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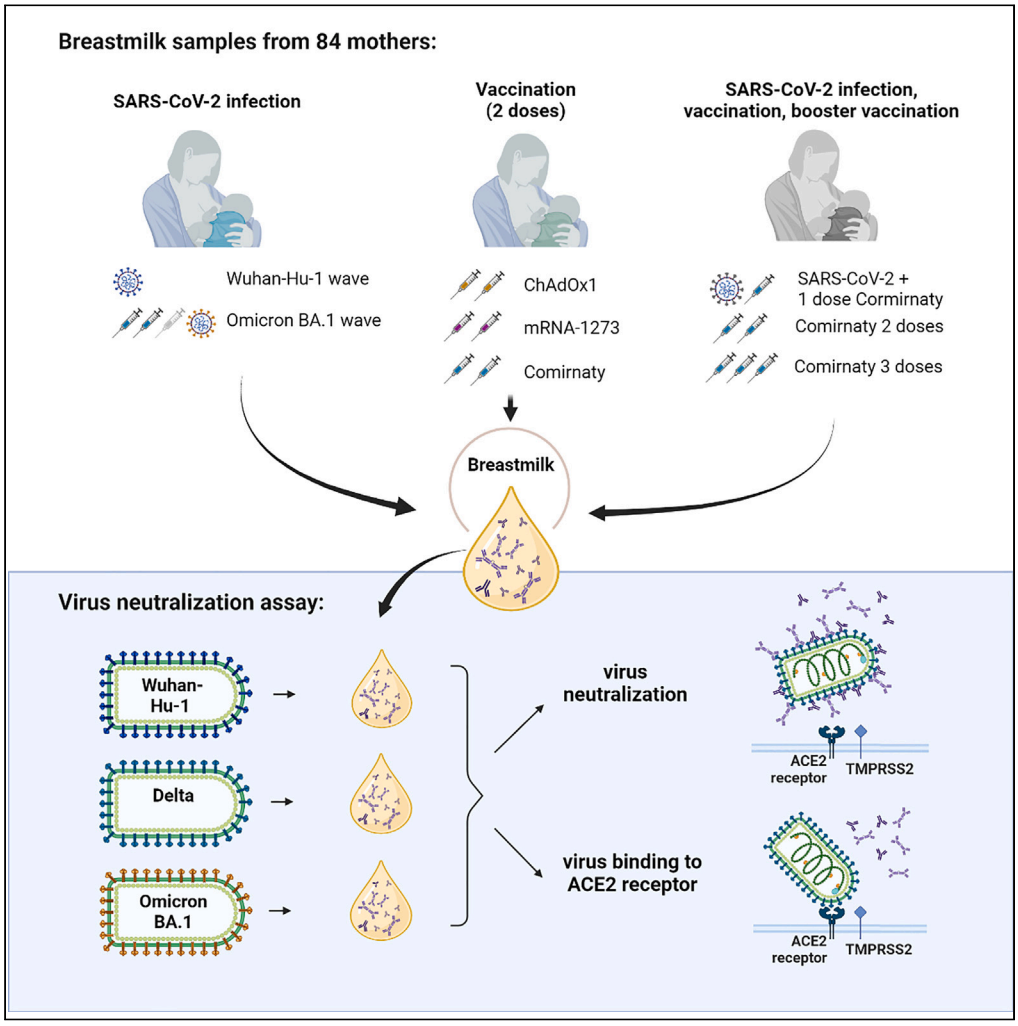

Christine Bäuerl, Joao Zulaica, Luciana Rusu, ..., Ron Geller, Maria Carmen Collado, MilkCORONA study team

ron.geller@uv.es (R.G.)
mcolam@iata.csic.es (M.C.C.)

Highlights

Most breastmilk contain neutralizing antibodies after infection or vaccination

ChAdOx1 vaccine fails to induce neutralizing antibodies

BA.1 Omicron evades antibodies generated after vaccination and infection

Natural infection resulted in higher neutralizing antibody titers than vaccination

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Assessment of SARS-CoV-2 neutralizing antibody titers in breastmilk from convalescent and vaccinated mothers

Christine Bäuerl,[1,10] Joao Zulaica,[2,10] Luciana Rusu,[2] Alicia Rodríguez Moreno,[2] Francisco J. Pérez-Cano,[3] Carles Lerin,[4,5] Desirée Mena-Tudela,[6] Laia Aguilar-Camprubí,[7] Anna Parra-Llorca,[8] Cecilia Martínez-Costa,[9] Ron Geller,[2,11,*] Maria Carmen Collado,[1,11,12,*] and on behalf of MilkCORONA study team

SUMMARY

Breastmilk contains antibodies that could protect breastfed infants from infections. In this work, we examined if antibodies in breastmilk could neutralize SARS-CoV-2 in 84 breastmilk samples from women that were either vaccinated (Comirnaty, mRNA-1273, or ChAdOx1), infected with SARS-CoV-2, or both infected and vaccinated. The neutralization capacity of these sera was tested using pseudotyped vesicular stomatitis virus carrying either the Wuhan-Hu-1, Delta, or BA.1 Omicron spike proteins. We found that natural infection resulted in higher neutralizing titers and that neutralization correlated positively with levels of immunoglobulin A in breastmilk. In addition, significant differences in the capacity to produce neutralizing antibodies were observed between both mRNA-based vaccines and the adenovirus-vectored ChAdOx1 COVID-19 vaccine. Overall, our results indicate that breastmilk from naturally infected women or those vaccinated with mRNA-based vaccines contains SARS-CoV-2 neutralizing antibodies that could potentially provide protection to breastfed infants from infection.

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic has shifted research priorities in several disciplines and affected vulnerable populations, including pregnant or lactating women and infants. In particular, the management of pregnant and breastfeeding women with COVID-19 was compromised during this pandemic by the lack of scientific evidence regarding potential severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) transmission during breastfeeding, the impact of the virus on milk composition, and the generation of specific antibodies and their persistence in breastmilk.[1] Now, nearly three years following the emergence of SARS-CoV-2, several studies have reported the presence of specific immunoglobulin (Ig) A, IgM, and IgG antibodies in milk after SARS-CoV-2 infection and after maternal COVID-19 vaccinations.[2–8] As breastmilk contains antibodies that could protect breastfeeding infants from infections, defining whether these antibodies are functional, persist after infant gastrointestinal digestion,[9,10] and can provide protection to infants is of key importance.

The spread of the pandemic and the vaccination campaigns occurred in parallel with the appearance of new SARS-CoV-2 variants, including Omicron, which is currently circulating among the population.[11] Despite clear evidence for differences between vaccines in their capacity to induce neutralizing antibodies in serum, our knowledge of how different vaccination strategies and platforms affect breastmilk antibody levels and neutralizing capacity remains limited.[3,12–14] In addition, there are no studies to date on the ability of breastmilk to neutralize different SARS-CoV-2 variants. Therefore, the present exploratory study aimed to assess the ability of breastmilk antibodies from women that were either vaccinated with different vaccine platforms (ChAdOx1, mRNA-1273, or Comirnaty), infected, or both infected and vaccinated, to neutralize SARS-CoV-2 Wuhan-Hu-1, Delta, or Omicron BA.1 spike (S) proteins.

[1]Institute of Agrochemistry and Food Technology-National Research Council (IATA-CSIC), 46980 Paterna, Valencia, Spain

[2]Institute for Integrative Systems Biology (I^{2}SysBio), University of Valencia-CSIC, 46980 Paterna, Valencia, Spain

[3]Physiology Section, Department of Biochemistry and Physiology, Faculty of Pharmacy and Food Science and Institute of Research in Nutrition and Food Safety (INSA), University of Barcelona (UB), 08028 Barcelona, Spain

[4]Endocrinology department, Institut de Recerca Sant Joan de Déu, Hospital Sant Joan de Déu, 08950 Barcelona, Spain

[5]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, 28029 Madrid, Spain

[6]Department of Nursing, Nursing Research Group, Universitat Jaume I, Castellón, Spain

[7]LactApp Women Health, Barcelona, Spain

[8]Health Research Institute La Fe, Neonatal Research Group, Spain and University and Polytechnic Hospital La Fe, Division of Neonatology, 46026 Valencia, Spain

[9]Department of Pediatrics, Hospital Clínico Universitario, University of Valencia, Spain. Nutrition Research Group of INCLIVA, 46010 Valencia, Spain

[10]These authors contributed equally

[11]Senior author

[12]Lead contact

*Correspondence: ron.geller@uv.es (R.G.), mcolam@iata.csic.es (M.C.C.)

