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RESEARCH ARTICLE

Proteome characterization of extracellular vesicles from human milk: Uncovering the surfaceome by a lipid-based protein immobilization technology

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Abstract

Breast milk is an essential source of nutrition and hydration for the infant. In addition, this highly complex fluid is rich in extracellular vesicles (EVs). Here, we have applied a microfluidic technology, lipid-based protein immobilization (LPI) and liquid chromatography with tandem mass spectrometry (LC-MS/MS) to characterize the proteome of human milk EVs. Mature milk from six mothers was subjected to EV isolation by ultracentrifugation followed by size exclusion chromatography. Three of the samples were carefully characterized; suggesting a subset enriched by small EVs. The EVs were digested by trypsin in an LPI flow cell and in-solution digestion, giving rise to two fractions of peptides originating from the surface proteome (LPI fraction) or the complete proteome (in-solution digestion). LC-MS/MS recovered peptides corresponding to 582 proteins in the LPI fraction and 938 proteins in the in-solution digested samples; 400 of these proteins were uniquely found in the in-solution digested samples and were hence denoted "cargo proteome". GeneOntology overrepresentation analysis gave rise to distinctly different functional predictions of the EV surfaceome and the cargo proteome. The surfaceome tends to be overrepresented in functions and components of relevance for the immune system, while the cargo proteome primarily seems to be associated with EV biogenesis.

KEYWORDS
breast milk, exosomes, extracellular vesicles, human milk, immune regulation, mass spectrometry, proteomics

1 | INTRODUCTION

Human milk is an essential source of nutrition and hydration for the infant. In addition, this highly complex biological fluid contains numerous immunologically active factors such as microorganisms, immunoglobulins, cytokines and microRNAs (miRNAs) (Andres et al., 2023). We have previously shown that some of these miRNAs are associated with frequencies of regulatory T cells in breastfed infants (Ahlberg, Marti, et al., 2023) and have further proposed that this may be of importance in oral tolerance development (Ahlberg, Al-Kaabawi, et al., 2023). Thus, human milk is supposedly important in promoting the normal development of infant immunity. To exert immunological effects, human milk miRNAs need to withstand the harsh environment of the infant digestive system for subsequent uptake by somatic cells. On the basis of previous research, it is mainly facilitated through transport by extracellular vesicles (EVs) (Tingo et al., 2021). EVs are nanosized, membrane-enclosed vesicles that are released

by cells into the extracellular space. They carry functional RNA, lipids, proteins, and DNA that can be shuttled to recipient cells and change their phenotype (Manca et al., 2018; Yanez-Mo et al., 2015; Zhou et al., 2022). Packing of the EV cargo involves sorting mechanisms that favour some cargos over others and is hence not random (Squadrito et al., 2014; Stevanato et al., 2016); emphasizing the biological relevance of their content.

In vitro studies have shown that milk-derived EVs survive when exposed to the simulated conditions of gastric digestion and are also able to subsequently enter intestinal crypt-like cells (Kahn et al., 2018; Liao et al., 2017; Zhou et al., 2012). Furthermore, in vivo animal experiments show that milk EVs can be absorbed through the digestive tract to be further distributed throughout the body of a suckling pup or piglet (Carrillo-Lozano et al., 2020; Manca et al., 2018; Zhou et al., 2022), or after oral gavage in adult mice (Lopez de Las Hazas et al., 2022). Cells seem to absorb EVs via different endocytic pathways, including clathrin-dependent endocytosis, phagocytosis, macropinocytosis and caveolin mediated uptake (Melnik et al., 2021), but the full mechanisms underlying EVs absorption, and the subsequent epigenetic regulation exerted by the miRNA cargo, remain to be discovered in detail.

When EVs are released from the parent cell, certain subsets of proteins from the endosome and the plasma membrane are retained (Kim et al., 2012; Thery et al., 2001). Giovanazzi et al. (2023), identified unique surface proteins for milk-derived EVs compared to serum-derived EVs using multiplexed flow cytometric bead assay (MACSPlex). It was concluded that EVs from different sources have distinct molecular signatures that reflect their biological functions and origins. Furthermore, the protein profile is not only affected by the cells producing the EVs; the formation of the protein corona is a dynamic process that begins as the EVs are secreted from the parental cell and come in contact with the proteins of the biological fluid into which they are released. This process is influenced by various factors, including the chemical properties of the EV surface and the composition of the biological fluid. The protein corona phenomenon was demonstrated by Toth et al. (2021), showing that EVs indeed pick up proteins from their surroundings in vivo. It, however, remains to be further studied how different EV properties and fluid compositions influence the corona formation. So far, a very limited number of studies have attempted to describe the surface composition of EVs on the basis of proteomic analysis; this question deserves attention and methodological development as there are clues to be found regarding EV origin, functional properties and potential recipient cells in the "surfaceome".

Here, we have applied a microfluidic technology, lipid-based protein immobilization (LPI) and liquid chromatography with tandem mass spectrometry (LC-MS/MS) to characterize the surfaceome of human milk EVs, with the overall aim of increasing the knowledge on potential immunological cues transferred from mother to infant *via* the milk.

2 | MATERIALS AND METHODS

2.1 | Human milk samples

Please refer to Figure 1 for a schematic overview of the methods used in this study. Mature milk (3 months postpartum) was collected from six healthy women enrolled in a double-blind, randomized, placebo-controlled trial. The intervention and design will not be further described here, as it is not of particular importance to the approach taken in this study, but is described in detail elsewhere (Ahlberg, Marti, et al., 2023). Milk was collected from both breasts by manual or electronic pumping (according to the mother's own choice) in the home of each participant directly after the first-morning feeding. The breast was wiped clean of infant saliva before pumping, and milk samples were then instantly cooled in the refrigerator and transported on ice to the clinic and centrifuged within 2 to 3 h, except for one sample which was centrifuged after 14 h. Complete maternal characteristics are described in Supporting Information S1. The study was approved by the Regional Ethics Committee in Linköping 2011-04-20 (identification no. 2011/45-31). The participants' written informed consent was obtained prior to inclusion.

2.2 | Isolation of extracellular vesicles

The milk samples were transferred from the clinic to the lab on ice, and directly centrifuged twice at 800 × *g*, for 10 min at 4°C (Sigma 4–16 KS with swing-out rotor 11150) to separate the watery phase of the milk from the milk fat and cells. The supernatant was further centrifuged at 3000 × *g* for 40 min at 4°C and filtered through a 100 µm cell strainer (Merck). The milk was mixed 1:2 with cold 2% pre-filtered sodium citrate to break down casein micelles and thereby prevent coprecipitation of caseins or other contaminating proteins (Benmoussa et al., 2020) and incubated for 15 min on ice under gentle rocking. This was followed by centrifugation at 10,000 × *g* for 60 min at 4°C (Sigma 3–30 KS, rotor 12156-H), and filtration through a 0.22 µm PVDF syringe filter (Merck) (with the purpose of removing any particles larger than 220 nm before the ultracentrifugation). EVs were then pelleted down from a total volume of 10 mL milk at 100,000 × *g* for 70 min at 4°C in an Optima L80-XP ultracentrifuge using a Ti70 rotor (Beckman Coulter), followed by a wash with 0.22 µm filtered PBS and subjected to a second ultracentrifugation at 100,000 × *g* for 70 min at 4°C. The EV pellet was resuspended in one mL pre-filtered PBS overnight at 4°C and stored at −70°C. After ultracentrifugation, size exclusion chromatography (qEV1 35 nm, IZON) was also used, according to the manufacturer´s

