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# Characterization of Extracellular Vesicles From Fresh vs. Frozen Human Milk Including the Vesicular microRNA Cargo

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## ABSTRACT

Human milk is rich in extracellular vesicles (EV) that may contribute to shaping neonatal immunity. Here, we evaluated whether freezing, and the addition of sodium citrate (SC), affect the characteristics of human milk EVs and their miRNAs. Freezing may compromise the milk EV population and their miRNA profile by creating artificial vesicles due to cell lysis. Furthermore, SC can be added to clear the EV fraction of micelles, that is, protein aggregates that co-isolate with milk EVs, and may affect certain downstream analyses. To investigate potential differences between milk EV and their miRNA cargo when isolated from fresh and frozen samples, mature milk samples were collected from 10 women and subjected to four different treatments: fresh and frozen; fresh<sup>SC</sup> and frozen<sup>SC</sup>. Ultracentrifugation was used for EV isolation, and subsequently characterized by Nanoparticle tracking analysis, flow cytometry, Western blot and electron microscopy. While freezing without SC has no impact on the evaluated EV parameters, freezing *with* SC significantly altered particle mean size as measured by NTA and protein levels as studied by MACSPlex flow cytometry. Importantly, neither freezing nor SC had an impact on the EV miRNA cargo, measured by qPCR. These findings also suggest that EV isolates from frozen samples, in comparison to freshly isolated ones, can produce valid results concerning morphology, size, surface markers and the EV miRNA profile.

**Trial Registration:** ClinicalTrials.gov-ID: NCT01542970

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## 1 | Introduction

Human milk is a complex fluid containing a wide variety of macromolecules with distinctive biological functions. In addition to its nutritional value for the infant, it possesses various bioactive properties that can influence the gut microbiome composition, eliminate pathogens, support intestinal barrier function and affect the development of the naïve immune system (Andres et al. 2023). Even though such beneficial effects of human milk have long been acknowledged, more studies are needed to explore additional milk components, such as extracellular vesicles (EV) and their microRNA (miRNA).

Increasing data suggest that EVs, released by numerous cell types and found in several body fluids, including human milk, may exert important immunological effects in the infant (Admyre et al. 2007). Smaller-sized EVs called exosomes (size from 30 to 150 nm in diameter) originating from endosomes, may be of particular interest in this regard (Veerman et al. 2021; Welsh et al. 2024). However, several EV populations, such as microvesicles and apoptotic bodies, overlap in size and density and may hence co-isolate with exosomes; it is thus more correct to refer to the broader term 'EVs' in studies like this one.

Human milk EVs are considered to primarily be produced by the mammary glands and immune cells (Li et al. 2022; Giovanazzi et al. 2023; Alsaweed et al. 2016; Ahlberg et al. 2024) and seem resistant to different digestion processes resembling the baby's gastrointestinal system (Liao et al. 2017; Kahn et al. 2018; Zempleni et al. 2017; Yung et al. 2024). Indeed, previous murine and porcine studies suggest that milk EVs can be traced to a range of different organs post-suckling (Manca et al. 2018; Macia et al. 2019). Hence, it is possible that human milk miRNA carried by the EVs can be absorbed in the intestine and subsequently be involved in gene regulation at multiple sites throughout the infant's body. The packing of EVs is not a random process as it involves sorting mechanisms that favour some cargos over others (Squadrito et al. 2014; Stevanato et al. 2016), suggesting biological relevance of their content. Several miRNAs, that is, short RNA sequences that regulate gene expression and are found in milk EVs, seem to be involved in the regulation of immune modulatory pathways (Ahlberg et al. 2024, 2023; Tingö et al. 2021; Perri et al. 2018; Rodríguez-Galán et al. 2018). Noteworthy, mammalian milk displays a similar abundance of evolutionary conserved immunomodulatory miRNAs across several species, inferring biological relevance (van Herwijnen et al. 2018). Interestingly, we have previously observed that some milk miRNAs correlated with the proportion of subpopulations of T regulatory cells (Treg) in breastfed children (Ahlberg et al. 2023). Hence, milk EVs and their miRNA cargo could be an additional route to facilitate immune programming in infants, yet less explored as compared to other commonly known macromolecules, for example, immunoglobulins (Kosaka et al. 2010; Vahkal et al. 2025).

It has been argued that milk samples intended for EV isolation should be processed as soon as possible after expression, as freezing before EV isolation risks lysing milk cells and thereby producing vesicular populations that are not true EVs (Zonneveld et al. 2014; Wijenayake et al. 2021), which can potentially affect the

EV miRNA profile. This may create a methodological challenge in many research studies, especially in larger clinical trials where many samples are collected simultaneously and often at locations outside of the research facility. Due to such logistical reasons, milk samples are commonly frozen without any prior processing; especially concerning trials that were started before the interest in EVs and their miRNA was sparked and standardized methods for their isolation were missing. As we have previously recognized that the EV research field is in shortage of studies investigating milk EVs and their miRNA cargo in relation to maternal and infant outcomes in a randomized controlled fashion, we believe it is of importance to address the matter of 'freezing or not' once again (Ahlberg et al. 2023; Tingö et al. 2021).

Several methods can be used to isolate EVs. One of the most common methods is ultracentrifugation, where high-speed centrifugation (above 100,000 × g) is used to pellet down small EVs. Depending on the biofluid, some proteins may co-isolate with the EVs; in milk it is primarily caseins. Caseins tend to form micelles under high centrifugal force, which can be problematic for certain downstream analyses, for example, mass spectrometry and functional studies, as these additional proteins may interfere with the readouts of such methods/experimental setups (Famelart et al. 1998; Benmoussa et al. 2020, 2019). Several methods of micelle removal have therefore been suggested (Cetinkaya et al. 2024), either by acidifying the milk with hydrochloric acid and acetic acid (Rahman et al. 2019), by enzymatic treatment (Gao et al. 2019; Zhang et al. 2017), or by calcium chelation with ethylenediaminetetraacetic acid (EDTA) (Weiskirchen et al. 2023) or sodium citrate (SC) (Benmoussa et al. 2020). The use of SC was first described by Benmoussa et al. (2020) in bovine milk, and this was further evaluated in human milk by Cetinkaya et al. (2024). Both studies showed that pre-treating milk with 1% sodium citrate, resulted in less micelle formation and that most of the casein proteins stayed monomeric.

In this study, we aimed to evaluate (1) if freezing whole milk at  $-70^{\circ}\text{C}$  before EV isolation alters EV characteristics, including the miRNA cargo, and (2) if the addition of SC would further affect any of the EV characteristics.

## 2 | Materials and Methods

### 2.1 | Milk Samples

Mature milk (three months postpartum) was collected from 10 women enrolled in a double-blind, randomized, placebo-controlled trial. The intervention and design have been described in detail elsewhere (Forsberg et al. 2020). Milk was collected from both breasts by manual or electric pumping (according to the mothers' own choice) in the home of each participant directly after the first morning feeding; the breast was wiped clean of infant saliva before pumping. Milk samples were then instantly cooled in the home refrigerator and transported on ice to the clinic, and from the clinic to the lab, as soon as possible. Full maternal characteristics (Table S1) and sample information (Table S2) are described in the Supplementary file. The study was approved by the Regional Ethics Committee in Linköping (identification no. 2011/45-31). The participants' informed consent was obtained before inclusion.

