Biodistribution of mRNA COVID-19 vaccines in human breast milk

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Summary

Background COVID-19 mRNA vaccines play a vital role in the fight against SARS-CoV-2 infection. However, lactating women have been largely excluded from most vaccine clinical trials. As a result, limited research has been conducted on the systemic distribution of vaccine mRNA during lactation and whether it is excreted in human breast milk (BM). Here, we evaluated if COVID-19 vaccine mRNA is detectable in BM after maternal vaccination and determined its potential translational activity.

Methods We collected BM samples from 13 lactating, healthy, post-partum women before and after COVID-19 mRNA vaccination. Vaccine mRNA in whole BM and BM extracellular vesicles (EVs) was assayed using quantitative Droplet Digital PCR, and its integrity and translational activity were evaluated.

Findings Of 13 lactating women receiving the vaccine (20 exposures), trace mRNA amounts were detected in 10 exposures up to 45 h post-vaccination. The mRNA was concentrated in the BM EVs; however, these EVs neither expressed SARS-COV-2 spike protein nor induced its expression in the HT-29 cell line. Linkage analysis suggests vaccine mRNA integrity was reduced to 12–25% in BM.

Interpretation Our findings demonstrate that the COVID-19 vaccine mRNA is not confined to the injection site but spreads systemically and is packaged into BM EVs. However, as only trace quantities are present and a clear translational activity is absent, we believe breastfeeding post-vaccination is safe, especially 48 h after vaccination. Nevertheless, since the minimum mRNA vaccine dose to elicit an immune reaction in infants <6 months is unknown, a dialogue between a breastfeeding mother and her healthcare provider should address the benefit/risk considerations of breastfeeding in the first two days after maternal vaccination.

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Introduction

The SARS-CoV-2 spike (S) protein was identified as the primary vaccine target for COVID-19 disease, as it contains the receptor-binding domain that allows for viral host cell entry.[1] This effort has led to the development of two effective nucleoside-modified mRNA vaccines encoding the SARS-CoV-2 spike (S) protein—BNT162b2 manufactured by Pfizer-BioNTech and mRNA-1273 manufactured by Moderna. Clinical trials for the COVID-19 vaccines were established in what seemed like record time; however, hundreds of scientists had worked on mRNA vaccines for decades before developing these life-saving vaccines.[2,3] However, several vulnerable groups, such as pregnant and lactating women, have been excluded from the initial vaccine clinical trials.[4,5] Nevertheless, based on favorable

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Research in context

Evidence before this study

COVID-19 mRNA vaccines are crucial in combating SARS-CoV-2 infection; however, most clinical trials have excluded lactating women. The previous assumption that the mRNA vaccine is rapidly broken down at its intramuscular administration site with no biodistribution to other organs in human subjects has been challenged. In murine models, the biodistribution of the mRNA-loaded lipid nanoparticles after intramuscular administration demonstrated transportation and active translation of the vaccine mRNA in several organs. Few human studies have evaluated the vaccine mRNA biodistribution during lactation, whether it reaches the human breast milk, and if it is intact and biologically active.

Added value of this study

Our findings suggest that the COVID-19 vaccine mRNA administered to lactating mothers can spread systemically to breast milk in the first two days after maternal vaccination. However, the mRNA was only occasionally detected in breast milk, in trace amounts, and mainly concentrated in the breast milk extracellular vesicles. Our proposed model suggests that after intramuscular administration, the vaccine mRNA enclosed in lipid nanoparticles is transported to the mammary glands through either hematogenous or lymphatic pathways. Within the mammary cells' cytosol, a portion of the released vaccine mRNA is recruited and packaged into the developing extracellular vesicles, which are then released into the breast milk. Furthermore, our analysis demonstrated that the vaccine mRNA detected in breast milk extracellular vesicles was largely fragmented, retaining only 12–25% of its original integrity. Although the vaccine mRNA appears to be translationally inactive, further investigation is required to determine the minimum amount of mRNA needed to elicit an immune response in newborns.

Implications of all the available evidence

This research would prompt a discussion among the experts responsible for formulating policies related to breastfeeding after mRNA vaccination. Although we believe breastfeeding after mRNA vaccination is safe, a dialogue between a breastfeeding mother and her healthcare provider should address the benefit/risk considerations of breastfeeding in the first two days after maternal mRNA vaccination. The significance of this research extends beyond the scope of COVID-19 mRNA vaccines. The findings provide valuable insights into the transport and presence of vaccine mRNA in breast milk, which can be relevant for assessing the safety and efficacy of future mRNA-based therapies administered to lactating women.

safety profiles and its high efficacy in non-lactating adults, the COVID mRNA vaccine was recommended for breastfeeding mothers.[6–9] However, the possible passage of the vaccine mRNA to breast milk (BM), resulting in neonatal exposure, was not investigated. Notably, the Centers for Disease Control and Prevention (CDC) does not recommend COVID-19 vaccination in infants <6 months of age because of the lack of safety studies and the possible interaction with other routine vaccinations in this age group.[10] The mRNA COVID-19 vaccines comprise lipid nanoparticles (LNPs) that contain mRNA coding the SARS-CoV-2 S protein as the active component.[1] At present, relatively little has been reported on the tissue localization of the LNPs after intramuscular administration of the vaccine.[11] The assumption that the mRNA vaccine is rapidly broken down at its intramuscular administration site with no biodistribution to other organs may not be accurate.[11,12] Following intramuscular administration, the vaccine LNPs were rapidly disseminated to several organs in the murine model.[13,14] Few studies have explored the biodistribution of mRNA vaccines in humans[15–17] and examined the impact of the protein corona on the modification of nanoparticles, which can potentially affect their biodistribution and mRNA release.[18–21] Our recently published research letter[22] demonstrated the presence of COVID vaccine mRNA in the BM extracellular vesicles (EVs); however, the exact mRNA quantification, its integrity (either intact or fragmented), and its potential translational activity were not evaluated. The primary objective of this study was to investigate, using a highly sensitive methodology, whether COVID-19 vaccine mRNA could be detected in the BM of lactating women and, if so, to evaluate their integrity and translational activity.