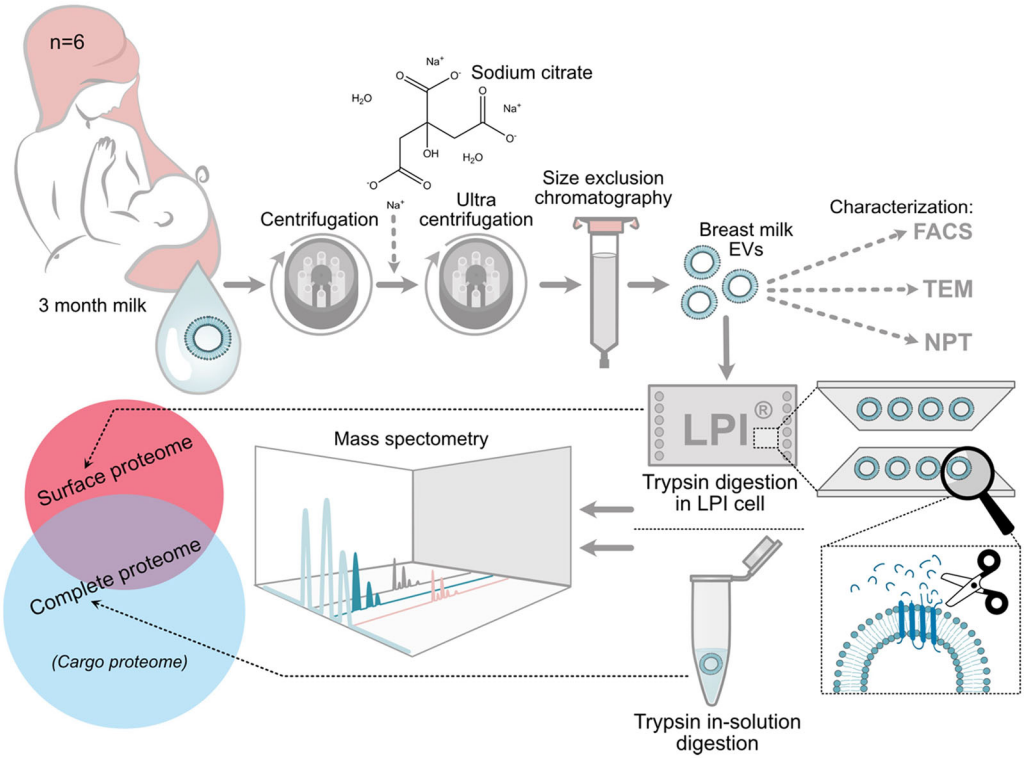

FIGURE 1 Schematic overview of the workflow for the six milk samples included in the experimental design (i.e., not depicting the preceding pilot study). EVs were isolated using several centrifugation steps including ultra-centrifugation. Prior to ultra-centrifugation, the samples were treated with 1% sodium citrate to remove milk micelles. Before centrifugation at 100,000 × *g* samples were also filtered (0.22 μm). After EV isolation, three samples were characterized by FACS (bead-based flow cytometry), TEM (transmission electron microscopy) and NTA (nanoparticle tracking analysis). All six samples were also subjected to mass spectrometry analysis following trypsin digestion in an LPI flow cell or in-solution. The latter gave insights into the complete proteome of the EVs, while the LPI cell allows for "surface shaving" by trypsin and hence gives the surface proteome.

protocol, to obtain a higher purity of EVs. EVs in one mL filtered-PBS were loaded on top of the column and fractions 5–10 were collected and concentrated at 3800 × *g*, for 45 min using an Amicon Ultra-15 3K centrifugal filter (Merck) to approximately 400 μL and stored at −70°C until protein analysis or EV characterization.

2.3 | Nanoparticle tracking analysis

The NanoSight NS300 (™NanoSight Technology, Malvern, United Kingdom) was used to measure particle concentration and EV size ($n = 3$). The instrument was equipped with a 488 nm laser beam and the NTA software 3.4. The camera level was set to 16 and the detection threshold to 5. The EV samples were further diluted in pre-filtered PBS (1:2000 or 1:8000), injected with a syringe pump (speed 60) and five videos of 30 s were recorded for each sample with a 5 s delay between each video.

2.4 | Bead-based flow cytometry

MACSPlex human Exosomes Kit (Miltenyi Biotec) was used to quantify 37 EV markers, including two isotype controls, as described by the manufacturer. In brief, three EVs samples equal to 10 μg protein (quantified using Qubit Protein Assay (Invitrogen) according to manufacturers' guidelines and measured on a Qubit 3.0 Fluorometer (Invitrogen)) were diluted in MACSPlex buffer to a total volume of 120 μL, including blank. 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 were added to the sample followed by incubation at RT for 60 min under rotation at 12 rpm. The sample was then washed twice with MACSPlex buffer and the 150 μL sample was transferred to a FACS tube before analysis, using a Gallios Flow Cytometer (Beckman Coulter, Miami, FL, USA).

The data was analysed with Kaluza 2.1 (Beckman Coulter). Calibration beads were used to set the gates; 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

(Wiklander et al., 2018). The median fluorescence intensity (MFI) of the APC signal within each bead population was calculated. All markers were background corrected by subtracting the MFI signal from the negative control (buffer + antibody).

2.5 | Transmission electron microscopy

2.5.1 | Negative staining

The EV samples ($n = 3$) were subjected to negative stain transmission electron microscopy (TEM), as described previously, with minor modifications (Cizmar & Yuana, 2017). In brief, a 5 μL sample (diluted 1:10) was placed on a Formvar-Carbon coated, copper, 300 mesh electron microscopy grids (TED PELLA, Inc.). Grids were washed, blotted, and negatively stained with 2% uranyl acetate (Polysciences Europe GmbH, Germany).

2.5.2 | Immunogold labelling

An EV sample ($n = 1$) was subjected to immuno-TEM. In brief, a 5 μL sample was placed on a Formvar-Carbon coated, copper, 300 mesh electron microscopy grid. The grid was blocked, 1:20 mouse anti-CD9 (0.5 mg/mL, HI9a, Biolegend), bridging antibody rabbit anti-mouse (1:50, ab6079, Abcam) 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.

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 | Protein digestion with LPI HexaLane FlowCell

2.6.1 | Pilot study of LPI digestion conditions

EVs from 3-months postpartum milk ($n = 4$), isolated by ultra-centrifugation (without further size exclusion isolation) were thoroughly characterized and used for optimizing the LPI digestion. EVs were loaded onto LPI FlowCells (Jansson et al., 2012; Karlsson et al., 2012), immobilized for 1 h and digested by trypsin (20 μg/mL), in PBS or ammonium bicarbonate (Ambic), for 15 or 45 min to determine the optimal digestion conditions. As a comparison, in-solution digestion overnight of the same samples were performed using trypsin (0.6 μg/mL) with a mass spectrometry-compatible detergent SDC (sodium deoxycholate, 1%). Peptides were subsequently eluted using 100 μL of either PBS or Ambic depending on conditions, and subsequently analysed by LC-MS/MS (described in detail in Supporting Information S2). The trypsin-ambic digestions produced more unique peptides per protein than trypsin-PBS, as did 45 min digestion compared to 15 min; the trypsin-ambic condition was hence regarded as superior to the trypsin-PBS and used in the following experiments. However, the 45 min digestions also produced more peptides of proteins with cytosolic origin, regardless of the digestion buffer composition. To balance the risk of membrane leakage, while still promoting peptide richness, the digestion time was set to 20 min in the following experiments.

