Identification of protein markers for extracellular vesicle (EV) subsets in cow’s milk

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ABSTRACT

Extracellular vesicles (EVs), like exosomes, are small membrane vesicles involved in cell-to-cell communications that modulate numerous biological processes. We previously discovered a new EV subset in milk (sedimenting at 35,000 g; 35K) that protected its cargo (RNAs and proteins) during simulated digestion and was more enriched in microRNAs than exosomes (sedimenting at 100K).

Here, we used LC-MS/MS to push further the comparison between these two pellets. Commonly used EV markers were not differentially enriched between the pellets, questioning their use with cow’s milk EVs. Similarly, the majority of the quantified proteins were equally enriched between the two pellets. Nevertheless, 20 proteins were specific to 35K, while 41 were specifically enriched in 100K (p<0.05), suggesting their potential use as specific markers. Loaded with these proteins, the EVs in these pellets might regulate translation, proliferation and cell survival for 35K, and metabolism, extracellular matrix turnover and immunity for 100K. This approach also brought new insights into milk EV-associated integrins and their possible role in specifically targeting recipient cell types. These findings may help better discriminate between milk EVs, improve our understanding of milk EV-associated protein function and their possible use as therapeutic tools for the management of immunity- and metabolism-associated disorders.

Keywords • Extracellular vesicles / protein markers / tetraspanins / mass-spectrometry / exosomes / cow milk
SIGNIFICANCE

This manuscript is in line with the view of the International Society for Extracellular Vesicles (ISEV) on the importance of characterizing the different EV subsets present in a given biological fluid [1]. Characterization of milk EVs by mass spectrometry unveiled new specific markers for different EV subsets, which may help (i) ensure reproducibility in EV research, (ii) promote their use for milk EV selection, (iii) predict their possible effects and functions in recipient cells, and (iv) envision their use as specific (e.g., drug) delivery vehicle to target specific cell types/organs.
1. INTRODUCTION

Extracellular vesicles (EVs) are small lipid membrane vesicles released by numerous cell types [1]. These EVs are among the factors exchanged by cells to ensure communication and homeostasis [2]. EV populations are heterogeneous, but two major subsets capture most of the interest. The first and most studied one comprise the exosomes, small vesicles of ~100 nm in diameter, generated within the multivesicular bodies (MVB) by the escort complex (ESCRT) and associated proteins, and released in the extracellular milieu upon the fusion of MVB with the cell membrane [3]. The second one is composed of several larger EVs, sometimes termed ectosomes or membrane vesicles (MVs), that are directly generated from budding of the cell membrane [3]. Other EV subsets have been described, including apoptotic bodies, high density lipoproteins [4], and milk fat globules [5].

EVs’ functions range from regulation of cell proliferation [6], modulation of inflammation, receptor mediated signaling [7] to regulation of metabolism-associated pathways [8].

In milk, EVs are relatively heterogeneous [9], with functional implications upon internalization or ingestion [10-12], including regulation of T-lymphocyte maturation [13], modulation of macrophage activity [12] and disease management in mouse models of rheumatoid arthritis [11].

In our previous work, we found that processed dairy cow’s milk EVs and associated microRNAs resisted degradation in an in vitro system that mimics human digestion [14]. We also described the existence of a new subset of milk EVs that pellet at 12,000 g (12K) and 35,000 g (35K), which contained the bulk of microRNAs present in milk [9].