## 2.2 | Isolation of Extracellular Vesicles

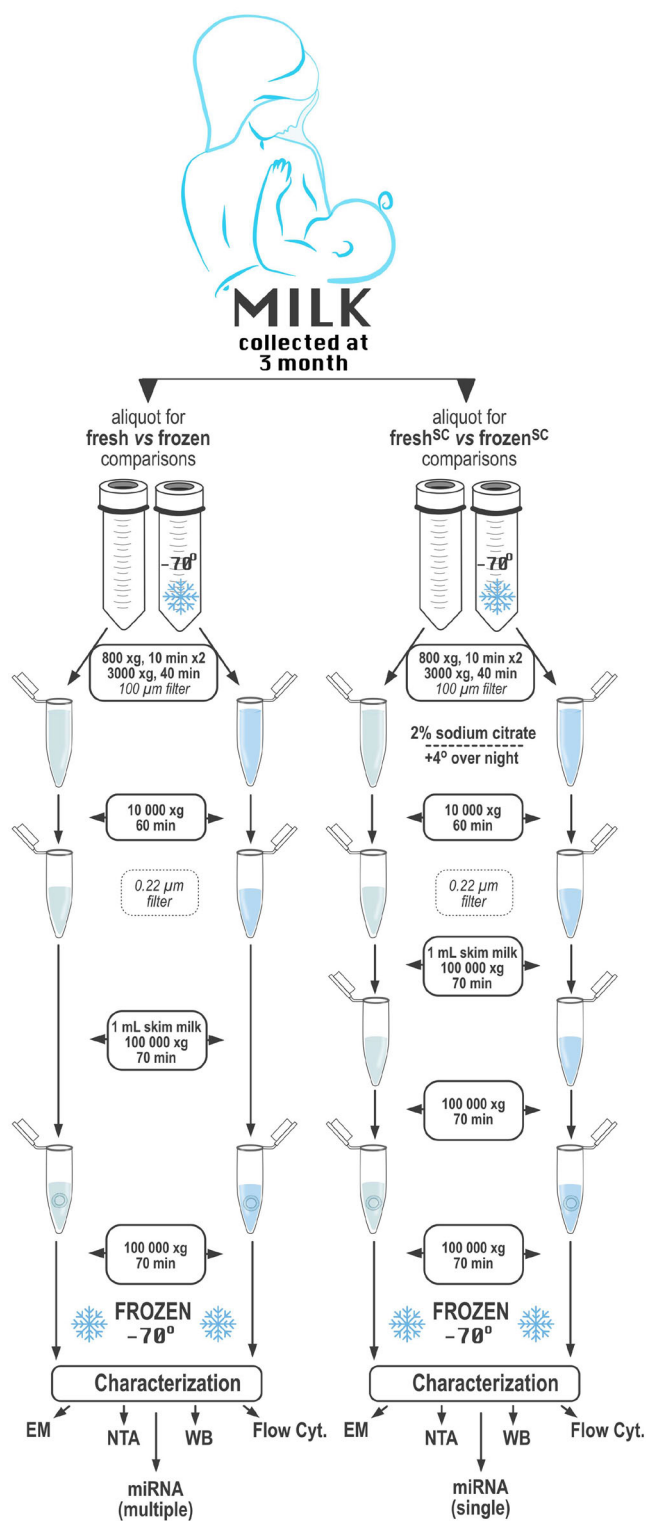
The milk samples were transferred from the clinic to the lab on ice as soon as possible and subsequently aliquoted. All samples were processed between 60 and 270 min (mean:146.5 min) after donation, see the experimental setup in Figure 1: (1) one aliquot was frozen directly as whole milk at  $-70^{\circ}\text{C}$  before EV isolation, and (2) a second aliquot was directly centrifuged twice at  $800 \times g$ , for 10 min at  $4^{\circ}\text{C}$  (Sigma 4–16KS with swing-out rotor 11150, Osterode am Harz, Germany) to separate the watery phase of the milk from the milk fat and cells. The supernatant was further centrifuged at  $3000 \times g$  for 40 min at  $4^{\circ}\text{C}$  and filtered through a  $100 \mu\text{m}$  cell strainer (Merck, Darmstadt, Germany). Half of the second aliquot was subsequently mixed 1:1 (vol:vol) with ice cold 2% pre-filtered sodium citrate (mixed in milli-Q water) and incubated 15 min on ice under gentle rocking, followed by centrifugation at  $10,000 \times g$  for 60 min at  $4^{\circ}\text{C}$  (Sigma 1–14K, fixed-angle rotor 12084), and later filtered through a  $0.22 \mu\text{m}$  PVDF syringe filter (Merck). The milk with sodium citrate (henceforth referred to as fresh<sup>SC</sup> and frozen<sup>SC</sup>) was left overnight at  $4^{\circ}\text{C}$  before being subjected to ultracentrifugation. Each milk sample was divided into five 1 mL, Open-Top Thickwall Polycarbonate Tubes (Beckman Coulter, Miami, FL, USA). One mL skim milk without sodium citrate was ultracentrifuged at  $100,000 \times g$  for 70 min at  $4^{\circ}\text{C}$  in an Optima MAX-XP ultracentrifuge with a TLA-120.2 rotor (Beckman Coulter). As the fresh<sup>SC</sup> and frozen<sup>SC</sup> samples were diluted 1:1 (vol:vol), the sample was ultracentrifuged twice to make the volume of milk equal between the conditions. For all four conditions, the EV pellet was washed with  $0.22 \mu\text{m}$  pre-filtered PBS and subjected to ultracentrifugation at  $100,000 \times g$  for 70 min at  $4^{\circ}\text{C}$ . The EV pellet was either resuspended in one mL (designated for Western blot and miRNA isolation) or  $250 \mu\text{L}$  (designated for flow cytometry, NanoSight and TEM) pre-filtered PBS and stored at  $-70^{\circ}\text{C}$  until further analysis. All samples, from all included women ( $n = 10$ ) and all four conditions were subsequently characterized using the methods described below, with the exception of EM and miRNA analyses, described in detail under respective subheadings below. Figure 1 shows a schematic overview of the workflow done on the 10 milk samples and the different isolation conditions.

## 2.3 | Nanoparticle Tracking Analysis

The NanoSight NS300 (NanoSight technology, Malvern, UK) was used to measure particle concentration and EV size. The instrument was equipped with a 488 nm blue laser beam and the NTA software (version 3.4, Malvern, UK). The camera level was set to 16 and the detection threshold to 5. The EV sample was thawed and further diluted in pre-filtered PBS (1:500 or 1:1000), injected with a syringe pump (syringe load 60) and five videos of 30 s were recorded for each sample with 5 s delay between each video.

## 2.4 | MACSPlex Flow Cytometry

The MACSPlex human Exosome Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to quantify 37 EV markers, including two isotype controls, as described by the manufacturer. The protein concentrations of the EV isolates were quantified



**FIGURE 1** | Schematic overview of the workflow for the 10 milk samples included in the experimental design. EVs were isolated by several centrifugation and ultracentrifugation steps. Prior to ultracentrifugation, one fresh and one frozen aliquot of milk were treated with 1% sodium citrate to remove casein micelles. After EV isolation, samples were characterized by MACSPlex (flow cytometry), electron microscopy (EM), NTA (nanoparticle tracking analysis), Western blot (WB) and EV-microRNAs were measured with qPCR.

using the Qubit Protein Assay (Invitrogen, Carlsbad, California, USA), according to the manufacturers' guidelines, and measured on a Qubit 3.0 Fluorometer (Invitrogen) (Table S3). The input to the flow cytometry analysis was standardized by the NTA measurements ( $5.5 \times 10^9$  particles). All samples were diluted in MACSPlex buffer to a total volume of 120  $\mu$ L, including a blank, according to protocol. MACSPlex Exosome Capture Beads were added to the sample and incubated overnight under rotation at 12 rpm. The EVs were washed in MACSPlex buffer, and a counterstaining cocktail with APC-conjugated anti-CD9, -CD63 and -CD81 was added to the sample, followed by incubation at room temperature (RT) for 60 min under rotation at 12 rpm. The sample was washed twice with MACSPlex buffer and 200  $\mu$ L sample was transferred to a FACS tube before analysis, using a Gallios Flow Cytometer (Beckman Coulter). The data was analyzed with Kaluza 2.1 (Beckman Coulter). Calibration beads were used to set the gates as follows (see Figure S1): single beads were gated based on side scatter and forward scatter, and each of the 39 bead populations was identified based on their PE and FITC signal. The median fluorescence intensity (MFI) of the APC signal within each bead population was calculated followed by subtracting the corresponding marker in the negative control. Proteins with an MFI signal lower than 1 after background subtraction were considered non-detectable and got a value of half the cutoff.

## 2.5 | Western Blotting

Western blotting was performed to compare the relative abundance of the EV proteins flotillin-1 (49 kDa) and lactadherin (46 kDa). Flotillin-1 was included as a 'positive EV-marker', as it is enriched in EVs (Welsh et al. 2024), while calnexin (90 kDa), an endoplasmic reticulum protein present in all eukaryotic cells, was included as a negative control to confirm the absence of cellular contamination in the EV pellets. Lactadherin was included as it has been reported to be enriched in milk fat globules, which may be co-isolated with the EV population, especially in frozen milk samples (Blans et al. 2017). EVs from the four isolation conditions were thawed and pelleted by ultracentrifugation as previously described. EV pellets and rat enteroglia cells (CRL-2690, ATCC, Manassas, Virginia, USA), that was used as a positive control for calnexin, were both resuspended in 100  $\mu$ L of lysing buffer containing radioimmunoprecipitation assay (RIPA) buffer (Sigma, Darmstadt, Germany), protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor (PhosSTOP, Sigma-Aldrich). As our aim was to compare overall differences between the four EV isolation methods, we chose to load equal volumes of EV lysates rather than normalizing by protein concentration or particle count, a decision consistent with the MISEV 2023 guidelines for Western blotting in EV research (Welsh et al. 2024). For protein preparation, 10  $\mu$ L of EV lysate was used for flotillin-1 detection, 10  $\mu$ L of cell lysate for calnexin detection and 2  $\mu$ L of EV lysate diluted with 8  $\mu$ L milli-Q water (10  $\mu$ L total) for lactadherin detection, ensuring comparable band intensities. The lysates were mixed with a sample buffer consisting of 4X Bolt LDS Sample Buffer (Invitrogen), 10X Bolt Sample Reducing Agent (Invitrogen), according to the manufacturer's instructions. Sample along with the PageRuler Prestained Protein Ladder (Thermo Scientific, Waltham, MA, USA) were loaded and run on a Bolt 4%–12% Bis-Tris Plus gel (Invitrogen), using the Bolt MES Sample

Buffer (Invitrogen) at 150 V for 50 min and transferred to a PVDF mini membrane (Invitrogen) using the iBlot 2 Gel Transfer Device (Invitrogen). The membranes were blocked with 5% skimmed milk for 1 h, following an overnight incubation (4°C with gentle shaking) with primary antibodies (anti-calnexin polyclonal antibody, 1:5000, PA5-34754, ThermoFisher; anti-flotillin-1 antibody, 1:1000, clone 18/Flotillin-1, BD Biosciences; anti-MFGE8 polyclonal antibody, 1:2000, PA5-109955, ThermoFisher). After washing, the membranes were incubated for 1 h at RT with the secondary antibodies (goat anti-mouse IgG, 1:2000, P0447, Dako, Glostrup, Denmark; goat anti-rabbit IgG, 1:5000, P0448, Dako). The proteins were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) using the ChemiDoc MP Imaging system (BioRad) and the high-quality autoexposure setting and analyzed with ImageJ software (version 1.52, NIH, Bethesda, MD, USA) to quantify the relative density of the protein bands. No unspecific binding of the secondary antibodies was observed (Figure S4). Since the four conditions were on the same membrane, background subtraction was not necessary.

