Comprehensive Proteomic Analysis of Human Milk-derived Extracellular Vesicles Unveils a Novel Functional Proteome Distinct from Other Milk Components*

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Breast milk contains several macromolecular components with distinctive functions, whereby milk fat globules and casein micelles mainly provide nutrition to the newborn, and whey contains molecules that can stimulate the newborn’s developing immune system and gastrointestinal tract. Although extracellular vesicles (EV) have been identified in breast milk, their physiological function and composition has not been addressed in detail. EV are submicron sized vehicles released by cells for intercellular communication via selectively incorporated lipids, nucleic acids, and proteins. Because of the difficulty in separating EV from other milk components, an in-depth analysis of the proteome of human milk-derived EV is lacking. In this study, an extensive LC-MS/MS proteomic analysis was performed of EV that had been purified from breast milk of seven individual donors using a recently established, optimized density-gradient-based EV isolation protocol. A total of 1963 proteins were identified in milk-derived EV, including EV-associated proteins like CD9, Annexin A5, and Flotillin-1, with a remarkable overlap between the different donors. Interestingly, 198 of the identified proteins are not present in the human EV database Vesiclepedia, indicating that milk-derived EV harbor proteins not yet identified in EV of different origin. Similarly, the proteome of milk-derived EV was compared with that of other milk components. For this, data from 38 published milk proteomic studies were combined in order to construct the total milk proteome, which consists of 2698 unique proteins. Remarkably, 633 proteins identified in milk-derived EV have not yet been identified in human milk to date. Interestingly, these novel proteins include proteins involved in regulation of cell growth and controlling inflammatory signaling pathways, suggesting that milk-derived EVs could support the newborn’s developing gastrointestinal tract and immune system. Overall, this study provides an expansion of the whole milk proteome and illustrates that milk-derived EV are macromolecular components with a unique functional proteome. Molecular & Cellular Proteomics 15: 10.1074/mcp.M116.060426, 3412–3423, 2016.

Breast milk is a complex body fluid containing a variety of macromolecular components with distinct functions. Breast milk does not only have a nutritional value for the newborn, it also has various bioactive properties, e.g. nurturing of commensal bacteria, killing of pathogens, promoting intestinal barrier function development, and supporting development and balancing of the newborn’s immune system (1–3). The beneficial effect of breastfeeding is reflected by the observation that breast milk intake results in a reduced risk for developing allergic disorders (2, 4). Although these functional aspects of breast milk have been observed, it is not yet clear which macromolecular components in milk perform these functions and via which molecular pathways they exert these effects.

The composition of milk is generally described as a mixture of macromolecular components which include cells, milk fat globules (MFG)¹, casein micelles, and whey (5–8). Although not mutually exclusive, these major components are often categorized into nutritional components and functional components (9). The primary nutritional components are MFG,
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which are the main source of lipids, and caseins, which provide most of the protein. Functionally bioactive properties are mainly allocated to the whey fraction, which contains carbohydrates, antimicrobial proteins, and immunomodulatory factors (6–8).

Apart from these well-described macromolecular structures, breast milk contains extracellular vesicles (EV) as well (10–13). EV are lipid bilayer enclosed vesicles that are released by cells as mediators of intercellular communication (14). EV released by viable cells are very heterogeneous in composition and size (ranging from \(\sim 50\) nm to \(>1\) \(\mu\)m), with the vast majority \(<200\) nm. EV can originate from either the endosomal route being released from multivesicular bodies as exosomes, or they are released as microvesicles by direct budding from the plasma membrane (15). Although the biogenesis of exosomes and microvesicles is different, once released into the extracellular milieu (as is the case with breast milk) there are no markers to distinguish these subsets of vesicles (16, 17). The molecular composition of EV (functional proteins, nucleic acids and lipids) is regulated by the producing cell and depends on the type and condition of the cell (18). Although for human milk-derived EV some microRNAs and proteins have been identified (12, 19), a detailed characterization of milk-derived EV is lacking. This makes milk-derived EV an understudied macromolecular component of milk.

Proteomic analysis has allowed for the exploration of the protein composition of distinct macromolecular structures. However, in a complex body fluid such as breast milk, which contains a variety of macromolecular components in the presence of a few highly abundant proteins, detection of low abundant proteins is easily obscured. Hence, to unravel the full milk proteome it is essential to define appropriate isolation methods for the individual components followed by high-resolution mass spectrometry. Recently, we developed a protocol to reliably isolate pure EV from other macromolecular structures in milk, allowing in-depth characterization of milk-derived EV (10).