Overall, the EVs present in the 12K and 35K pellets were comparable and likely a population of diverse small EVs, whereas the 100,000 g (100K) pellet is trusted to contain mostly milk exosomes [15]. Previous non-quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) and associated Western Blots suggested that some proteins are specifically enriched, either in the 12K/35K pellets (e.g. Xanthine dehydrogenase, XDH) or in the 100K (e.g. Tumor susceptibility gene 101, TSG101) pellet [9].
EV subtype classification is a complicated matter, and there is no consensus about the terminology for naming EVs [16]. The most frequently used term that refers to milk EVs is “exosomes”, which derives specifically from MVB [17]. This denomination is based on (i) the current practices in milk EV studies, which are mostly modeled on other biological fluids [18], (ii) isolation methodology [19], and/or (iii) the presence of some protein markers usually enriched in exosomes [20], although not being specific to this EV subset [21]. In the context of functional studies, this has led to the exclusion of many other EV subpopulations found in bovine milk [10, 11, 22-25]; some of those are highly enriched in bioactive compounds, like microRNAs [9], and resist digestion in vitro [14]. Therefore, there are very few resources available providing markers to compare EV subsets in milk and to ensure purity of milk exosomes in isolation procedures or to define other EV subsets present in milk. In this study, we aimed to discriminate milk EV subtypes based on their content, rather than on their possible cellular origin. This discrimination is of high importance when studying the biological activity of EVs, because it is only by ensuring reproducible isolation procedures and relative purity of EVs that one can associate a function to a specific EV subset [1, 15, 19, 26]. It is also of importance to determine which integrins are present on the surface of these EVs, since these might lead to the accumulation of specific EV subsets in specific cell types/organs; milk EVs may thus be used as a vehicle to develop strategies based on their potential ability to deliver therapeutics to specific diseased cell types/organs [27-29].

Here, we used quantitative LC-MS/MS to push our investigations further and provide a full comparison of the EV-associated proteins found in the 35K and 100K pellets of dairy milk, with the aim to define their content and comparative enrichment in specific proteins, including EVs markers and integrins. This work may help (i) identify protein markers specific to each EVs subsets, (ii) define their nature, cellular origin and possible cell type/organ targets, (iii) provide insights into the function of the proteins they contain and the pathways they might impact upon cellular internalization, and (iv) guide researchers interested in functional studies of milk EVs and their potential for disease management.
2. MATERIALS AND METHODS

2.1 Dairy milk samples

For all experiments, we used commercially available, filtered, skimmed dairy milk (Lactantia PurFiltre brand; product: http://www.lactantia.ca/food_product/lactantia-purfiltre-skim-milk/) bought at a local grocery store in Quebec City, QC in biological triplicates (three milk tetra packs with different expiry dates).

2.2 Sedimentation of dairy milk extracellular vesicles (EVs) by differential ultracentrifugation

Milk EV pellets were obtained by following a previously described protocol [9, 30], with slight modifications. One hundred (100) mL of dairy milk samples were mixed with 1 volume of 2% sodium citrate (in MilliQ water) that had been filtered with 0.22 µm membrane microfilters (Corning). The samples were subjected to successive differential ultracentrifugation steps at 35,000 g (35K) for 2 h, then 70,000 g and 100,000 g (100K) for 1 h each at 4°C in a Sorvall® WX TL-100 ultracentrifuge, equipped with a T-1250 rotor (ThermoFisher). After each step, the pellets were suspended in 1 mL of 0.22 µm filtered sterile phosphate buffered saline (PBS) containing 100 nM ethylenediaminetetraacetic acid (EDTA) pH 7.4 and processed for subsequent LC-MS/MS analysis of the 35K and 100K pellets.

2.3 Trizol LS protein isolation and resuspension

Proteins from the 35K and 100K pellets were precipitated with Trizol-LS by following the manufacturer’s protocol, with slight modifications. Briefly, interphase and organic phase obtained after adding chloroform to the pellets homogenized with Trizol-LS were mixed with 2 volumes of isopropanol and centrifuged at 13,000 g. Pellets were rinsed thrice with absolute ethanol before being solubilized in 25 µl of 50 mM ammonium bicarbonate containing 1% sodium deoxycholate, and heated at 95°C for 5 min. Protein concentration of each sample was determined by colorimetric Bradford assay (Thermo-Fisher Scientific).
2.4 Sample preparation for liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Sample preparation and LC-MS/MS analyses were performed by the Proteomics Platform (Genomics Center, CHU de Québec Research Center/CHUL Pavilion). Protein samples (10 µg) in 50 mM ABC / 1% DOC were reduced with DTT (0.2 mM) at 37°C for 30 min, and cysteins were alkylated by iodoacetamide (0.8 mM) at 37°C for 30 min. Trypsin (0.2 µg) was added and the samples were incubated overnight at 37°C. Following the digestion, an acidification of the samples was performed with 2% ACN / 1% TFA / 0.5% acetic acid in order to stop the trypsin reaction and precipitated the deoxycholate. After centrifugation at 16000g for 5 min, peptides contained in the supernatants were purified on stage tip C18 reversed phase (3M Empore C18 extraction disks), and vacuum dried. Prior to MS injection, the peptides were solubilized at 0.2 µg/µL in 2% acetonitrile / 0.05% TFA.