2.6.2 | Proteomic analysis of extracellular vesicles fromhuman milk

The samples were processed both using the LPI flowcell and in-solution digestion.

For the LPI flowcell process, the EVs were digested using 20 μg/mL trypsin for 20 min and the resulting peptides were eluted using PBS (100 μL). The eluted peptides were digested for 1 h at 37°C by the addition of an additional 10 μL trypsin (20 μg/mL stock) and then acidified with 10% formic acid (40 μL).

For the in-solution digestion, the EVs were digested by adding SDC to a final concentration of 1% to each sample (45 μL). Trypsin added to a concentration of 2 μg/mL (10 μL of 20 μg/mL stock) was added and the samples were digested overnight at 37°C. The samples were acidified by the addition of 20 μL 10% formic acid and SDC was removed by centrifugation, supernatant was frozen at −20°C until analysis.

2.7 | Proteomic analysis

The samples were purified using HiPPR detergent removal kit and Pierce peptide desalting spin columns (both Thermo Fisher Scientific), according to the manufacturer´s instructions, dried and reconstituted in 15 μL (3% acetonitrile, 0.2% formic acid).

The LC-MS analysis was performed on an Orbitrap Exploris mass spectrometer interfaced with an Easy-nLC1200 liquid chromatography system (both Thermo Fisher Scientific). Peptides were trapped on an Acclaim Pepmap 100 C18 trap column (100 μm × 2 cm, particle size 5 μm, Thermo Fischer Scientific) and separated on an in-house packed analytical column (35 cm × 75 μm, particle size 3 μm, Reprosil-Pur C18, Dr. Maisch) at a flow of 300 nL/min using a gradient from 5% to 80% acetonitrile in 0.2% formic acid over 60 min or 90 min for the LPI and in-solution digest samples, respectively. The injection volumes were 2 or 5 μL for LPI samples, while 1 μL for the more complex in-solution digested sample. The instruments operated in data-dependent mode where the precursor ion mass spectra were acquired at a resolution of 120,000, m/z range 380–1500. The most intense ions with charge states 2 to 6 were selected for fragmentation using HCD at collision energy settings of 30 and dynamic exclusion 30 s, 10 ppm. The isolation window was set to 1.4 Da and MS2 spectra were recorded at a resolution of 30,000.

2.8 | Identification and data processing of proteomics data

The raw files were searched for identification using Proteome Discoverer version 2.4 (Thermo Fisher Scientific). The data were matched against SwissProt Human (April 2023, 20,422 entries) using Sequest as a search engine. The precursor mass tolerance was set to 5 ppm and the fragment mass tolerance to 0.06 Da. Tryptic peptides were accepted with one missed cleavage and variable modifications for methionine oxidation were selected. FixedValue PSM (peptide spectra matches) Validator was set for LPI samples. For in-solution digested samples, Percolator in combination with Protein FDR Confidence at least medium (5% FDR) were selected.

2.8.1 | Data curation

The protein lists produced from the LPI and the in-solution digested were curated to remove proteins denoted by less than two peptides in less than one sample, that is, a protein would have to be discovered at a minimum by one peptide in two samples or by two peptides in one sample to be further processed. After this step, all proteins discovered by only one peptide were discriminated between on the basis of their PSM; all single peptide discoveries with a PSM < 10 were excluded.

One sample had to be excluded from the data interpretation as it was deemed non-representative due to substantial deviations from the other five LPI samples; it had a much higher number of peptides for practically all protein, as compared to the rest of the LPI samples. We could not see any reason for this in the sample handling, but the results for this particular sample more resembled that of the in-solution digested samples, hence lysis of the exosomes might have occurred. In most instances, the sample had two-fold higher peptide counts than the second highest sample, but at quite some occasions as much as 15–20 times higher. Furthermore, neither of the five samples followed the same protein expression pattern.

2.8.2 | Databases and overrepresentation predictions

Accession numbers for the two protein lists, LPI and in-solution digested samples, were compared, and duplicates were removed from the in-solution list to produce a separate list for "cargo proteins". The LPI list (i.e., surface proteome) and the list of cargo proteins were then separately pasted into the UniProt database (release 2023-05) for ID mapping and predictions of cellular location of the surface versus cargo proteins. The two lists were also subjected to predictions of Cellular component, Molecular function and Biological processes in the GeneOntology database (Ashburner et al., 2000), release 2023-01-05 (Gene Ontology et al., 2023) using the PANTHER Overrepresentation Test (including Fisher's exact test and false discovery rate calculations), release 20231017 (Thomas et al., 2022). Furthermore, the FunRich software (version 3.1.4) was used to compare our data set with the ExoCarta and Vesiclepedia (version 5.1).

2.9 | Data repository

The raw data will be made available through ProteomeXchange and PRoteomics IDEntifications (PRIDE) Archive database.

3 | RESULTS

To confirm successful EV isolation and purity, nanoparticle tracking analysis (NTA), bead-based flow cytometry, and TEM were performed on samples isolated from three women, in addition to the mass spectrometry data from all individuals that describe cargo and potential contaminating proteins, as recommended by Minimal Information for Studies of Extracellular