In this study, in-depth proteomic analysis on purified human milk-derived EV from seven individual donors was performed and the first comprehensive proteome for milk-derived EV was established. We identified 198 novel EV-associated proteins not present in the EV database Vesiclepedia. By comparing the milk-derived EV proteome to the total milk proteome, which was manually constructed using previously published proteomics data of various isolated milk components, 633 proteins were identified in the milk-derived EV proteome that have not been identified in milk previously. Based on GO analysis, these 633 newly identified milk proteins have distinct functions from other milk proteins, indicating that milk-derived EV are a separate bioactive component of breast milk.

**EXPERIMENTAL PROCEDURES**

**Breast Milk Collection**—Breast milk was collected as previously described (10). Briefly, fresh, mature milk samples were collected by seven healthy mothers (between 3 and 9 months after delivery) who were not actively terminating breast feeding. Additional donor information can be found in Table I. Milk was prevented from cooling down and transported to the lab in order to start EV isolation within 20 min after collection. Informed consent was signed by all donors and this study was approved by the local ethics committee.

**Isolation of Milk-derived EV and High-density Complexes**—Isolation of milk-derived EV was done as previously described (10). Whole milk was centrifuged at RT for 10 min at 3000 \(\times\) g (Beckman Coulter Allegra X-12R, Fullerton, CA) (Fig. 1A) in which a white pellet and cream layer were formed. The cream layer, containing MFG, was removed and milk supernatant was harvested without disturbing the cell pellet. Supernatant was transferred to new tubes and centrifuged at 3000 \(\times\) g at RT. The supernatant was stored at 80 °C until further processing. Later, frozen supernatant was thawed and transferred immediately to polyyllomer SW40 tubes or SW28 tubes (Beckman Coulter) and centrifuged at 5000 \(\times\) g for 30 min at 4 °C and subsequently at 10,000 \(\times\) g. Next, 6.5 ml aliquots of the 10,000 \(\times\) g supernatant were loaded on top of individual gradients in a SW40 tube. For donor 1, a total of 6.5 ml was used, and for donors 2–7 the total volume was 32.5 ml (Table I). The gradient consisted of 15 successive layered sucrose fractions of 350 µl (ranging from 2.9 µl to 0.4 µl sucrose, with a decrement of 0.114 µl per fraction) on top of 700 µl 2.5 µl sucrose. Ultracentrifugation was done at 192,000 \(\times\) g (in a Beckman Coulter Optima L-90K with a SW40 rotor) for 15–18h. After ultracentrifugation, fractions of 500 µl were harvested from the density gradient starting from the bottom of the SW40 tube and collected in Eppendorf tubes. The density of the collected fraction was measured by refractometry in order to identify the fractions containing milk-derived EV (densities of 1.12–1.18 g/ml sucrose) or high-density complexes (bottom 3 fractions with densities 1.23–1.28 g/ml). After collection of all fractions, the residual pellet was resuspended in 500 µl PBS. Fractions containing EV and fractions containing high-density complexes were pooled (the resuspended pellet was added to the high density complex sample). Pooled fractions were transferred to SW28 tubes and diluted with PBS and samples were pelleted at 100,000 \(\times\) g for 65 min. After centrifugation, supernatant was removed and pellets were stored at –80 °C.

**Protein Extraction**—For protein isolation, pellets were suspended in lysis buffer containing 8 µl urea in 50 µl ammonium bicarbonate, 1 tablet of protease inhibitors Complete mini (Roche ref. 11836170001, Sigma, St. Louis, MO) and 1 tablet of PhosSTOP phosphatase inhibitor mixture (Roche ref. 4908645001). Sample sonication was carried out using a UP100H sonicator (Hielcher Ultrasound Technology, Teltow, Germany) for 6 cycles of 1 s pulse with a 30 s gap in between at 60% amplitude. Clear supernatant was collected after centrifugation of the sample at 20,000 \(\times\) g for 15 min at 4 °C for isolation of soluble proteins.