2.5 LC-MS/MS

Samples were analysed by nanoLC-MS/MS. For each injection, 1 µg of peptide digest were separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analysed by electrospray mass spectrometry (ESI MS/MS). The experiments were performed with a Dionex UltiMate 3000 nanoRSLC chromatography system (Thermo Fisher Scientific / Dionex Softron GmbH, Germering, Germany) connected to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) driving with Orbitrap Fusion Tune Application 2.0 and equipped with a nanoelectrospray ion source. Peptides were trapped at 20 µL/min in loading solvent (2% acetonitrile, 0.05% TFA) on a 5 mm x 300 µm C18 PepMap cartridge pre-column (Thermo Fisher Scientific / Dionex Softron GmbH, Germering, Germany) during 5 minutes. Then, the pre-column was switch online with a 50 cm length, 75 µm ID Acclaim PepMap 100 C18 analytical column (Thermo Fisher Scientific / Dionex Softron GmbH, Germering, Germany) and the peptides were eluted with a linear gradient from 5-40% solvent B (A: 0.1% formic acid, B: 80% acetonitrile,
0.1% formic acid) in 90 minutes, at 300 nL/min. Mass spectra were acquired using a data dependent acquisition mode using Thermo XCalibur software version 3.0.63. Full scan mass spectra (350 to 1800 m/z) were acquired in the orbitrap using an AGC target of 4e5, a maximum injection time of 50 ms and a resolution of 120 000. Internal calibration using lock mass on the m/z 445.12003 siloxane ion was used. Each MS scan was followed by acquisition of fragmentation MSMS spectra of the most intense ions for a total cycle time of 3 seconds (top speed mode). The selected ions were isolated using the quadrupole analyser in a window of 1.6 m/z and fragmented by Higher energy Collision-induced Dissociation (HCD) with 35% of collision energy. The resulting fragments were detected by the linear ion trap in rapid scan rate with an AGC target of 1e4 and a maximum injection time of 50 ms. Dynamic exclusion of previously fragmented peptides was set for a period of 20 sec and a tolerance of 10 ppm.

2.6 Database searching and Label Free Quantification

Spectra were searched against a Bos taurus proteins database (Uniprot BosTaurus TaxID 9913) using the Andromeda module of MaxQuant [31] software v. 1.5.5.1. Trypsin/P enzyme parameter was selected with two possible missed cleavages. Carbamidomethylation of cysteins was set as fixed modifications, and methionine oxidation and acetylation of protein N terminus as variable modifications. Mass search tolerances were set to 7 ppm and 0.6 Da for MS and MS/MS respectively. For protein validation, a maximum False Discovery Rate (FDR) of 1% at peptide and protein level was used based on a target/decoy search. MaxQuant was also used for Label Free Quantification (LFQ). The 'match between runs' option was used with a 20 min value as an alignment time window and 5 min as a match time window. Only unique and 'razor' peptides were used for quantification. The LFQ intensity values (non-normalized values from the ProteinGroups file) extracted by MaxQuant for each protein in each sample replicate were normalized with the median value and were used to calculate the ratio between the two samples. When LFQ intensity values were missing, they were replaced by a noise value corresponding to the first percentile of...
LFQ values of all proteins of the sample replicate. A protein was considered to be quantifiable only if at least two of the three replicate values were present in one of the compared samples.

