S. Ojeda[2,3†*], Rocio B. Antivero Battistelli[1,3], Lautaro Sanchez[2], Santiago Oviedo Rouco[2], Daniel Albano[4], Eleonora Guevara[4], Vanesa Valls[4], María A. Pando[1,3] and Andrea V. Gamarnik[2*]

[1]CONICET – Universidad de Buenos Aires, Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS), Buenos Aires, Argentina, [2]Laboratorio de Virología Molecular, Fundación Instituto Leloir-CONICET, Buenos Aires, Argentina, [3]Universidad de Buenos Aires, Facultad de Medicina, Departamento de Microbiología, Parasitología e Inmunología, Buenos Aires, Argentina, [4]Banco de Leche Humana – Hospital Materno Infantil Ramón Sardá, Ciudad Autónoma de Buenos Aires, Argentina

Background: Recent studies have shown the presence of SARS-CoV-2-specific antibodies in the milk of breastfeeding mothers vaccinated with mRNA and convalescent. However, limited information is available in lactating women receiving other vaccine platforms used in developing countries, such as the inactivated SARS-CoV-2 vaccine BBIBP-CorV (Sinopharm) and the non-replicating adenovirus vaccines Sputnik V (Gamaleya Institute) and ChAdOx1-S (Oxford AstraZeneca).

Methods: Here, we evaluated anti-SARS-CoV-2 IgG and IgA levels in both serum and milk samples using a longitudinal and a cross-sectional cohort of 208 breastfeeding vaccinated women from Argentina with or without previous SARS-CoV-2 infection.

Results: The analysis showed that IgA levels remain constant in serum and milk of breastfeeding mothers between the first and second doses of vector-based vaccines (Sputnik V and ChAdOx1-S). After the second dose, anti-spike IgA was found positive in 100% of the serum samples and in 66% of breastmilk samples. In addition, no significant differences in milk IgA levels were observed in participants receiving BBIBP-CorV, Sputnik V or ChAdOx1-S. IgG levels in milk increased after the second dose of vector-based vaccines. Paired longitudinal samples taken at 45 and 120 days after the second dose showed a decrease in milk IgG levels over time. Study of IgA levels in serum and milk of vaccinated naïve of infection and vaccinated-convalescent breastfeeding participants showed significantly higher levels in vaccinated-convalescent than in participants without previous infection.

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Conclusion: This study is relevant to understand the protection against SARS-CoV-2 by passive immunity in newborns and children who are not yet eligible to receive vaccination.

KEYWORDS

SARS-CoV-2, breastmilk, COVID-19 vaccine, immune response, Sputnik V, BBIBP-CorV, ChAdOx1-S

Introduction

The World Health Organization recommends exclusive breastfeeding for six months and to continue breastfeeding for two years or more due to the great benefits on babies' and mothers' health (1). Along with the transfer of enough nutrients to satisfy growth requirements during the first months, human milk contains both adaptive and innate immune components. In particular, breast milk immunoglobulins are essential players during the maturation of the newborn's immune system and provide protection against pathogens (2). Research studies have shown a high concentration of immunoglobulins in breast milk also during prolonged lactation (4 years) (3). Human milk antibodies are derived primarily from B cells primed in the mucosa, resulting in high concentrations of secretory antibodies that offer a prolonged period of immune transfer to confer immunity against mucosal pathogens such as respiratory syncytial virus, pneumococcus, influenza, and meningococcus (4, 5). In particular, IgA is the dominant antibody that is transferred to infants through breast milk and is thought to play a critical role in mucosal defense (6, 7).

During the global spread of Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), studies have shown that milk produced by infected mothers contains detectable levels of anti-SARS-CoV-2 IgA and IgG during and after acute infection (8–12).The presence of these specific antibodies potentially provides passive immunization to the infant (13, 14). However, SARS-CoV-2 infection during pregnancy was associated with an increased risk of a composite outcome of maternal mortality or serious morbidity from obstetric complications (15). This highlights the importance of vaccination, since vaccines induce a strong antibodies production by pregnant women.