**SDS-PAGE, In-gel Digestion and Western Blotting**—For high-resolution LC-MS/MS analysis samples were further processed after sonication. For this, 20 µl or 40 µl of each sample was added to 10 µl 200 mM DTT (Sigma ref. 43815) and 10 µl sample buffer (Bio-Rad ref. 161–0791, Hercules, CA) and incubated for 5 min at 95 °C. After a short centrifugation step, the samples were separated on a 12% Tris-HCl gel next to a protein ladder (Thermo ref. 10747012, Waltham, MA). The gels were fixed with 50% ethanol/10% acetic acid, stained with Gelcode Blue Stain (Thermo ref. 24592) and destained with MilliQ water. Tris-HCl gel next to a protein ladder (Thermo ref. 10747012, Waltham, CA) and incubated for 5 min at 95 °C. After a short centrifugation step, the samples were separated on a 12% Tris-HCl gel next to a protein ladder (Thermo ref. 10747012, Waltham, MA). The gels were fixed with 50% ethanol/10% acetic acid, stained with Gelcode Blue Stain (Thermo ref. 24592) and destained with MilliQ water. The gels were fixed with 50% ethanol/10% acetic acid, stained with Gelcode Blue Stain (Thermo ref. 24592) and destained with MilliQ water. The gels were fixed with 50% ethanol/10% acetic acid, stained with Gelcode Blue Stain (Thermo ref. 24592) and destained with MilliQ water. The gels were fixed with 50% ethanol/10% acetic acid, stained with Gelcode Blue Stain (Thermo ref. 24592) and destained with MilliQ water. The gels were fixed with 50% ethanol/10% acetic acid, stained with Gelcode Blue Stain (Thermo ref. 24592) and destained with MilliQ water. The gels were fixed with 50% ethanol/10% acetic acid, stained with Gelcode Blue Stain (Thermo ref. 24592) and destained with MilliQ water. The gels were fixed with 50% ethanol/10% acetic acid, stained with Gelcode Blue Stain (Thermo ref. 24592) and destained with MilliQ water.
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For Western blotting (Fig. 4, Fig. 5E, and Fig. 6B), 1 ml of the pooled fractions containing EV and pooled fractions containing high-density complexes from donors 4 to 7 were taken and transferred to SW60 tubes (Beckman Coulter) and diluted with PBS. Next, samples were pelleted at 100,000 × g for 65 min (in a Beckman Coulter Optima L-90K with a SW60 rotor) and pellets were taken up in reducing buffer (containing 350 mM β-mercaptoethanol) for EHD3 detection, or nonreducing sample buffer for the detection of flotillin-1, CD9, OLAH, PTHLH and MPZL1. Samples were loaded on a 4–20% TGX-Criterion gel (Bio-Rad, Hercules, CA) and separated proteins were transferred to PVDF membranes followed by blocking in PBS containing 0.2% fish skin gelatin and 0.1% Tween-20. Proteins of interest were detected by immunoblotting using mouse anti-human flotillin-1 (clone 18, BD Biosciences, San Jose, CA), mouse anti-human CD9 (H9252, BioLegend, Fell, Germany), rabbit anti-human OLAH (HPA037948, Atlas Antibodies, Stockholm, Sweden), rabbit anti-human PTHLH (PAS-40799, Thermo Fisher, Rockford, IL), mouse anti-human MPZL1 (SAB1406763, Sigma), mouse anti-human EHD3 (ab194512, Abcam, Cambridge, UK). Secondary antibodies used were goat anti-mouse-HRP (Jackson Immuno Research, Suffolk, UK) and goat anti-rabbit-HRP (Dako, Everlee, Belgium). Labeled antibodies were detected using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Landsmeer, Netherlands) and blots were imaged using a ChemiDoc XRS (Bio-Rad).

High-resolution LC-MS/MS Analysis—Samples were individually acquired in two separate experiments (milk-derived EV or non-floating complexes from donor 1 and 2 in the first experiment). Milk-derived EV or non-floating complexes from donor 3 to 7 in the second experiment). Experiment 1 was analyzed by nanoflow LC-MS/MS using a Proxeon EASY-nLC 1000 (Thermo Scientific, Odense, Denmark) with an analytical column heater (40 °C) coupled to an LTQ-Orbitrap Elite. Trapping was performed at a flow rate of 5 μl/min for 10 min and 400 μl/min for 60 min gradient from 0 to 40% mobile phase B (0.1 M acetic acid in water). Next, peptides eluted from the trap column were loaded onto an analytical column (Poroshell 120 EC-C18 (Zorbax, Agilent Technologies); 40 cm length, 50 μm inner diameter, packed in-house (at the Netherlands Proteomics Centre) at 5 μl/min in 100% solvent A (0.1 M acetic acid in water). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (20) partner repository with the data set identifier PXD004423.