Methods

This cohort study included 13 post-partum mothers with no significant past medical history or comorbidities who received either the BNT162b2 (Pfizer) or mRNA-1273 (Moderna) COVID-19 vaccine during lactation from February to October 2021. Mothers were asked to collect and immediately freeze expressed BM samples at home until samples were transported to the laboratory. BM samples were collected before vaccination (used as negative control samples) and daily (at least twice/day if possible) for at least 5 days post-vaccination or longer when possible. Seven mothers provided BM samples after both the first and second vaccine doses (Table 1). For this study, we considered each vaccine dose as a separate exposure (13 mothers with 20 exposures). Mothers were instructed to write the "hour of collection post-vaccination" on each container and store the expressed BM in the freezer immediately after collection. The research team transported the samples to the

Participant No.	Exposure	Maternal Age (years)	Ethnicity	Mode of Delivery	Gestational age at birth, wk	Vaccine adminstration after delivery (wk)	Vaccine type	Vaccine dose
P1	E1	33	White	Vaginal	39.3	22.4	BNT162b2	1
	E2	33	White	Vaginal	39.3	25.4	BNT162b2	2
P2	E3	36	White	Vaginal	37.4	5.3	BNT162b2	1
	E4	36	White	Vaginal	37.4	5.3	BNT162b2	2
P3	E5	34	Asian	Cesarean	39.6	15	BNT162b2	1
	E6	34	Asian	Cesarean	39.6	18.2	BNT162b2	2
P4	E7	32	White	Vaginal	38.1	27.1	BNT162b2	1
P5	E8	36	White	Cesarean	39.2	1.4	BNT162b2	1
	E9	36	White	Cesarean	39.2	4.6	BNT162b2	2
P6	E10	29	White	Vaginal	38.6	26.3	BNT162b2	2
P7	E11	38	Black	Vaginal	39.5	23	BNT162b2	1
P8	E12	33	White	Vaginal	26.6	10.1	mRNA-1273	2
P9	E13	37	Asian	Cesarean	39	50.1	mRNA-1273	2
P10	E14	37	White	Cesarean	39.5	2.2	mRNA-1273	1
	E15	37	White	Cesarean	39.5	7.1	mRNA-1273	2
P11	E16	37	White	Vaginal	32.1	1.4	mRNA-1273	1
	E17	37	White	Vaginal	32.1	6.4	mRNA-1273	2
P12	E18	34	White	Cesarean	39.4	3	mRNA-1273	1
P13	E19	36	White	Cesarean	26.2	1.1	mRNA-1273	1
	E20	36	White	Cesarean	26.2	5.1	mRNA-1273	2

Shaded rows indicate subjects that had detectable vaccine mRNA. mRNA-1273 manufactured by Moderna and BNT162b2 manufactured by Pfizer-BioNTech. For the week value, the digit after the decimal point represents the additional days beyond the whole number of weeks. For example, a gestational age of 39.3 represents 39 weeks and 3 days gestation.

Table 1: **Breast milk (BM) samples collected from 13 lactating mothers after receiving COVID-19 mRNA vaccine.**

laboratory on ice for analysis. Before BM collection, all participants tested negative for COVID-19 using a SARS-CoV-2 nasal swab test. No participant reported any unusual vaccine side effects or allergic reactions other than the usual mild discomfort in the arm. Also, no mothers reported COVID-19 disease within a week before the vaccination or during the samples' collection. Despite the instructions given to the mothers to provide a minimum of 5 mL of BM for each sample, the actual amounts collected were often below this threshold. Our primary focus was on detecting vaccine mRNA using droplet digital PCR (ddPCR), which was performed for all samples. Following this step, we prioritized the isolation and characterization of BM EVs for further experimentation, as detailed below. However, in several cases, the inadequate volume of BM collected hindered the completion of all intended experiments, as specified below.

Isolation of extracellular vesicles from breastmilk

As described previously,[22] BM EVs were isolated by sequential centrifugation (Supplemental Methods). The EVs number and characterization were determined by ZetaView (Particle Metrix, Ammersee, Germany), and the EVs recovery rate after the isolation procedure was calculated. Expression of exosome markers CD63 and CD9 was confirmed by anti-CD63 antibody (cat# ab134045, RRID: AB-2800495, Abcam, Waltham, MA)[23] and anti-CD9 antibody (cat# 13403, RRID: AB-2732848, Cell Signaling Technology, Danvers, MA)[24] detection using automated capillary western blot system.

Detection of COVID-19 vaccine mRNA

The level of COVID-19 vaccine mRNA was assayed by ddPCR, which provides higher precision, ultrasensitive mRNA detection, and absolute quantification by providing the absolute count of target mRNA copies per input sample and is superior to RT-qPCR in detection and quantifying low-level mRNA.[25,26] Total RNA was isolated from 0.6 mL of the whole BM. Whenever enough BM samples were available, EVs were isolated (requiring 2.3 mL of whole BM) by miRNeasy mini kit (cat# 217004, Qiagen, Germantown, MD) according to manufacturer instructions. One-third of the eluted RNA was used for reverse transcription reaction (cat# 4368814, ThermoFisher, Waltham, MA) with random primers. Based on the putative sequences of vaccines BNT162b2 (Pfizer) and mRNA1273 (Moderna)[22] two sets of vaccine mRNA detection assays were designed to target two different regions of each vaccine mRNA (Supplemental Table S1), the primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA). These primer sets are specific to the respective codon-modified vaccine mRNA sequences and do not amplify wild-type S-gene (Supplemental Table S2). ddPCR was performed with the QX200 Droplet Digital PCR system (Bio-Rad, Hercules, California, USA) using

2X Supermix for Probes (Bio-Rad, USA, cat# 1863024) following the manufacturer's instruction. RNA from BM spiked with vaccine solution was used as a positive control and for setting the positive droplet threshold. Samples with 3 or more positive droplets were considered vaccine mRNA positive. The copy number of the vaccine mRNA template in the PCR reaction was used to derive the copy number per mL of whole milk, or in the case of EVs, the whole milk equivalent corresponding to the whole milk volume used for EVs isolation.

Detection of S protein in skimmed milk, BM cells, and BM EVs

BM cell pellets were collected by centrifugation at 2000×*g* for 10 min at 4 °C and were lysed in RIPA buffer with proteinase inhibitor cocktail (cat# 32955, Thermo Scientific, Rockford, IL, USA). The resulting supernatant was transferred into new tubes and centrifuged again at 17,000*g* for 60 min at 4 °C to obtain the skimmed acellular sample. The cell pellets and BM EVs were lysed in RIPA buffer with proteinase inhibitor cocktail (cat# 32955, Thermo Scientific, Rockford, IL, USA). The S protein expression was assayed by anti-S antibody (cat# 99423, Cell Signaling Technology, Danvers, MA, USA)[27] detection using an automated capillary western blot system. The presence of the SARS-CoV-2 Spike protein in the skimmed milk was determined using the COVID-19 S-Protein (S1RBD) ELISA Kit (Cat # ab284402, Abcam). The sensitivity of the kit is 4.5 pg/mL. In addition to the Spike protein standard included in the kit, vaccine mRNA translated Spike protein in the cell lysate of BNT162b2 and mRNA1273 treated HT-29 cells were used to validate further the specificity of the ELISA kit.