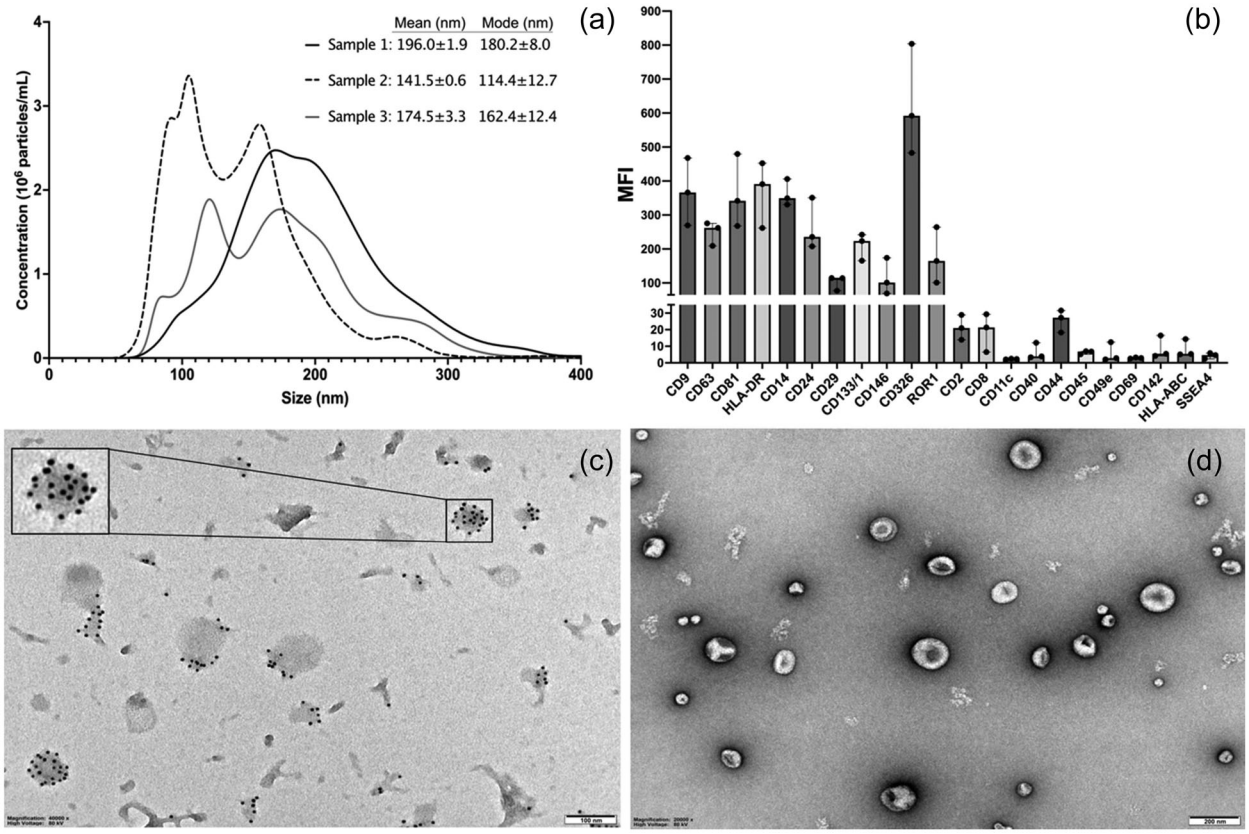

FIGURE 2 Characterization of isolated extracellular vesicles (EV) from mature human milk ($n = 3$). (a) Nanoparticle tracking analysis was performed to confirm particle concentration (10^6 particles/mL) and size (nm). Sample 1 was diluted 1:8000 and samples 2 and 3 1:2000 in pre-filtered PBS. The dilution factor has not been considered in the displayed data. (b) Common EV protein markers were confirmed with MACSPlex human Exosomes flow cytometry kit, presented as background corrected MFI, and an MFI above 2 in all three samples were considered positive. (c) The morphology, size and the presence of CD9 were evaluated using immunogold labelled transmission electron microscopy (TEM). CD9 was identified using antibodies (mouse anti-CD9 and rabbit anti-mouse) and protein A-gold (5 nm), scale bar 100 nm. (d) The morphology and size were further confirmed using negative stain TEM, scale bar 200 nm.

Vesicles (MISEV) 2023 guidelines, as proposed by The International Society for Extracellular Vesicles (Welsh et al., 2024). The results presented in Figure 2 suggest a population enriched by small EVs. The NTA suggest that the size range of the EVs are between 141.5 and 196.0 nm (Figure 2a) and the flow cytometry results describe several "exosome"-associated proteins, such as the tetraspanins CD9, −63 and −81 (Figure 2b). Analysis by TEM and immunolabeling further supports the presence of EVs, as they had a common morphology, as well as the presence of CD9 on the surface (Figure 2c,d). A majority of the proteins from category 1, described in the MISEV2023, were present in the mass spectrometry data set, that is, CD55, CD73, MHC I/II, and the Sonic hedgehog protein. Furthermore, cytosolic proteins of category 2 were represented in our EV samples, such as flotillin-1, syntenin-2 and the heat shock proteins 70 and 90. Lastly, calnexin could be found in the in-solution digestion fraction.

3.1 | Proteomics

In the LPI fractions, representative of the EV surfaceome, 582 proteins were discovered identified by at least 1 peptide with a PSM (peptide spectral match) > 10 in at least two samples. Out of these proteins, UniProt identified 112 as transmembrane and 5 as intramembrane. In the in-solution digested fractions, *i.e.* global/overall proteome, we discovered 938 unique proteins of which 176 were defined as transmembrane and 8 as intramembrane. Please refer to Figure 3 for a schematic overview.

Overlap between the two digestions was extensive: 538 proteins from the LPI were also discovered in the in-solution digested samples. However, 44 were uniquely found in the LPI and 400 existed only in the in-solution. These 400 may, hence, be considered cargo proteins. The 44 that were uniquely found in the LPI fraction were mostly discovered by low number of peptides (<5 unique peptides), with five exceptions; the hornerin protein (UniProt accession no Q86YZ3), hormonally up-regulated neu-associated kinase (P57058), small ribosomal subunit protein uS9 (P62249), large ribosomal subunit protein eL8 (P62424) and titin (Q8WZ42).

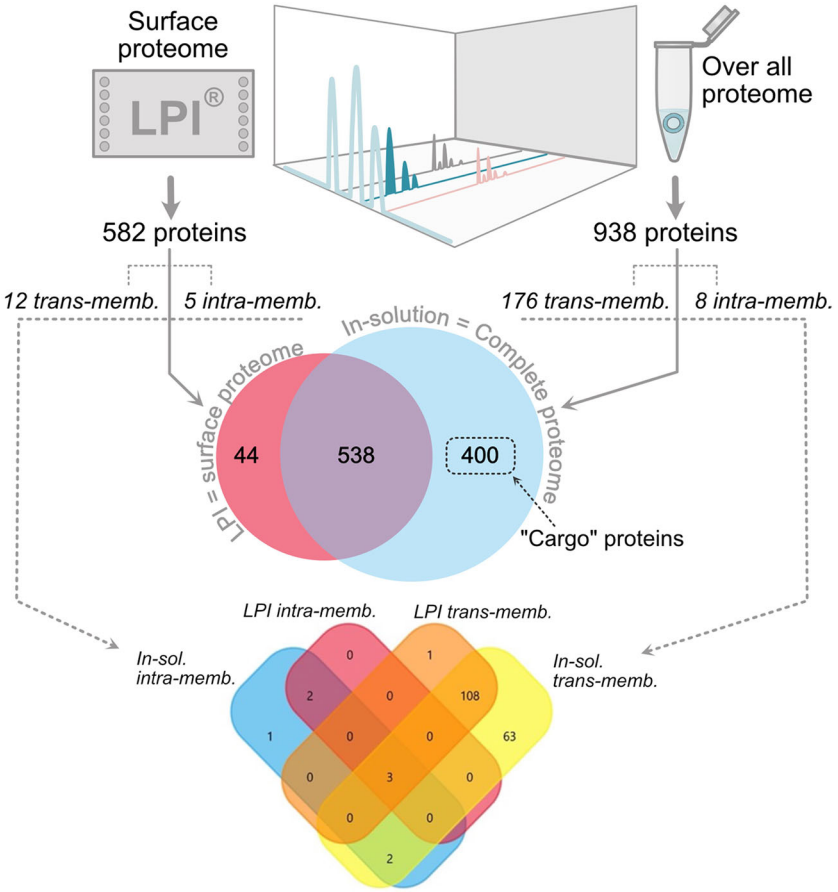

FIGURE 3 Schematic overview of the number of proteins discovered by LPI and in-solution digestion, including overlaps and number of intra- and transmembrane proteins.

TABLE 1 Top predictions of cellular components.

Fraction	GeneOntology cellular component complete	Fold enrichment	*p*-value	*q*-value
Surfaceome	prominosome (GO:0071914)	35.56	4.32E-03	3.08E-02
	amphisome membrane (GO:1904930)	26.67	1.76E-09	3.12E-08
	muscle thin filament tropomyosin (GO:0005862)	26.67	6.56E-04	5.51E-03
Cargo proteome	retromer, cargo-selective complex (GO:0030906)	38.71	2.25E-04	3.49E-03
	F-actin capping protein complex (GO:0008290)	32.26	2.75E-06	6.98E-05
	chaperonin-containing T-complex (GO:0005832)	28.15	4.71E-07	1.39E-05

Note: Fold enrichment based on PANTHER overrepresentation analysis. *P*-value = raw *p*-value, *q*-value = false discovery corrected *p*-value.