2.7 Statistical analysis

All statistical analysis were performed using R. Experiments were conducted in biological triplicates (n=3) for the 35K and 100K pellets. A protein was considered to be variant if its z-score is higher than 1.96 and had a Benjamini-Hochberg-corrected Welch p-value below 0.05.

2.8 Cellular component analysis

For cellular component analysis, we used the Gene-Ontology (GO) PANTHER Overrepresentation Test (Released 20171205, PANTHER version 13.1 Released 2018-02-03) [32] and Bos taurus (all genes in database) as a reference list. More specifically, we used Panther GO-slim cellular component analysis with Fisher's Exact multiple test correction with FDR, and selecting only the results with FDR < 0.05.

2.9 Pathway analysis

Biological pathways potentially involving milk pellets' proteins were determined using Reactome database (release 63) with Pathways Analysis Tool (v3.5) [33], converting all non-human identifiers to their human equivalents in order to get a hint at the possible impact of milk pellets’ proteins on recipient human cells.

2.10 Figures and illustrations

Figures displayed in this manuscript were generated using R (Free Software Fondation) along with the ggplot2(volcano) and gplot(heatmap) packages, Inkscape software (Free Software Fondation) and Prism 7 (GraphPad Software, Inc.).
3. RESULTS

Milk 35K and 100K pellets have similar protein content, but specific protein markers

We subjected our cow milk samples to differential ultracentrifugation (Fig. 1A), except that we skipped the 12,000 g centrifugation, since the 12K and 35K pellets contain closely related EV subsets [9]. The 70K pellet was excluded from this analysis, as it contains a mixture of the two EV subsets previously reported [9].

We thus isolated all the proteins from the 35K and 100K pellets (n=3), and used quantitative LC-MS/MS to determine their protein content and enrichment profiles (Fig. 1A). We identified and quantitated a total of 1,974 different proteins with a FDR below 1% at the peptide and protein level across all samples (Supplementary file S1). The complete list of the common and specific proteins, with their related p-values, Z-scores and fold enrichment, are available in Supplementary File S1.

Some proteins were found in all samples, while others were identified only in some of the replicates or only in one of the pellets (Fig. 1B). There were 1,974 different identified proteins in these samples and 1899 quantified proteins Supplementary File S1.

There were 1,838 proteins common to both pellets, 20 proteins specific to the 35K pellet and 41 proteins specific to the 100K pellet (Fig. 1B). The 35K and 100K pellets contained several proteins with a z-score above threshold (1.96) and an adjusted p-value below 0.05, making these proteins possible specific markers (Fig. 2A).

Cellular localization analysis, through the Gene Ontology Panther tools, suggested that the proteins the two pellets share in common were part of the Golgi apparatus, lysosomes, vesicular coating, vacuoles, plasmic membrane, cytoplasm, endoplasmic reticulum or mitochondria, thereby supporting the enrichment of extracellular vesicles in these pellets (Fig. 1B). Specific cellular origin could be defined for 35K proteins, with the most significant ones involving actin cytoskeleton, peroxisome and plastid, which support the presence of membrane-derived extracellular vesicles in this pellet (Fig. 1B). Most of the proteins specific to the 100K pellet originate from endosomal
compartment, vacuole and extracellular region, which is in accordance with exosome enrichment in this fraction (Fig. 1B).

The three most abundant proteins across all samples were Beta-lactoglobulin (LGB), bovine progestagen-associated endometrial protein analog (PAEP) and Alpha-S1-casein (CSN1S1). These proteins were equally enriched in all the samples (Fig. 1C). Most of the top 15 proteins include common milk contaminant proteins (e.g. caseins, lactoglobulins) and half of them were more enriched in the 100K compared to the 35K pellet (Fig. 1C).