Expression of S protein in HT-29 cells treated with BM EVs

HT-29 cells, a human colorectal adenocarcinoma cell line with epithelial morphology (ATCC HTB-38 RRID: CVCL-0320), were seeded in a 48-well plate, and after attachment for 24 h, cells were treated with a suspension of BM EVs (2×10^{10} particles/well) and incubated for 24 h. Thereafter, the cells were lysed in RIPA buffer, and the expression of S protein was assayed as described above. As a positive control, HT-29 cells were treated with mRNA-1273 at various dilutions ($1{:}10^4$, $1{:}10^6$, and $1{:}10^7$). The concentration of the vaccine mRNA at the dilution of $1{:}10^7$ is similar to the average concentration detected in BM EVs. Cells were lysed, and the expression of spike protein was assayed using automated capillary western blotting.

Linkage duplex assays

The above-mentioned PCR-based assays detect the presence of very short mRNA sequences but do not distinguish whether these sequences are derived from the full-length vaccine mRNA present in the sample or a fragmented mRNA segment. Linkage studies can provide information on the quality of the vaccine mRNA in a sample and allow the determination of how degraded or fragmented the mRNA is. The ddPCR duplex assay[28,29] uses two probes targeting the flanks of the intact vaccine mRNA. By quantifying the proportion of droplets in which both assays yield amplification, samples containing intact vaccine mRNA (positive linkage) can be distinguished from samples containing only fragmented mRNA.[30]

To investigate the integrity of vaccine mRNA in our samples, linkage ddPCR was performed using a ONE Step RT ddPCR advanced Kit for Probes (cat# 1664021, BioRad, Hercules, CA) following the manufacturer's instructions. Two 20X assays (mRNA-1273-FAM and mRNA1273-2-HEX) spanning 1598nt of the vaccine mRNA (nt876–nt2474) were combined with Supermix, reverse transcriptase, DTT, and RNA to a 20 µL reaction. RT-PCR amplification was carried out on a T100 Touch thermal cycler (Bio-Rad, USA) using a thermal profile beginning with reverse transcription at 46 °C for 60 min, followed by Taq polymerase activation at 95 °C for 10 min; amplification for 40 cycles of 95 °C for 30 s and 59 °C for 60 s; and concluding with 98 °C for 10 min. After PCR, the plate was analyzed on a droplet reader (Bio-Rad, Hercules, California, USA). Values for the copies/µL of linked molecules were derived using a method described previously.[28,29] The percent linkage of each sample was expressed as the percentage of linked molecules in relation to the total molecules detected, normalized to the original vaccine stock solution. The concentration of the target molecule sequence was determined by using the ratio of negative partitions to the total number of partitions and applying the Poisson distribution accomplished by the QX Manager Software.[31] Linkage was calculated by QX Manager Software, which determined the excess of double-positive droplets over the expected due to random colocalization of unlinked targets. Percent linkage of each sample was expressed as the percentage of linked molecules in relation to the total molecules detected, normalized to the original vaccine stock solution. QX Manager Software makes two assumptions to fit the Poisson distribution: a) all the partitions are of equal volume, and b) target molecules are randomly distributed across partitions.[32] Software algorithm by QX Manager Software developed by BioRad was used to ensure the validity of the Poisson distribution assumption.

Cytokines secretion in vaccine-stimulated cord blood mononuclear cells and HT-29 cells

Cord blood mononuclear cells (CBMCs) were isolated from umbilical cord blood collected from pregnant women with no COVID-19 disease or COVID-19 vaccination history. CBMCs were isolated using Lymphoprep™ Tube (cat# 1019818, Alere Technologies AS, Oslo, Norway) following the manufacturer's instructions. Isolated CBMCs were aliquoted and stored in

the gas phase above the liquid nitrogen until use. One day before vaccine stimulation, aliquots of CBMCs were thawed and recovered in RPMI-1640 supplemented with 10% human serum from AB Plasma (H3367, Sigma) and penicillin-streptomycin. On the day of vaccine treatment, CBMCs and HT-29 cells were seeded at a density of 1×10^6/well in 24-well plates. BNT162b2 and mRNA-1273 at $1{:}10^3$ and $1{:}10^6$ dilution; LPS (3 EU/mL, *E. coli* 026; B6 cat# L8274, Sigma Aldrich, St. Louis, MO), Poly (I:C) (10 μg/mL, catalog code tlrl-pic, Invivogen, San Diego, CA), R848 (2 μg/mL catalog code tlrl-r848, Invivogen, San Diego, CA) were added as a positive control for TLR4, TLR3, and TLR7/8 agonists, respectively. After 24 h treatment, condition media were collected and centrifugated at 12,000*g*, 10 min at 4 °C. Cytokines in the supernatant was assayed by commercial ELISA kits: TNFa, Invitrogen™ TNF alpha Human Uncoated ELISA Kit (cat# 88-7346, ThermoFisher); IL6, Invitrogen™ IL-6 Human Uncoated ELISA Kit (cat# 88-7066, ThermoFisher); IFNr, Human IFN-gamma DuoSet ELISA (cat# DY285B, RnDSystems); IFNa, ProQuantum Human IFNa immunoassay Kit (cat# A42897, ThermoFisher). In addition, as a positive control to confirm the functional activity of the vaccine used, we incubated CBMCs with the mRNA vaccine at $1{:}10^3$ and $1{:}10^6$ dilution for 24 h. Thereafter, the cells were lysed in RIPA buffer, and the expression of S protein was assayed using automated capillary western blotting. The $1{:}10^6$ mRNA vaccine dilution is similar to the maximum levels detected in the BM EVs, and the $1{:}10^3$ dilution (100 ng/mL) represents the maximum possible level detected in the serum of vaccinated women.[33] Yeo et al.[33] have detected the presence of COVID-19 vaccine mRNA in 20 serum samples collected from lactating mothers who received the vaccine, with maximum levels reaching approximately 70 ng/mL.