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RESULTS

Study population characteristics

This study included 84 lactating women, eight of which had a confirmed infection during the primary SARS-CoV-2 wave from March to May 2020 and 13 with a confirmed breakthrough infection during the emergence of the BA.1 Omicron variant from January to mid-February 2022. The presence of neutralizing antibodies in vaccinated lactating mothers with two doses was assessed in 16 participants in each of the three vaccine groups (ChAdOx1, mRNA-1273, and Comirnaty). To further understand the effect of a third vaccine booster or vaccination following SARS-CoV-2 infection, 10 additional samples were included at seven days post-third Comirnaty vaccine dose, and five additional samples from mothers infected with SARS-CoV-2 before Comirnaty vaccination (infections during March to December 2020) were included at seven (2 samples) and 15 days (3 samples) post-first dose (Tables 1, S1). Significant differences between groups were detected for the mode of birth ($p = 0.035$), due to the absence of C-section in the ChAdOx1 group and for infant age at maternal vaccination ($p = 0.011$), which was significantly lower in convalescent mothers infected during the Wuhan-Hu-1 wave than the Omicron wave. SARS-CoV-2 infected study participants during the Wuhan-Hu-1 wave were primarily recruited in hospitals at birth, when SARS-CoV-2 PCR diagnostic tests were performed as part of routine surveillance before labor.

Breastmilk samples from COVID-19 convalescent mothers neutralize less effectively Omicron BA.1 than the Wuhan-Hu-1 variant

We first examined the ability of breastmilk samples from SARS-CoV-2 convalescent mothers to neutralize the Wuhan-Hu-1, Delta, and Omicron BA.1 S variants using a pseudotyped vesicular stomatitis virus. Specifically, eight breastmilk samples from convalescent mothers infected during the first wave (Wuhan-Hu-1 wave) of SARS-CoV-2 from March to May 2020 (predominance Nextstrain Clade 19 A, 20A [D614G mutation in the spike protein], and descendants[15,16]), and 13 samples from convalescent mothers that were infected during the Omicron wave in January to mid-February 2022 (predominance of Omicron BA.1 variant[17]) were evaluated. Most or all of the samples neutralized the Wuhan-Hu-1 S variant (reciprocal IC50 > 10; 100% and 84.6% detection rate in samples from infected donors during Wuhan and Omicron wave, respectively), with no significant differences in neutralizing antibody (NtAb) titers between the two groups ($p > 0.05$; Figure 1, S1 and Table S2). A somewhat lower fraction of samples in each group neutralized the Delta variant (~50%) than the Wuhan-Hu-1 variant, although this reduction was not statistically significant within the groups or between them ($p > 0.05$). Finally, the fraction of samples neutralizing the BA.1 Omicron S variant was significantly lower than the Wuhan-Hu-1 in both samples, with none of the samples from mothers infected during the Wuhan-Hu-1 wave neutralizing the BA.1 Omicron S variant (0/8 versus 8/8 for the Wuhan-Hu-1 variant; $p < 0.001$) and 38.5% of the samples from Omicron-infected mothers showing neutralization (5/13 versus 11/13 for the Wuhan-Hu-1 variant; $p = 0.041$; Figure 1). Hence, significant escape from neutralization is observed for Omicron BA.1 S than Wuhan-Hu-1 S, even in mothers that were likely infected with the BA.1 S variant.

As breastmilk samples contained neutralizing antibodies, we next investigated the class of SARS-CoV-2 specific antibodies present in these samples. No differences were observed in the levels of IgA antibodies targeting the S receptor-binding domain (RBD) between samples from mothers infected during the Wuhan-Hu-1 and Omicron BA.1 wave ($p > 0.05$; Figure 2A). In contrast, the titers of IgG antibodies targeting the RBD were significantly higher in infected participants during the Omicron BA.1 wave, which can most likely be attributed to previous vaccination with at least two, and in five cases three, vaccine doses ($p < 0.001$; Figure 2B). Of note, NtAb titers did not correlate with RBD-IgG titers (rho = 0.04, $p = 0.868$), whereas a positive correlation could be observed for RBD-IgA titers (rho = 0.73, $p < 0.001$), suggesting the latter are likely to drive the virus-neutralizing activity (Figure S2).

ChAdOx1 fails to induce neutralizing antibodies in breastmilk

We next analyzed the ability of breastmilk from SARS-CoV-2 vaccinated mothers to neutralize SARS-CoV-2. The frequency of samples neutralizing the Wuhan-Hu-1 ancestral strain (reciprocal IC50 > 10) was similar between mRNA-1273- and Comirnaty-vaccinated participants (56.3% and 50% of samples, respectively), and no significant differences were detected in the magnitude of NtAb titers between both mRNA-based vaccines ($p > 0.05$; Figure 3A and Table 2). Strikingly, none of the breastmilk samples from ChAdOx1-vaccinated participants neutralized the Wuhan-Hu-1 ancestral strain (0/16). For the Delta variant, 25% (4/16) of human milk samples from mRNA-1273-vaccinated participants could neutralize the Delta variant versus

Table 1. Characteristics of the participants included in the study

	SARS-CoV-2 infection			Vaccine type			
	Wuhan-Hu-1 (n = 8)	Omicron BA.1 (n = 13)	p value	ChAdOx1 (n = 16)	mRNA-1273 (n = 16)	Comirnaty (n = 31)	p value
Maternal characteristics							
Age (years)	36.9 ± 4.7[g]	35.0 ± 2.9	**0.295**[a]	35,1 ± 3.5	34.9 ± 3.4	34.7 ± 3.7	**0.788**[b]
Gestational age (weeks)	39.2 ± 0.93[h]	39.5 ± 1.3	**0.549**[a]	40.4 ± 0.8	39.4 ± 1.4	39.7 ± 1.2	**0.078**[b]
SARS-CoV-2-positive before vaccine	8	0		0	0	5 (16.1%)	
Sample collection after							
1st dose	0	0		0	0	5	
2nd dose	0	8		16	16	16	
3rd dose	0	5		0	0	10	
Days after vaccination	–	N.A.		7	7	7–15	
Infant characteristics							
Gender (female, %)	4 (50%)	7 (53.8%)	**> 0.999**[c]	8 (50%)	7 (43.8%)	12 (38.7%)	**0.757**[d]
Mode of birth (C-section, n)	1 (14.3%)[g]	2 (15.4%)	**> 0.999**[c]	0 (0%)	5 (31.3%)	10 (32.3%)	**0.035**[d]
Weight at birth (g)	3199 ± 318.7	3409 ± 301.2[g]	**0.154**[a]	3388 ± 321.4	3470 ± 540.7	3338 ± 377.5	**0.581**[e]
Length at birth (cm)	49 ± 1.3	50 ± 1.5[h]	**0.160**[a]	50.6 ± 1.5	50.6 ± 2.6	50.6 ± 1.9	**0.998**[b]
Infant age at maternal vaccination (month)	3.9 ± 7.6	13.3 ± 8.8[i]	**0.011**[f]	11.8 ± 6.1	10.8 ± 5.6	15.8 ± 10.7	**0.252**[b]

Categorical data are presented as positive cases (% of the total population).
N.A. data not available.
[a]Student's *t* test.
[b]Kruskal-Wallis test.
[c]Fisher's exact test.
[d]Chi-squared test.
[e]One-Way ANOVA.
[f]Mann-Whitney-test.
[g]1 data missing.
[h]2 data missing.
[i]3 data missing.

6.25% (1/16) or 0% (0/16) from Comirnaty- and ChAdOx1-immunized mothers, respectively ($p > 0.05$ for both). Finally, only one sample in the mRNA-1273-immunized group was able to neutralize the Omicron BA.1 variant. Overall, NtAb titers from vaccinated mothers were significantly lower when compared to samples from infected mothers during the Wuhan-Hu-1 wave or breakthrough infections during the Omicron BA.1 wave (Figure 3B).

We previously evaluated RBD-specific IgA at 7 days following the second vaccine dose in breastmilk[6] and in this work, we extended our analysis to RBD-specific IgG at the same time point. We next analyzed whether different levels of RBD-specific IgA and IgG antibodies could be observed in breastmilk samples in response to the different vaccine types. All samples from mRNA-1273- and Comirnaty-vaccinated donors contained RBD-specific IgA antibodies above the positive cut-off value and endpoint titers above 1 (16/16 for both; Table S3); in contrast, only 4 out of 16 samples (25%) used in this study in the ChAdOx1 group reached these criteria. Moreover, in positive samples, the titer of RBD-specific IgA antibodies was significantly lower in ChAdOx1-vaccinated donors versus both mRNA-1273- ($p < 0.001$) and Comirnaty-vaccinated ($p < 0.001$; Figure S3 and Table S3). For RBD-specific IgG, 93.8% of participants vaccinated with ChAdOx1 were positive, but endpoint titers were still significantly inferior to both mRNA-based vaccines ($p < 0.001$ for both) (Figure S3 and Table S3).