Altogether, these results suggest that both 35K and 100K pellets have a very similar protein content, with the most enriched proteins found more enriched in 100K pellet. Cellular origin analysis suggests the presence of multiple EV subsets in these pellets, with exosomes dominating the 100K pellet and cytoplasmic membrane-derived vesicles the 35K pellet.

Can usual EV markers be used to classify commercial dairy cow milk EVs?

We assessed whether widely accepted EV protein markers [21, 34] could be used to discriminate the nature and subsets of the EVs isolated from commercial cow milk. Two previous studies, performed on milk or another biological fluid (e.g. cell culture supernatant), suggested several protein markers for different EV subsets, based either on isolation procedures and physical properties of the EVs (light, dense, large or multiple EVs) [21], or on cellular origin of the proteins associated with those EVs – proteins from the endosomal sorting complexes required for transport (ESCRT) and proteins from the Rab family strongly associated to MVB-derived EVs (i.e. exosomes) [34]. The concomitant presence of the three tetraspanins CD9, CD63 and CD81 was also suggested to be primordial to define an EV subset as exosomes [21]. Samuel et al. [34] proposed that the presence of integrins is an important marker for EV internalization and biological activity, and those could be used to predict possible cell targets. We thus looked for the relative enrichment of each of these markers and proteins of interest between the two pellets (Fig. 2 and Supplementary File S3).
The tetraspanins CD9, CD63 and CD81 tended to be more enriched in the 100K pellet, compared to the 35K pellet (Fig. 2).

For ESCRT-associated proteins, there was no discriminatory pattern between the two EV populations: some components were equally enriched between the pellets (e.g., VPS4A, VTA1 and CHMP6), some tended to be more enriched in the 100K pellet (e.g., VPS37C, MVB12A and VPS28), while others (e.g., STAM, CHAMP4A and PTPN6) tended to be in higher abundance in the 35K pellet (Fig. 2).

When we looked at the Rab family, the majority was found in comparable or close enrichment between the 35K and 100K pellets (e.g., Rab22A, RAB13 and RAB10). However, the 35K pellet tended to be more enriched in some of the Rab family proteins (e.g. RABGAP1, RAB3IP and RAB9A). Only one Rab family protein (RAB33B) was slightly more enriched in the 100K pellet versus the 35K pellet (Fig. 2).

Altogether, these results support the enrichment of CD9\textsuperscript{high}, CD81\textsuperscript{high} and CD63\textsuperscript{high} exosomes with some ESCRT proteins in the 100K pellet, but not without questioning the use of Rab proteins as exosome markers. On the contrary, the enrichment of these proteins in the 35K pellet suggests their importance in the biogenesis of EVs sedimenting at 35,000 g.

However, following our stringent methodology, none of these tendencies fell within the significance threshold defined in the Materials and Methods section. The lack of statistically different enrichment of these usual EV markers precludes their use to discriminate the nature and subsets of cow milk EVs sedimenting at 35,000 and 100,000 g.

Further analyses of markers described for different EV subsets (light, dense, large or multiple EVs) revealed that 3 of the 4 proteins associated to light EVs (including TSG101) were more enriched in the 100K pellet, compared to the 35K pellet. EH Domain Containing 4 (EHD4), however, was slightly more enriched in the 35K pellet (Fig. 2). Together, these findings suggest that the 100K EVs may be termed “light EVs”.

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On the other hand, 3 of the 4 proteins associated to dense EVs (e.g., complement proteins C2, C6 and C7) were also more enriched in the 100K pellet, compared to the 35K pellet, with Fibronectin 1 (FN1) having comparable levels between the two pellets (Fig. 2). These observations indicate the presence of multiple EV subsets in the 100K pellet.

Proteins usually found in large EVs and proteins associated to multiple EVs were slightly more represented within the 35K pellet than in the 100K pellets, with a tendency for enrichment of Actin 1 (ACTN1), Heat Shock Protein 90 Alpha Family Class B Member 1 (HSP90AB1) and Major Vault protein (MVP) in the 35K pellet (Fig. 2).