## 2.6 | Electron Microscopy

### 2.6.1 | Immunogold Labelling

EV samples from two donors were subjected to immunogold labelling against CD9. In brief, 5  $\mu$ L sample was placed on a Formvar-Carbon coated, copper, 300 mesh electron microscopy grid (TED PELLA, Inc., Redding, CA, USA). The grid was blocked, mouse anti-CD9 (1:20, HI9a, Biolegend, San Diego, CA, USA), bridging antibody rabbit anti-mouse (1:50, ab6079, abcam, Cambridge, UK) and Protein A-gold (5 nm, 1:50, University Medical Center Utrecht, the Netherlands) were added to the grid with PBS wash in between, followed by staining with 0.75% uranyl acetate (Polysciences Europe GmbH, Hirschberg an der Bergstraße, Germany).

### 2.6.2 | Negative Staining

EV samples from two individuals with all four conditions were subjected to negative stain transmission electron microscopy (TEM), as described previously, with minor modifications (Cizmar and Yuana 2017). In brief, a 5  $\mu$ L sample was placed on a Formvar-Carbon coated, copper, 300 mesh electron microscopy grids. Grids were washed, blotted, and negatively stained with 2% uranyl acetate.

Images were taken using an 80 kV transmission electron microscope (JEOL JEM1400 Flash, JEOL Ltd., Tokyo, Japan) equipped with a XAROSA camera and RADIUS image analysis software (EMSIS GmbH, Münster, Germany).

### 2.6.3 | RNA Isolation and Quantification

Total RNA, including miRNA, was isolated using the Exosomal RNA isolation kit (Norgen, Thorold, ON, Canada), as described by the manufacturer, with some modifications. Briefly, three tubes with EVs (originating from a total of 3 mL skim milk before

ultracentrifugation) were thawed and pelleted by ultracentrifugation as described above. Approximately 100  $\mu$ L supernatant was left on each pellet and the three aliquots were subsequently pooled together. EVs were mixed with 300  $\mu$ L Lysis A and 37.5  $\mu$ L Lysis B buffer, and incubated for 10 min at RT. The miRNA was later eluted in 50  $\mu$ L elution solution A and kept at  $-70^{\circ}\text{C}$  until further analysis. The total EV-miRNA concentration was quantified using the Qubit microRNA Assay, following the manufacturer's guidelines, and measured on a Qubit 3.0 Fluorometer (Table S3).

## 2.6.4 | Quantitative Real-time PCR

The miRNA cargo was extensively investigated in the fresh vs. frozen (without SC) conditions. We applied the TaqMan advanced miRNA human A low-density array 384-well microfluidic card (Applied Biosystems) for a simultaneous analysis of 384 miRNAs (including exogenous and endogenous controls), and a single assay for miR-148a-3p. In the fresh<sup>SC</sup> and frozen<sup>SC</sup> we settled with performing only the single assay of miRNA-148a-3p; the miRNA cargo is a main point to be addressed concerning the fresh vs. frozen EV isolates, but as we did not expect the reduction of micelles in the SC treated sample to have any major effects on the EV miRNA cargo we refrained from extensive evaluation of all 375 miRNAs in the fresh<sup>SC</sup> and frozen<sup>SC</sup>. The reason for selecting miRNA-148a-3p to be evaluated as a *single assay* for all samples is that this miRNA is one of the topmost expressed miRNAs across mammals and should be rather consistently present in any milk (Ahlberg et al. 2023, 2023; Tingö et al. 2021).

Accordingly, isolated miRNA from all four conditions was reversely transcribed into cDNA using the TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. All samples were then subjected to real-time PCR *single assay* using TaqMan Fast Advanced Master Mix (Applied Biosystems), and the TaqMan Advanced miRNA Assay primers for hsa-miR-148a-3p (477814\_mir), according to instructions. The reactions were processed using a 7500 Fast real-time PCR instrument (Applied Biosystems) with the following settings:  $95^{\circ}\text{C}$  for 20 s, followed by 40 cycles of  $95^{\circ}\text{C}$  for 3 s and  $60^{\circ}\text{C}$  for 30 s. All assays, including the non-template control, were run in duplicates.

The fresh and frozen conditions *without* SC were then additionally subjected to analysis by TaqMan advanced miRNA human A low-density array 384-well microfluidic card (Applied Biosystems). The reactions were processed following the manufacturer's instructions, using a QuantStudio 7 PCR instrument (Applied Biosystems), with the following settings:  $95^{\circ}\text{C}$  for 20 sec, followed by 40 cycles of  $95^{\circ}\text{C}$  for 1 s and  $60^{\circ}\text{C}$  for 20 s. Amplification curves and cycle threshold (Ct) values were generated using the Thermo Fisher Connect Software (Life Technologies Corp) for the qPCR data generated. The threshold was set automatically for all miRNAs and the automatic baseline was kept (baseline start cycle: 3, baseline end cycle: 15). The raw qPCR curves underwent quality control; each individual reaction was inspected manually. Ct values above 35 were considered non-detectable and for statistical purpose all undetectable samples got the value of 35.

## 2.6.5 | Statistics

Statistical analyses were performed using non-parametric tests due to the small sample sizes and no normal distribution of the data. The Wilcoxon matched pairs signed rank test for paired data was used for NTA, Western blot and qPCR data for the single assay of miR-148a-3p. For flow cytometry, the Wilcoxon test was followed by False Discovery Rate (FDR) correction using the Benjamini-Hochberg correction method to account for multiple comparisons (Benjamini and Hochberg 1995). For the statistical analysis of miRNAs quantified using the low-density 384-well microfluidic cards, only miRNAs with detectable Ct values ( $\text{Ct} < 35$ ) in at least one condition (fresh or frozen) across all milk samples were included. Raw Ct values were used for both statistical testing and data visualization without any normalization of the data. Differences between conditions were assessed using the Wilcoxon matched pairs signed rank test and Permutation test, followed by FDR correction. The correlation between the miRNA Ct values in the fresh and frozen conditions was assessed using the Spearman rho ( $r_s$ ) correlation coefficient.

Statistical significance was defined as  $p$  value  $< 0.05$ , and FDR-adjusted  $p$  value ( $q$ )  $< 0.05$ . GraphPad Prism 10 (GraphPad Software, Inc., La Jolla, CA, USA) was used for graphs and statistical analysis of NTA, Western blot, flow cytometry, and the miR-148a-3p data. Graphs and statistical analyses of the microfluidic card data were conducted in R version 4.4.3 using the ggplot2 package (R Core Team 2024).

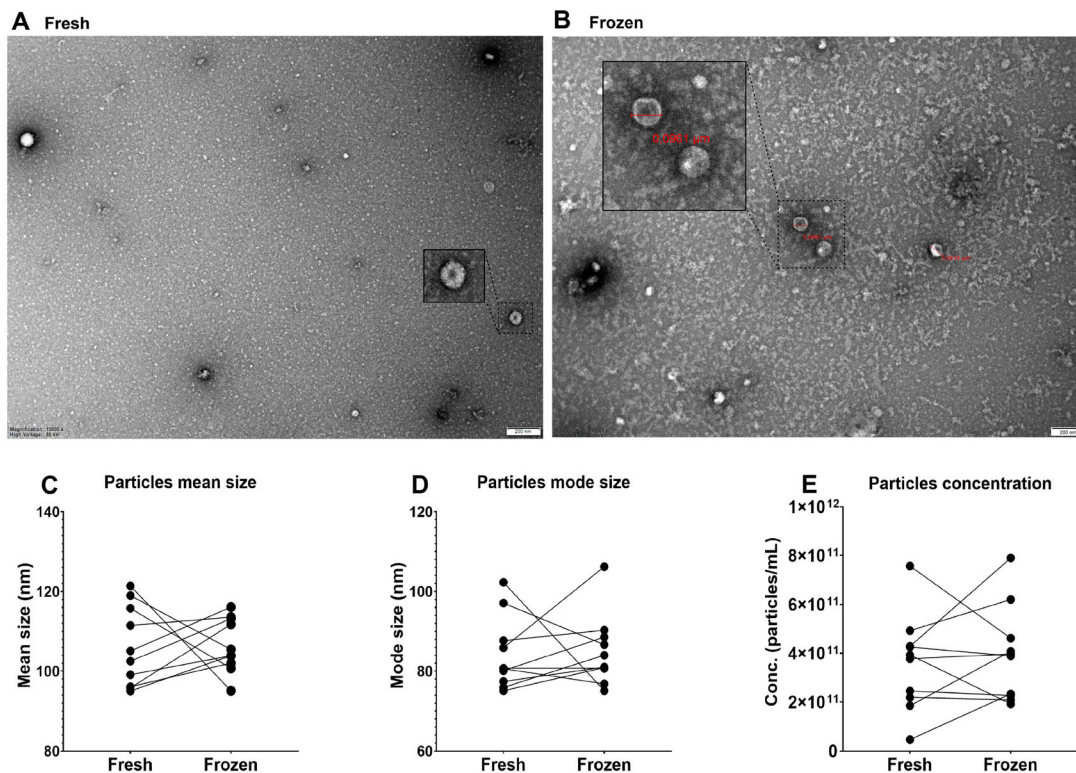
## 3 | Results

Here, we analyzed EVs by comparing isolates from fresh vs. frozen milk *without* SC, and fresh (fresh<sup>SC</sup>) vs. frozen milk *with* SC (frozen<sup>SC</sup>), separately, in line with our aim to assess (1) whether freezing at  $-70^{\circ}\text{C}$  alters EV characteristics, including miRNA cargo, and (2) whether SC addition further modifies these characteristics. The results are structured accordingly. In this section, we compared the conditions fresh vs. frozen without SC separately from fresh<sup>SC</sup> vs. frozen<sup>SC</sup>. This is because each set of conditions addresses a different research question. Moreover, since different isolation protocols were used for samples with and without SC, comparing all four conditions together is not meaningful. Methodological differences in EV isolation can lead to recovery of different EV populations, increasing the risk of Type 1 errors.