Finally, the correlation between RBD-specific antibody titers and NtAb titers against the Wuhan-Hu-1 strain was examined in breastmilk from vaccinated mothers. In contrast to our findings with breastmilk from convalescent mothers, the end-point titers of both antibody isotypes in breastmilk correlated significantly

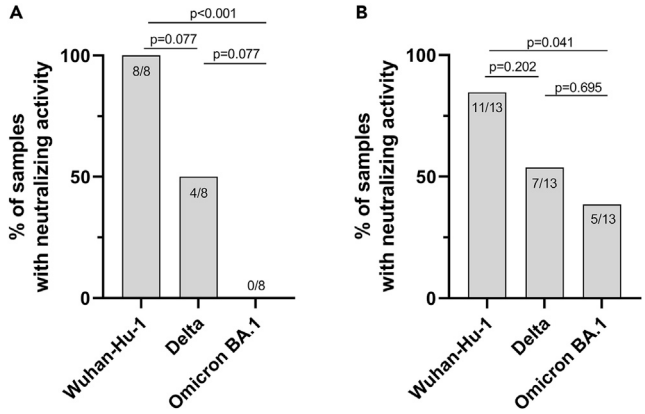

Figure 1. Frequency distribution of neutralizing antibody titers against Wuhan-Hu-1, Delta, and Omicron BA.1 S variants in breastmilk samples from convalescent mothers

(A) Breastmilk samples from mothers infected during March to May 2020 (Wuhan-Hu-1 wave).

(B) Breastmilk samples from mothers infected during January to mid-February 2022 (Omicron wave). Fisher's exact test was used to assess statistical significance. Numbers in the bars indicate the number of samples with detectable NtAb/ total number of each group.

with NtAb titers, although RBD-specific IgA titers showed better correlation (Spearman's rho = 0.74 and 0.54 for RBD-specific IgA and IgG, respectively; $p < 0.001$ for both; Figures 4 and S4). Moreover, total IgA levels correlated significantly with both NtAb titers (rho = 0.38, $p = 0.008$) and lactational stage (rho = 0.46, $p = 0.002$), respectively (Figure S5), indicating a time-dependent rise of IgA concentrations along lactation. Overall, these results indicate that IgA is likely more relevant for the neutralization of SARS-CoV-2 in breastmilk, although, in contrast to natural infection, IgG titers are also correlated with neutralization activity following vaccination.

SARS-CoV-2 infection prior to vaccination results in higher neutralizing activity against the ancestral Wuhan-Hu-1 variant in breastmilk

The effect of booster vaccination in participants receiving the mRNA-based Comirnaty vaccine was analyzed next. Booster vaccination did not significantly change SARS-CoV-2 specific IgA and IgG antibody titer (Figures 5A and 5B). The relative number of samples that contained antibodies neutralizing the Wuhan-Hu-1 variant increased slightly (50% and 70% after the second and third dose, respectively), although this was not statistically significant ($p = 0.428$). Remarkably, samples collected from participants

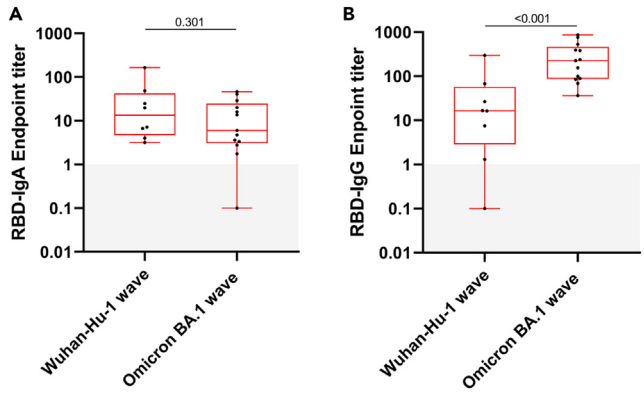

Figure 2. Virus-specific-RBD antibody endpoint titers in breastmilk samples from convalescent mothers infected with SARS-CoV-2 during Wuhan-Hu-1 wave and the first Omicron BA.1 wave

(A) RBD-IgA Endpoint titer.

(B) RBD-IgG Endpoint titer. Mann-Whitney test was used to assess for statistical significance. Box-plots show the median (horizontal line inside box), interquartile range (box), and whiskers depict the minimum and maximum value. Endpoint titers above 1 are considered positive for SARS-CoV-2-specific RBD-IgG and IgA.

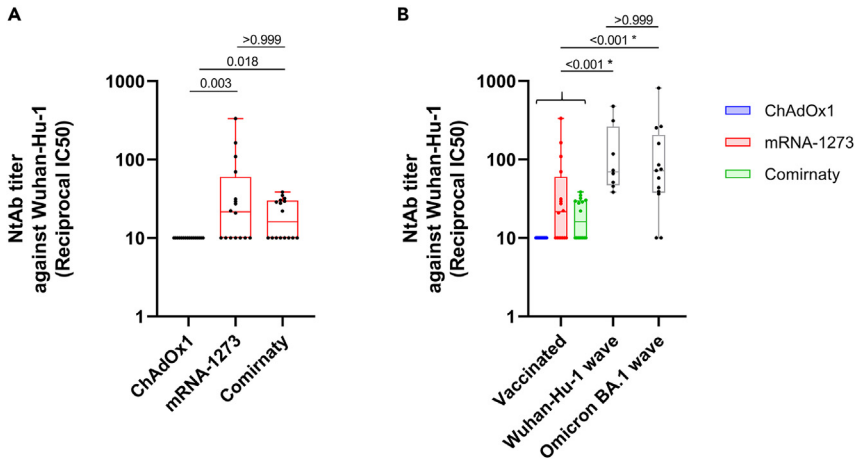

Figure 3. ChAdOx1 fails to induce neutralizing antibodies in breastmilk

(A) Neutralizing antibody titer (reciprocal IC50) against SARS-CoV-2 ancestral Wuhan-Hu-1 S variant in breastmilk samples of ChAdOx1, mRNA-1273, and Comirnaty-vaccinated lactating mothers at seven days after the second dose.

(B) Neutralizing antibody titer (reciprocal IC50) against SARS-CoV-2 ancestral Wuhan-Hu-1 S variant in breastmilk samples of vaccinated lactating mothers compared to convalescent mothers infected during the Wuhan-Hu-1 wave in 2020 (March to May 2020) and during Omicron BA.1 wave (January to mid-February 2022). * Statistical significance to the vaccinated group was calculated with all data from the different vaccine types. Kruskal-Wallis test followed by a Dunn's multiple comparison test was used to assess for statistical significance. Box-plots show the median (horizontal line inside box), interquartile range (box), and whiskers depict the minimum and maximum value.

that were infected with SARS-CoV-2 before receiving the first vaccination had a significantly higher fraction of samples that neutralized both Wuhan-Hu-1 and the Delta variant at 7–15 days after vaccine administration (Table 3). Moreover, NtAb titers against Wuhan-Hu-1 were significantly higher in this group than in samples from participants receiving two ($p < 0.001$) and three doses ($p = 0.019$; Figure 5C). In contrast to Wuhan-Hu-1 and Delta, none of the samples contained antibodies capable of neutralizing Omicron BA.1 S. Finally, NtAb titers against Wuhan-Hu-1 correlated with IgA and IgG RBD-targeting antibodies (RBD-IgA: rho = 0.36, $p = 0.047$; RBD-IgG: rho = 0.40, $p = 0.026$), as well as with total IgA concentrations (rho = 0.36, $p = 0.046$). As observed in the analysis of breastmilk from vaccinated donors with 2 doses, total IgA concentration showed a significant positive correlation with the lactational stage (rho = 0.43, $p = 0.030$) (Figure S6).