Finally, most integrin proteins of importance in EVs internalization (ITGA1, ITFG1, ITGA6, ITGAM and ITGB2) were found in equal levels between the two pellets. Only Integrin alpha-V (ITGAV) was slightly, but not significantly, more enriched in the 100K pellet (Fig. 2), suggesting that both pellets contain multiple EV subsets that can be internalized, albeit with a differential capacity to bind extracellular matrix.

We observed a slight enrichment of some protein markers usable to discriminate at least between the 35K and 100K pellets and their associated EVs, with STAM, CHMP4A, PTPN6, RAB3IP and other Rab proteins being possible markers for 35K EVs, and TSG101, SDCBP, CD9, CD63, CD81, Complement proteins C2, C6 and C7 and ITGAV being the ones most markedly enriched in the 100K EV subsets. However, although the p-values of those markers were significantly lower than 0.05, none of these canonical EV markers passed the z-score threshold, suggesting that they may not be used alone to discriminate between commercial milk EVs. Their possible use in combination, as previously suggested [21], could circumvent such limitations.

Unraveling specific protein markers for the 35K and 100K pellets, and their associated EVs

Unable to use canonical EV markers, we turned to the 20 proteins that were significantly more enriched in the 35K pellet and on the 41 proteins specific to the 100K pellet to find specific markers
capable of discriminating between milk EV subsets (**Fig. 1B** and **3**). All the proteins present in both pellets are listed in **Supplementary File S1**.

The five proteins most specific to the 35K pellet included Epidermal growth factor receptor substrate 15 (EPS15), Phosphoglycerate Dehydrogenase (PHGDH), dynactin subunit 2 (DCTN2), Protein Kinase CAMP-Dependent Type II Regulatory Subunit Beta (PRKAR2B) and Glutaredoxin-3 (GLRX3), in decreasing order of significance (**Fig. 3A** and **3B**).

Concerning the 100K pellet, the most specific proteins were Complement C8 Beta Chain (C8B), C1GALT1-specific chaperone 1 (C1GALT1C1), Cartilage-associated protein (CRTAP), Alpha-mannosidase 2x (MAN2A2) and Procollagen-lysine 2-oxoglutarate 5-dioxygenase 3 (PLOD3) (**Fig. 3A** and **3B**).

These proteins may thus be used, alone or in combination, as markers to discriminate between the EV subsets found in the 35K and 100K milk pellets.

**Molecular function analysis unveils functional role of 100K milk EVs in translation regulation, galactose and protein metabolism**

Use of the GO Panther analysis tool revealed that proteins found at comparable levels in both milk pellets (common) may be involved mainly in the regulation of translation initiation, protein modification, peroxidase activity and metabolism-associated functions (**Fig. 4A**).

No defined function could be found for the 20 proteins specific to the 35K milk pellet.

For the proteins specifically enriched in the 100K pellet, three functions reached statistical significance: galactosidase activity and, to a lesser extent, glycosyl transferase and peptidase activity (**Fig. 4B**).

Together, these results suggest that the proteins found in the milk pellets are mainly involved in translation regulation, protein maturation and cell structural maintenance, with 100K EVs and associated proteins concealing specific metabolic activity.
Milk EVs and associated proteins may impact metabolism, translation and immunity

modulating pathways upon internalization

Milk EVs can be internalized by multiple human cells [22, 23, 25, 35, 36] and have been suggested to enter blood stream along with their RNA cargo [37, 38] or to release their protein load into recipient cells, thereby impacting their function [22, 23, 25, 35, 36]. Several studies previously underlined the potential impact of milk EVs on immunity, although most of them used 100K EVs [22, 23, 36, 39, 40] or a crude mixture of milk EVs [13].

Here, we used the Reactome Pathways Analysis Tool to analyze the pathways potentially impacted by a surge of milk EVs within human cells. We found that both 35K and 100K pellets contained proteins capable of impacting translation regulation (e.g. 60S and 40S ribosomal subunits modulation, translation initiation and elongation, etc.), immunity (e.g. neutrophil degranulation, innate immune system), vesicular trafficking (e.g. vesicle-mediated transport, membrane trafficking) and cell migration (e.g. axon guidance, signaling by ROBO receptors) (Table 1).