Although vaccination against COVID-19 is the most effective way to prevent SARS-CoV-2 infection and transmission, pregnant and breastfeeding women were not included in the original vaccine trials. However, as this group has been associated with high rates of preterm birth and neonatal morbidity (16, 17), pregnant and lactating women were included in subsequent vaccination trials. Recommendations to prioritize these groups are supported by the effectiveness (18, 19) and safety (20–23) of different COVID-19 vaccines. Several studies evaluated the presence of anti-SARS-CoV-2 IgA and IgG in the breast milk of lactating mothers vaccinated with mRNA and non-replicating adenovirus vaccines (24–30), however, scarce information is available with inactivated virus platforms widely used in many regions of the world (31).

Different anti-SARS-CoV-2 vaccines are currently used in Argentina, including the non-replicating adenovirus vaccines Sputnik V (Gamaleya Institute), ChAdOx1-S (Oxford AstraZeneca), and Ad5-nCoV (CanSino); the mRNA vaccines BNT162b2 (Pfizer) and mRNA-1273 (Moderna); and the inactivated SARS-CoV-2 vaccine BBIBP-CorV (Sinopharm). As of today, August 2022, 91% of the total population have received at last one dose of the COVID-19 vaccine, 84% have received two doses and 60% have received three or four doses. The national vaccination plan included the entire population from 3 years of age. Unfortunately, stratified data on vaccination coverage by age is not available (32). Due to the lack of information regarding immunogenicity in breast milk in lactating women after the application of vaccine platforms based on viral vectors (Sputnik V and ChAdOx1-S) or inactivated viruses (BBIBP-CorV), we evaluated the presence of specific IgA and IgG anti-SARS-CoV-2 in maternal blood and breast milk of vaccinated lactating participants without prior infection and convalescent lactating participants who were vaccinated with diverse vaccine platforms.

Material and methods

Population

Breastfeeding mothers from the Human Milk Bank (HMB) at the Hospital Materno-Infantil Ramón Sardá were donors in this study. We expanded this cohort with volunteers outside the HMB, including breastfeeding women that were enrolled by social network advertisements. Serum and breast milk samples were obtained from lactating mothers before and after

vaccination against SARS-CoV-2. From February 2021 to February 2022, samples were taken from 226 breastfeeding women from Buenos Aires City and surroundings. During the study period, vaccination for COVID-19 advanced substantially in Argentina, registering two waves of contagion (May-June 2021 and January 2022). Of this, 171 naïve participants received one or two doses of the vaccines available at that time in Argentina (Sputnik V, ChAdOx1-S or BBIBP-CorV). None of these vaccinated mothers reported clinical COVID-19 infection before immunization. Participants were also separated as naïve or convalescent by measuring the presence of anti-nucleocapsid antibodies. Additionally, we evaluated the National COVID-19 Surveillance System, which includes many asymptomatic cases tested during surveillance and as close contacts of symptomatic cases, to identify if there were previously infected volunteers (33).

For this longitudinal and cross-sectional study, 208 breastfeeding mothers' serum and breast milk samples were analyzed. We evaluated three data sets. Al first, we analyzed 44 paired serum and breast milk samples from 22 vaccinated breastfeeding mothers without prior SARS-CoV-2 infection. In this case, the samples were obtained longitudinally after the first and second dose of Sputnik V and ChAdOx1-S vaccine application. Then, we analyzed a longitudinal cohort of 27 naïve vaccinated mothers with two doses of Sputnik, ChAdOx1-S or BBIBP-CorV vaccines. These samples were collected as a function of time after the second dose, at 40 and 120-day after completing the vaccination schedule. Finally, the third group was a cross sectional cohort composed of 122 vaccinated mothers without previous SARS-CoV-2 infection (Sputnik V, N=32, ChAdOx1-S, N=45 and BBIBP-CorV N=45), in addition this group included 26 vaccinated convalescents, with SARS-CoV-2 infection confirmed by molecular diagnosis before vaccination and 11 convalescents non vaccinated breastfeeding mothers (Figure 1).

Clinical data collection

Inclusion criteria included women ≥ 18 years of age who were breastfeeding at any infant age. Data collected included age of mother and infant, vaccine type (Sputnik V, ChAdOx1-S and BIBP-CorV), vaccination dates and history of SARS-CoV-2 infection. Ethical approval was obtained from the Institutional Review Board (IRB) of the Faculty of Medicine of Buenos Aires University (Comité de Ética en Investigación Biomédica, Instituto Alberto C. Taquini de Investigaciones en Medicina Traslacional (IATIMET), Facultad de Medicina, Universidad de Buenos Aires). Informed consent was obtained from all study participants.