### 3.1 | The Effects of Freezing on Milk EV Characteristics and Their miRNA Cargo

#### 3.1.1 | Transmission Electron Microscopy

Milk EV size and morphology were assessed using TEM, which confirms the presence of the characteristic cup-shaped EVs (Figure 2A,B). Moreover, immune-labelled TEM images confirmed the presence of the EV marker CD9 on vesicles in both conditions, fresh and frozen (Figure S2A,B).



**FIGURE 2** | The morphology, size and concentration of milk-derived EVs in fresh and frozen conditions *without* SC. (A) Electron microscopy images showing the size and the characteristic cup-shaped EVs isolated from fresh milk samples. (B) Electron microscopy images showing the size and the characteristic cup-shaped EVs isolated from frozen milk samples. The image's scale bar is 200 nm, and the magnification is 15,000 $\times$ . Additional magnification in is 1.65 $\times$  and 2.20 $\times$  in insert A and B respectively. (C) Comparison of the particle mean size, measured by NTA, shows no significant difference between the fresh and frozen conditions. (D) Comparison of the particle mode size, measured by NTA, shows no significant difference between the fresh and frozen conditions. (E) Comparison of the particle concentration, measured by NTA, shows no significant difference between the fresh and frozen conditions.

### 3.1.2 | Nanoparticle Tracking Analysis

The size distribution and particle concentration of the fresh and frozen milk EVs were determined using NTA (Figure S3 and Table S4). Statistical analysis showed no significant differences between fresh and frozen paired samples in particle mean size, mode size, or particle concentration (Figure 2C–E).

### 3.1.3 | Western Blot

The relative abundance of the EV proteins, flotillin-1 and lactadherin, was compared across the fresh and frozen conditions using Western blot (Figures S4–S6). No significant differences in band densities for either protein were observed between the fresh and frozen conditions (Figure 3A–C). EV contamination with cellular proteins was assessed using the cellular marker calnexin, which was positive in all cell lysates and faintly present in two fresh and one frozen EV lysates, indicating minimal cellular contamination of the EV preparations (Figure S7).

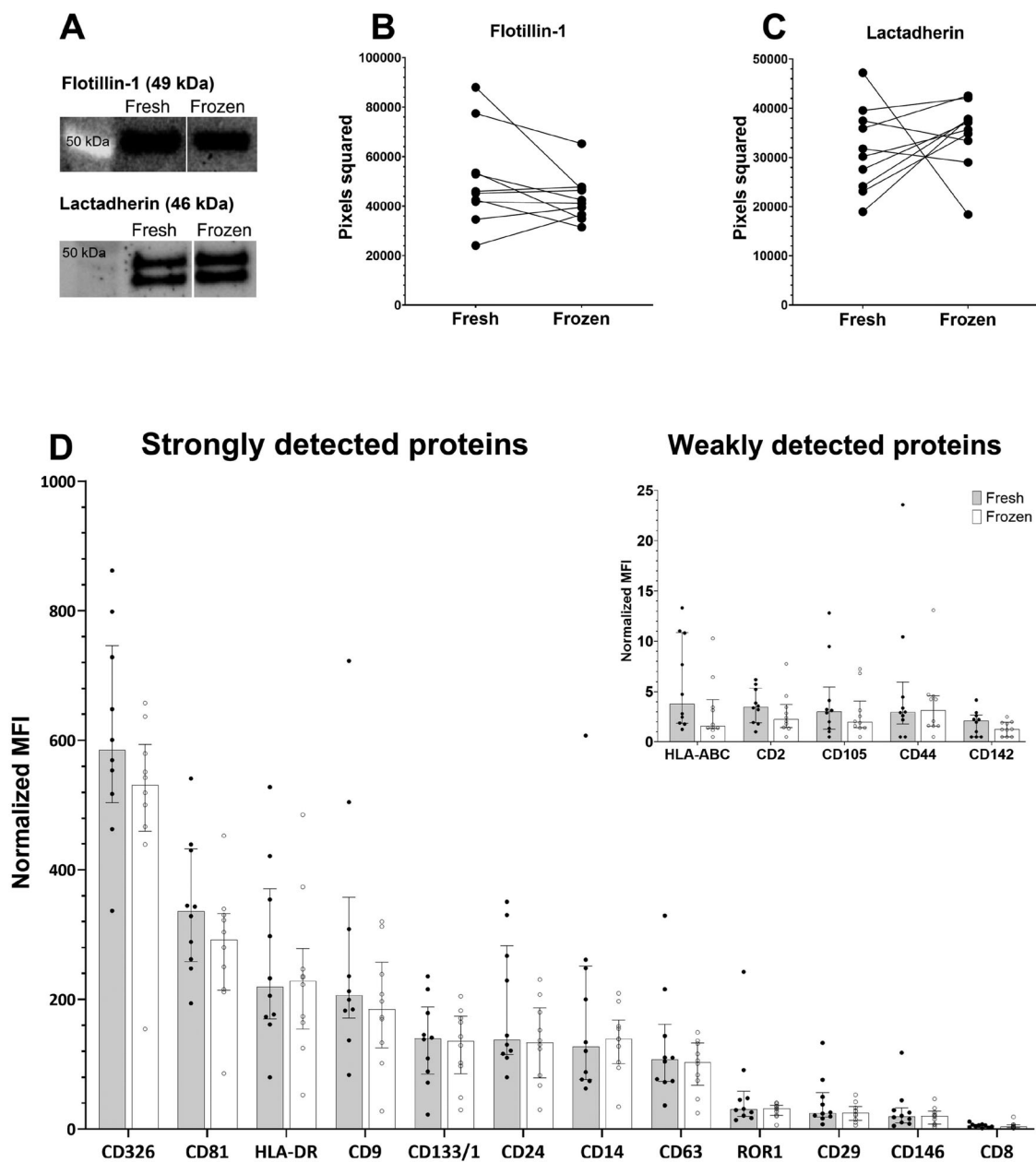
### 3.1.4 | Flow Cytometry

The surface proteins of the fresh and frozen milk EVs were assessed using the MACSPlex Exosome Kit. We classified pro-

teins as strongly detected if their MFI signal, after background subtraction, exceeded 1 in all samples from both conditions, and as weakly detected if the signal exceeded 1 in most samples from both conditions (Table S5). Statistical analysis showed no significant differences between the fresh and frozen conditions for the 12 strongly detected proteins (CD8, CD9, CD14, CD24, CD29, CD63, CD81, CD133/1, CD146, CD326, HLA-DR and ROR1) or the five weakly detected proteins (CD2, CD44, CD105, CD142 and HLA-ABC) (Figure 3D).

### 3.1.5 | EV-miRNA Analysis

The milk EV-miRNAs from fresh and frozen conditions were measured using the TaqMan advanced miRNA human A microfluidic cards. The qPCR cards included 375 miRNA assays, out of which 226 were detected (*Ct* value < 35) in at least one sample of any condition (Table S6). Of those 226 detected, 93 miRNAs were detected in at least one condition, fresh or frozen, of all 10 milk samples, and 43 were detected in both conditions, fresh and frozen, across all 10 milk samples (Table S7). The Venn diagram shows the number of detected EV-miRNAs only in fresh milk samples, the number of detected EV-miRNAs only in frozen milk samples, and the number of detected EV-miRNAs in both conditions for each mother (Figure 4A). A median number of 79.5 out of the 93 miRNAs (~85%) included in the