Concerning the proteins denoted trans- or intramembrane in the two digestions, the overlap was almost complete: 5 intramembrane and 111 transmembrane proteins were shared between the two fractions. Please refer to Figure 3. Furthermore, when comparing our data with other existing resources, we covered 86.4% of the EV proteins listed for human milk EVs in ExoCarta and 86.0% of the top-100 listed in Vesiclepedia. Please observe that in both these databases proteins are listed as their corresponding genes, hence, to compare our data set to these resources we used the gene names generated for our protein accession numbers when pasted into FunRich. However, 16 proteins lacked corresponding genes in FunRich and were instead complemented by the gene information listed in UniProt. Considering all 1990 unique gene/protein entries specific for human milk in Vesiclepedia, we covered 796 proteins.

3.1.1 | Surfaceome versus cargo proteins

The top three GeneOntology predictions by PANTHER Overrepresentation Test for "Cellular component", "Molecular function" and "Biological processes" are displayed in Tables 1–3. See complete lists in Supporting Information S4–7.

TABLE 2 Top predictions of molecular function.

Fraction	GeneOntology molecular function complete	Fold Enrichment	*p*-value	*q*-value
Surfaceome	MIT domain binding (GO:0090541)	35.56	3.83E-04	0.016
	protein kinase C inhibitor activity (GO:0008426)	35.56	3.83E-04	0.0159
	type 3 metabotropic glutamate receptor binding (GO:0031800)	35.56	3.83E-04	1.57E-02
Cargo proteome	purine nucleobase binding (GO:0002060)	51.61	1.31E-04	8.36E-03
	nucleobase binding (GO:0002054)	38.71	2.25E-04	1.36E-02
	1,4-alpha-oligoglucan phosphorylase activity (GO:0004645)	30.97	3.56E-04	2.00E-02

Note: Fold enrichment based on PANTHER overrepresentation analysis. *P*-value = raw *p*-value, *q*-value = false discovery corrected *p*-value.

TABLE 3 Top predictions of biological processes.

Fraction	GeneOntology biological processes complete	Fold Enrichment	*p*-value	*q*-value
Surfaceome	protein localization to cell leading edge (GO:1902463)	35.56	3.83E-04	8.69E-03
	ubiquitin-independent protein catabolic process via the multivesicular body sorting pathway (GO:0090611)	35.56	3.38E-06	1.51E-04
	positive regulation of viral budding via host ESCRT complex (GO:1903774)	35.56	3.83E-04	8.68E-03
Cargo proteome	cysteine transport (GO:0042883)	22.13	> 100	2.85E-05
	cysteine transmembrane transport (GO:1903712)	22.13	> 100	2.85E-05
	L-aspartate import across plasma membrane (GO:0140009)	22.13	> 100	5.70E-07

Note: Fold enrichment based on PANTHER overrepresentation analysis. *P*-value = raw *p*-value, *q*-value = false discovery corrected *p*-value.

Interestingly, 11 of the top-50 GO predicted cellular components in the surfaceome were immunoglobulin complexes (pentameric IgM immunoglobulin complex (ic) (GO:0071756); IgM ic (GO:0071754); secretory dimeric IgA ic (GO:0071752); dimeric IgA ic (GO:0071750); secretory IgA ic (GO:0071751); polymeric IgA ic (GO:0071749); IgA ic (GO:0071746); ic (GO:0042571); IgA ic (GO:0071745); IgG ic (GO:0071735); IgM ic (GO:0071753) (all FDR < 0.05); the cargo proteome shared none of these proteins. Shared between the surface and the cargo proteome were, however, the GO terms for extracellular exosome (GO:0070062); extracellular vesicle (GO:1903561); ESCRT complex (GO:0036452); ESCRT I complex (GO:0000813); the ESCRT III complex (GO:0000815) was however only discovered in the surfaceome (all FDR < 0.05). In the surfaceome, MHC class I protein complex (GO:0042612) and MHC protein complex (GO:0042611) were also predicted (FDR < 0.001) as cellular components, while not being present in the cargo fraction.

Concerning the predicted molecular functions of the surfaceome, T cell receptor binding (GO:0042608), MHC class II protein complex binding (GO:0023026), Toll-like receptor binding (GO:0035325), MHC protein complex binding (GO:0023023) (all FDR < 0.05), were all found top-50; none of these functions were predicted for the cargo proteome.

4 | DISCUSSION

Here, we have applied a microfluidic LPI technique followed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis to characterize the surfaceome (Jansson et al., 2012; Karlsson et al., 2012) of human milk EVs, with the aim of increasing knowledge on potential immunological cues transferred from mother to infant *via* the milk. However, first, we extensively characterized our milk EVs. The NTA suggest that the EVs had a size range between 141.5 and 196.0 nm (Figure 2a) and the flow cytometry results describe several "exosome"-associated proteins, such as the tetraspanins CD9, −63 and −81 (Figure 2b). In line with previous data by Giovanazzi et al. (2023), we could see a similar protein expression pattern in our flow cytometry data. This is interesting as Giovanazzi et al. used another approach to isolate the milk EVs, suggesting that the protein expression on milk-derived EVs is not sensitive to the isolation method or the presence of sodium citrate. Furthermore, analysis by TEM and immunolabeling further support the presence of EVs in our isolates as they display the common morphology, as well as the presence of CD9 on the surface (Figure 2c,d). Also, most of the proteins from category 1, described in the MISEV2023, were present in the mass spectrometry data set, for example, CD55, CD73, MHC I/II, and the Sonic hedgehog protein, with the exception of CD63 (which however was detected by flow cytometry, as expected). Furthermore, cytosolic proteins of category 2 were represented in our EV samples, such as flotillin-1, syntenin-2 and the heat shock proteins 70 and 90. We could, however, identify calnexin in the *in-solution* digestion fraction, suggesting some potential impurity.

To answer our aim, that is, characterize the surfaceome of human milk EVs, we used two ways of digesting the vesicular proteins (1) 20 min trypsin digestion in an LPI FlowCell, to target the surface-related proteins and (2) trypsin in-solution digestion to recover the complete proteome. As expected, there was a significant, although not complete, overlap between the two digestions. Given that the in-solution digestion would recover the proteome of the entire vesicle, including the surfaceome, one would, in theory, anticipate an all-inclusive overlap. In this study, data-dependent acquisition of tandem mass spectra was used, where the topmost intense peptide ions were selected for fragmentation, favouring highly abundant peptides over low abundant peptides. The lack of some peptides in the complete digestions is hence likely due to the discrimination between peptides that occurs in the data-driven spectrometry analysis, favouring the more abundant peptides in a sample. Hence, the LPI fraction containing fewer peptides will be more sensitive to detect surface-associated proteins, whose peptides are overlooked in the in-solution digestion due to their lower abundance compared to other peptides.