The 35K pellet proteins are likely more involved in cell proliferation and survival (e.g. apoptosis-induced DNA fragmentation, G2/M transition, apoptotic execution phase), intercellular structure (e.g. gap junction trafficking, formation of annular gap junctions, gap junction degradation) and vesicle formation and vesicular communication (cell-extracellular matrix interactions, clathrin-mediated endocytosis, membrane trafficking) (Table 2). As for the 100K pellet proteins, they may have a wider range of impact on metabolism and extracellular matrix turnover (e.g. glycosphingolipid metabolism, HS-GAG metabolism, extracellular matrix organization), hemostasis and immunity modulation (e.g. platelet degranulation, innate immune system, terminal pathway of complement) (Table 3).

Together, these results support a role for the EVs and associated proteins, found in both pellets, in the modulation of immunity and translation, with additional implications of the proteins specific to each of the milk pellets; 35K pellet proteins are potentially more involved in translation regulation, cell migration, proliferation and survival, whereas those in the 100K pellet are possibly...
oriented towards an enhanced metabolic and extracellular matrix turnover and immunity modulation fate. The multiple EV subsets and associated proteins present in milk may thus constitute a complex system that ensures the delivery of bioactive regulatory molecules naturally selected to modulate digestive tract integrity and inflammatory processes.

4. DISCUSSION

There is an ever increasing interest in EVs among laboratory scientists and clinicians [41, 42], with tremendous opportunities for EVs to be used as markers for disease [42] and product quality assessment [43, 44], and as carriers for drug delivery and therapeutics [27-29]. With an isolation method that is scalable virtually to the industrial level [45-48], milk EVs are among the most interesting and promising EV population, as they may be used for inflammatory disease management [11] and, even more, as drug carrier for cancer treatment [49].

In this study, we characterized further two distinct EV populations present in cow milk, including the well-studied exosomes and a new EV population that we recently reported [9], by LC-MS/MS. These analyses unveiled that this latter new EV population bears a wider variety of proteins than canonical milk exosomes. However, both EV subsets share as many as 1,838 different proteins, including XDH, BTN1A1 and MFGE8, strongly suggesting that they have a common mammary gland cell origin [50, 51] – both EV subsets may come from the same cell type [20].

Some of these proteins, like XDH, are highly enriched in milk fat globules (MFG). Comparison of the milk proteins associated with EVs versus MFGs, using a similar approach, could help define whether milk EVs derive from MFGs upon processing or if they are indeed secreted during lactation. Several of these proteins are well-known for their immunity modulating properties [52] and their implication in health and disease management, such as ulcerative colitis for MFGE8 [53].

These properties of dietary (milk) EVs may thus provide an additional mechanism by which nutrition may help prevent inflammatory diseases and improve health.
The complete list of the new markers of interest that we have found for each of the milk 35K and 100K pellets (Supplementary Files S1 and S3) may help discriminate the EV subsets under study and ensure reproducibility of milk EV isolation and functional analyses [1, 15, 19, 26].

Because of its low enrichment in microRNAs [9], its high volumes and its relatively high enrichment in caseins and whey proteins, the residual sample obtained after ultracentrifugation (supernatant, SN) was not analyzed in this study. Nevertheless, we systematically kept the SN fraction and used it as a control for the milk EVs tested in our experimental settings the same way as proposed by the ISEV position paper for EV RNA functional studies [54].

Based on these results, the 100K milk pellet likely contains the EV subset closest to “exosomes”, as they are usually referred to in the literature [55]. However, considering the presence of ESCRT and Rab family proteins in both pellets, it would be more prudent to stick to the generic terminology for milk EVs, fully describe the isolation methodology and provide a full description of those EVs based on their content in specific markers (e.g. CD63\textsuperscript{high or +}, CD81\textsuperscript{high}, CD9\textsuperscript{high}, bta-miR-223\textsuperscript{low} EVs), as it is the common practice for immune cell types. In fact, the relatively high enrichment of CD9, CD63 and CD81 proteins in the 100K pellet support their combined use as “exosomes” markers – their concomitant presence is likely necessary to define EVs as exosomes, as previously suggested [21].