Data Processing—MS raw data were processed with Proteome Discoverer (version 1.3, Thermo Scientific). Generated peak lists were searched against the Uniprot database; Homo sapiens (July 2014, 546,121 entries), supplemented with frequently observed contaminants using Mascot software version 2.3 (Matrix Science, UK). Trypsin was chosen as enzyme and two missed cleavages were allowed. Carbamidomethylation (C) was set as a fixed modification and oxidation (M) was set as variable modification. The searches were performed using a peptide mass tolerance of 50 ppm and a product ion tolerance of 0.05 Da (HCD), followed by data filtering using percolator, resulting in 1% false discovery rate (FDR). Only ranked 1 PSMs with Mascot scores >20 were accepted. Keratins were not considered contamination and therefore were not removed from the data set, because mammary epithelial cells (as possible EV producers) express keratins. The data from this study can be found in supplemental File S1 and has been submitted to the Vesiclepedia database, http://www.microvesicles.org/(accession number: Vesiclepedia_574).

Experimental Design and Statistical Rationale—Mass spectrometry was performed on seven biological replicates with milk-derived EV and high-density complexes, whereas Western blot analysis was performed on four biological replicates. Functional enrichment analysis for Gene Ontology (GO) terms and comparison of data sets (Fig. 2, Fig. 3, Fig. 5, and Fig. 6) was done using FunRich (with the human FunRich database as background; as recent as November 2015) (21). Comparison between this study and EV database Vesiclepedia was done by importing the online protein data from the Vesiclepedia database (as recent as November 2015) into FunRich. This data set included all proteins listed in Vesiclepedia (from all tissues and cell types; all cell lines; all isolation methods; all detection methods; all vesicle types). GO analysis of annotated proteins was performed for cellular component, molecular function, biological processes and site of expression. Enriched terms were ranked by p value (Hypergeometric test) by FunRich. GraphPad Prism software (La Jolla, CA) was used to plot the graphs when comparing data sets. In order to show the highest diversity of enriched GO terms, duplicate or highly similar terms were removed, selecting those with the highest statistical power or with the highest number of genes in the background data set. For cellular component in Fig. 2B and Fig. 6C, GO terms for 11 major cellular components were selected in order to limit results (nucleus, ribosome, vesicle (named exosomes in FunRich), endoplasmic reticulum (ER), Golgi apparatus, cytoskeleton, mitochondrion, cytoplasm, lysosome, centrosome, and plasma membrane) and supplemented with extracellular to include secreted proteins. For site of expression analysis, primary cells reported to reside in the mammary gland or cells known to be present in the circulation were selected. Cell lines, other tissues than breast tissue, or fluids were excluded from the analysis. In comparisons for molecular function and biological process between milk proteins or milk-derived EV proteins in Fig. 6C, enriched GO terms that were significant (GO terms with a p value <0.05 corrected for multiple comparisons by hyper geometric test) in at least one data set were selected and compared with the other data set. The number of genes in the background data set was at least 15 in order to remove underrepresented GO terms. For statistical comparison of data sets within a single GO-term (Fig. 2B and Fig. 6C), the number of proteins linking to the GO term were tested for significant difference in distribution with chi-square test
using GraphPad prism software. Data sets with a \( p \) value \( \leq 0.05 \) were regarded as significant.

Comparison of identified proteins between all seven individual donors (Table III) was done using the Venn diagrams web tool from http://bioinformatics.psb.ugent.be/webtools/Venn/.

Construction of the Human Milk Proteome—Previously published raw data was obtained from eight studies, with six primary studies and two review studies that contained a total of 38 individual studies. Supplemental File S2 contains the raw data from these eight studies and supplemental Table S1 contains an overview of the individual 38 studies. Data sets from Beck et al. (22) and D’Alessandro et al. (23), which summarized previous published work, were used instead of each individual study reviewed. Data sets were converted to the common gene name format in order to construct one data set that could be compared with our data set. Genes listed under multiple (alternative) names were condensed to the common gene name as listed in the UniProt database. Proteins without a common gene name (“N/A”), or entries no longer existing in the UniProt database, or listed under cDNA entries were excluded from the milk proteome data set. The common gene names from all studies were imported into FunRich as a data set and FunRich was used to compare this data set in a Venn diagram.

