Text S1. Extended Material and Methods

Study population

A control group of women not exposed to SARS-CoV-2 and from prepandemic time was included. Those women were randomly selected from the MAMI birth cohort in Spain [1] (ClinicalTrials.gov Identifier: NCT03552939). This protocol was approved by the Ethical Committee of the Hospital Clínico Universitario of Valencia (Ref. 2015/0024) and by the local Ethical Committee of Atención Primaria-Generalitat Valenciana (CEIC-APCV).

Human milk collection and processing

Breast milk collection was performed following a standardized protocol described elsewhere [2]. In brief, breast skin was cleaned with water and soap and the first drops were discarded. Then, milk was collected either by use of a sterile pump or manually extracted. Samples were collected in sterile bottles to normalize collection among participants. Morning collection was recommendable. Finally, breast milk samples were immediately stored at −20 °C in deep freezers and sent to the hospital to be stored at -80°C until further analysis.

Whole milk was used for SARS-CoV-2 RNA detection. Whey milk samples were used for antibody determination and were prepared as follows: samples were thawed and centrifuged at 14,000 rpm at 4 °C for 10 min to remove fat and the resulting supernatant was transferred into new tubes. Centrifugation was repeated twice to ensure removal of all cells and fat. Skimmed acellular milk was then aliquoted and frozen at -80 °C until further use. Pre-pandemic control milk samples were stored at -80°C before processing exactly as described for COVID-19 milk samples.

Validation of SARS-CoV-2 RNA extraction, detection, and quantification in breast milk samples

A manual column-based commercial kit (referred as MN) and an automated assisted method based on magnetic beads (referred as Max) were adapted following previous recommendations [3] and compared to assess their sensitivity for detecting viral particles in breast milk samples. Main modifications of providers' official protocols included: 150 µL of whole breast milk were treated with Plant RNA Isolation Aid (Ambion, USA) prior to extraction with MN (Nucleospin RNA virus Kit, Macherey-Nagel GmbH & Co., Germany), while 300 µL of whole breast milk were used for nucleic acid isolation with Max (Maxwell® RSC Instrument coupled with Maxwell RSC Pure Food GMO and authentication kit, Promega, Spain). RNA was finally eluted in 100 µL nuclease-free water in both extraction protocols.

Initially, to characterize the viral recovery of both methods, porcine epidemic diarrhoea virus (PEDV) strain CV777 (an enveloped virus member of the *Coronaviridae* family and surrogate for SARS-CoV-2), and also, mengovirus (MgV) vMC0 (CECT 100000, non-enveloped member of the *Picornaviridae* designated in the ISO 15216-1:2017 standard method as process control) were spiked in phosphate buffer solution (PBS) and in breast milk. Detection ratios and limits of detection ($LoD_{95\%}$ and $LoD_{50\%}$) were also characterized for both extraction methods by spiking serial dilutions of PEDV.

Finally, serially diluted viral suspensions of gamma irradiated SARS-CoV-2 (Bei Resources; NR-52287), and human coronavirus (HCoV) 229E (ATCC-VR740) were used to further define the analytical performances of Max extraction. Detection limits were calculated using the PODLOD calculation program v.9 according to [4].

Viral RNA detection was performed by reverse transcription polymerase chain reactions (RT-qPCR) using One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Takara Bio, USA), targeting the N1 region of the nucleocapsid gene for SARS-CoV-2 [5], the membrane gene for HCoV 229-E [6] and PEDV [7], and the region from 110 to 209 nucleotides for MgV [8]. The human RNase P gene [5] was used as quality control parameter for extraction. Reaction mixes, thermal cycling conditions, sequences for primers and probes, and standard quantification curves are detailed elsewhere [3,7]. Those for HCoV 229-E detection are included as supplementary material (Figure S1). Genome copies (gc) were calculated by using standard curves of 10-fold serial dilutions of SARS-CoV-2 genomic RNA (ATCC VR-1986D) or HCoV 229-E, PEDV and MgV suspensions in quintuplicates.

All RT-qPCR assays were performed in duplicate on a LightCycler 480 instrument (Roche Diagnostics, Germany). Positive (genomic RNA), negative (nuclease-free water), and inhibition (either 10-fold diluted RNA or RP gene) controls were included in each assay.

Breast milk SARS-CoV-2-specific antibody detection
Levels of antibodies directed to structural proteins like the RBD of the SARS-CoV-2 spike protein and to non-structural viral proteins like the cysteine-like protease, also known as the main protease (Mpro) or 3CLpro, were analyzed.

RBD-specific antibodies were determined using a previously published and validated ELISA protocol for use in human plasma and serum samples [9,10], modified for its use in human milk samples. RBD protein was produced under HHSN272201400008C and obtained through BEI Resources, NIAID, NIH: Spike Glycoprotein RBD from SARS-CoV-2, Wuhan-Hu-1 with C-Terminal Histidine Tag, Recombinant from HEK293T Cells, NR-52946. Briefly, 96-well ELISA immunoplates (Costar) were coated with RBD protein at 2 μg/mL and incubated at 4 °C overnight. Coated plates were blocked in 3 % (w/v) milk powder in PBS containing 0.1 % Tween 20 (PBS-T) for 1 h. Then, 4-fold dilution of samples in 1 % (w/v) milk powder in PBS-T were added, incubated for 2 h at room temperature and washed with PBS-T before addition of horseradish peroxidase-conjugated secondary antibodies. For detection of the different antibody isotypes, anti-human IgA (α-chain-specific) HRP antibody (Thermo-Fisher Scientific; A18781; 1:6.000), anti-human IgM (μ-chain-specific) HRP antibody (Sigma-Aldrich; A0420; 1:4.000), 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 antigen-specific antibodies were detected with 100 μL 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. For detection of MPro-reactive antibodies, a commercial ELISA Kit (ImmunoStep, Salamanca, Spain) was used. Samples were incubated 1:4 diluted, and remaining steps of the protocol were performed according to manufacturer´s instructions. For ELISA studies, milk samples were considered positive when OD values from undiluted samples exceeded the positive cut-off values for each assay and isotype calculated from prepandemic control samples and defined as the mean + two standard deviations (SD). Values from dilution curves were used for determining the area under the curve (AUC) to get a better quantitative impression between COVID-19 and control group. Endpoint titers were calculated from log-transformed titration curves using 4-parameter non-linear regression function in GraphPad Prism 8.0 and the positive cut-off values obtained from the prepandemic control group for each antigen and isotype.

2

Breast milk Total IgA quantification

Total IgA, including secretory IgA (sIgA), was measured in whey milk using a sandwich ELISA quantitation kit from Bethyl Laboratories (Montgomery, TX) following manufacturer's instructions as previously detailed [11]. Briefly, an anti-human IgA antibody pre-adsorbed to the plate allowed to capture the IgA, which was later detected by the addition of a biotinylated detection antibody and streptavidin-conjugated horseradish peroxidase that catalyzed the colorimetric reaction with the chromogenic substrate TMB. All whey milk samples were analyzed at a 1:8,000 dilution rate. Data were expressed as mg/L of milk. Duplicate determinations were performed on each plate.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 8.0. After Shapiro-Wilk normality test, non-parametric t-test (Mann-Whitney) was used to detect significant differences between groups and Spearman correlation analysis to assess correlations between variables.

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3

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