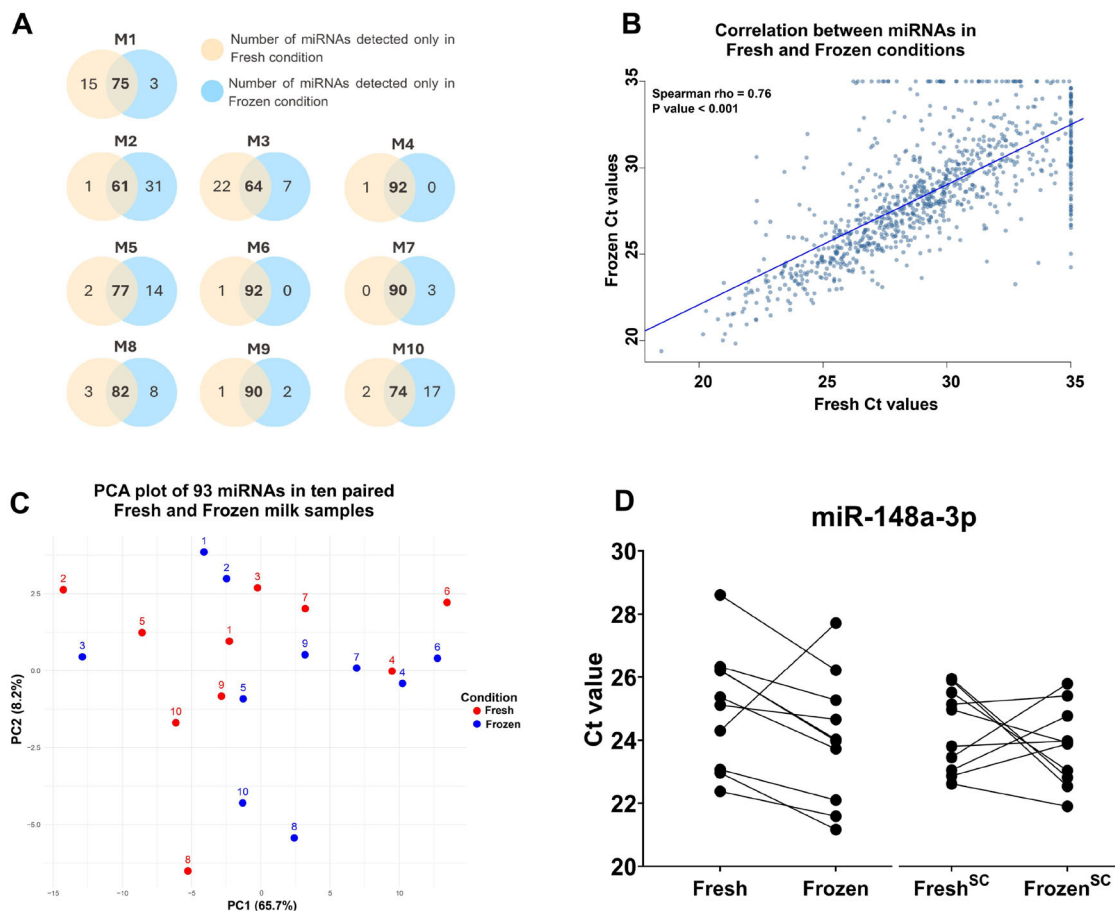


**FIGURE 3** | Comparisons of EV proteins between the fresh and frozen conditions. (A) A representative Western blot image showing similar flotillin-1 and lactadherin bands between the fresh and frozen conditions. (B) Comparison of the flotillin-1 Western blot band densities shows no significant differences between the fresh and frozen conditions. (C) Comparison of the lactadherin Western blot band densities shows no significant differences between the fresh and frozen conditions. (D) Comparison of EV surface proteins, measured by MACSPlex flow cytometry, shows no significant differences between the 12 strongly detected or the 5 weakly detected proteins across the fresh and frozen conditions.

analysis were detected in both conditions, fresh and frozen. The median number of miRNAs detected only in the fresh condition was 1.5, while the number of miRNAs detected only in the frozen condition was 5. However, statistical analysis showed no significant differences in the number of detectable miRNAs of the ten milk samples between the fresh and the frozen conditions.

To investigate the effect of freezing on the 93 included miRNAs, the Wilcoxon matched pairs signed rank test for paired samples was used. After adjusting for multiple comparisons, no significant differences were found in the miRNA Ct values between the fresh and frozen conditions (Table S8). Since the Wilcoxon matched

pairs signed rank test assumption of a symmetrical distribution of differences might be violated, we additionally conducted a paired samples permutation test, a nonparametric and distribution-free alternative. After FDR correction, the permutation test showed no significant differences in the median Ct values between fresh and frozen paired samples (Table S8). Moreover, the 43 miRNAs detected in both conditions and in all samples showed no significant differences between the fresh and frozen conditions (Table S9). Furthermore, Spearman correlation analysis showed a strong association between the miRNA Ct values in the fresh and frozen conditions ( $r_s = 0.76$ ,  $p < 0.001$ ) (Figure 4B). Moreover, a PCA plot of the 93 miRNA Ct values in the fresh and



**FIGURE 4** | EV-miRNA analysis from the milk samples across different isolation conditions. (A) Venn diagram of the 93 miRNAs detected in at least one condition, fresh or frozen, and in all milk samples. This diagram shows the number of miRNAs detected only in EVs from fresh condition, frozen condition or both, for each mother (M1-M10). A median number of 79.5 out of 93 miRNAs are detected in both conditions. (B) Spearman correlation analysis shows a strong association ( $r_s = 0.76$ ,  $p < 0.001$ ) between the 93 miRNA Ct values in the fresh and frozen conditions. (C) PCA plot of the 93 miRNAs in the fresh and frozen conditions of the ten milk samples shows no visible clustering of the data points between conditions. (D) Comparisons of miR-148a-3p Ct values between the fresh vs. frozen, and the fresh<sup>SC</sup> vs. frozen<sup>SC</sup>, show no significant differences across conditions.

frozen conditions showed no visible clustering between the two conditions (Figure 4C). Additionally, similar correlation analysis and PCA plot results were observed for the 43 miRNAs detected in both conditions and in all samples (Figure S8).

In addition, levels of miRNA-148a-3p were investigated in the fresh and frozen conditions using a single assay analysis and found no significant differences in the Ct values between the two conditions (Figure 4D).

### 3.2 | The Effects of Sodium Citrate on EV Characteristics and miRNA-148a-3p Isolated From Fresh and Frozen Milk

#### 3.2.1 | Transmission Electron Microscopy

The size and morphology of milk EVs isolated with sodium citrate (SC) were assessed with TEM, which confirms the presence of the characteristic cup-shaped EVs in both fresh<sup>SC</sup> and frozen<sup>SC</sup> conditions (Figure 5A,B). In addition, the immune-labelled TEM images confirmed the presence of the EV marker CD9 on vesicles,

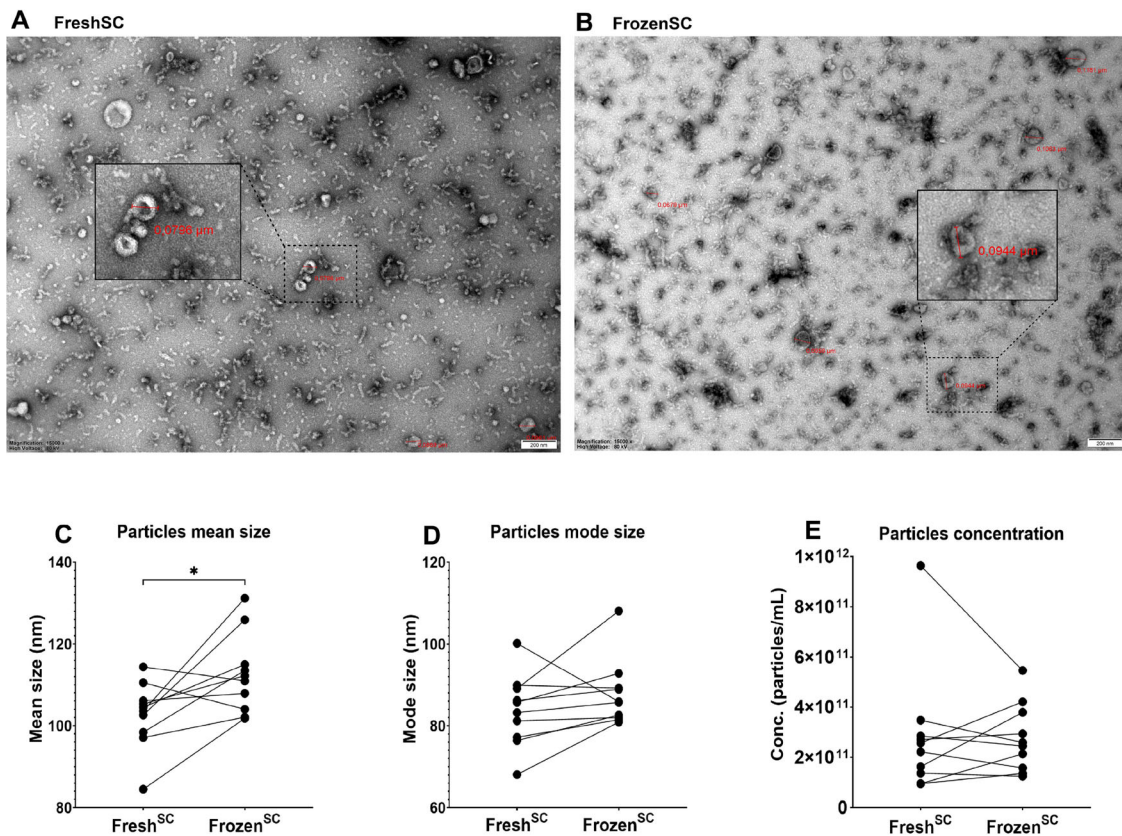
with some background contamination, in both conditions (Figure S2C,D).

#### 3.2.2 | Nanoparticle Tracking Analysis

The size distribution and particle concentration of the EVs were determined using NTA (Figure S3 and Table S4). The Wilcoxon matched pairs signed rank test showed that the vesicles' mean size in the fresh<sup>SC</sup> condition was significantly smaller than the vesicles' mean size in the frozen<sup>SC</sup> condition (Figure 5C). However, the vesicles' modal size and particle concentration showed no significant differences between the two conditions (Figure 5D,E).