Automated capillary western blot (WES)

Proteins in cell lysate and EV were analyzed with a WES system (ProteinSimple) according to the manufacturer's instructions, using a 12–230 kDa Separation Module (ProteinSimple SM-W002). The proteins of interest were assayed with rabbit monoclonal antibody detection as described above, and the signal was detected using the Anti-Rabbit Detection Module (ProteinSimple DM-001). Data were analyzed using Compass™ software (V.2.6.5, Protein Simple).

Ethics committee approval

New York University institutional review board approval (approval number: s18-01725) was obtained before initiating the study. Written informed consent was obtained from all volunteers before enrollment in the study.

Statistical analysis

Statistical analysis was performed by using GraphPad Prism v9.00. The data's normality was tested by the Shapiro–Wilk test and visual assessment of Q–Q plot. If the samples followed a normal distribution, we chose the appropriate parametric test; otherwise, the non-parametric counterpart was chosen. For multiple groups comparison, repeated-measures one-way analysis of variance (ANOVA) with posttest Holm-Šídák's multiple comparisons test or Friedman with posttest Dunn's multiple comparisons were used as indicated. Sphericity was assessed by Mauchly's test using Mauchly package in Stata version 18.0. When the significance level of the Mauchly's test is >0.05, sphericity is assumed, if $p \leq 0.05$, Geisser-Greenhouse correction will be implemented in repeated-measures one-way ANOVA. The choice of each test was dependent on the underlying distribution and is indicated in the legend of the figures.

Role of funding source

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Results

A total of 13 subjects (representing 20 exposures) were enrolled, with 11 exposures to the BNT162b2 vaccine and 9 exposures to the mRNA-1273 vaccine, as detailed in Table 1. Daily collection of BM samples (2–5 samples/day) for the first 5 days post-vaccine exposure was achieved for 11 of the 20 exposures. Daily sample collections were not feasible for the other exposures because of the scant BM produced by the lactating mothers. A total of 154 samples were collected from the 20 vaccine exposures.

Detection of COVID-19 vaccine mRNA in whole BM and BM EVs using quantitative ddPCR

All pre-vaccination BM samples were negative for COVID-19 vaccine mRNA. Small amounts of vaccine mRNA were detected in the whole BM in 15 samples from 10 exposures at 3–45 h post-vaccination (Fig. 1). No vaccine mRNA was detected in any collected whole BM samples beyond the 48-h post-vaccination time point. Also, no vaccine mRNA was detected in the BM fat fraction or the BM cell pellets (data not shown). We also investigated if the vaccine mRNA could be packaged and detected in EVs secreted in BM. Isolated BM EVs were analyzed using Particle Metrix ZetaView Nanoparticle Tracking Analysis (NTA) (Supplemental Figure S1). The yield of EVs from BM was a median of 8.08×10^9 (IQR 2.66×10^9–3.02×10^{10}) particles/mL, and the mean (SD) particle size was 110.0 (3.0) nm. No

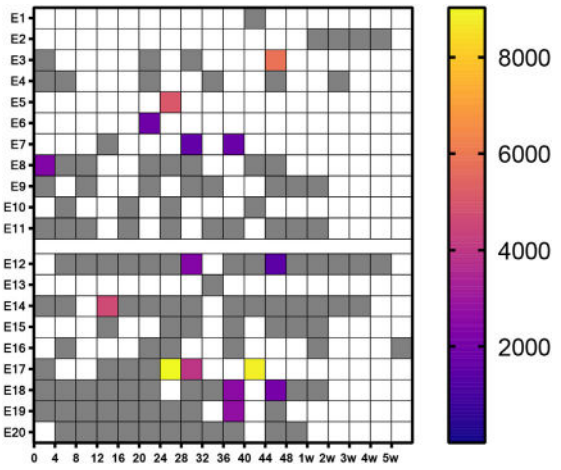


***Fig. 1:* COVID Vaccine mRNA detected in breast milk (BM) measured by ddPCR.** The heat map represents the vaccine mRNA concentrations in the BM. The X-axis represents the time points between vaccination and sample collection (hours/weeks). The Y axis represents different individual exposures (as detailed in Table 1) to BNT162b2 (upper panel) and mRNA1273 (lower panel). White cells indicate there were no samples collected in that time interval. Gray cells indicate vaccine mRNA was not detected. The amount of mRNA (copies/mL BM) in the sample is indicated by the color gradient.

vaccine mRNA was detected in the pre-vaccinated BM EV samples. Whenever sufficient BM samples were available, EVs were isolated from BM samples that tested positive for the vaccine mRNA. Owing to the scant amount of BM supplied by some mothers in the first week of life, only a few samples contained sufficient BM to isolate EVs. Approximately 90% of the EVs from BM were recovered with our method. The vaccine mRNA was consistently detected in EVs whenever their corresponding whole BM samples were positive. The vaccine mRNA copy numbers in whole BM samples and their corresponding EV fractions, normalized to the starting BM volume (mL), are presented in Table 2. As shown in Table 2, vaccine mRNA in the BM was concentrated in the EVs, with approximately 12–90% of vaccine mRNA found in the EV fraction, even though the EVs only account for a very small fraction of the whole BM volume. The effect of the milk type (colostrum vs. transitional vs. mature milk) on EV count and mRNA content could not be evaluated since the mRNA-positive samples included only one colostrum and three transitional milk samples. Although the number of EVs was similar in the transitional samples compared to mature milk samples, the small sample size hindered a meaningful comparison.

S protein was not detected in skimmed BM, cell pellets, or EV samples positive for vaccine mRNA

Using the automated capillary western blotting, the S protein was not detected in EV samples derived from pre-vaccination BM samples nor in the post-vaccination BM EV samples that tested positive for vaccine mRNA. Furthermore, all skimmed and cell pellets of the BM samples tested were negative for S protein expression (data not shown).

Vaccine mRNA-positive EVs did not induce S protein expression in HT-29 cells

Isolated EVs from BM samples that tested negative for vaccine mRNA (pre-vaccination BM samples) and EVs from BM samples that tested positive for vaccine mRNA were incubated with HT-29 cells for 24 h (samples E5, E7, and E17). As shown in Fig. 2, no S protein was detected in any of the samples tested. However, positive control samples used in concentrations similar to those of BM EVs also failed to induce S protein expression.