This is the first study to analyse the EV surfaceome by utilizing the microfluidic LPI approach preceding LC-MS/MS. However, a previous study used a combination of proteinase K and biotin tagging followed by mass spectrometry to separate the surface and luminal proteins of mast cell EVs (Cvjetkovic et al., 2016). In this study, they eventually arrived at a separation of the surface-associated proteins from the membrane-bound and the luminal proteins. Unlike this, we have considered all proteins originating from the LPI digestion as "surfaceome", including trans- ($n = 112$) and intramembrane ($n = 5$) proteins. We came to this division as membrane-associated proteins tend to have surface-accessible parts, as evident by the degradation caused by trypsin treatment of the vesicle surface. We, however, discovered trans- ($n = 176$) and intramembrane ($n = 8$) proteins also in the in-solution digestions (i.e., the complete proteome). Surprisingly, some of these proteins were not found in the LPI digestions and were hence ascribed to the cargo protein fraction; 65 denoted a transmembrane localization and 3 denoted an intramembrane. For the three intramembrane proteins this is reasonable as they may not have been affected by the surface trypsin treatment and are more protected by their membrane integration, alternatively that they miss trypsin available cleaving sites. Interestingly, all but three of the 65 transmembrane proteins are, according to Protter tool http://wlab.ethz.ch/protter/, version 1.0, January 2024, (Omasits et al., 2014), supposed to have an extracellular part which intuitively should have been accessed by the trypsin and hence appeared in the LPI fraction; but they do not. Previous studies have suggested that EVs with double membranes (i.e., double lipid bilayered membranes, as suggested in Figure 4) exists (D'Acunzo et al., 2021; Emelyanov et al., 2020; Hoog & Lotvall, 2015; Poliakov et al., 2009; Zabeo et al., 2017; Zonneveld et al., 2014). In such cases, as previously suggested by Cvjetkovic et al. (2016), the proteins of the inner membrane are protected from digestion by the outer vesicular membrane. This may explain why transmembrane proteins occur *only* in the cargo protein fraction. Another puzzling observation by Cvjetkovic et al. (2016) is that some of the surface-anchored proteins seem to have an inside-out topology; our data indicate the same. The inside-out orientation of six proteins were specifically validated in the prior study; we have three of them in our LPI fraction (i.e., surfaceome): flotillin-1, GADPH and TSG101. According to the Protter Tool, their topology is oriented towards the cytosolic side of the membrane, and they should hence intuitively appear in the in-solution digestion rather than the LPI one; but they do not. We believe this finding may indicate an in-side out topology, in support of Cvjetkovic et al.; they speculate that this conformational change may appear due to sudden differences in the lipid environment during different steps of the EV biogenesis. Furthermore, Cvjetkovic et al. (2016) hypothesize that the proteins of cytosolic or nuclear origin appearing in the surfaceome may be bound to the EV surface by non-covalent binding and thus shuttled between cells as an extra-vesicular cargo; a "hitchhiking" proteome. They point out that it is unclear whether these hitchhiking proteins are natural EV surface molecules or whether they are a result of in vitro culture conditions; we, however also find this fraction of non-membrane-associated proteins in our in vivo recovered milk EVs.

Furthermore, given our immune-related aim, we found it very intriguing that several of the predicted cellular components were immunoglobulin complexes, such as IgM, IgG and IgA, including secretory IgA. The fact that the cargo proteome shared none of these proteins gives reason to consider these proteins as either "inherited" from the cell of origin, or alternatively that the milk EVs acquire these proteins from their milieu. Toth et al. (2021) describe how EVs recovered from plasma form an external "protein corona" made up of common plasma proteins, including immunoglobulin heavy constant $\gamma 2$ and $\gamma 4$ chains. As human milk is inherently rich in immunoglobulins it is quite conceivable that these soluble proteins are acquired by the milk EVs, that is, are part of the corona. Interestingly, they also showed that EVs with an external plasma protein corona induced an increased expression of TNF, IL-6, CD83, CD86 and HLA-DR in human monocyte-derived dendritic cells, while EV-free protein aggregates had no effect; implying increased immune activation by corona EVs. Previous research from our group has shown that children with allergic manifestations, particularly asthma, during childhood have a lower proportion of IgA bound to fecal bacteria at 12 months of age compared with healthy children (Dzidic et al., 2017); the early IgA antibodies are predominantly maternally derived in breastfed children. Moreover, the ample number of immunoglobulins in the surfaceome of the milk EVs may suggest a B-cell origin; B-cells were predicted as "tissue of origin" by both FunRich and STRING (https://string-db.org, version 12.0). However, we believe it to be more likely that there is an "after-coating" of the vesicles due to the large amounts of immunoglobulins in human milk; earlier proteomics data from milk EVs has similarly discovered immunoglobulins (Admyre et al., 2007; Freiria-Martinez et al., 2023; Yang et al., 2017).

Whether or not some human milk EVs have a B-cell origin it is not farfetched to consider milk EVs to, at least to some extent, be produced by immune cells with antigen-presenting properties; because MHC II is present in our data sets. In fact, it has been previously proposed that EVs could take part in antigen presentation and lymphocyte activation (Xie et al., 2022). We, however,

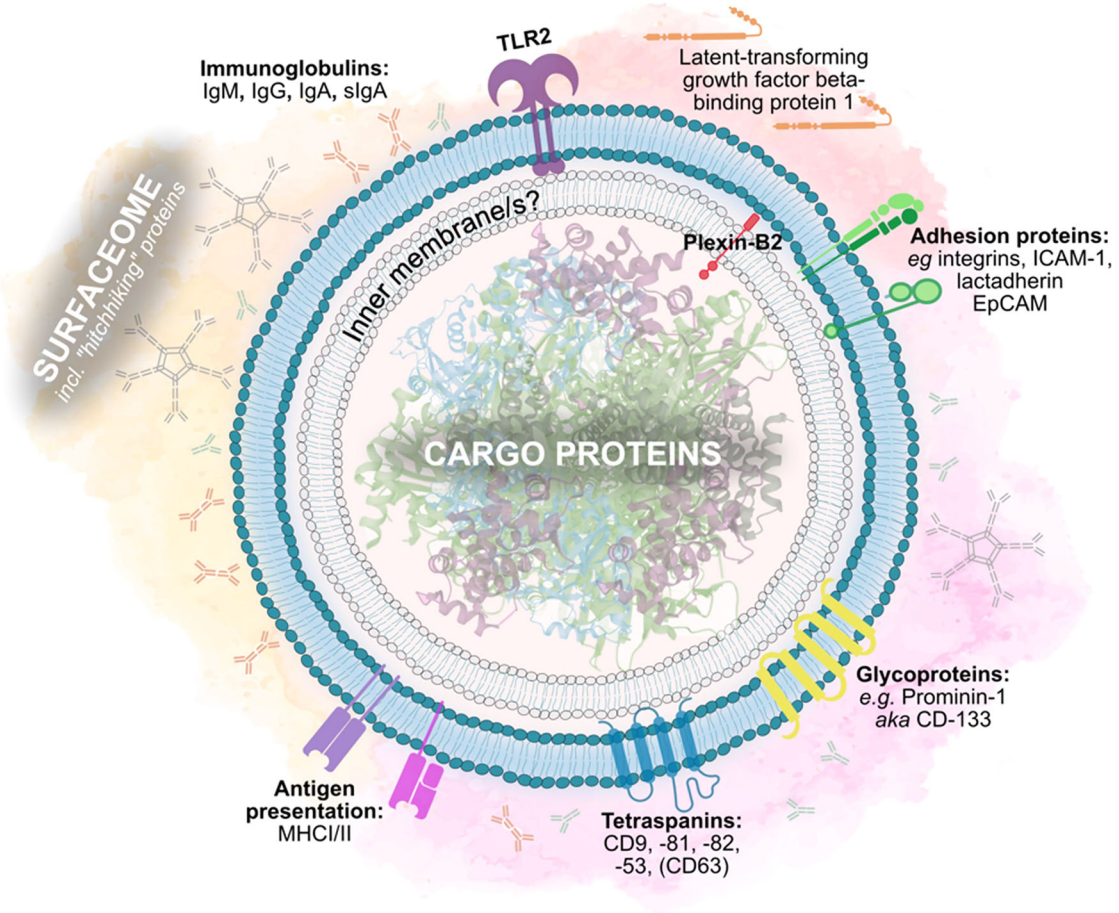

FIGURE 4 Illustrative overview of a human milk EV showing selective parts of the proteome, focusing on immune-relevant surface proteins and proteins commonly discovered on EVs, such as tetraspanins and adhesion proteins. Also, we included prominin-1 (CD133), one of the transmembrane proteins that are present in surfaceome as measured both by proteomic analysis and bead-based flow cytometry, and Latent-transforming growth factor beta-binding protein 1 (LTBP1). LTBP1 is expressed in the surfaceome, but regarded as a secreted protein found in the extracellular space and are thus most likely one of the corona proteins of our milk EVs. LTBP1 controls TGF-beta activation by maintaining it in a latent state during storage in extracellular space. Moreover, Plexin-B2, a transmembrane protein, was discovered as a high-ranking protein of the cargo proteome. The presence of this protein and other trans- and intramembrane proteins in this fraction leads us to consider the existence of multiple bilayered membranes in breast milk EVs.