RESULTS

Isolation of Milk-derived EV and Workflow of the Proteomics Approach—Isolation of milk-derived EV from breast milk of seven donors was performed using the protocol by Zonneveld et al. (10) (Fig. 1A). First, fat (containing MFG), cells and cellular debris were removed by differential centrifugation resulting in 10,000 g milk supernatant. The milk supernatant was loaded on top of a sucrose gradient and ultracentrifuged to allow for the separation and concentration of EV. This top-down density gradient ultracentrifugation was used because pelleting EV from milk supernatant via 100,000 \( \times \) g centrifugation results in an insoluble pellet (10). After ultracentrifugation, individual fractions were collected. To prepare the milk-derived EV sample, EV enriched fractions with densities 1.12 to 1.18 g/ml were pooled. Additionally, high-density fractions (density 1.23 to 1.28 g/ml), enriched in nonmembrane-associated protein complexes, were pooled and used as a control.

Next, the isolated milk-derived EV and high-density complexes from the individual donors were further processed (Fig. 1B). Although differences in total protein amount between donors were observed, SDS-PAGE analysis showed large similarities between different donors in the protein patterns obtained for both milk-derived EV and high-density complexes. Milk-derived EV had a more complex banding pattern than high-density complexes, which exhibited a few high abundant proteins (Fig. 1B). For each individual donor, protein bands were excised for in gel digestion and these samples were subjected to LC-MS/MS analysis.

Identification of the Milk-derived EV Proteome—In-depth proteomic analysis was performed to determine the proteome...
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Information regarding age, stage of lactation (months after delivery) and number of pregnancies to a viable gestational age (parity) of the individual donors. Additionally, volumes of milk collected and amount of 10,000 g milk supernatant that was used for further isolation of milk-derived EV and non-floating complexes is given, as described in the experimental procedures.

| Donor | Age donor | Months after delivery | Parity | Amount of milk (ml) | Amount of 10,000 g sup (ml) |
|-------|-----------|-----------------------|--------|---------------------|-----------------------------|
| 1     | 35        | 6                     | 3      | 10                  | 6.5                         |
| 2     | 35        | 4                     | 1      | 35                  | 32.5                        |
| 3     | 34        | 4                     | 1      | 30                  | 32.5                        |
| 4     | 32        | 5                     | 1      | 33                  | 32.5                        |
| 5     | 32        | 5                     | 1      | 30                  | 32.5                        |
| 6     | 39        | 9                     | 3      | 30                  | 32.5                        |
| 7     | 34        | 3                     | 2      | 30                  | 32.5                        |

The number of identified proteins in the milk-derived EV samples and the high-density complexes samples per individual donor

| Donor | Number of identified proteins milk-derived EV | Number of identified proteins high-density complexes |
|-------|-----------------------------------------------|-----------------------------------------------|
| Donor 1 | 857                                           | 495                                           |
| Donor 2 | 1615                                          | 358                                           |
| Donor 3 | 817                                           | 194                                           |
| Donor 4 | 650                                           | 157                                           |
| Donor 5 | 783                                           | 260                                           |
| Donor 6 | 814                                           | 236                                           |
| Donor 7 | 964                                           | 304                                           |

of milk-derived EV and high-density complexes. The total number of proteins identified was higher for milk-derived EV than high-density complexes. For milk-derived EV, proteins numbered between 650 and 1615, whereas for high-density complexes this ranged from 194 to 495 individual proteins (Table II). Remarkably, in the milk-derived EV from donor 2 there were more proteins identified, although this donor was similar to the other donors (Table I and high-density complexes in Table II). In total, 1963 unique proteins were identified in milk-derived EV in all samples, whereas 739 unique proteins were determined in the high-density complexes (Fig. 2A). To get more insight into the subcellular origin of the identified proteins, functional enrichment analysis for the cellular component was done. These analyses showed that in both milk-derived EV and high-density complexes a high percentage of proteins linked to GO terms like “exosomes” (which are a subclass of extracellular vesicles), “lysosome,” and “cytoplasm” (Fig. 2B). We next determined the influence of the overlapping proteins for the enrichment analysis (Fig 2A: 606 proteins). Within the samples, we compared nonoverlapping proteins (1357 in milk-derived EV and 133 for high-density complexes) to the proteins identified in the entire sample (1963 for milk-derived EV and 739 for high-density complexes). When the overlapping proteins were excluded for the GO analysis, then especially exosomes were significantly lower for milk-derived EV (Fig. 2B, left figure). For high-density complexes especially GO-terms “exosomes,” “lysosomes,” “cytoplasm” and “plasma membrane” were significantly less enriched when the overlapping proteins were excluded for analysis. This suggests that the overlapping proteins most likely are derived from some high-density EV present in the high-density complexes fractions, whereas the 133 proteins unique in the high-density complexes are most likely not EV-associated proteins. Therefore, we selected all proteins identified in the milk-derived EV sample (1963) for further analysis.