Sample collection and processing

Mothers were virtually instructed by study staff in clean techniques to obtain milk samples. Women collected the milk in sterile containers that were immediately frozen until shipment in a cooler to INBIRS (Instituto de Investigaciones Biomédicas en Retrovirus y Sida). Once at the laboratory, human milk samples were stored at −20 ∘C until use. The volunteers assisted to the laboratory were blood was drawn.

Samples consisted of 15 mL of milk and 5 mL of venous blood without anticoagulants. Both types of samples were collected on the same day. Blood was centrifuged at 2500

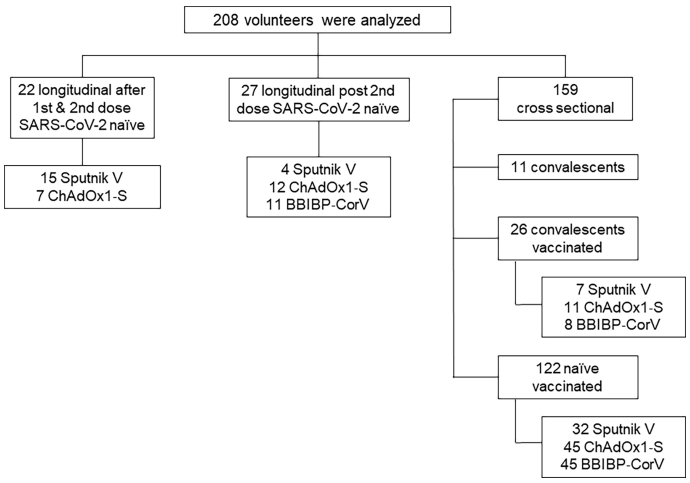

FIGURE 1
Flowchart of the study cohort. For longitudinal analysis, the geometric mean time interval for the samples obtained was 65 and 21 days after first and second doses of Sputnik V and ChAdOx1-S vaccines.

revolutions per minute (rpm) for 10 min at room temperature, and sera were aliquoted in cryogenic vials and stored at −20 ∘C until use. Prior to processing, breast milk samples were thawed centrifuged at 1500 rpm for 15 min, fat was removed, and supernatant was transferred to a new tube. Centrifugation was repeated 2× to ensure removal of all cells and fat, and the supernatant was aliquoted into cryogenic vials and stored at −20 ∘C until use. All serum and breast milk samples were tested in parallel on two different SARS-CoV-2 antibodies testing platforms, which are described in detail below. Evaluation of possible previous asymptomatic SARS-CoV-2 infection was assessed by measuring the presence of IgG anti nucleocapsid by ELISA.

Detection of specific SARS-CoV-2-antibodies in serum and breast milk

Antibodies to SARS-CoV-2 spike protein were detected using an established commercially available two-step ELISA (COVIDAR) for IgG in serum samples. We have previously described the development of the ELISA for IgG in serum samples (34). Modifications of the ELISA for IgA in serum and IgG/IgA in breast milk samples are described below. Serum and breast milk samples diluted in PBS-T containing 0.05% Tween and 0.8% casein were added to the plate (200 µl of a 1:50 dilution for IgG and IgA determination in serum and 200 µl of a 1:8 dilution for IgG and IgA in breast milk), and incubated for 1 h at 37°C for serum samples and for 2h at 24°C for breast milk samples. Following a washing step with PBS-T, 100 µl of diluted horseradish peroxidase (HRP)-conjugated with goat anti-human IgA (Sigma), or with mouse anti-human IgG antibodies (BD pharmingen), was added to plates and incubated for 30 min at 37°C for serum, or 1 h at 37°C for milk. The conjugated monoclonal antibody used for human IgG detection in the COVIDAR ELISA is G18-145, which specifically binds to the heavy chain of all four human immunoglobulin G subclasses: IgG1, IgG2, IgG3, and IgG4. The conjugate employed for IgA detection in human specifically binds to α-chain specific of human immunoglobulin A (SIGMA, Cat A0295-1ML). Subsequently, the plates were washed with PBS-T, and the peroxidase reaction was visualized by incubating the plates with 100 µl of TMB solution for 30 min. at 37°C for serum samples and for 1 h at 24°C in breast milk samples. The reaction was stopped by adding 100 µl of 1M sulfuric acid, and optical densities (OD) were immediately measured at 450 nm. Cut-off for serum and breast milk samples resulted from the mean of OD450 values from negative controls plus 3 times the standard deviation. All the assays, IgG and IgA determinations in serum and breast milk samples, were performed simultaneously with the same plate batch. The IgG concentration of serum sample, expressed in international units per milliliter (BAU/ml), was calculated by extrapolation of the optical density at 450 nm (OD450) on a calibration curve built using serial dilutions of the WHO International Standard for anti-SARS-CoV-2 immunoglobulin.