#### 3.2.3 | Western Blot

The relative abundance of flotillin-1 and lactadherin was compared between the fresh<sup>SC</sup> and frozen<sup>SC</sup> conditions using Western blot (Figures S4-S6). The results showed no significant differences in band densities for either protein between conditions



**FIGURE 5** | The morphology, size, and concentration of milk-derived EVs in fresh<sup>SC</sup> and frozen<sup>SC</sup> conditions. (A) Electron microscopy images showing the size and the characteristic cup-shaped EVs isolated from fresh<sup>SC</sup> milk samples. (B) Electron microscopy images showing the size and the characteristic cup-shaped EVs isolated from frozen<sup>SC</sup> milk samples. The image's scale bar is 200 nm, and the magnification is 15,000×. Additional magnification is 2.06× and 1.90× in insert A and B respectively. (C) Comparison of the particle mean size, measured by NTA, shows that the mean size in the fresh<sup>SC</sup> condition is smaller than that in the frozen<sup>SC</sup> condition. (D) Comparison of the particle mode size, measured by NTA, shows no significant difference between the fresh<sup>SC</sup> and frozen<sup>SC</sup> conditions. (E) Comparison of the particle concentration, measured by NTA, shows no significant difference between the fresh<sup>SC</sup> and frozen<sup>SC</sup> conditions.

(Figure 6A–C). EV contamination with cellular proteins was assessed using the cellular marker calnexin, which was faintly visible in two fresh<sup>SC</sup> and two frozen<sup>SC</sup> EV lysates, suggesting low cellular contamination of the EV pellet (Figure S7).

### 3.2.4 | Flow Cytometry

The MACSPlex analysis with flow cytometry of the fresh<sup>SC</sup> and frozen<sup>SC</sup> samples showed that 11 proteins were strongly detected (CD9, CD14, CD24, CD29, CD63, CD81, CD133/1, CD146, CD326, HLA-DR and ROR1) and six proteins were weakly detected (CD2, CD8, CD44, CD105, CD142 and SSEA4) (Table S5). Statistical analysis showed that all detected proteins in the fresh<sup>SC</sup> samples had significantly lower normalized MFI signals compared to the frozen<sup>SC</sup> samples, except CD24 and ROR1, which showed no significant differences between conditions (Figure 6D).

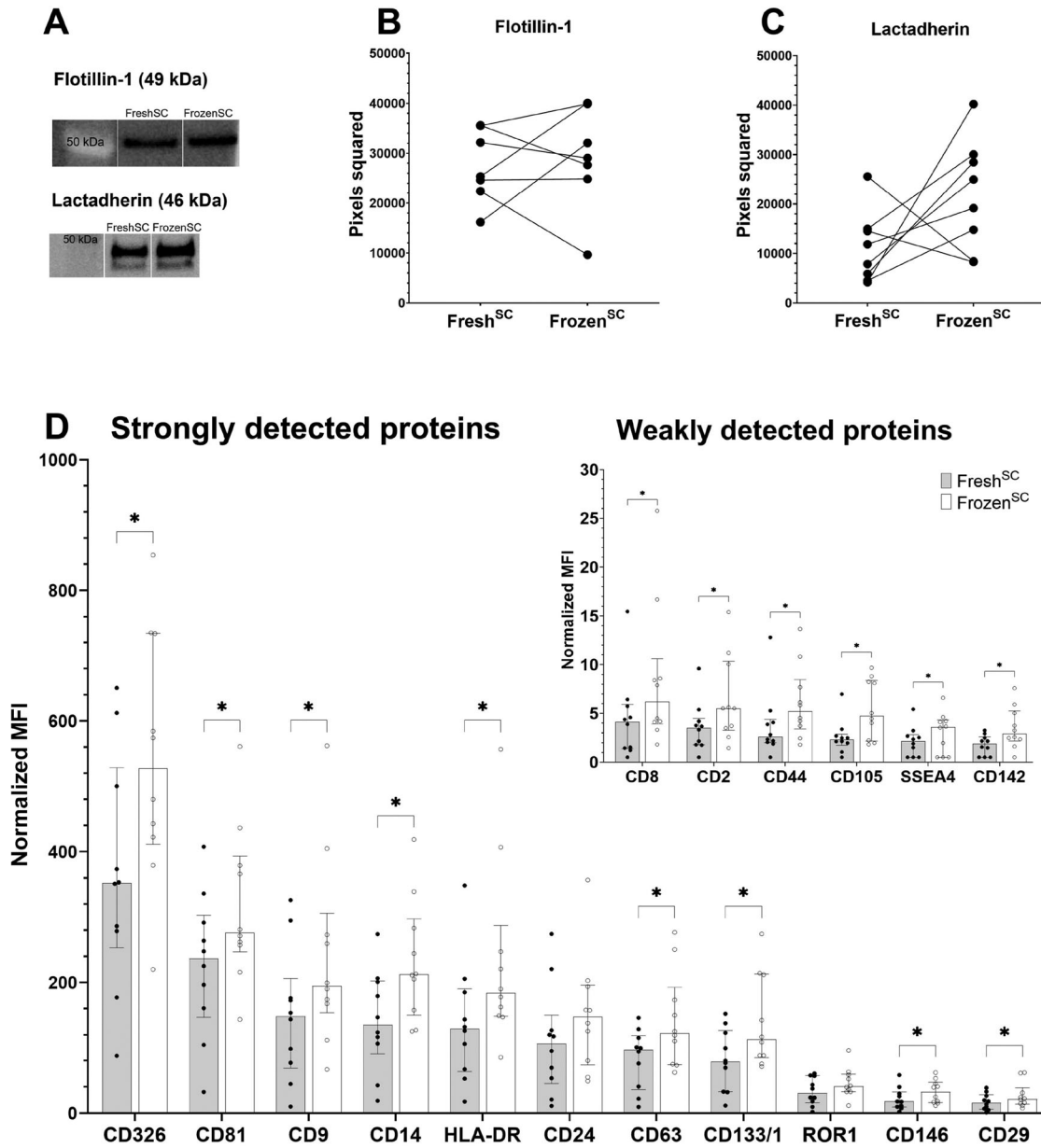
### 3.2.5 | EV-miRNA Analysis

Levels of miRNA-148a-3p were investigated in the fresh<sup>SC</sup> and frozen<sup>SC</sup> conditions, which showed no significant differences in the Ct values (Figure 4D).

## 4 | Discussion

Here, we evaluated how freezing human whole milk at  $-70^{\circ}\text{C}$  prior to EV isolation affects certain EV characteristics or their miRNA cargo. The study has a unique set up using samples from a clinically relevant context, combining investigations of EV characteristics with a comprehensive miRNA analysis. Our findings suggest that freezing does not significantly alter either EV composition or miRNA cargo, supporting the use of frozen whole milk as a viable alternative for EV studies. This is valuable knowledge for research conducted in clinical or biobank-based settings, where fresh samples are not necessarily accessible. We also assessed the effects of SC addition to fresh and frozen milk samples prior to EV isolation; a common strategy to reduce casein micelle formation. SC treatment altered particle size (Figure 5C) and EV surface protein detection (Figure 6D), suggesting that SC-mediated micelle removal may interact with prior freeze-thaw processes. Together, our findings offer practical guidance for researchers working with human milk EVs and contribute to the ongoing methodological standardization of EV research in line with MISEV guidelines.

This study was conceived in consideration of previous studies suggesting that freezing of whole milk before EV isolation may



**FIGURE 6** | Comparisons of EV proteins between the fresh<sup>SC</sup> and frozen<sup>SC</sup> conditions. (A) A representative Western blot image showing similar flotillin-1 and lactadherin bands between the fresh<sup>SC</sup> and frozen<sup>SC</sup> conditions. (B) Comparison of the flotillin-1 Western blot band densities shows no significant differences between the fresh<sup>SC</sup> and frozen<sup>SC</sup> conditions. (C) Comparison of the lactadherin Western blot band densities shows no significant differences between the fresh<sup>SC</sup> and frozen<sup>SC</sup> conditions. (D) Comparison of EV surface proteins, measured by MACSPlex flow cytometry, shows that all strongly and weakly detected proteins were significantly lower in the fresh<sup>SC</sup> condition compared to the frozen<sup>SC</sup> condition, except CD24 and ROR1, which show no significant difference.

affect the yield and purity of the isolated vesicles (Zonneveld et al. 2014; Wijenayake et al. 2021; Cetinkaya et al. 2024; Leiferman et al. 2019). To investigate this further, we designed a study with more extensive evaluation than, to the best of our knowledge, any prior study. For this purpose, we included multiple methods for EV characterization: NTA, EM, MACSPlex flow cytometry, Western blotting and real-time qPCR for miRNA analysis. These methods were used on milk samples from ten women, across four different EV isolation protocols based on ultracentrifugation: fresh and frozen; fresh<sup>SC</sup> and frozen<sup>SC</sup>, as described in the Section 2.

A previous study conducted by Zonneveld et al. (2014), also compared EVs isolated from fresh vs. frozen milk and found that storing unprocessed human milk spiked with murine cells, especially at freezing temperatures ( $-80^{\circ}\text{C}$  for 2 h), led to a significant cell death and an increased release of murine-EVs. These findings suggest that freezing milk prior to EV isolation could introduce non-native vesicles released due to cell damage. In addition, the study found that cell death also occurs at room temperature and  $4^{\circ}\text{C}$  after 2 h. However, since the study used murine cells spiked into human milk, which may be more fragile than native human cells, the relevance of these results to naturally occurring human milk EVs may be limited. To characterize the

EVs, Zonneveld et al. (2014) used cryo-EM (immunolabelling of annexin V) and protein analysis by Western blot (CD9, CD63, MHC). In contrast to their findings, none of our investigated parameters differ between the EVs isolated from fresh and frozen samples, suggesting that freezing under our conditions does not introduce detectable contamination from vesicles released due to cell death or stress.