Identification of Common Milk-derived EV Proteins—Because milk composition is known to vary among individuals we determined the variation of identified proteins in milk-derived EV between individual donors. This revealed that a total of 367 proteins were shared among all seven donors, which is 19% of the total of 1963 unique proteins in the milk-derived EV proteome (Table III). Although variation exists whereby some proteins are uniquely identified in specific donors, a substantial number of proteins is shared between the different milk-derived EV samples, indicating that there is a common milk-derived EV proteome. Importantly, from the proteins shared among all donors, several are considered as EV-associated proteins and are used as markers for EV (as reviewed by Lötvall et al. (17)). These EV-associated proteins can be categorized according to their subcellular location (Table IV). For instance, transmembrane or lipid-bound extra-
cellular proteins (e.g. tetraspanins CD9, CD63, and CD81), and cytosolic proteins (e.g. annexins and Ras-related proteins) were identified in all milk-derived EV samples, whereas some intracellular proteins (e.g. histone) that are not expected to be enriched in EV were indeed lacking. In contrast to the milk-derived EV proteome, EV associated proteins are under-represented or lacking in the proteome of the high-density complexes (Table IV). These data were confirmed by Western blotting (Fig. 4), which showed the presence of Flotillin-1 in milk-derived EV while being absent in high-density complexes. CD9 was more abundantly present in milk-derived EV than in high-density complexes.

**Table III**

The number of identified proteins shared among donors. The milk-derived EV proteomes from all seven individual donors were compared in an overlay. From these data the number of shared proteins between donors was calculated. Percentage of total relates to the total of 1963 unique proteins identified in all seven milk-derived EV samples.

| Number of shared proteins | Percentage of total |
|---------------------------|--------------------|
| Identified in all 7 donors | 367                | 19                 |
| Identified in at least 6 donors | 527          | 27                 |
| Identified in at least 5 donors | 653          | 33                 |
| Identified in at least 4 donors | 789          | 40                 |
| Identified in at least 3 donors | 954          | 49                 |
| Identified in at least 2 donors | 1245         | 63                 |
Milk-derived EV Contain Proteins Not Listed in the Vesiclepedia Database—Apart from common EV proteins (Table IV), EV contain subset-specific proteins because of different cells of origin and biogenesis pathways. To identify any known EV proteins in milk-derived EV, we compared the 1963 proteins identified in the milk-derived EV with previously published EV studies included in the (human) Vesiclepedia database (25). Vesiclepedia contains 8450 unique proteins identified in EV from an extensive number of studies (currently 188 individual human studies) that included the characterization of EV from a wide variety of cellular sources (e.g., bodily fluids, primary cells, and cell lines) and vesicle types.

When comparing the overlap we found that 1765 proteins were already reported in Vesiclepedia (which is 90% of the 1963 proteins in the milk-derived EV proteome), however 198 proteins have been identified in this study that have not been listed in Vesiclepedia (Fig. 5A). With the results from this analysis, we can append the current “total” EV proteome to 8648 unique proteins.

Among the proteins that have not previously been reported in other EV were Oleoyl-ACP Hydrolase (OLAH) and Parathyroid Hormone-related Protein (PTHHLH). In our proteomic analysis OLAH was detected with a coverage of 27.9% with five unique peptides, whereas PTHHLH was detected with a coverage of 40.7% with nine unique peptides (supplemental File S1). Using Western blot analysis, we verified the presence of both proteins on all donors tested (Fig. 5B). Interestingly, various isoforms of PTHHLH have been identified in human milk (26), which were detected with varying intensities between donors.

Milk-derived EV Contain Proteins Previously Not Identified in Milk Proteomic Studies—Breast milk is a complex body fluid that contains a variety of macromolecular structures that harbor proteins. The most frequently studied milk components abundant in proteins are MFG, caseins, and whey (6, 7, 27). Apart from whole milk and skim milk, the proteomes of milk fat globule membrane (MFGM), caseins and whey have been extensively characterized. Based on these data, we
constructed the human milk proteome by combining the data from eight previously published studies (22, 23, 28–33), which represent a total of 38 individual studies on whole milk, skim milk, caseins, whey, and MFGM (supplemental Table S1). This “total” milk proteome has a sum of 2698 unique proteins (supplemental File S2).