IgG antibodies against SARS-CoV-2 nucleocapsid protein were detected using a in house two-step ELISA test. The assay uses plates coated with 100 ng of the full-length nucleocapsid protein, expressed in E. Coli and purified using HisTrap excel columns and the conjugated monoclonal antibody was the same as the one used for COVIDAR. The assay was validated using a panel of 170 healthy blood donors obtained pre-pandemic as negative control. The cut off was set as the mean of negative control plus 3 standard deviations and was defined to maximize specificity (Figure S1A). We measured anti SARS-CoV-2 nucleocapsid IgG of serum samples included in this study (Figure S1B). Samples from individuals vaccinated with Sputnik V or ChAdOx1-S yielded negative results. In addition, convalescent, vaccinated convalescent and vaccinated with BBIBP-CorV groups yielded 100, 92 and 88% positive results, respectively (see Figure S1).

Quantification and statistical analysis

All statistical tests and plots were performed using GraphPad Prism 8.0 software. Comparisons of antibody concentration were made using two-tailed Wilcoxon matched-pair test in Figures 2, 3. Comparison on non-paired determinations of antibody concentration was made using the One Way ANOVA Krustal-Wallis test in Figure 4. Statistical significance is shown in the figure legends with the following notations: ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant. Geometric means with 95% confidence intervals were calculated for Figures 2–4. Spearman correlation coefficient was used to calculate correlations between serum and human breast milk IgG and IgA. A two-tailed p-value lower than 0.05 was considered as significant.

Results

IgA levels are constant between the 1st and 2nd doses of adenoviral-based vaccines in serum and breast milk of lactating women

We evaluated SARS-CoV-2-specific IgG and IgA responses in human serum and breast milk paired samples of 22 volunteers without prior SARS-CoV-2 infection after one and two doses of Sputnik V (N=15) or after one or two doses of ChAdOx1-S (N=7). Application of the second dose increased the IgG level in both serum and breast milk samples ($p<0.0001$ and $p<0.001$ respectively) (Figure 2). In serum, the IgG levels (measured as geometric means by OD at 450 nm, GMOD) were 1.1 after the first dose, and 2.6 after the second dose (95% confidence interval [CI], 0.8 to 1.5 and 2.1 to 3, respectively); and in breast milk, the levels were 0.21 after the first dose and 0.61 after the second dose

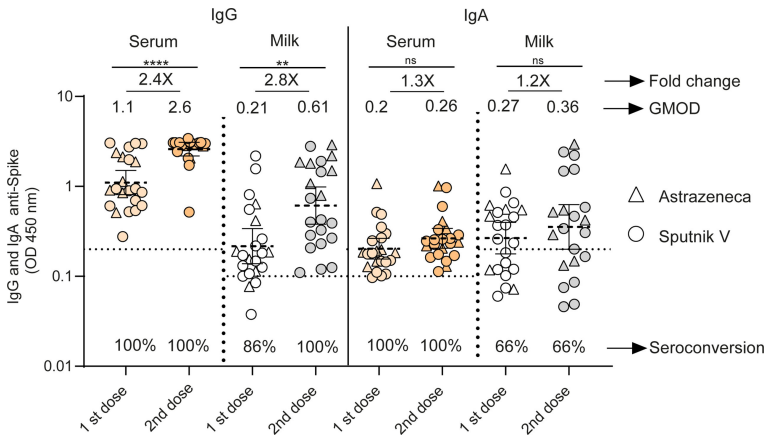

FIGURE 2
Longitudinal antibodies measurements between the 1st and 2nd doses of adenoviral-based vaccines in serum and breast milk of lactating women. Anti-spike IgG and IgA antibody levels measured as geometric means by OD at 450 nm receiving Sputnik V C1 and C2 vaccine (Gamaleya, N= 15) and ChAdOx1-S vaccine (AstraZeneca, N= 7). Cut-off for serum and breast milk samples resulted from the mean of OD450 values from negative controls plus 3 times the standard deviation and is shown as dotted line. Samples were obtained as a function of time at 65 (95%CI: 54 to 79 days) and 21 (95%CI: 18 to 26 days) days after first and second dose of Sputnik V and ChAdOx1-S vaccines. Wilcoxon matched-pair test was used. Statistical significance is shown with the following notations: ****, $P < 0.0001$; **, $P < 0.01$; ns, not significant.