Table 2. Detectable NtAbs against SARS-CoV-2 variants in breastmilk samples from mothers vaccinated with ChAdOx1, mRNA-1273 and Comirnaty

SARS-CoV-2 variant	Vaccine type	Number of samples with detectable NtAb/total number (%)	Comparison	p value[a]
Wuhan-Hu-1	ChAdOx1	0/16 (0%)	ChAdOx1 vs. mRNA-1273	< 0.001
	mRNA-1273	9/16 (56.2%)	mRNA-1273 vs. Comirnaty	> 0.999
	Comirnaty	8/16 (50%)	ChAdOx1 vs. Comirnaty	0.002
Delta	ChAdOx1	0/16 (0%)	ChAdOx1 vs. mRNA-1273	0.101
	mRNA-1273	4/16 (25%)	mRNA-1273 vs. Comirnaty	0.333
	Comirnaty	1/16 (6.25%)	ChAdOx1 vs. Comirnaty	> 0.999
Omicron BA.1	ChAdOx1	0/16 (0%)	ChAdOx1 vs. mRNA-1273	> 0.999
	mRNA-1273	1/16 (6.25%)	mRNA-1273 vs. Comirnaty	> 0.999
	Comirnaty	0/16 (0%)	ChAdOx1 vs. Comirnaty	> 0.999

[a]Fisher's exact test (two-sided).

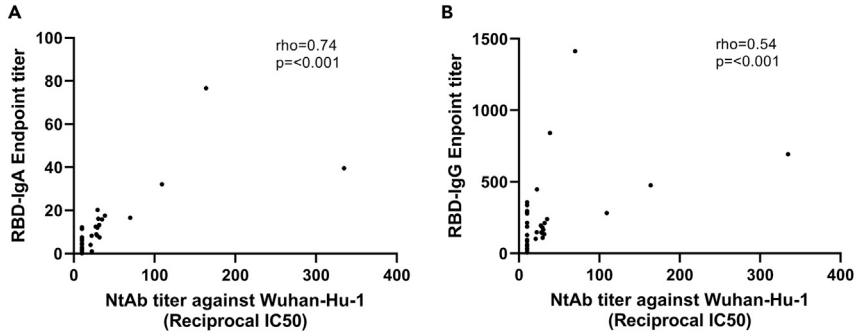

Figure 4. NtAb titers against the ancestral SARS-CoV-2 Wuhan-Hu-1 S variant correlate with RBD-specific antibodies in breastmilk from vaccinated mothers

Spearman's correlation analysis between NtAb titers (reciprocal IC50) against the SARS-CoV-2 Wuhan-Hu-1 variant and virus-specific-RBD antibody titers were performed for:

(A) RBD-IgA Endpoint titer.

(B) RBD-IgG Endpoint titer. Data from different vaccine types were grouped due to the small dataset (n = 16 in each vaccine group: ChAdOx1, mRNA-1273, and Cormirnaty); individual correlations for vaccines are shown in Figure S4.

DISCUSSION

The presence of SARS-CoV-2-specific antibodies in breastmilk after infection and vaccination has been demonstrated in a number of studies.[3,5,6,8,18] However, the functionality and neutralizing activity of these antibodies—whether generated after natural infection or vaccination—is not well defined.[3,13,19,20] Furthermore, antibody-conferred protection against COVID-19 declined over time, largely due to viral antigenic evolution leading to escape from neutralizing antibodies.[21] Hence, we compared the ability of breastmilk samples from both SARS-CoV-2-infected and vaccinated mothers to neutralize the spike protein from the ancestral Wuhan-Hu-1 strain, as well as the Delta and Omicron BA.1 variants.

The emergence of virus variants during the pandemic was observed with great concern, particularly for the Omicron variant. This was due to a reduction in protection and escape from neutralization by antibodies generated in response to the first approved vaccines that used the Wuhan-Hu-1 Spike protein for immunization.[22,23] We observed in our study that antibodies generated during the beginning of the pandemic, when the Wuhan-Hu-1 strain predominated, neutralized the ancestral virus variant in all samples analyzed (100% prevalence). While some of these samples were also able to neutralize the Delta variant, none were found to neutralize Omicron BA.1. This is in contrast to breastmilk samples from vaccinated and infected mothers during the Omicron wave that contained neutralizing antibodies against Omicron BA.1 (prevalence of NtAbs of 38.5%). These results are in line with studies in blood samples where the BA.1 Omicron variant evades antibodies generated in response to vaccination and infection with previous virus variants.[22–25]

Virus-specific antibody titers were significantly higher for RBD-IgG in the group of breakthrough infection during Omicron BA.1, likely due to these mothers having been vaccinated with two and, in some individuals, three doses. It has been previously shown that vaccination elicits an IgG-dominant antibody response that differs from natural infection, where higher amounts of secretory IgA (sIgA) are induced.[2,6] Interestingly, NtAb titers correlated significantly with RBD-IgA, underlining the role of IgA in breastmilk, where it is the most abundant isotype (about 70–90% of all immunoglobulins) and present predominantly in its dimeric sIgA form.[26] sIgA displays higher anti-viral activity than in its monomeric form and is present on mucosal surfaces such as the nasopharynx, where sIgA is the primary form of IgA.[27] Thus, sIgA contained in breastmilk may be particularly valuable for providing protection against SARS-CoV-2, which infects the epithelium of the respiratory tract. Additionally, longer persistence for SARS-Cov-2-specific IgA than IgG was shown recently in concentrated saliva of breastfed infants after feeding.[28]

Comparing the three different vaccine types (ChAdOx1, mRNA-1273, Comirnaty) we observed that vaccination with ChAdOx1 did not induce neutralizing antibodies against any of the studied SARS-CoV-2 virus variants. The lack of neutralizing activity of breastmilk samples from ChAdOx1-vaccinated study participants is in line with the absence or low titers of virus-specific antibodies, particularly IgA, reported for vector-based vaccines.[2,6,7,29] On the contrary, both mRNA vaccines displayed neutralization ability in

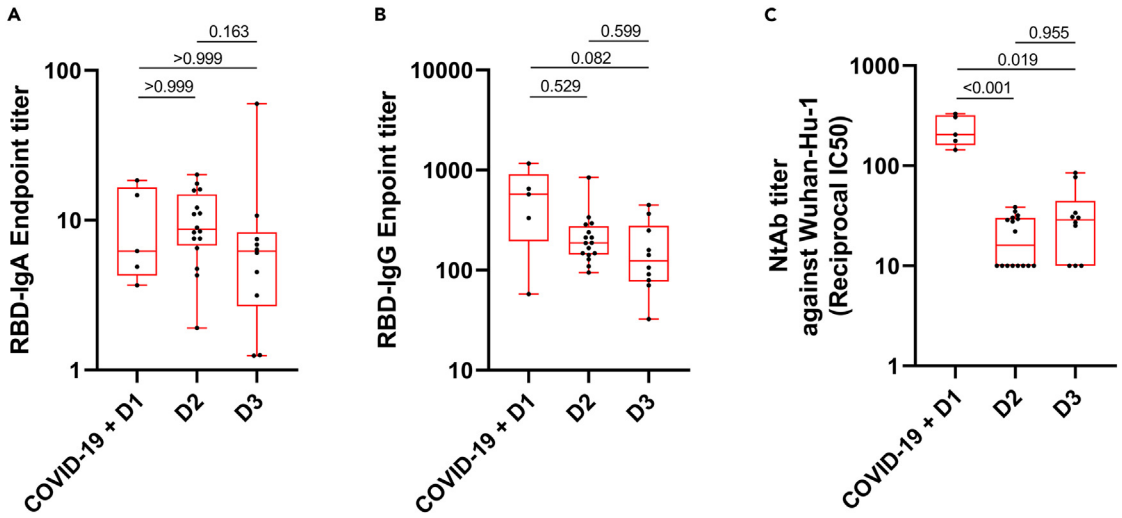

Figure 5. SARS-CoV-2 infection before vaccination results in higher neutralizing activity against ancestral Wuhan-Hu-1 variant in breastmilk

Breastmilk samples from mothers with a past COVID-19 infection and vaccinated with one dose (COVID-19 + D1; n = 5), and mothers vaccinated with two (n = 16) and three doses (n = 10) of Comirnaty vaccine were analyzed for:

(A) RBD-IgA Endpoint titer.

(B) RBD-IgG Endpoint titer.