Next, we investigated whether the proteins identified in milk-derived EV have been identified in previous milk studies (because whole milk and skim milk will contain EV), or whether the differential centrifugation of milk followed by density gradient-based isolation of EV improved the detection of proteins that would otherwise be masked by highly abundant milk proteins. When comparing the milk-derived EV proteome from this study to the total milk proteome, 1330 proteins were shared (which is 49% of the total milk proteome of 2698 proteins) between milk-derived EV and the composed milk proteome (Fig. 6A). The 1368 non-EV milk proteins were significantly enriched for cellular component terms like “ER,” “centrosome,” “ribosome,” and “extracellular.” Subsequently, we compared the molecular function of proteins and found that complement, cytokine, and chemokine activity, and “translation regulator activity” was significantly enriched in the non-EV milk proteome. Interestingly, proteins exclusive to milk-derived EV are significantly enriched in other molecular functions e.g. cytoskeletal/structural activity, “transmembrane receptor protein tyrosine kinase activity,” and “cell adhesion.” Finally, the annotated biological processes of proteins was assessed. Although significantly enriched non-EV milk proteins were involved in “immune response” and “protein metabolism,” the milk-derived EV proteins were enriched for biological processes related to, “signal transduction” and “cell growth and/or maintenance.” Interestingly, the protein EHD3, which was verified with Western blotting (Fig. 6B), was annotated under signal transduction and “cytoskeletal protein binding.” Together, the GO analysis indicates that proteins from milk-derived EV come from different cellular compartments and have other molecular functions and are involved in distinct biological processes compared with non-EV milk proteins.

DISCUSSION

To unveil the proteome of milk-derived EV, we performed a comprehensive proteomic analysis of purified EV isolated from breast milk from seven different donors. For EV isolation we used our recently established protocol optimized to purify EV from human milk (10). Proper sample collection and storage are critical for accurate analysis of EV that are naturally present in body fluids (34). For breast milk, removal of cells and MFG by differential centrifugation at low speed before long term storage is crucial (10). Next, isolation of EV from milk supernatant should involve density gradient-based ultracentrifugation for further EV enrichment. An alternative approach often used for EV isolation is the direct pelleting of EV via ultracentrifugation (with at least 100,000 × g), however this approach yields less pure EV (35) and leads to an insol-
Milk-derived EV are a Distinct Bioactive Component of Milk

A

Milk-derived EV (this study) 198 1765 6685 Vesiclepedia

B

![Comparison of the milk-derived EV proteome from this study to the EV database Vesiclepedia.](image)

A, All protein entries from Vesiclepedia were downloaded and imported into FunRich for overlay analysis with the milk-derived EV proteome from this study, which is shown in the Venn diagram. B, Western blot analysis was performed for OLAH (~30 kDa) and PTHLH (~20 kDa and ~24 kDa) on milk-derived EV from donors 4, 5, 6, and 7.

Milk composition of an individual changes during lactation (7, 36–38). This is influenced by a variety of factors including diet (39) and age of the mother (40). We did observe that the milk-derived EV proteome from donor 2 was considerably larger than from the other donors (1615 proteins, whereas the average of the other donors was 814 proteins), whereas the high-density complex proteome was similar. We therefore performed additional GO analyses excluding donor 2 and found that this gave similar results and conclusions for all data presented in this report (data not shown). Using the current approach it remains to be determined whether the total number of EV changes, or that the composition of milk-derived EV is altered during lactation.