(95% CI, 0.13 to 0.33 and 0.37 to 0.98, respectively). The increase in IgG was statistically significant (2.4- and 2.8-fold in serum and milk, respectively). In contrast, IgA level remained constant in serum and breast milk between the two doses (Figure 2). The seroconversion of IgG was 100% in serum and milk after the second dose, while the seroconversion of IgA in serum and milk was 100 and 66%, respectively.

Vaccination in breastfeeding women is associated with sustained IgA level in milk over time

We then evaluated IgG and IgA responses across both compartments in 27 paired samples from lactating women without prior SARS-CoV-2 infection. For this group, samples

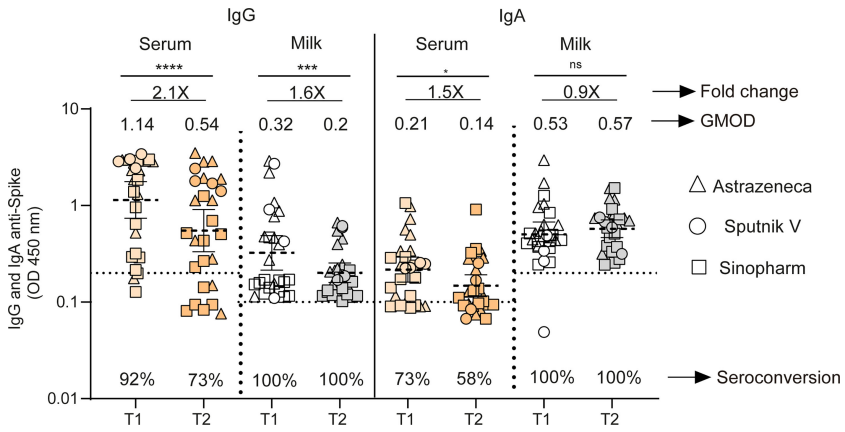

FIGURE 3
Longitudinal antibodies measurements in fully vaccinated breastfeeding without previous infection. IgG and IgA anti-spike antibody levels measured as geometric means by OD at 450 nm in naïve mothers receiving Sputnik V C1 and C2 vaccine (Gamaleya, N= 4), ChAdOx1-S vaccine (AstraZeneca, N= 12) and BBIBP10 CorV vaccine (Sinopharm, N=12). Samples were obtained as a function of time at 44 (T1) and 120 (T2) days after second doses of Sputnik V, ChAdOx1-S and BIBP-CorV vaccines. Cut-off for serum and breast milk samples resulted from the mean of OD450 values from negative controls plus 3 times the standard deviation and is shown as dotted line. Wilcoxon matched-pair test was used. Statistical significance is shown with the following notations: ****, $P < 0.0001$; ***, $P < 0.001$; *, $P < 0.05$; ns, not significant.