Vaccine mRNA in the EVs is partially intact

Vaccine mRNA integrity was assayed in a duplex ddPCR using two probes targeting the flanks of the intact mRNA vaccine. Positive linkage indicates vaccine mRNA integrity, and negative linkage indicates fragmented vaccine mRNA.[28,29,34] Due to limited availability, only five samples were assayed (Fig. 3). Percent linkage of each sample was expressed as the percentage of linked molecules in relation to the total molecules detected, normalized to the original vaccine stock solution.[31,32,34] As demonstrated in Fig. 3c, the vaccine

Sample ID	Vaccine type	Time of sample collection (h)	Vaccine RNA (copies/mL)		% of mRNA in EVs (%)
			Whole BM	EV fraction	
E5	BNT-162b2	27	5035	595	12
E7	BNT-162b2	37	1544	340	22
E12	mRNA-1273	44	1247	1120	90
E17	mRNA-1273	42	7604	1953	26

EVs of breast milk (BM) from 4 positive samples were isolated by differential centrifugation method. Vaccine mRNA copy numbers in whole milk and EV fraction were normalized to the starting BM volume (mL). All positive samples were detected within the first 48 h after vaccination. Vaccine mRNA in the BM was concentrated in the EVs, with approximately 12–90% of total vaccine mRNA found in the EV fractions even though the EVs only account for a very small fraction of the whole milk volume.

***Table 2:* Distribution of vaccine mRNA in whole milk and extracellular vesicles (EVs) from vaccinated women.**

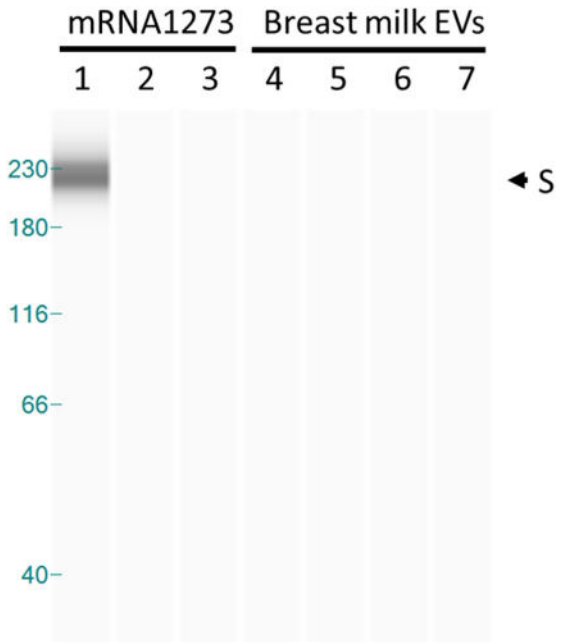


Fig. 2: **Vaccine mRNA-positive BM EVS did not induce spike protein expression when incubated with intestinal HT-29 cells for 24 h.** HT-29 cells treated with vaccine mRNA1273 at different dilutions were used as positive controls (1:10^4, 1:10^6, and 1:10^7, lanes 1–3, respectively). Lane 4 represents EVs from pre-vaccination BM. Lanes 5–7 represent EVs positive for the vaccine mRNA (lane 5, E5; lane 6, E7; lane 7, E17, respectively). Cells were lysed, and the expression of spike protein was assayed by automated capillary western blot (WES). S: Full-length spike protein. No S protein was detected in any of the BM EV samples tested. However, positive control samples in concentrations similar to those of BM EVs (lane 3) also failed to induce S protein expression. The only positive control sample that induced spike protein was the HT-29 cells treated with a higher concentration of stock mRNA vaccine (1:10^4, lane 1).

mRNA in BM samples retained only 12–25% of its original mRNA vaccine integrity.

Vaccine mRNA did not induce significant cytokines secretion in CBMCs or HT-29 cells

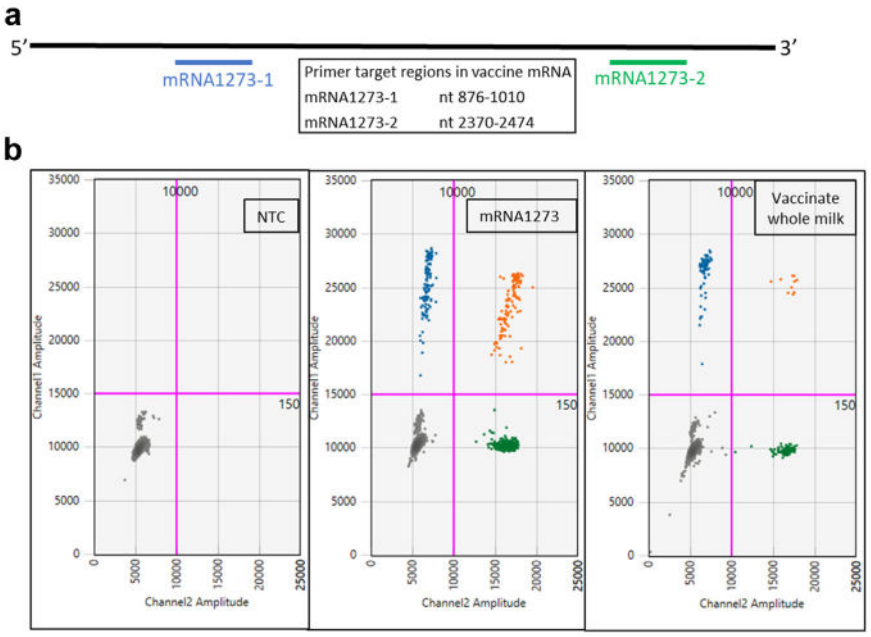
As shown in Fig. 4a, ssRNA-sensing toll-like receptor TLR7/8 (ssRNA) agonists R848 induced the secretion of TNFa, IL-6, and IFNa in CBMCs. Double-stranded RNA-sensing TLR3 agonist Poly (I:C) induced TNFa secretion in HT-29 cells. However, cells treated with 1:10^3 and 1:10^6 vaccine mRNA dilution did not induce cytokines secretion. However, only 1:10^3 and not 1:10^6 vaccine mRNA dilution induced S protein expression in CBMCs (Fig. 4b).

Discussion

Our findings suggest that the COVID-19 vaccine mRNA administered to lactating mothers can spread systemically to the BM in the first two days after maternal vaccination. However, the mRNA was only occasionally detected in BM, in trace amounts, and mainly concentrated in BM EVs. The linkage analysis showed that the vaccine mRNA detected in BM was largely fragmented and retained only 12–25% of the original vaccine mRNA integrity. While the vaccine mRNA seems to be translationally inactive, further investigation is required to determine the minimum amount of mRNA needed to elicit an immune response in newborns.