(C) NtAb titers (reciprocal IC50) against SARS-CoV-2 Wuhan-Hu-1 variant. Kruskal-Wallis test followed by a Dunn's multiple comparison test was used to assess for statistical significance. Box-plots show the median (horizontal line inside box), interquartile range (box), and whiskers depict the minimum and maximum value. Endpoint titers above 1 are considered positive for SARS-CoV-2-specific RBD-IgG and IgA.

about 50% of samples against Wuhan-Hu-1 and weaker or no activity against the Delta and Omicron BA.1 variants, without significant differences between both mRNA vaccines. The tendency for higher neutralizing antibody titers against Wuhan-Hu-1 and detection rates for mRNA-1273 could be due to the higher dose of mRNA employed in this vaccine type.[30] In contrast to breastmilk from convalescent mothers, neutralizing antibody titers and RBD-antibody titers correlated significantly for both isotypes, highlighting differences in the immune responses elicited by natural infection and vaccination. Nevertheless, despite higher titers of RBD-specific IgG antibodies, a better correlation was observed for IgA, again highlighting a key role for IgA in the virus-neutralization ability of breastmilk. To date, there are only a few studies that evaluated the neutralizing activity of different vaccine types in breastmilk samples. One study determined the neutralizing effect of breastmilk samples from mothers vaccinated with the mRNA vaccine from Pfizer, or two replication-incompetent adenovirus vector vaccines, Ad26.COV2S (Johnson & Johnson/Jansen) and Ad5-nCoV (CanSino Biologics). This study found the Pfizer vaccine to induce the strongest neutralizing capacity, with the Ad26.COV2S vaccine showing lower neutralization and the Ad5-nCoV vaccine being similar to the non-vaccinated group.[13] Yeo and colleagues[12] conducted a study evaluating breastmilk from 35 Pfizer-vaccinated mothers and found that neutralizing antibody titers peaked at 7 days following the second dose and that breastmilk from only 3 mothers failed to show any neutralizing activity until 42 days following the first dose. Another study conducted in 9 mothers vaccinated with Pfizer and one mother after a single dose of Ad26.COV2.S and boosted with one dose of mRNA-1273, showed that SARS-CoV-2-specific IgG, IgA and neutralizing activity were higher 1-month after the third dose than after the initial primary vaccine series with 2 doses.[14]

Our study also compared NtAb titers after vaccination with Comirnaty 7 days post-second and third dose and we observed an increase, although not reaching significance, in both NtAb titers and detection rates. Interestingly, a small subset of samples from SARS-CoV-2 infected and vaccinated mothers showed significantly higher detectable NtAb titers against both the ancient Wuhan-Hu-1 and the Delta variant, respectively, at 7–15 days post-first dose. These results agree with studies describing good protection against SARS-CoV-2 as a result of hybrid immunity[24,31,32]; however, no neutralizing activity against Omicron BA.1 could be detected.

To the best of our knowledge, this is the first study analyzing neutralizing activity of breastmilk against SARS-CoV-2 variants. While the evasion of antibody neutralization by SARS-CoV-2 has been heavily studied

Table 3. Detectable NtAbs against SARS-CoV-2 variants in breastmilk samples from participants recovered from COVID-19 and vaccinated with 1 dose (COVID-19 + D1), and participants vaccinated with two (D2) and three doses (D3) of Comirnaty

SARS-CoV-2 variant	Group	Number of samples with detectable NtAb/total number (%)	Comparison	p value[a]
Wuhan-Hu-1	COVID-19 + D1	5/5 (100%)	COVID-19 + D1 vs. D2	0.111
	D2	8/16 (50%)	D3 vs. D2	0.428
	D3	7/10 (70%)	COVID-19 + D1 vs. D3	0.506
Delta	COVID-19 + D1	5/5 (100%)	COVID-19 + D1 vs. D2	<0.001
	D2	1/16 (6.25%)	D2 vs. D3	>0.999
	D3	0/10 (0%)	COVID-19 + D1 vs. D3	<0.001
Omicron BA.1	COVID-19 + D1	0/16 (0%)	COVID-19 + D1 vs. D2	>0.999
	D2	0/16 (0%)	D2 vs. D3	>0.999
	D3	0/16 (0%)	COVID-19 + D1 vs. D3	>0.999

[a]Fisher's exact test (two-sided).

in blood, and although virus-specific antibodies levels are generally reflected in breastmilk at lower quantities but in similar dynamics,[12,20] more studies are needed addressing the neutralizing ability of breastmilk against new emerging virus variants. This is of particular importance for knowing the protection that may be provided to neonates by passive immunity through breastfeeding or transplacental transfer, where some studies have shown SARS-CoV-2 antibodies in cord blood.[33,34] Although virus-specific and neutralizing antibody titers are lower in breastmilk than blood serum,[12,20] breastfeeding represents a continuous source of antibodies to the neonate. In addition, the ingested anti-SARS-CoV-2 antibodies from milk are mainly in a sIgA form and therefore not-absorbed but rather are directly located at mucosal surfaces of the whole digestive system, including the mouth and the throat. In our study, the levels of immunoglobulin A in breastmilk correlated positively with NtAb titers following natural infection with SARS-CoV-2 and also, higher NtAb titers have been observed than those induced by mRNA-based vaccines. This should encourage efforts to improve responses after vaccination in pregnant and lactating women in order to augment passive immunity to neonates via the transfer of neutralizing antibodies during breastfeeding.

In summary, we found that mRNA-based vaccines elicited in breastmilk higher SARS-CoV-2 antibody levels and that these antibodies had neutralizing activity, unlike the vector-based ChAdOx1 vaccine. Breastmilk antibodies produced in response to vaccination or infection showed significant escape from neutralization for BA.1 S compared to Wuhan-Hu-1 S. Breastfeeding is considered the gold standard in infant nutrition and has been shown to provide protection against respiratory infections.[35,36] Therefore, considering the absence of an approved vaccine for infants against COVID-19, our results support the recommendation to pregnant and lactating women for vaccination in order to provide potential health benefits through passive immunity to the neonate.

Limitations of the study

Our study is a preliminary study that has some limitations. Larger sample sizes are necessary to confirm the different NtAb titers in response to different vaccination types, booster vaccination, as well as SARS-CoV-2 infections in naive and experienced individuals. Development of antibody induction after booster vaccine should be monitored longitudinally in the same study subjects. Of note, breastmilk samples from vaccinated mothers were not tested for the presence of SARS-CoV-2 antibodies targeting epitopes other than the Spike protein used for immunization (e.g., N protein, MPro, etc.) to exclude previous asymptomatic infections. Furthermore, our study lags behind the epidemiological landscape where Omicron BA.1 has been widely replaced by other Omicron sublineages, such as BA.5 and BQ.1 in Spain.[37] Finally, no SARS-CoV-2 variant sequencing was performed in the SARS-CoV-2 infected groups and therefore the assignment of the infecting virus is based on the epidemiological situation of variants in Spain at the respective sample collection time points.[17] Of note, we cannot rule out that samples from convalescent

mothers infected during the Omicron BA.1 wave have been previously infected with other virus variants or with the Delta variant, which in Spain in the first week of January 2022 was still circulating with a prevalence of 16.1%, and decreased to 0.4% at the end of January 2022.[17,38]

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106802.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.G. and M.C.C.; methodology, J.Z., L.R., A.R.M., and C.B.; investigation, C.B., J.Z., L.R., and A.R.M.; formal analysis, C.B., J.Z., and R.G.; Writing—original draft, C.B., and M.C.C.; writing—review & editing, C.B., J.Z., F.J.P.C., C.L., D.M.T., L.A.C., A.P.L., C.M.C., R.G., and M.C.C.; resources, D.M.T., L.A.C., A.P.L., and C.M.C.; funding acquisition, R.G. and M.C.C.; supervision, R.G. and M.C.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

1. Spatz, D.L., Davanzo, R., Müller, J.A., Powell, R., Rigourd, V., Yates, A., Geddes, D.T., van Goudoever, J.B., and Bode, L. (2020). Promoting and protecting human milk and breastfeeding in a COVID-19 world. Front. Pediatr. *8*, 633700. https://doi.org/10.3389/fped.2020.633700.

2. Yang, X., Fox, A., DeCarlo, C., Norris, C., Griffin, S., Wedekind, S., Flanagan, J.M., Shenker, N., and Powell, R.L. (2022). Comparative profiles of SARS-CoV-2 spike-specific human milk antibodies elicited by mRNA- and adenovirus-based COVID-19 vaccines. Breastfeed. Med. *17*, 638–646. https://doi.org/10.1089/bfm.2022.0019.

3. Pace, R.M., Williams, J.E., Järvinen, K.M., Belfort, M.B., Pace, C.D.W., Lackey, K.A., Gogel, A.C., Nguyen-Contant, P., Kanagaiah, P., Fitzgerald, T., et al. (2021). Characterization of SARS-CoV-2 RNA, antibodies, and neutralizing capacity in milk produced by women with COVID-19. mBio *12*. https://doi.org/10.1128/mBio.03192-20.