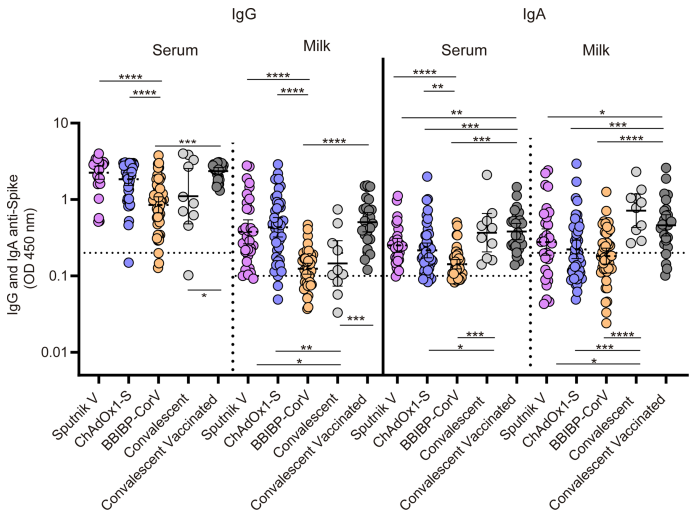

FIGURE 4
Cross sectional analysis of antibody responses in serum and breast milk in vaccinated naïve, vaccinated convalescent and convalescent breastfeeding women. IgG and IgA anti-spike antibody levels measured as geometric means by OD at 450 nm. Cut-off for serum and breast milk samples resulted from the mean of OD450 values from negative controls plus 3 times the standard deviation and is shown as dotted line. Samples were obtained at 30 (95%CI: 23 to 47 days), 35 (95%CI: 29 to 42 days) and 52 (95%CI: 45 to 62 days) days after second dose in mothers without previous SARS-CoV-2 infection vaccinated with Sputnik V, ChAdOx1-Sand BIBP-CorV respectively. In addition, samples from convalescents vaccinated and convalescents non vaccinated breastfeeding mothers were obtained at 36 (95%CI: 29 to 46 days) days after second dose and at 163 (95%CI: 93 to 285 days) days after symptoms onset. For no paired samples analysis, Kruskal-Wallis One-Way ANOVA was performed to compare antibody response. Statistical significance is shown with the following notations: ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

were obtained as a function of time at 44 (95% CI 34 to 56 days) and 120 (95% CI 114 to 124 days) days after second doses of Sputnik V, ChAdOx1-S and BIBP-CorV vaccines. For the vaccinated participants, the IgG level waned significantly across both serum and breast milk, showing a 2.1- and 1.6-fold decrease over time, respectively ($p<0.0001$ and $p<0.001$). IgA level declined slightly over time in serum, while it was sustained in breast milk (Figure 3). In addition, the seropositive rate declined in circulating IgG and IgA, while no significant changes in this rate was observed in breast milk compartments over time after the second dose (Figure 3).

Antibody responses in serum and breast milk in vaccinated naïve, unvaccinated convalescent and vaccinated convalescent breastfeeding women

The IgG and IgA responses in both serum and breast milk after the second dose of Sputnik V (N=32), ChAdOx1-S (N=45) and BIBP-CorV (N=45) vaccine application were compared with those of unvaccinated convalescent (N=11) and convalescent fully vaccinated (N=26) breastfeeding women. IgG levels in serum and milk from participants receiving the adenoviral-based vaccines reached higher levels than those observed in those vaccinated with inactivated SARS-CoV-2 Sinopharm vaccine (Figure 4). Specific-IgA response in breast milk showed no significant differences after vaccination with Sputnik V, ChAdOx1-S or BIBP-CorV. The subset of vaccinated mothers with previous SARS-CoV-2 infection showed the highest IgG level in serum and milk. Finally, a robust IgA response in both serum and breast milk was evidenced in unvaccinated and vaccinated convalescent mothers, showing a significant difference compared to vaccinated naïve participants (Figure 4).

Correlation of SARS CoV-2 specific IgG and IgA antibodies in paired breast milk and serum samples

Comparison of paired SARS CoV-2 IgG antibodies in serum and breast milk shows high correlation in the two groups analyzed: vaccinated naïve (IgG correlation coefficient $r = 0.73$, $P < 0.0001$; Figure 5A) and convalescents volunteers (IgG correlation coefficient $r = 0.66$, $P < 0.0001$; Figure 5A). In contrast, low correlation was observed when specific IgAs were analyzed in vaccinated naïve volunteers (IgA correlation coefficient $r = 0.20$, $P = 0.0062$; Figure 5B) and convalescents volunteers (IgA correlation coefficient $r = 0.23$, $P = 0.05$; Figure 5B).

Discussion

Comparison of SARS-CoV-2-specific antibodies in breast milk and serum after vaccination has been mainly described for mRNA and non-replicating adenovirus vaccines (24–30) used in most developed countries. However, little information was available on widely used inactivated virus platforms in many regions of the world (31). This study provides data about longitudinal antibody responses to adenoviral-based vaccines (Sputnik V and ChAdOx1-S) and inactivated SARS-CoV-2 vaccine (BIBP-CorV) in SARS-CoV-2 naive and previously infected breastfeeding mothers.