More in line with our results, Cetinkaya et al. (2024) found that EVs from fresh and frozen milk do not differ in particle concentration and size (assessed by NTA); their sample preparation and protocol are however not clearly described. Furthermore, Leiferman et al. (2019) evaluated if storage of milk affected the concentration and size of what is claimed to be 'EV-like' vesicles. In pursuit of these EV-like vesicles, they diluted whole milk with water and ran NTA analysis directly on this solution, thus they evaluated *all* particles present in the whole milk without any prior EV isolation. By this method, they see no significant differences between fresh and frozen milk samples, however, milk that was stored for four weeks at 4°C presented with fewer particles. This may in part support our findings of no differences between frozen and freshly isolated EVs. However, their conclusions are based on total milk particles and not isolated EVs, limiting direct comparison.

Casein is one of the most abundant proteins in mammal milk. In comparison to bovine milk, human milk consists of less casein proteins in relation to whey (Hernell 2011). The human casein micelles are also generally smaller than the bovine (Meng et al. 2021). Casein may be problematic in milk EV studies because of its abundance, potentially overshadowing more low-expressed proteins in proteomic investigations, and because of its tendency to form micelles that can co-isolate with the milk EVs. Several methods can be used to remove micelles (Cetinkaya et al. 2024); SC treatment is a widely used approach. The use of SC has been described by Benmoussa et al. (2020) in bovine milk and further evaluated in human milk by Cetinkaya et al. (2024). In the current study, we treated the milk with 1% SC before ultracentrifugation. Due to logistical reasons, we could not process the SC treated samples on the same day as the samples without SC, rendering comparisons with the EV samples without SC unfeasible. The samples were also subjected to a third ultracentrifugation, as the milk was diluted 1:1 with SC, while the samples without SC were only subjected to ultracentrifugation twice. We observed a slightly larger particle mean size in the frozen<sup>SC</sup> condition in comparison to fresh<sup>SC</sup>, but this increase was not accompanied by a decrease in particle concentration. An increase in particle size could potentially stem from increased vesicle aggregation, which would typically lead to fewer detectable particles. However, the lack of a reduction in particle concentration suggests that other mechanisms, for example, simultaneous formation of new particles, could be involved.

Furthermore, SC treatment of fresh and frozen milk samples seems to affect the protein levels, at least based on the flow cytometry data reported here; we did not see any such differences between the fresh and frozen conditions *without* SC (see Figure 3D). Statistical comparison of the fresh<sup>SC</sup> vs. frozen<sup>SC</sup> conditions identified a majority of the proteins as significantly more abundant in the frozen<sup>SC</sup>, see Figure 6D. This increase in detection of surface proteins may suggest that more EVs, or

vesicle-like structures, are present in the frozen<sup>SC</sup>. If aggregation mask a simultaneous increase in particle number, as speculated above, this may imply that some aspects of the sample handling for the frozen<sup>SC</sup> condition, such as the addition of SC to frozen samples, extra ultracentrifugation or overnight storage at 4°C, has contributed to the formation of vesicle-like particles not present in the fresh samples.

Considering the general protein profile of the milk EVs, all four conditions (regardless of SC) showed a quite similar protein repertoire as a previous study of ours using the same MACSPlex method (Ahlberg et al. 2024), but a combination of SC treatment with ultracentrifugation and size exclusion chromatography (SEC) for EV isolation. A similar EV protein profile is also evident in a study by Giovanazzi et al. (2023), using the same sample type but a different EV isolation method: density gradient in combination with SEC. This speaks for a consistency in the surface proteins of milk EVs regardless of differences in isolation methods, at least when evaluated by flow cytometry. Giovanazzi et al. (2023) however detected CD3, a T cell marker, although with low MFI. In our study, CD3 was mostly absent in the fresh and frozen conditions *without* SC, only one sample in the fresh condition presented with this protein (MFI 1.69). In the fresh<sup>SC</sup> and frozen<sup>SC</sup> conditions, however, CD3 was present in four and five samples, respectively, although with rather low MFI (range 1.01–1.99 and 1.39–2.82, refer to Table S5).

Cetinkaya et al. (2024) showed that casein depletion may lead to an increase in the number of EV proteins identified, at least when the proteins are analyzed by mass spectrometry. It is reasonable that removing a significant portion of casein from the sample increases the ability of the mass spectrometer to detect more low-abundant proteins; why micelle removal is supposedly of particular importance in EV samples intended for such methods. However, Cetinkaya et al. (2024) found remaining caseins in all conditions regardless of depletion attempts with several different methods; the remaining caseins were then suggested to be a part of the EV corona. We observed similar findings in a previous study of ours (Ahlberg et al. 2024), where mass spectrometry analysis of the EV surface proteome showed that a-, b- and k-casein were all still present even after SC treatment (Ahlberg et al. 2024). Interestingly, Leiferman et al. (2019) did not detect any remaining casein proteins in their EV isolates after ultracentrifugation. Compared to the EV isolation protocols of our studies, they however include an additional ultracentrifugation (at 83,000 × g) before subjecting the skim milk to ultracentrifugation at 130,000 × g to pellet the EVs. One might speculate that the 83,000 × g centrifugation sedimented most casein micelles, hence explaining the absence of casein proteins in the EV Western blot. Furthermore, in another study employing a similar protocol that is, adding an additional centrifugation step (at 21,500 × g), Wijenayake et al. (2021) claim that they successfully removed all casein proteins. They, however, do not provide experimental evidence to confirm this claim. The ultimate protocol for micelle removal is yet to be determined, and so is whether or not caseins can be considered a naturally occurring part of the EV protein corona. As previously suggested by us and others, attempts to deplete or remove excess caseins/micelles may be of particular importance in preparing samples for certain downstream analysis, such as mass spectrometry, as their mere abundance may interfere with its results. In more targeted approaches, like the flow cytometry

analysis we used in this paper, caseins should pose less of a problem.

Moreover, we found no significant differences in the EV proteins detected by Western blot, neither concerning the fresh vs. frozen nor the fresh<sup>SC</sup> vs. frozen<sup>SC</sup> conditions. Flotillin-1, a common EV marker, was present in most samples and conditions (Figures 3B and 6B), however, two samples in the fresh<sup>SC</sup> did not show a clear band. The slight calnexin detection in a few samples from two of the included individuals may point to some contamination of cell debris in the EV pellet of these specific samples. Furthermore, we included lactadherin, also known as milk fat globule epidermal growth factor 8, as it is abundantly present in milk fat globules (MFG), approximately 20% of the MFG protein content (Blans et al. 2017). The presence of MFGs may be particularly important to consider in EV preparations from frozen milk as MFGs are prone to collapse during freezing and thawing procedures, leading to the formation of contaminating vesicle structures (Keenan et al. 1970; Kobylka and Carraway 1972). In this study, we however, did not detect any differences in lactadherin levels in the fresh vs. frozen or fresh<sup>SC</sup> vs. frozen<sup>SC</sup> EV preparations, suggesting that there is no pronounced increase in structures carrying lactadherin in the frozen conditions. Table 1 gives an overview about the different studies investigating the effect of storage conditions/freezing and SC on EVs.

Regarding our investigations of EV miRNAs, miRNA-148a-3p levels (as analyzed in *single assay*) did not differ significantly in either fresh vs. frozen or fresh<sup>SC</sup> vs. frozen<sup>SC</sup>, see Figure 4D. Albeit these results from comparisons of a single miRNA, they align with the preconceived logic that micelle removal by SC would not affect the miRNA expression, as the vesicular cargo supposedly remains intact. Neither our screening of the 375 miRNAs in the EV preparations *without* SC shows any significant differences between the fresh and frozen conditions. In addition, the Venn diagram showed that the majority of the miRNAs included in the analysis (93 miRNAs) were detectable in both conditions (~85%) (Figure 4A). Also, the raw Ct values of these miRNAs from the two conditions showed high correlation ( $r_s$  value = 0.76) (Figure 4B). The PCA plot results complemented these findings by showing no clustering of the 10 milk samples based on their fresh or frozen condition (Figure 4C). In contrast, Leiferman et al. (2019) found freezing to reduce miRNA recovery as compared to miRNA recovery from fresh milk EVs. It is hard to speculate on the reason for this discrepancy, but it probably stems from differences in the isolation protocols used, either concerning the isolation of the EVs or the miRNAs. Of note, the Leiferman study compared the general miRNA yield, while we compare specific miRNAs, which may play a significant role in the result interpretation. In a study of whole milk, Kim et al. (2023) showed that the storage condition may affect miRNA recovery. They, however, quantified miRNAs from fresh and frozen milk that had been thawed by heating, either in the microwave or in a bottle warmer, and it is a far stretch to allude to this saying anything about milk EV miRNAs. As long as the EVs stay intact throughout the sample handling and preparation prior to their isolation, the miRNA contents should not be significantly impacted. Few studies so far have however, investigated this matter, and it hence remains to be further studied.