Initially, it was thought that the vaccine mRNA encapsulated in LNPs would remain localized at the injection site and quickly degrade. However, several reports suggest that the LNPs/mRNA can enter the bloodstream and accumulate in distant tissues.[14,35] The Pfizer and Moderna Assessment Reports provided to the European Medicines Agency[16,17] concluded that a small fraction of the administered mRNA dose was distributed to distant tissues, mainly the liver, adrenal glands, spleen, and ovaries. Additionally, mRNA constructs persisted for 1–3 days in tissues other than the injection site. For lactating mothers receiving the vaccine, our results suggest that the vaccine LNPs will reach the breast tissue. However, since the intact blood-milk barrier prevents an uncontrolled exchange of soluble and cellular components between blood and milk in the mammary gland[36] it is unlikely that intact LNPs will pass the blood-milk barrier to the BM. Using the fraction of RNA we detected in breast milk/mL, we calculated that the expected level of lipids in the same volume of milk was below the level of detection using the currently available analytical methodology. Our model (Fig. 5) proposes that following intramuscular administration, the LNPs containing the vaccine mRNA are likely carried to mammary glands via hematogenous or lymphatic transport.[13,14] The LNPs will release their mRNA content into the cytosol of the mammary gland cells, and a portion of this mRNA will be recruited, packaged, and released in the BM EVs (exosomes or microvesicles). This can be significant as the BM EVs act as natural LNPs, protecting the mRNA from degradation. Milk-derived EVs are resistant to proteolysis by gastric and pancreatic secretions and can be readily absorbed by intestinal epithelial cells.[37] Because of their ability to transfer and protect the mRNA, milk EVs have been tested as a vehicle for COVID mRNA oral vaccine.[38] Since the cells likely to encounter BM EVs-loaded mRNA are intestinal epithelial cells, our study used intestinal epithelial HT-29 cells, a human colorectal adenocarcinoma cell line with epithelial morphology. Due to their similarities with enterocytes of the small intestine, it has been used as an in-vitro model to study absorption, transport, and secretion by intestinal cells and has been used to study intestinal cell response to human milk factors and human milk oligosaccharides.[39,40]

Notably, the detected mRNA in whole BM samples includes both mRNA in the EVs and the mRNA outside the EVs. These results indicated that the vaccine mRNA was concentrated mainly in the BM EVs.



c

Sample ID	Sample type	Time of sample collection	% linkage
E17	Whole milk	27 hrs	25.7
E17	Whole milk	42 hrs	19.5
E17	EVs	42 hrs	19.6
E18	Whole milk	45 hrs	12.1
E18	EVs	45hrs	23.3

Fig. 3: **Integrity of vaccine mRNA in breast milk from vaccinated women. (a)** Vaccine mRNA integrity was assayed in a duplex ddPCR assay using a two-primer set targeting 1602 nt of the mRNA1273 sequence. **(b)** Representative dot plot profiles of FAM-labeled mRNA1273-1 primer and probe (channel 1, amplitude) and HEX-labeled mRNA1273-2 primer and probe (channel 2, amplitude). Droplets emitting 2D signals were separated into four groups (Gray, double negative for mRNA1273-1 and mRNA1273-2; Blue, positive for mRNA1273-1, negative for mRNA1273-2; Green, positive for mRNA1273-2, negative for mRNA1273-1; Orange, double positive for both mRNA1273-1 and mRNA1273-2). Left panel, No template control; middle panel, RNA isolated from vaccine mRNA1273 stock (positive control); right panel, a representative BM sample from a vaccinated woman. **(c)** The number of droplets in each single or double positive group was derived by QX Manager Software. Percent linkage of each sample was expressed as the percentage of linked molecules in relation to the total molecules detected, normalized to the original vaccine stock solution.

These results confirm our previous findings.[22] Furthermore, our results demonstrated that the vaccine mRNA-positive EVs did not induce S protein expression in HT-29 cells. However, positive control samples used in concentrations similar to those of BM EVs also failed to induce S protein expression. Although this may indicate that the vaccine mRNA in the EVs is not translationally active, it may also indicate that the methodology used is not sensitive enough to detect S protein expression. Thus, confirming the lack of translational activity needs further investigation. Our finding that the COVID-19 mRNA vaccines do not induce cytokine secretion in cord blood immune cells is in agreement with previous reports demonstrating that mRNA vaccine does not induce various cytokine secretion in adults.[16,17,41]

Other studies have also detected the COVID-19 vaccine mRNA in BM.[22,33,42] Low et al.[42] detected the vaccine mRNA in BM samples at a maximum concentration of 2 ng/mL, which is much higher than the concentrations we observed. Yeo et al.[33] detected vaccine mRNA in BM and serum samples in comparable concentrations. One published study[43] did not detect the COVID-19 vaccine mRNA in BM in a limited number of samples (15 BM samples, compared to 154 samples in our study). Similar to our study, self-collected post-vaccination BM