We observed sustained levels of IgA between the first and second doses of adenoviral-based vaccines Sputnik V and ChAdOx1-S. These levels were maintained over time up to 120 days after vaccination. We also showed that the IgA response in breast milk did not show significant differences after vaccination with Sputnik V, ChAdOx1-S or BIBP-CorV. In contrast, the adenoviral-based vaccines achieved higher IgG levels than those observed in individuals vaccinated with the inactivated BBIBP-CorV vaccine in both serum and milk. Regarding previously infected volunteers, convalescent and vaccinated convalescents lactating women showed a robust IgA response in both serum and breast milk compared to unvaccinated volunteers. Also, vaccinated mothers with previous SARS-CoV-2 infection showed the highest IgG level in serum and milk.

We observed that the IgG levels increase after the second dose of the Sputnik V and ChAdOx1-S, with 100% of the participants showing IgG positivity in milk and serum. Similar observations were previously reported for both, mRNA and adenoviral based vaccines (27, 28). This rapid increase of IgG after the second dose is consistent with a specific B lymphocyte memory that will prime a faster response with higher antibodies levels (33). In contrast, we observed that the IgA levels remained constant between the two doses, as it was reported in previous studies with adenoviral-based vaccines (Ad26.COV2.S and ChAdOx1-S) (27, 28, 35). Heterogeneous dynamics in IgG and IgA antibody levels can be associated to their diverse functions. IgA shows a key role dominated in the early SARS-CoV-2–specific antibody response and IgG is predominantly important in the secondary immune response (36).

Analysis of paired longitudinal samples taken at 45 and 120 days after second vaccination dose showed that, while IgG levels waned over time in milk, the IgA levels were maintained and 100% of the participants displayed IgA in milk. We detected a

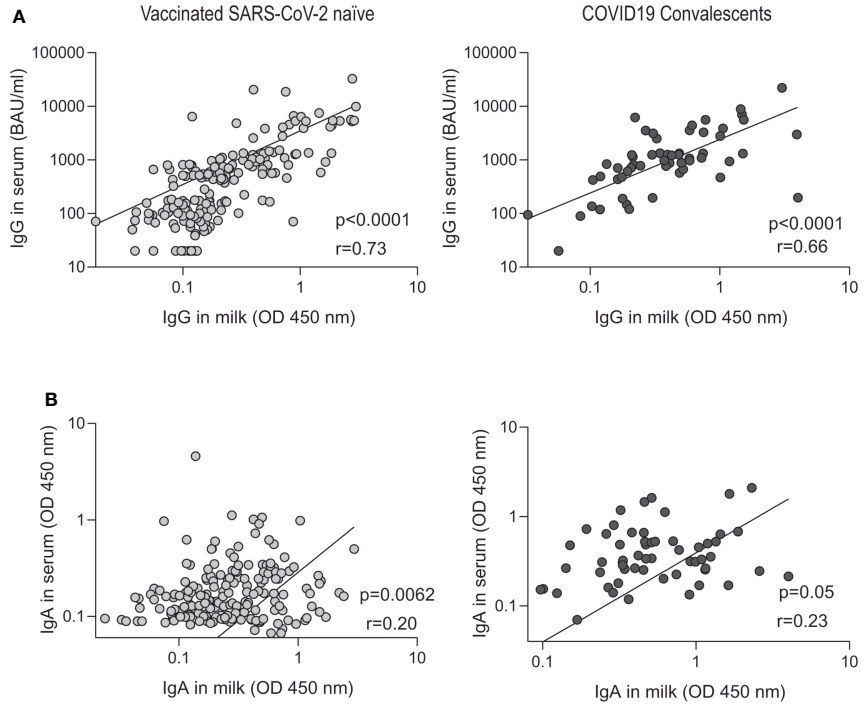

FIGURE 5
Correlation between paired breast milk and serum SARS-CoV-2-specific antibody in vaccinated naïve and convalescent breastfeeding women. Correlation of IgG (quantified according to the WHO International Antibody Standard in serum) and IgA anti-spike antibody levels measured by OD at 450 nm in vaccinated naïve (n=122) and convalescents plus vaccinated convalescents (n = 47). The specific IgG **(A)** and IgA **(B)** serum levels are correlated to the breast milk levels. In the inset the r Spearman and p values from linear regression are shown.

slight reduction of IgA titers in serum relative to paired breast milk samples obtained 120 days after the second dose of Sputnik V, ChAdOx1-S and BBIBP-CorV vaccines, suggesting a more sustained IgA level in mucosal secretions. Previous studies observed that IgA antibody levels slightly decreased 70 days after the second dose of mRNA vaccine administration (37). It is important to mention that other studies showed a decrease in IgA after 90 days of a second dose of the inactivated SARS-CoV-2 vaccine (30). In contrast to that observed for IgA, a significant decreased was observed in IgG levels both in serum and breastmilk pared samples. These results are in agreement with previous studies using mRNA-based vaccines that showed increased IgG levels after the first and second doses, with a significant reduction thereafter (35, 37, 38).