4. Peng, S., Zhu, H., Yang, L., Cao, L., Huang, X., Dynes, M., Narayan, A., Xia, J., Chen, Y., Zhang, P., et al. (2020). A study of breastfeeding practices, SARS-CoV-2 and its antibodies in the breast milk of mothers confirmed with COVID-19. Lancet Reg. Health. West. Pac. *4*, 100045. https://doi.org/10.1016/j.lanwpc.2020.100045.

5. Bäuerl, C., Randazzo, W., Sánchez, G., Selma-Royo, M., García Verdevio, E., Martínez, L., Parra-Llorca, A., Lerin, C., Fumadó, V., Crovetto, F., et al. (2022). SARS-CoV-2 RNA and antibody detection in breast milk from a prospective multicentre study in Spain. Arch. Dis. Child. Fetal Neonatal Ed. *107*, 216–221. https://doi.org/10.1136/archdischild-2021-322463.

6. Selma-Royo, M., Bäuerl, C., Mena-Tudela, D., Aguilar-Camprubí, L., Pérez-Cano, F.J., Parra-Llorca, A., Lerin, C., Martínez-Costa, C., and Collado, M.C. (2022). Anti-SARS-CoV-2 IgA and IgG in human milk after vaccination is dependent on vaccine type and previous SARS-CoV-2 exposure: a longitudinal study. Genome Med. *14*, 42. https://doi.org/10.1186/s13073-022-01043-9.

7. Juncker, H.G., Mulleners, S.J., Coenen, E.R.M., van Goudoever, J.B., van Gils, M.J., and van Keulen, B.J. (2022). Comparing human milk antibody response after 4 different vaccines for COVID-19. JAMA Pediatr. *176*, 611–612. https://doi.org/10.1001/jamapediatrics.2022.0084.

8. Fox, A., Marino, J., Amanat, F., Krammer, F., Hahn-Holbrook, J., Zolla-Pazner, S., and Powell, R.L. (2020). Robust and specific secretory IgA against SARS-CoV-2 detected in human milk. iScience *23*, 101735. https://doi.org/10.1016/j.isci.2020.101735.

9. Calvo-Lerma, J., Bueno-Llamoga, P., Bäuerl, C., Cortés-Macias, E., Selma-Royo, M., Pérez-Cano, F., Lerin, C., Martínez-Costa, C., and Collado, M.C. (2022). Persistence of anti SARS-CoV-2 antibodies in breast milk from infected and vaccinated women after in vitro-simulated gastrointestinal digestion. Nutrients *14*, 2117. https://doi.org/10.3390/nu14102117.

10. Pieri, M., Maniori, M.-A., Shahabian, L., Kanaan, E., Paphiti-Demetriou, I., Pipis, S., Felekkis, K., Nicolaidou, V., and Papaneophytou, C. (2022). Survival of vaccine-induced human milk SARS-CoV-2 IgG, IgA and SIgA immunoglobulins across simulated human infant gastrointestinal digestion. Nutrients *14*, 3368. https://doi.org/10.3390/nu14163368.

11. World Health Organization. Tracking SARS-CoV-2 Variants. https://www.who.int/activities/tracking-SARS-CoV-2-variants.

12. Yeo, K.T., Chia, W.N., Tan, C.W., Ong, C., Yeo, J.G., Zhang, J., Poh, S.L., Lim, A.J.M., Sim, K.H.Z., Sutamam, N., et al. (2021). Neutralizing activity and SARS-CoV-2 vaccine mRNA persistence in serum and breastmilk after BNT162b2 vaccination in lactating women. Front. Immunol. *12*, 783975. https://doi.org/10.3389/fimmu.2021.783975.

13. Cabanillas-Bernal, O., Cervantes-Luevano, K., Flores-Acosta, G.I., Bernáldez-Sarabia, J., and Licea-Navarro, A.F. (2022). COVID-19 neutralizing antibodies in breast milk of mothers vaccinated with three different vaccines in Mexico. Vaccines 10. https://doi.org/10.3390/vaccines10040629.

14. Bender, J.M., Lee, Y., Cheng, W.A., Marentes Ruiz, C.J., and Pannaraj, P.S. (2022). Coronavirus disease 2019 vaccine booster effects are seen in human milk antibody response. Front. Nutr. *9*, 898849. https://doi.org/10.3389/fnut.2022.898849.

15. Hadfield, J., Megill, C., Bell, S.M., Huddleston, J., Potter, B., Callender, C., Sagulenko, P., Bedford, T., and Neher, R.A. (2018). Nextstrain: real-time tracking of pathogen evolution. Bioinformatics *34*, 4121–4123. https://doi.org/10.1093/bioinformatics/bty407.

16. Troyano-Hernáez, P., Reinosa, R., and Holguín, Á. (2022). Evolution of SARS-CoV-2 in Spain during the first two years of the pandemic: circulating variants, amino acid conservation, and genetic variability in structural, non-structural, and accessory proteins. Int. J. Mol. Sci. *23*, 6394. https://doi.org/10.3390/ijms23126394.

17. Spanish Health Ministry. Actualización de la situación epidemiológica de las variantes de SARS-CoV-2 en España - 14 de febrero de 2022. https://www.sanidad.gob.es/profesionales/saludPublica/ccayes/alertasActual/nCov/documentos/COVID19_Actualizacion_variantes_20220214.pdf.

18. Perl, S.H., Uzan-Yulzari, A., Klainer, H., Asiskovich, L., Youngster, M., Rinott, E., and Youngster, I. (2021). SARS-CoV-2–Specific antibodies in breast milk after COVID-19 vaccination of breastfeeding women. JAMA *325*, 2013–2014. https://doi.org/10.1001/jama.2021.5782.

19. Young, B.E., Seppo, A.E., Diaz, N., Rosen-Carole, C., Nowak-Wegrzyn, A., Cruz Vasquez, J.M., Ferri-Huerta, R., Nguyen-Contant, P., Fitzgerald, T., Sangster, M.Y., et al. (2022). Association of human milk antibody induction, persistence, and neutralizing capacity with SARS-CoV-2 infection vs mRNA vaccination. JAMA Pediatr. *176*, 159–168. https://doi.org/10.1001/jamapediatrics.2021.4897.

20. Rosenberg-Friedman, M., Kigel, A., Bahar, Y., Werbner, M., Alter, J., Yogev, Y., Dror, Y., Lubetzky, R., Dessau, M., Gal-Tanamy, M., et al. (2021). BNT162b2 mRNA vaccine elicited antibody response in blood and milk of breastfeeding women. Nat. Commun. *12*, 6222. https://doi.org/10.1038/s41467-021-26507-1.

21. Collie, S., Champion, J., Moultrie, H., Bekker, L.-G., and Gray, G. (2022). Effectiveness of BNT162b2 vaccine against Omicron variant in South Africa. N. Engl. J. Med. *386*, 494–496. https://doi.org/10.1056/NEJMc2119270.

22. Sullivan, D.J., Franchini, M., Joyner, M.J., Casadevall, A., and Focosi, D. (2022). Analysis of anti-SARS-CoV-2 Omicron-neutralizing antibody titers in different vaccinated and unvaccinated convalescent plasma sources. Nat. Commun. *13*, 6478. https://doi.org/10.1038/s41467-022-33864-y.

23. Cele, S., Jackson, L., Khoury, D.S., Khan, K., Moyo-Gwete, T., Tegally, H., San, J.E., Cromer, D., Scheepers, C., Amoako, D.G., et al. (2022). Omicron extensively but incompletely escapes Pfizer BNT162b2 neutralization. Nature *602*, 654–656. https://doi.org/10.1038/s41586-021-04387-1.

24. Carreño, J.M., Alshammary, H., Tcheou, J., Singh, G., Raskin, A.J., Kawabata, H., Sominsky, L.A., Clark, J.J., Adelsberg, D.C., Bielak, D.A., et al. (2022). Activity of convalescent and vaccine serum against SARS-CoV-2 Omicron. Nature *602*, 682–688. https://doi.org/10.1038/s41586-022-04399-5.

25. Planas, D., Saunders, N., Maes, P., Guivel-Benhassine, F., Planchais, C., Buchrieser, J., Bolland, W.-H., Porrot, F., Staropoli, I., Lemoine, F., et al. (2022). Considerable escape of SARS-CoV-2 Omicron to antibody neutralization. Nature *602*, 671–675. https://doi.org/10.1038/s41586-021-04389-z.