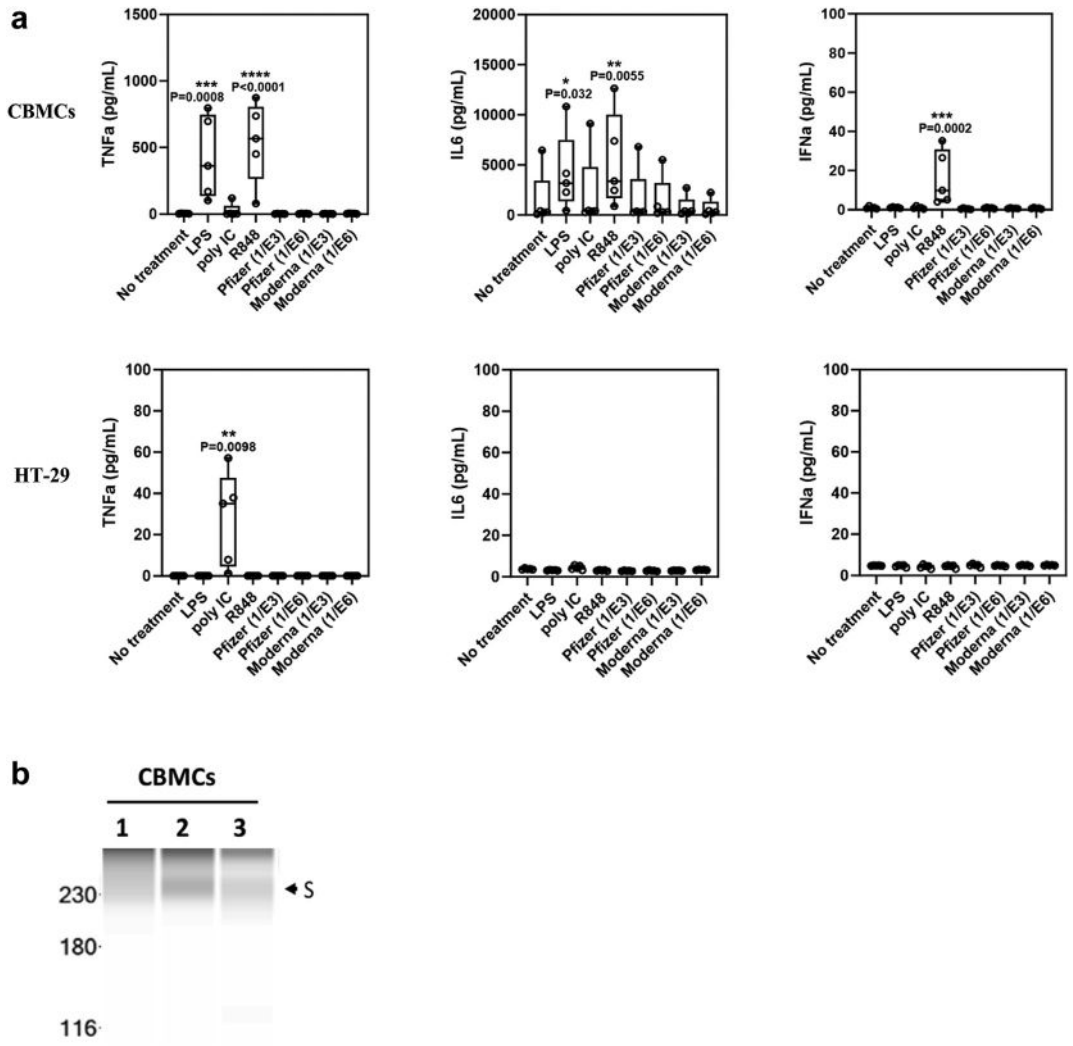


Fig. 4: **Cytokine levels in COVID-19 mRNA vaccines stimulated CBMCs and HT-29 cells. (a)** Following 24 h stimulation with 1–10^3 and 1–10^6 diluted reconstituted vaccine product, cytokine concentrations in supernatant from CBMC (5 biological replicates) and HT-29 cells (n = 5) were measured by ELISA as described in Material and Methods. TLR agonists LPS, Poly IC, and R848 were used as positive control. Cytokine concentrations of CBMC (Upper panel) and HT-29 cells (Lower panel) are presented as box-and-whisker plot showing the median and IQR with minimum and maximum whiskers. p values were computed using repeated measure one-way ANOVA with posttest Holm-Šídák's multiple comparisons test (CBMC: TNFa, IFNa and HT-29: IL6, IFNa) or Friedman with posttest Dunn's multiple comparisons (CBMC: IL6 and HT-29: TNFa). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. **(b)** Representative sample of S protein expression in vaccine BNT162b2 treated CBMCs. 1) CBMCs received no treatment, 2) CBMCs treated with 1:10^3 diluted BNT162b2, and 3) treated with 1:10^6 diluted BNT162b2. S: full-length spike protein.

samples were frozen at home until transported to the laboratory. However, the study did not evaluate vaccine mRNA in BM EVs and used a single primer set, which covers only 131 bases of the 5′ end of the vaccine mRNA sequence. This primer carries a one-base mismatch for mRNA-1273, which might have reduced RT-qPCR sensitivity. In our study, considering the differences in nucleotide sequence between BNT162b2 and mRNA1273, two distinct sets of primers were designed, each specifically targeting the respective vaccines' mRNA. Also, each pair of primers covers approximately 1.5 kb of the full-length vaccine mRNA, increasing assay specificity and sensitivity. In addition, using quantitative ddPCR significantly improves the sensitivity of the detection.

Based on the sporadic detection of trace amounts of the vaccine mRNA in BM, the likelihood that the vaccine might be biologically inactive, the excellent safety profile of the COVID-19 mRNA vaccines thus far, and their efficacy in protecting lactating women, the