hold this for unlikely as the EVs are evidently missing the necessary co-stimulatory molecules for antigen presentation. It is, however possible that EVs have a role in antigen presentation and activation of naïve T cells if captured and presented by other cells, such as dendritic cells, as previously suggested by (Bobrie et al., 2011). Further considering themilk EV origin, mammary epithelium has previously been suggested to as a primary producer of milk EVs (Alsaweed et al., 2016; Jiang et al., 2021; Larssen et al., 2017; Modepalli et al., 2014). We, however, believe our data aligns quite well with previous research from van Herwijnen et al. (2016), suggesting immune cells as major contributors to milk EV production. Nevertheless, it should be noted that it is possible for an inflamed epithelium to also express MHC class II, which may, for example, occur in mastitis. We, however, consider this a less likely explanation as the women recruited for our study where all free from mastitis symptoms at the time of milk expression (refer to Supporting Information S1). Furthermore, MHC class II is mainly found on antigen presenting cells that belong to the innate immune system. Similarly, the presence of Toll-like receptor 2 (TLR2) in our data indicates innate origin; as it is primarily myelomonocytic cell lines that express surface TLR2 (Flo et al., 2001). Also, the presence of CD14, a common monocyte marker, in the surfaceome implies innate origin. Noteworthy tough, it has been found that also T cells occasionally can be HLA-DR (i.e., MHC class II) positive (Holling et al., 2004; Kang et al., 2023).

Moreover, a recent paper from Lischnig et al. (2022), compared the proteome of large and small EVs isolated using density gradient, concluding that they have distinct protein profiles. Small EVs appear to be enriched in tetraspanins, ADAMs and ESCRT proteins. Specifically, syntenin-1 and ADAM10 were suggested as markers of small EVs (from breast cancer cell lines); both proteins are present in our data. We also detected ADAM9, 12 and 18. Furthermore, we find syndecan-4 and ALIX. These proteins have previously been suggested to be involved in exosomes biogenesis (Baietti et al., 2012) potentially indicative of the presence of this particular EV subset in our milk samples. In addition, mitochondrial proteins such as TIM and TOM complexes

were found by Lisching et al. to be enriched in larger EVs. In our dataset, we only find TIM16, which is not mentioned in the Lisching et al. paper. We, however, find several other proteins with mitochondrial origin/association; for example, ribosomal protein 28S; found by Lisching et al. to be enriched in larger EVs. Cytochrome C is also present in our dataset, a highly conserved mitochondrial protein that is often detected in EV isolates as denoted in the (Welsh et al., 2024) MISEV 2023 guidelines. As cytochrome C, histones are taken up as "Transmembrane, lipid-bound and soluble proteins associated to other intracellular compartments than PM/endosomes" in the guidelines. We find histone H1, H2B, H3 and H4 in the *cargo* fraction of our milk EVs; in the surface faction we find histone deacetylase 3, 4 and 6. Histone H2B, H3 and H4 have previously been discovered in EVs from dendritic cells (Thery et al., 2001). The actual mechanisms or circumstances by which histones appear in EVs remain to be further studied, but previous research has identified histone H1 as a major extracellular candidate that causes neurotoxicity and activation of the innate immune system in neurodegenerative conditions (Gilthorpe et al., 2013). Extracellular histone may act as damage-associated molecular patterns, causing immune activation primarily via TLRs as previously reviewed (Richards et al., 2023). Despite their histone cargo, human milk EVs, however seem to be primarily involved in anti-inflammatory mechanisms (Admyre et al., 2007; Ahlberg, Al-Kaabawi, et al., 2023; Ahlberg, Marti, et al., 2023; Reif et al., 2020).

Among the top-three predicted cellular components and molecular functions of the surfaceome are the "prominosome" and the "amphisome membrane", and "protein kinase C inhibitor activity" and "type 3 metabotropic glutamate receptor binding", respectively; all with involvement in immune-related pathways. The top expressed cargo proteins, however, generated predictions that have to do with nucleobase binding, protein folding and vesicular trafficking. We interpret this as the vesicular enclosed proteins, which are mainly remnants of vesicular formation and packing rather than cargo with specific biological purposes for any recipient cell. In a recent paper from us we show associations between infant immune cells and milk miRNAs (Ahlberg, Marti, et al., 2023), drawing from these findings and our current proteome analyses we would propose that the surface-related proteome of milk EVs indeed have implications for the regulation and function of recipient cells. Concerning the luminal content of the EVs, however, cargo such as small RNAs rather than proteins have biological potency. The prominosome prediction of the surfaceome involves prominin 1 and 2. Prominin 1 is an integral membrane glycoprotein found on neuroepithelial and hematopoietic stem cells (Fargeas et al., 2003) and prominin-2 is a glycoprotein structurally related to prominin 1. The structural similarities between prominin-1 and prominin-2 are, to some extent, reflected by their biochemical properties; both proteins are selectively concentrated in specific plasma membrane subdomains that protrude into the extracellular space and are released in small extracellular membrane vesicles (Fargeas, 2013). Previous research has found prominin 1, also known as CD133, to be closely associated with oncogenic signalling pathways (Mak et al., 2012; Won et al., 2015). We find CD133 in both our proteomic analysis as well as in the flow cytometry, refer to Figure 2b. EVs enriched by this protein are effectively transferred to non-cancerous recipient cells; a suggested mechanism in oncoprotein trafficking (Kang et al., 2019). Furthermore, CD133 positive microvesicles contribute to immunosuppression in cancerous tissue, potentially by stimulating M2-like macrophage polarization (Kim et al., 2023). Interestingly, for our approach such immunosuppressive macrophages are of utter importance for the fetomaternal immunological tolerance (Parasar et al., 2021). Decidual macrophages modulate the activity of T cells contributing to dampened immune responses during early human pregnancy (Grozdics et al., 2014; Sayama et al., 2013). Furthermore, macrophages also interact with regulatory T (Tregs) cells leading to immunosuppressive functions (Grozdics et al., 2014). This series of events is likely important for establishing tolerance in the maturing infant, with Tregs at the hub (Ahlberg, Al-Kaabawi, et al., 2023). Hence, we would argue that these mechanisms are meant for adequate maturation of the developing baby; the tumour-promoting effects may be yet another example of cancers "high jacking" normal physiological mechanisms for their own purposes.