26. Rio-Aige, K., Azagra-Boronat, I., Castell, M., Selma-Royo, M., Collado, M.C., Rodríguez-Lagunas, M.J., and Pérez-Cano, F.J. (2021). The breast milk immunoglobulinome. Nutrients *13*, 1810. https://doi.org/10.3390/nu13061810.

27. Wang, Z., Lorenzi, J.C.C., Muecksch, F., Finkin, S., Viant, C., Gaebler, C., Cipolla, M., Hoffmann, H.-H., Oliveira, T.Y., Oren, D.A., et al. (2021). Enhanced SARS-CoV-2 neutralization by dimeric IgA. Sci. Transl. Med. *13*, eabf1555. https://doi.org/10.1126/scitranslmed.abf1555.

28. Golan, Y., Ilala, M., Gay, C., Hunagund, S., Lin, C.Y., Cassidy, A.G., Jigmeddagva, U., Li, L., Ozarslan, N., Asiodu, I.V., et al. (2022). Milk antibody response after 3rd dose of COVID-19 mRNA vaccine and SARS-CoV-2 breakthrough infection and implications for

infant protection. Preprint at medRxiv. https://doi.org/10.1101/2022.12.12.22283367.

29. Juncker, H.G., Mulleners, S.J., Ruhé, E.J.M., Coenen, E.R.M., Bakker, S., van Doesburg, M., Harinck, J.E., Rood, R.D., Bouhuijs, J.H., Oomen, M., et al. (2022). Comparing the human milk antibody response after vaccination with four COVID-19 vaccines: a prospective, longitudinal cohort study in The Netherlands. eClinicalMedicine *47*, 101393. https://doi.org/10.1016/j.eclinm.2022.101393.

30. Heinz, F.X., and Stiasny, K. (2021). Distinguishing features of current COVID-19 vaccines: knowns and unknowns of antigen presentation and modes of action. NPJ Vaccines *6*, 104. https://doi.org/10.1038/s41541-021-00369-6.

31. Andeweg, S.P., de Gier, B., Eggink, D., van den Ende, C., van Maarseveen, N., Ali, L., Vlaemynck, B., Schepers, R., Hahné, S.J., Reusken, C.B., et al. (2022). Protection of COVID-19 vaccination and previous infection against Omicron BA.1, BA.2 and Delta SARS-CoV-2 infections. Nat. Commun. *13*, 4738. https://doi.org/10.1038/s41467-022-31838-8.

32. Wang, Z., Muecksch, F., Schaefer-Babajew, D., Finkin, S., Viant, C., Gaebler, C., Hoffmann, H.-H., Barnes, C.O., Cipolla, M., Ramos, V., et al. (2021). Naturally enhanced neutralizing breadth against SARS-CoV-2 one year after infection. Nature *595*, 426–431. https://doi.org/10.1038/s41586-021-03696-9.

33. Gray, K.J., Bordt, E.A., Atyeo, C., Deriso, E., Akinwunmi, B., Young, N., Baez, A.M., Shook, L.L., Cvrk, D., James, K., et al. (2021). Coronavirus disease 2019 vaccine response in pregnant and lactating women: a cohort study. Am. J. Obstet. Gynecol. *225*, 303.e1–303.e17. https://doi.org/10.1016/j.ajog.2021.03.023.

34. Zdanowski, W., and Waśniewski, T. (2021). Evaluation of SARS-CoV-2 spike protein antibody titers in cord blood after COVID-19 vaccination during pregnancy in polish healthcare workers: preliminary results. Vaccines *9*, 675. https://doi.org/10.3390/vaccines9060675.

35. Frank, N.M., Lynch, K.F., Uusitalo, U., Yang, J., Lönnrot, M., Virtanen, S.M., Hyöty, H., and Norris, J.M.; TEDDY Study Group (2019). The relationship between breastfeeding and reported respiratory and gastrointestinal infection rates in young children. BMC Pediatr. *19*, 339. https://doi.org/10.1186/s12887-019-1693-2.

36. Pandolfi, E., Gesualdo, F., Rizzo, C., Carloni, E., Villani, A., Concato, C., Linardos, G., Russo, L., Ferretti, B., Campagna, I., and Tozzi, A. (2019). Breastfeeding and respiratory infections in the first 6 Months of life: a case control study. Front. Pediatr. *7*, 152. https://doi.org/10.3389/fped.2019.00152.

37. Spanish Health Ministry. Actualización de la situación epidemiológica de las variantes de SARS-CoV-2 en España - 27 de diciembre de 2022. https://www.sanidad.gob.es/profesionales/saludPublica/ccayes/alertasActual/nCov/documentos/COVID19_Actualizacion_variantes_20221227.pdf.

38. Spanish Health Ministry. Actualización de la situación epidemiológica de las variantes de SARS-CoV-2 en España - 17 de enero de 2022. https://www.sanidad.gob.es/profesionales/saludPublica/ccayes/alertasActual/nCov/documentos/COVID19_Actualizacion_variantes_20220117.pdf.

39. McGuire, M.K., Seppo, A., Goga, A., Buonsenso, D., Collado, M.C., Donovan, S.M., Müller, J.A., Ofman, G., Monroy-Valle, M., O'Connor, D.L., et al. (2021). Best practices for human milk collection for COVID-19 research. Breastfeed. Med. *16*, 29–38. https://doi.org/10.1089/bfm.2020.0296.

40. Giménez, E., Albert, E., Zulaica, J., Torres, I., Rusu, L., Moreno, A.R., Burgos, J.S., Peiró, S., Salas, D., Vanaclocha, H., et al. (2022). Severe acute respiratory syndrome coronavirus 2 adaptive immunity in nursing home residents following a third dose of the comirnaty coronavirus disease 2019 vaccine. Clin. Infect. Dis. *75*, e865–e868. https://doi.org/10.1093/cid/ciac223.

41. Camacho, J., Giménez, E., Albert, E., Zulaica, J., Álvarez-Rodríguez, B., Torres, I., Rusu, L., Burgos, J.S., Peiró, S., Vanaclocha, H., et al. (2023). Cumulative incidence of SARS-CoV-2 infection in the general population of the Valencian Community (Spain) after the surge of the Omicron BA.1 variant. J. Med. Virol. *95*, e28284. https://doi.org/10.1002/jmv.28284.

42. Berger Rentsch, M., and Zimmer, G. (2011). A vesicular stomatitis virus replicon-based bioassay for the rapid and sensitive determination of multi-species type I interferon. PLoS One *6*, e25858. https://doi.org/10.1371/journal.pone.0025858.

43. Yang, X., Fox, A., and Powell, R.L.R. (2022). Qualitative immunoassay for the detection of anti-SARS-COV-2 spike antibody in human milk samples. STAR Protoc. *3*, 101203. https://doi.org/10.1016/j.xpro.2022.101203.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-Human IgA Secondary Antibody, HRP	Thermo-Fisher Scientific	A18781; RRID: AB_2535558
Anti-Human IgG (Fc specific)–Peroxidase antibody produced in goat	Sigma Aldrich	A0170-1 ML; RRID: AB_257868
Chemicals, peptides, and recombinant proteins		
Spike Glycoprotein Receptor Binding Domain (RBD) from SARS-Related Coronavirus 2, Wuhan-Hu-1 with C-Terminal Histidine Tag, Recombinant from HEK293T Cells	BEI Resources	NR-52946
1-step-Ultra TMB-ELISA Substrate Solution	Thermo Scientific	34028
Critical commercial assays		
IgA Human Uncoated ELISA Kit	Invitrogen	88-50600-86
Experimental models: Cell lines		
A549 hACE2-TMPRSS2	InvivoGen	a549-hace2tps
Software and algorithms		
GraphPad Prism 8.4.3	GraphPad Software	Version 8.4.3
R Statistical Software		drc package

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Maria Carmen Collado (mcolam@iata.csic.es).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- No original code has been generated in this work.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study population and design

Three different populations were analyzed. Group 1 comprised eight convalescent lactating women with either PCR or serology-confirmed SARS-CoV-2 infection during the primary Wuhan-Hu-1 wave in Spain (infections from March to May 2020, Table S1). These were included from a prospective observational, longitudinal, and multicenter study (ClinicalTrials.gov Identifier: NCT04768244), which were recruited from seven healthcare centers from different areas in Spain (Valencia, Barcelona, Granada, and Zaragoza). Participants were pregnant women intending to breastfeed and breastfeeding women with positive PCR for SARS-CoV-2 based on nasopharyngeal swabs or the presence of SARS-CoV-2 antibodies in serum determined in hospitals. Group 2 included 13 convalescent lactating and vaccinated women with breakthrough SARS-CoV-2 infection during Omicron BA.1 wave in Spain (infected from January to mid-February