## 4.1 | Methodological Limitations

As for all methods, they have their advantages and disadvantages to consider. First, NanoSight it is a method used to determine all particles, EVs or not, within range, size and concentration using the movement of nanoparticles in a solution according to the Brownian motion and laser light scattering microscopy (Bachurski et al. 2019; Vestad et al. 2017). According to the company, the NanoSight instrument can measure particles in the size range of 10–2000 nm, depending on the source material. This might hold true if the particles are homogeneous with a similar refractive index, which is not the case for EVs from biofluids. For this kind of sample, it is rather around 50 nm at best, resulting in a skewed size distribution and particle concentration (Bachurski et al. 2019). Importantly, it cannot discriminate between EVs, micelles, liposomes or protein aggregates. In addition, the instrument itself, software settings and the operator strongly affect the outcome of the analysis, which should be taken into consideration (Vestad et al. 2017). In this study, the same instrument operator performed the analysis, adopting the same instrumental settings and handling the samples within a short time frame. Furthermore, we used a bead-based flow cytometry (MACSPlex assay), which can capture EVs using antibodies against 37 surface markers followed by an antibody cocktail against the tetraspanins CD9, CD63 and CD81 for detection. As such, the detection of EVs is dependent on the presence of at least one of these tetraspanins, making it more likely to detect EVs (Welsh et al. 2024; Giovanazzi et al. 2023). EVs from biofluids, such as human milk, will be produced from various cell types, including epithelial and immune cells (Giovanazzi et al. 2023; Ahlberg et al. 2024). Hence, giving rise to different tetraspanin distributions on the EV surface, which could potentially affect the outcome of the analysis. Furthermore, the differences we observe across EV characteristics may, in part, reflect differences in how EV input was standardized across methods. For example, MACSPlex analysis was based on particle number, whereas miRNA quantification and Western blotting were performed using total RNA and protein extracted from the entire EV pellet; the input to the MACSPlex was standardized on the basis of NTA particle count, while the Western blot was standardized on the basis of volume. This approach reflects typical workflows in EV research, though it may contribute to variation in sensitivity across different assays. Regarding the interpretation of our Western blot results, we acknowledge the importance of using well-validated antibodies with demonstrated specificity. The flotillin-1 antibody used in our study has been employed in several previous publications, including a study using a flotillin knock-out model where the Western blot signal was completely abolished (Meister et al. 2017), supporting its specificity. Similarly, the lactadherin antibody has shown consistent and condition-dependent binding in Western blotting (Tao et al. 2022) as well as in other molecular assays (Xu et al. 2024). While we did not include full antibody-specific positive and negative controls in this study—aside from calnexin—these prior validations provide support for the reliability of the antibodies used. Moreover, the TEM negative staining showed nicely ‘cup-shaped’ EVs (Figures 2A,B and 5A,B), while the EVs stained by immuno-EM gold labelling appear to lack this signifying feature, most likely attaining to the differences in procedures, see Figure S2 (for details refer to the method section for Electron

**TABLE 1** | Overview of studies investigating fresh and frozen conditions or casein removal methods on extracellular vesicles from milk.

| Conditions       | Author                   | Samples and storage                                                                                                                                                                                            | Methods                                                                                                                                                                                                                                                          | Main finding                                                                                                                                                                                                                                                   |
|------------------|--------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Fresh vs. frozen | Zonneveld et al. (2014)  | Mature breast milk samples collected from 17 healthy mothers were spiked with murine (m) bone marrow-derived dendritic cells and processed freshly, and after freezing at $-80^{\circ}\text{C}$ for 2–8 weeks. | EVs were isolated with a top-down sucrose density gradient. EV protein markers were analysed with western blot (WB).                                                                                                                                             | Storing unprocessed milk at $-80^{\circ}\text{C}$ introduced storage-induced EVs into the milk. However, no significant differences in mCD9 and mCD63 WB bands were observed between fresh and frozen milk supernatants devoid of cells and fat.               |
|                  | Leiferman et al. (2019)  | Mature breast milk samples from five donors were pooled and stored at $4^{\circ}\text{C}$ and $-80^{\circ}\text{C}$ .                                                                                          | Ultracentrifugation at $83,000 \times g$ was used to remove milk fat globules and microvesicles, followed by $130,000 \times g$ to isolate exosome-sized vesicles. Particle size and concentration from 3 replicates were assessed using NTA weekly for 4 weeks. | The vesicle count decreased progressively over time in the $4^{\circ}\text{C}$ storage condition. In contrast, there was no significant reduction in vesicle count after 4 weeks of freezing. The size of vesicles was not affected by the storage conditions. |
|                  | Cetinkaya et al. (2024)  | Breast milk sample from one donor and processed fresh and after freezing at $-80^{\circ}\text{C}$ for 18 h.                                                                                                    | EVs were isolated with a 35 nm size exclusion column (qEV), and NTA was used to measure EV size and concentration.                                                                                                                                               | No significant differences in EV size or concentration between the fresh and frozen samples.                                                                                                                                                                   |
| Casein removal   | Wijenayake et al. (2021) | Unpasteurized bovine colostrum samples were collected from three healthy cows. The samples were pooled and frozen unprocessed or after casein removal at $-80^{\circ}\text{C}$ .                               | The bovine milk casein was removed using multiple centrifugations at $21,500 \times g$ , while EVs were isolated with ultracentrifugation at $100,000 \times g$ .                                                                                                | The removal of milk cream and fat globules before freezing improved the yield of milk EVs. However, the additional removal of casein proteins and cellular debris did not confer further benefits in terms of EV yield or purity.                              |
|                  | Cetinkaya et al. (2024)  | Breast milk samples were collected at 2 months postpartum from four mothers and pooled together. Skimmed milk samples were aliquoted and stored at $-80^{\circ}\text{C}$ .                                     | The human milk casein was removed with either sodium citrate (1%), EDTA (20 mM), acetic acid (1%) or chymosin with calcium chloride (1%). EVs were isolated with a 35 nm qEV size-exclusion column.                                                              | All treatments effectively reduced casein micelle contamination in EV preparations. Chymosin demonstrated the highest efficiency in casein depletion, followed by EDTA, acetic acid and sodium citrate.                                                        |

(Continues)

TABLE 1 | (Continued)

| Conditions            | Author                                                                                                                              | Samples and storage                                                                                                                                                                                                                                                                                                                                            | Methods                                                                                                                                                 | Main finding |
|-----------------------|-------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|
| Ahlberg et al. (2024) | Breast milk samples were collected at 3 months postpartum from six mothers and processed before freezing at $-70^{\circ}\text{C}$ . | Sequential centrifugation and filtration were used to remove fat and cellular debris from the milk samples. Sodium citrate (1%) was used to remove casein micelles from the samples before freezing. EVs were isolated with ultracentrifugation, followed by a 35 nm size exclusion chromatography. Proteomic analysis was performed on the isolated milk EVs. | The EV surface proteome analysis revealed that $\alpha$ -, $\beta$ - and $\kappa$ -casein remained detectable even after sodium citrate (SC) treatment. |              |

microscopy). We have observed the same pattern in a prior study of ours that adopted both EM techniques (Ahlberg et al. 2024). Lastly, our study design prioritized individual-level resolution. While it could be argued that pooling samples and including technical replicates would provide a more controlled assessment of handling-related variability, our approach was chosen to reflect clinically relevant conditions and to allow for future correlations with donor-specific outcomes. Additionally, the rarity and limited volumes of clinically collected milk samples inherently restrict opportunities for extensive technical replication. These considerations shaped our methodological choices and reflect the balance between experimental control and clinical applicability.

## 5 | Conclusion

Our results did not reveal any significant differences between the fresh and the frozen milk samples *without* SC across any of the parameters studied. This suggests that frozen milk samples could be a viable alternative to fresh ones in studies of EVs and their miRNA cargo. However, this conclusion may not generalize to *all* EV isolation protocols or downstream analyses/applications, and each individual study should assess the suitability of frozen samples in its specific context. Importantly, we did not evaluate the impact of freezing on EV performance in functional studies, which should be addressed in future studies.

Adding SC to the protocol appeared to affect fresh and frozen samples differently, altering EV size and protein detection. This suggests that casein removal strategies like SC treatment may be less compatible with frozen whole milk in some protocols. Nonetheless, casein depletion may still be essential for certain downstream applications, such as mass spectrometry, where high-abundance milk proteins like casein could mask detection of low-abundance EV proteins.

Finally, we emphasize the importance of detailed reporting of sample handling, isolation and processing methods in all EV studies, in line with the MISEV guidelines (Welsh et al. 2024), to ensure reproducibility and interpretability across the field.

### Author Contributions

**Emelie Ahlberg:** writing - original draft, methodology, funding acquisition, formal analysis, conceptualization, investigation, visualization, data curation. **Ahmed Al-kaabawi:** writing - original draft, methodology, investigation, visualization, formal analysis, conceptualization. **Maria Eldh:** writing - review and editing, methodology. **Susanne Gabriellson:** methodology, writing - review and editing. **Lina Tingö:** visualization, writing - original draft, writing - review and editing, supervision, conceptualization, methodology.

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### Conflicts of Interest

The authors E.A., A.A.-K., M.E., M.C.J and L.T. report no conflict of interest. S.G reports a relationship with Anjarium Biosciences that includes consulting or advisory, equity or stocks, and holds a patent on B cell targeting extracellular vesicles.

### Data Availability Statement

Data is available from the corresponding author upon reasonable request.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section.

**Supporting Figures:** jex270092-sup-0001-FigureS1-

S8.docx **Supporting Tables:** jex270092-sup-0001-Tables.xlsx