Using the EV database Vesiclepedia (25), which included 8450 unique proteins identified in EV from a broad range of different origins, we were able to determine a large overlap of 1765 proteins between this milk-EV study and previously documented EV-proteins in Vesiclepedia, illustrating a common protein profile of EV regardless of the cell type that produces them, or bodily fluid from which EV are isolated. Additionally, this study reveals 198 proteins identified in milk-derived EV that have not been identified in other EV studies before. The discovery of these EV proteins could be because milk-derived EV are underrepresented in this database, because only few studies have characterized human milk-derived EV (12, 13, 41), contributing only 81 proteins to Vesiclepedia. Indeed, some of the proteins we identified in milk-derived EV that are not listed in Vesiclepedia, OLAH, and PTHLH, have been described in milk proteomics studies on whole milk or skim milk (22, 42–44). These proteins are thus specific to EV derived from milk (and have not been identified in other EV preparations). Moreover, OLAH could potentially be used to differentiate between milk-derived EV and MFG, as some studies performed on MFG have reported the presence of PTHLH, whereas OLAH has never been detected in this milk component (31–33, 37). Both OLAH and PTHLH have been detected in whey (36, 45), which contains EV but not MFG, further supporting the idea that milk-derived EV express both OLAH and PTHLH. Most likely, OLAH and PTHLH will be derived from the EV subset produced by cells in the mammary gland, because these proteins were exclusively mapped to "breast" and "mammary gland" respectively in the GO anal-
Fig. 6. **Comparison of the milk-derived EV proteome to the milk proteome.** A. Comparison of the proteins identified in milk-derived EV from all 7 donors in the current study with the milk proteome constructed from previously published milk studies. Thirty-eight published (or reviewed) proteomes described in Beck (22), D’Alessandro (23), Dallas (28), Guerrero (29), Hettinga (30), Spertino (31), Yang (32) and Lu (33) were combined such that each protein is represented by its common gene name. The milk proteome was compared with the milk-derived EV proteome from this study and is depicted in the Venn diagram. B, MPZL1 (~29 kDa) and EHD3 (~61 kDa) were detected on milk-derived EV from donors 4, 5, 6, and 7 using Western blot analysis. C, Functional annotations of proteins uniquely identified in milk-derived EV compared with non-EV milk proteins. The 633 proteins exclusively identified in milk-derived EV (shown as milk-derived EV in green) were compared with the 1368 proteins from the milk proteome that were not shared with milk-derived EV (shown as milk proteome in blue). Enriched terms for cellular compartment, molecular function and biological process term were ranked by p value (Hypergeometric test) for either data set. Those GO terms that were not significantly enriched are indicated with “ns.” Within individual GO-terms, annotated proteins were compared for distribution via chi-square test. Those data sets that were significantly differently distributed are indicated with “*.” For both the Hypergeometric test and the chi-square test, a p value <0.05 is regarded as significant.
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ysis for site of expression in this study. However, besides EV produced by cells in the mammary gland we found by GO analysis that also immune cells might be the origin of milk-derived EV. Taken together the cellular origin of milk-derived EV is diverse and it remains to be determined what the contribution of individual cell types is to the number and composition of milk-derived EV.

Although the analysis of breast milk composition has received considerable attention, milk-derived EV have not yet been recognized as a major component of milk. In order to compare and assess the relative contribution of the milk-derived EV proteome to proteins previously identified in other milk components, we combined the data from previous milk studies (including analysis of whole milk, skim milk, casein, whey and MFG) to construct the proteome of total human milk. Overlap analysis revealed that 1330 proteins that were identified in our study have also been identified in other milk proteome studies and reflect 49% of the total milk proteome. Because the total milk proteome contains proteins identified in whole milk and skim milk, which both contain milk-derived EV, indeed a considerable overlap was expected. Why in previous milk proteomics studies the milk-derived EV proteome was not completely unrevealed could be caused by the relative low number of EV in the whole milk analyzed. This is in contrast to our study, where EV were purified and concentrated. Additionally, milk-derived EV contain proteins also present in other milk components. For instance, CD9 and MUC-1 have also been identified in MFG (37). Because the isolation procedure of MFG is considerably different from milk-derived EV the most likely explanation for this finding is that the EV-proteins are not derived from contaminating EV in milk-derived EV. Importantly, this study demonstrates that milk contains much more different proteins than previously known and that a substantial number of these proteins is EV-associated. Remarkably, whereas the proteins of the total milk proteome that are not shared with milk-derived EV are primarily linked to nutritional pathways (reflecting the nutritional characteristics of milk) and immune function based on complement activity and cytokine/chemokine function, the unique milk-derived EV proteins are linked to other bioactive biological pathways like cell signaling and cell growth and/or maintenance.

In conclusion, by using an optimized EV isolation procedure together with in-depth proteomic analysis we unraveled the milk-derived EV proteome in detail and identified novel bioactive proteins associated with milk-derived EV. This indicates that besides commonly described milk components, milk-derived EV need to be recognized as yet another important bioactive macromolecular component in breast milk, which might be involved in transmitting signals from the mother to the newborn.

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