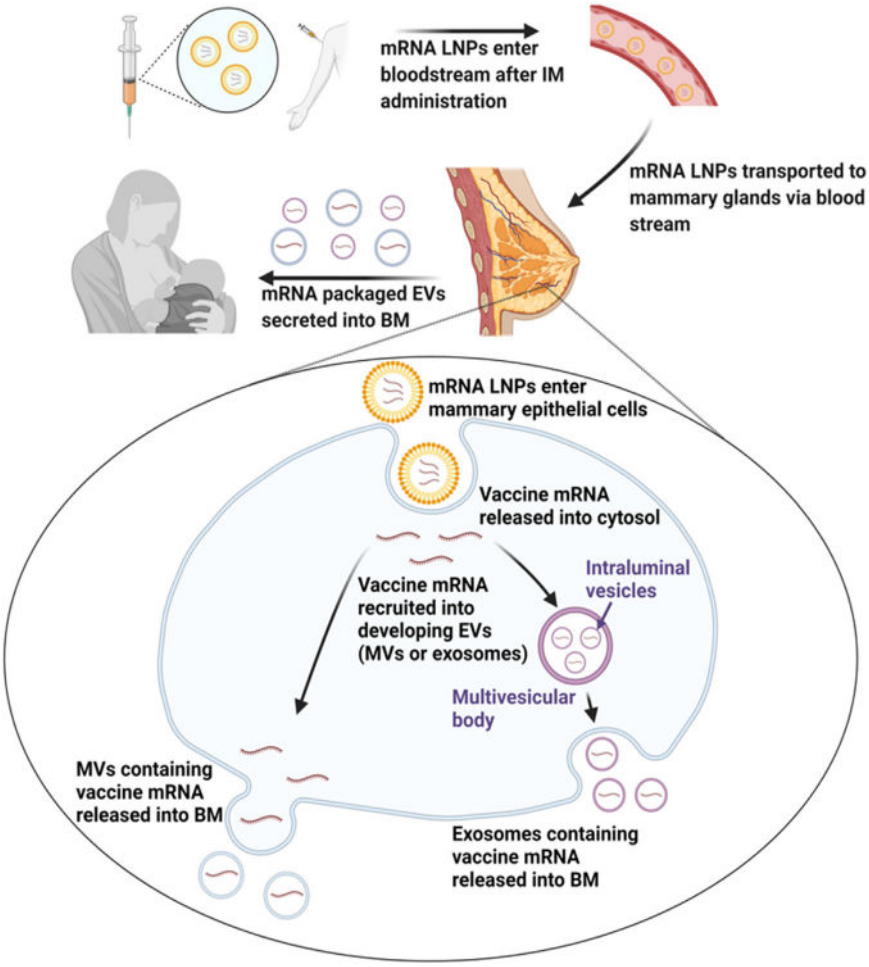


Fig. 5: **Proposed model of biodistribution of vaccine mRNA to breast milk (BM).** Following intramuscular administration, the vaccine mRNA enclosed in lipid nanoparticles (LNPs) is transported to the mammary glands through either hematogenous or lymphatic pathways. Within the mammary cell cytosol, a portion of the released vaccine mRNA is recruited and packaged into developing extracellular vesicles (EVs). The vaccine mRNA can be packaged into multivesicular bodies as intraluminal vesicles that will fuse with the mammary cell's plasma membrane, resulting in the release of mRNA-containing exosomes/EVs into breast milk. Some vaccine mRNA can also be packaged into microvesicles (MVs) formed by the outward budding of the mammary cell's plasma membrane and released into BM. This illustration was created with BioRender.com

benefits of these vaccines in protecting lactating women appear to outweigh the potential risks, especially after 48 h post-vaccination. However, for the first 48 h, when mRNA can be detected in the BM, a risk/benefit assessment is warranted. The potential bioactivity of the mRNA vaccine in BM will depend on several factors, including the concentration of biologically active mRNA and the specific cell type responding to the mRNA vaccine. The COVID mRNA serum levels required for vaccine efficacy after intramuscular vaccination are not defined. A recent report[5] suggested that the dose of COVID-19 vaccine detected in BM is of no concern because it is 0.002% of the intramuscular vaccine dose. However, this assumption may not be accurate. After vaccine mRNA biodistribution, the effective serum concentration is expected to be a very small fraction of the intramuscular dose. For example, the peak ampicillin serum level after intramuscular administration of 1000 mg is approximately 10 mcg/mL after 1 h[44]; this is 0.001% of the dose. Another study demonstrated that a lactating mother's vaccine mRNA serum concentration was comparable to that in her BM.[33] This can be of concern in breastfed infants, considering the minimum mRNA dose needed to elicit an immune reaction in infants <6 months of age is unknown. A recent report demonstrated an inflammatory response to COVID mRNA vaccines 48 h following the second vaccine dose.[45] As the risk/benefit balance of the COVID-19 vaccine can change over time, information transparency is imperative. A discussion between a breastfeeding mother and her healthcare provider will address the benefit/risk considerations of

continuing breastfeeding or withholding it temporarily (but continuing feeding her infant pre-vaccination collected BM) for 48 h after vaccination. This is consistent with the CDC's position not recommending COVID-19 vaccine exposure in infants <6 months of age because of the lack of safety studies. Concerns regarding vaccine exposure in BM are not unprecedented. The Yellow Fever live-attenuated vaccine was detected in BM; hence, the CDC recommends against breastfeeding in women until the vaccine exposure risks are evaluated.[46] It was suggested to temporarily withhold breastfeeding in the 10 days following the Yellow Fever vaccination, during which time the vaccine content is detectable in BM.[47] Notably, passive antibody transfer via BM does occur after maternal COVID-19 vaccination on the order of days to weeks post-vaccination and minimally in the first 48 h.[33]

Our study has some limitations, including the small sample size. Given the novelty of the vaccine, the narrow focus of our cohort, and the rarity of women receiving the vaccine during lactation, there were inherent limitations to achieving a larger sample size. Other limitations include the potential underestimation of the mRNA concentrations due to differences in the mothers' collection techniques and storage conditions following self-collection, which may contribute to mRNA degradation. Another limitation includes the limited volume of BM provided by mothers, which limited the feasibility of further experiments. Also, we did not test the possible cumulative vaccine mRNA exposure following frequent breastfeeding in infants, which can add up to 150–200 mL/kg/day of BM.

Conclusion

Our findings suggest that vaccine mRNA is not localized to the injection site but spreads systemically and can be packaged into BM EVs. While the mRNA vaccine seems to be translationally inactive, further investigation is required to determine the minimum amount of mRNA needed to elicit an immune response in newborns. This research would prompt a discussion among the experts responsible for formulating policies related to breastfeeding after mRNA vaccination. Although we believe breastfeeding after mRNA vaccination is safe, a dialogue between a breastfeeding mother and her healthcare provider should address the benefit/risk considerations of breastfeeding in the first two days after maternal mRNA vaccination. This is particularly important given the currently limited data on the effectiveness of booster mRNA vaccines, the varied health statuses of lactating women, and the diverse risk perceptions within our society. Furthermore, the significance of this research extends beyond the scope of COVID-19 mRNA vaccines. The findings provide valuable insights into the transport and presence of vaccine mRNA in BM, which can be relevant for assessing the safety and efficacy of future mRNA-based treatments administered to lactating women. Although there is a theoretical risk for the biodistribution of the mRNA vaccine in the BM, it also may provide a vaccination-protection benefit to the infant. Enhancing our understanding of the distribution patterns, factors that alter the LNPs, such as the corona protein, and the cellular responses to mRNA vaccines can potentially enhance the development of LNP designs and the duration of action of these therapies in lactating and pregnant women. Ultimately, this will contribute to the creation of safer and more effective mRNA therapies for lactating and pregnant women. Regulatory agents should establish comprehensive regulations and allocate necessary resources to facilitate the inclusion of lactating and pregnant women in clinical research, ensuring equal opportunities to benefit from advancements in new therapies and medical science.

Contributors

Dr. Hanna had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis.

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Obtained funding: Nazeeh Hanna.

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Study supervision: Amrita Nayak and Nazeeh Hanna.

All authors read and approved the final version of the manuscript.

Data sharing statement

The data or materials for the experiments reported here can be available at reasonable request and within relevant legal constraints.

Declaration of interests

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104800.

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