2022). These participants with breakthrough infections (2) were recruited by social media (i.e., Twitter and MilkCORONA study members' webpages), and SARS-CoV-2 infection was confirmed by a positive qualitative antigen test for COVID-19, and samples were collected within 28 days following positive antigen test. Finally, group 3 comprised 58 lactating women that received vaccination against SARS-CoV-2 infection (ClinicalTrials.gov Identifier: NCT04751734; URL: https://clinicaltrials.gov/ct2/show/NCT04751734) (recruitment period January 2021 to February 2022). Participants in this group were breastfeeding women within the vaccination priority groups (frontline health care workers) established by the Spanish Government. Participants were recruited at hospitals and health care centers as well as by using specific tools such as LactApp (an app dedicated to breastfeeding and motherhood) and social media (i.e., Twitter and MilkCORONA study members' webpages). Women were excluded if the cessation of breastfeeding and/or vaccine side effects required specific treatment and/or hospitalization. Participants received two doses of mRNA vaccines (Comirnaty from BioNTech/Pfizer and mRNA-1273 from Moderna) or of adenovirus-vectored vaccine (ChAdOx1nCoV-19 from Oxford/AstraZeneca). Samples at 7 days following the second dose were collected for analysis of both neutralizing antibody titer and virus-specific antibody titer, respectively. For the Comirnaty mRNA booster vaccine, 10 breastmilk samples were collected at 7 days post third dose.

All participants received information and written consent was obtained before enrollment. All procedures conformed to the principles of the Helsinki Declaration, and they were performed in accordance with the ethical standards approved by the Ethical Committee of the Hospital Clinico Universitario (ref. 2020/133), the Hospital Sant Joan de Deu (Ref. PIC-94-21), and the Spanish National Research Council-CSIC (ref. 061/2021). Maternal, pregnancy, and birth characteristics were collected as covariates for descriptive purposes and matched on maternal characteristics for associations between SARS-CoV-2 convalescent and vaccinated women (Table 1).

Cell lines

HEK293 cells were used for virus production and A549 hACE2-TMPRSS2 cells (InvivoGen) were used for virus neutralization assays. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum, Pen-Strep, and l-glutamine. In addition, A549 hACE2-TMPRSS2 cells selected with the appropriate antibiotics as indicated by the manufacturer. Both cell lines were regularly tested for mycoplasma and were found negative.

METHOD DETAILS

Sample collection and processing

Breastmilk collection was performed for each woman following a recommended standard procedure.[6,39] Breast skin was cleaned with water and soap, and after discarding the first drops, breastmilk was collected mainly by the use of a sterile pumper in sterile bottles or was manually extracted. Morning collection was recommended. Samples were stored immediately at −20°C and later stored at −80°C. Breastmilk samples were thawed, and fat was removed by centrifugation at 14,000 rpm at 4°C for 10 min. The resulting supernatant was transferred into new tubes and centrifuged again to ensure the removal of all cells and fat. Whey milk was aliquoted and stored at −80°C until further use in ELISA protocols for total IgA determination, SARS-CoV-2-specific, and neutralizing antibody detection.

Virus neutralization assay

The neutralization capacity of breastmilk against the SARS-CoV-2 ancestral (Wuhan-Hu-1), Delta (B.1.617.2), and Omicron BA.1 spike variants was assessed using a GFP-expressing VSV pseudotyped virus on A549 hACE2-TMPRSS2 cells (InvivoGen), as previously described.[40] Briefly, pseudotyped VSV were produced by transfection of HEK293 cells by calcium phosphate with a plasmid encoding the indicated S genes (the generation of the S plasmids used for pseudotyping have been previously described[41]). Following 24 h, cells were infected at a multiplicity of infection of three with VSV lacking the VSV-G protein and encoding both GFP and firefly luciferase[42] that was previously pseudotyped with the VSV-G protein. Following infection, the virus was removed by washing, a monoclonal antibody targeting VSV-G (a kind gift of Rafael Sanjuan, University of Valencia, Spain) was added to neutralize any VSV-G bearing viruses, and infected cells were incubated for 18 h. Subsequently, the supernatant containing the virus was clarified and the virus was concentrated by centrifugation at 50,000xg for 4 h. Finally, the virus was resuspended in PBS, aliquoted, and frozen. The titer of the virus (focus forming units [FFU] per mL) was obtained by serial

dilution on A549 hAce2-TMPRSS2 cells in 96 well plates and counting of GFP-expressing cells using a live cell microscope following 24 h (Incucyte SX5, Sartorius).

For virus neutralization assay, all tests were performed in duplicates on delipidated milk using 5-fold dilutions starting at an initial dilution of 1:20, with ~1000 FFU per well. Following 16 h of infection, the GFP signal in each well was quantified using a live-cell microscope (Incucyte SX5, Sartorius). Background fluorescence from uninfected wells was subtracted from all infected wells, and the GFP fluorescence in each antibody-treated dilution was standardized to the average fluorescence observed in mock-treated wells. Any value resulting in a relative GFP signal of <0.001 was assigned a value of 0.001 to eliminate negative values. Finally, the reciprocal antibody dilution resulting in 50% virus neutralization was calculated using the drc package (version 3.0-1) in R via a three-parameter log-logistic regression model (LL.3 model). Sera testing negative (undetectable) were arbitrarily ascribed a titer of 1/10 (limit of quantitation of the assay for all variants).

SARS-CoV-2-specific antibody detection

Levels of antibodies directed against the receptor-binding-domain (RBD) of the SARS-CoV-2 spike protein were analyzed as previously described.[5,6,43] Briefly, RBD protein was obtained through BEI Resources and was used to coat 96-well ELISA immunoplates (Costar) at 2 μg/mL and incubated at 4 °C overnight. Plates were blocked in 3% (w/v) milk powder in PBS containing 0.1% Tween 20 (PBS-T) for 1 h. For titration curves, 4-fold dilution of samples in 1% (w/v) milk powder in PBS-T was added and incubated for 2 h at room temperature. For detection of the different antibody isotypes, anti-human IgA (α-chain-specific) HRP antibody (Thermo-Fisher Scientific; A18781; 1:6.000) and anti-human IgG (Fc-specific) HRP antibody (Sigma-Aldrich; A0170; 1:4.000) were used and incubated for 1 h in 1% (w/v) milk powder in PBS-T. Bound RBD-specific antibodies were detected with 100 μL of 3,3′,5,5′-Tetramethylbenzidine (TMB) and reactions were stopped with 50 μL of 2M sulfuric acid. Absorbance at 450 nm was read in a ClarioStar (BMG Labtech) microplate reader using the path length correction mode.

Milk samples were considered positive when OD values from undiluted samples exceeded the positive cut-off values for each assay and isotype calculated from pre-pandemic control samples used in a previous study[6] and defined as the mean + two standard deviations (SD). Values from titration curves were used for determining endpoint titers using a 4-parameter non-linear regression function in GraphPad Prism Version 8.4.3, GraphPad Software, San Diego, California USA, www.graphpad.com, and the positive cut-off values obtained from the pre-pandemic control group for each isotype. Low endpoint titers that could not be modelized (n = 3 for ChAdOx1, n = 1 for Omicron BA.1 wave and n = 1 for Wuhan-Hu-1 wave) were assigned a value of 0.1 for plotting and calculation purposes.

Total IgA quantitation

Total IgA was quantified using the human IgA uncoated ELISA Kit (Invitrogen) according to the manufacturer's instructions. Briefly, anti-human IgA was coated onto an immunoplate (Costar) O/N at 4°C and whey milk samples were diluted in 1X Assay buffer 1:40.000. Bound IgA was detected using an anti-human IgA conjugated to HRP that catalyzed the colorimetric reaction with TMB.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were conducted in GraphPad Prism Version 8.4.3, GraphPad Software, San Diego, California USA, www.graphpad.com. Prior to evaluation and comparison of the data, normality was determined by the Shapiro-Wilk normality test. Statistical differences were assessed using either Student's *t* test and One-Way ANOVA (normal distribution) or the Mann-Whitney and Kruskal-Wallis test with Dunn's multiple comparison test. Categorical data were analyzed using Fisher's exact test Chi-squared test. Differences were considered significant with $p < 0.05$.