A cross sectional study with 159 samples showed that the mean IgA levels in the milk of breastfeeding women who received three different vaccine platforms were similar. No significant differences were observed in IgA levels after the application of technologies based on vectors or viruses inactivated vaccines. When this cohort was compared to samples from convalescent or convalescent/vaccinated participants, a significant difference in milk IgA levels was observed, indicating that infection results in a higher IgA response. In agreement with previous studies, COVID-19 convalescents were associated with an elevated IgA response in human milk and these levels were higher than those observed in vaccinated groups (37, 39). Furthermore, the IgA response in milk was not significantly different when convalescents unvaccinated and convalescents vaccinated participants were compared.

Regarding the IgG response in breast milk, vaccination of infected volunteers resulted in a strong and long-term IgG response. Differences in milk IgG levels were observed with all three vaccines platforms, showing higher levels when the vector-based vaccines were used compared to the inactivated virus vaccine. Previous studies have demonstrated greater IgG levels in the serum of volunteers (general population cohort) vaccinated with adenoviral vector vaccines when compared to that with virus inactivated vaccines (40–42). However, there are no reports of antibody response comparing adenoviral vector-based and inactivated virus vaccines in paired samples of milk and serum from lactating mothers. Regarding convalescent volunteers, significantly lower levels of IgG were detected in milk and serum in unvaccinated convalescents compared to vaccinated convalescents. In this regard, previous studies also provided data showing that IgG levels in vaccinated SARS-CoV-2 naïve and vaccinated convalescents mothers were higher than those observed in unvaccinated convalescents (28, 43).

We also found a higher correlation with IgG than with IgA in breast milk and serum paired samples, which demonstrates a greater accumulation of IgA in breast milk as reported in other studies with mRNA vaccines (7). This lack of IgA correlation between serum and breast milk is in agreement with the results shown in Figures 2, 3 and with previous studies demonstrating that the IgA response was greater in breastmilk than in serum (44). Even more, these results are consistent with a previous work that demonstrated that IgA in breast milk is produced by plasma cells that are accumulated in the lactating mammary glands (45). These plasma cells are primed in the lymph nodes and in the Peyer's patches of the mucosal tissues and home to the mammary glands in lactation (46). A similar Spearman´s correlation coefficient was observed for IgG in serum and milk samples from vaccinated and convalescent donors.

This study includes the largest cross-sectional analysis of human serum and breast milk samples after Sputnik V, ChAdOx1-S and BIBP-CorV vaccination in breastfeeding women compared with unvaccinated-convalescent and vaccinated-convalescent participants. Our data add to previous information generated with mRNA vaccine platforms to support the idea that SARS-CoV-2 vaccination in lactating women provides passive immunity for the recipient infant against this virus.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Comité de Ética en Investigación Biomédica del Instituto Alberto C. Taquini de Investigaciones en Medicina Traslacional (IATIMET), Facultad de Medicina. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AG, MP and DO are the principal investigator, designed and performed research, and coordinated the study. DA, VV and EG recruit's mothers included in the Human Milk Bank of the Hospital Materno Infantil Ramón Sardá´s as regular donors to participated in the study. RB recruit the volunteers through social networks advertisements. YL, RB and MP coordinated the study. YL, RB and MP were involved in sample processing and collection. DO and LS were involved in ELISA protocols modifications for IgA in serum and IgG/IgA in breast milk samples. DO and SR validated ELISA anti-nucleocapsid IgG. DO and LS built of the data base. DO and LS performed the statistical analysis and interpretation of the data. YL, DO, RB and LS were involved in data collection, organization, coordination, and technical support of the study. AG, MP, YL

and DO wrote and edited the manuscript. All authors had full access to all data in the studies, critically reviewed the manuscript, approved the final version and had final responsibility for the decision to submit for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.909995/full#supplementary-material

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