The amphisome membrane was another top-predicted cellular component. The amphisome is a transitional hybrid organelle formed by the fusion of autophagosomes and multivesicular bodies (MVBs). This is a route by which the autophagy system may get rid of cell debris such as misfolded proteins and damaged organelles. In fact, autophagy—as in the initial phase of EV formation—is initiated by the formation of double-membrane organelles that capture cytosolic material; based on several pathway termination alternatives, autophagy may be degradative or secretory (Claude-Taupin et al., 2018). During secretory autophagy, cytoplasmic cargo is sequestered and secreted from the cell for the purpose of extracellular signalling, for example, IL-1β release. This proinflammatory cytokine is produced by activated macrophages, monocytes and some dendritic cells, as recently reviewed (Yaseen et al., 2023). The biogenesis of autophagy organelles destined for secretion and EVs seems to be very closely linked, as exemplified by amphisome formation. Indeed, the autophagy-EV crosstalk, including amphisome formation, has previously been suggested to cause small EVs to contain autophagy-associated proteins, such as LC3-II and p62 (Peng et al., 2021); these proteins both occur in our data (note that p62 represented by SQSTM1, which according to the NIH gene card for Gene ID: 8878 (update 26 January 2024) constitutes a major part of the p62 protein, was only found in the in-solution digestion).

Concerning the predicted molecular function of the surfaceome, protein kinase C inhibitor activity was prominent. Protein kinase C (PKC) is involved in signalling pathways that specifically phosphorylates substrates at residues of the amino acids serine/threonine. PKCs act as key mediators during immune cell signalling through the immunological synapse, that is, the organization of membrane proteins that occurs at the interface between lymphocytes and antigen-presenting cells during the antigen-presenting/recognition phase (Lim et al., 2015). In the immune system, PKCs are also involved in regulating major signal transduction pathways important for both innate and adaptive immunity, ultimately resulting in the expression of key immune genes, such as the MyD88 dependent and MyD88-independent pathway of innate immune cells (Faisal et al., 2008; McGettrick

et al., 2006) and the NF-κB, and STAT3 of the cells in the adaptive immune system (Kwon et al., 2012; Lim et al., 2015). PKCθ also plays an important role in down-regulation of inflammation by inhibition of STAT4, Tbet (Wachowicz et al., 2014) and the AKT/Foxo1/3a pathway (Ma et al., 2012), thereby affecting the equilibrium of T cell subpopulations important in the immune maturation of infants (Abelius et al., 2011, 2015).

Another top-predicted molecular function was Type 3 metabotropic glutamate receptor binding. Glutamate and its receptors are involved in the regulation of different immune cells' development and function, as suggested by the frequent glutamate receptor expression on immune cells (Ganor & Levite, 2012). The activation of glutamate receptors can, for example, enhance the effectiveness of the effector T cells, or decrease the cytokine production in immunosuppressive myeloid-derived suppressor cells. Because of these immune activating and suppressive effects, glutamate receptors have received ample attention as important players in the regulation of antitumor immune responses as recently reviewed (Koda et al., 2023). Interestingly, given our interest in the potential effects ofhuman milk EVs in infant immune maturation, a role for glutamine and glutamate in neonatal allergy and infection prevention has also been suggested (van Sadelhoff et al., 2020).

Moreover, among the top-three predictions of cellular component and molecular function of the surfaceome we also found "muscle thin filaments" and "MIT domain binding", respectively. According to previous research, we would regard these findings as related to EV biogenesis pathways. Previous research has, for example, shown elective incorporation of actin-associated proteins into osteoclast EVs suggesting a role in their formation (Holliday et al., 2019); the MIT domain binding seems to be an essential part of protein sorting and vesicle formation, involving ESCRT III (Scott et al., 2005). As expected, the cellular components shared between the surface and the cargo proteome were also GO terms related to EV biogenesis such as "extracellular exosome", "extracellular vesicle" and several of the ESCRT proteins; among them ESCRT III.

Although we did not invest space in describing the 'biological processes' (a third option available for GeneOntology predictions, top-three predictions as displayed in Table 3 and fully reported on in Supporting Information S7–8) it may be worth noting, that several of the biological processes predicted on the basis of our LPI proteomics data (*i.e.*, surfaceome) were related to immune function. For example, positive regulation of inflammatory response to antigenic stimulus, cellular response to interleukin-4, positive regulation of T cell-mediated cytotoxicity, myeloid leukocyte mediated immunity, leukocyte mediated cytotoxicity *and* leukocyte migration. Interestingly, a biological process termed 'intestinal absorption' were also found overrepresented in our data, which may be of particular interest with regards to human milk EVs, as they will need to interact with the intestinal wall to exert effects in the infant.

Finally, as briefly mentioned at the beginning of this discussion, there was a significant, although not complete, overlap between the two digestions, which is likely due to the discrimination between peptides that occurs in the data-driven spectrometry analysis; this is something to consider when interpreting the data. We were, for example, surprised not to discover CD63 in our LPI digestion as this is a common EV surface marker; which is also present in our flow cytometry-based MACSplex analysis (Figure 2). To find out what peptides would theoretically be formed by trypsin degradation of the CD63 amino acid sequence, we performed an in silico analysis, refer to S2. Another thing to take into account is that proteins that are glycosylated or phosphorylated may be missed in the mass spectrometry analysis, since the peptides would be modified (post-translational modification), which could also skew the data.

5 | CONCLUSION

This paper contributes with new insights into the proteome of EVs from human milk. For example, we found that the surfaceome gives rise to different functional predictions than do the cargo proteome. The surfaceome tends to be overrepresented in functions and components of relevance for the immune system, and we thus hypothesize that EVs delivered from mother to baby via the milk indeed have implications for infant immune regulation and tolerance development. The cargo proteome, on the other hand, gives rise to predictions such as nucleobase binding, protein folding and vesicular trafficking. Hence, we hypothesize that the vesicular enclosed proteins are mostly remnants of vesicular formation, rather than cargo with specific biological purposes for recipient cells; this remains to be clarified. What also remains to be further studied is what proteins are natively expressed on the EV surface and which are acquired from the external milieu. We would, for example, propose that the EV-associated immunoglobulins likely are an acquired part of the surfaceome, due to the ample amounts found in milk. Moreover, the surface-associated proteome suggests thathuman milk EVs originate from several sources, such as the mammary epithelium and different immune cell subset; the data primarily suggests immune cells of the innate immune system. Further, we would propose that a part of the EV population inhuman milk is excreted by cells with antigen-presenting capabilities; their potential role in antigen presentation, however, remains to be studied.

AUTHOR CONTRIBUTIONS

Emelie Ahlberg: Conceptualization (equal); investigation (equal); methodology (equal); writing—original draft (equal); writing—review and editing (equal). **Maria C Jenmalm**: Funding acquisition (lead); supervision (equal); writing—review

and editing (equal). **Roger Karlsson**: Data curation (equal); investigation (equal); methodology (equal); writing—review and editing (equal). **Anders Karlsson**: Data curation (equal); investigation (equal); methodology (equal); writing—review and editing (equal). **Lina Tingö**: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (equal); supervision (equal); visualization (equal); writing—original draft (lead); writing—review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

Emelie Ahlberg, Maria C. Jenmalm and Lina Tingö report no conflict of interest. Anders Karlsson and Roger Karlsson are affiliated to a company, Nanoxis Consulting AB. The company did not have influence on the study design, data collection, data analysis or interpretation of data, the writing of the paper or the decision to submit for publication.

DATA AVAILABILITY STATEMENT

The raw data will be made available through ProteomeXchange and PRoteomics IDEntifications (PRIDE) Archive database.

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