Common EV type markers, such as ESCRT complex proteins, Rab family proteins and tetraspanins, could not be used alone to distinguish these EV subsets. The presence of ESCRT machinery-associated proteins in both EV subsets does not go without reminding of a non-canonical type of EVs that are generated by a cellular mechanism very close to the one ensuring exosome biogenesis, but occurring at the cell membrane instead of the MVB membrane [56].

It may be interesting to note that membrane proteins bared at the surface of EVs may become surface receptors upon EV fusion with the recipient cells’ membrane and have functional implications [57]. In the case of MFGE8, such a transfer would confer the ability to recipient cells to link integrin αvβ3-5 and modulate immune and inflammatory processes [58-67]. Similarly, the
relatively high enrichment of integrin ITGAV in 100K milk EVs suggest the possible enhanced internalization of milk exosomes harboring it [34] by gastrointestinal tissues and cells that are enriched in the ITGAV ligand vitronectin (GeneAtlas, U133A, gcrma) [68]. 35K EVs, which are impoverished in this specific integrin, might not bind to the gut cells as efficiently and end up in the colon, in contact with gut bacteria, and impact the host-bacteria crosstalk through extracellular vesicles [69]. Therefore, the use of LC-MS/MS to determine the integrin enrichment between different EV subset could be applied to specifically identify and select an EV subset that bears a specific integrin profile targeting a specific tissue, which may allow its use as a targeted drug delivery vehicle [27-29].

We have identified more than 20 and 40 new possible protein markers specific to the EVs present in the 35K and 100K pellets, respectively, which will help researchers identify their milk EVs of interest and facilitate data dissemination and comparison. Admittedly, the population of milk EVs is likely more complex than the two subsets that we have isolated and characterized, and our data may help optimize the selection of specific EV subset and their study.

Intriguingly, the 35K EVs, which are most often readily discarded when isolating exosomes, seem to be more abundant and more enriched in microRNAs [9] than the canonical exosomes found in the 100K milk pellet. We have shown previously that the bulk of microRNAs that they carry resist digestion [14], supporting a protective effect conferred upon EV association. Containing comparable amounts of immunity modulating proteins and harboring surface integrins of importance for cellular internalization, milk EV subsets, which are likely heterogeneous in nature, may cooperate towards an enhanced regulation of immunity and health status. Whereas 100K EVs may exert more pronounced effects on the regulation of metabolism and inflammation, 35K EVs may be involved more closely in digestive tract maintenance and integrity, as previously reported for milk exosomes [34].

Providing anti-inflammatory proteins, growth factors and gene regulatory microRNAs, the relatively abundant EVs present in maternal milk [10] likely play an essential role in infant gut
development. Supplementation of infant formulations of milk, with specific EV subsets and protein content, as previously done with milk fat globule membranes [5], should be considered in order to better simulate maternal milk composition and properties, and improve the nutritional value and health benefits of the formulation.

The relative scarcity of reliable antibodies recognizing the most interesting milk proteins of bovine origin may hamper validation of our results and further projects based on them. Nevertheless, now that these proteins have been identified, approaches like Targeted Proteomics by Selected Reaction Monitoring (SRM) or Parallel- Reaction Monitoring (PRM) might be used to confirm the relevance of these markers in different biological samples [70]. Therefore, the present report does provide the milk EV research community and manufacturers with a list of bovine proteins of interest for monitoring milk EVs and possibly producing and developing antibodies aimed to detect specific milk EV subsets.

In this study, we chose to use the z-score approach instead of setting an arbitrary 2 or 3 fold change, like previously described [21, 34]. The z-score approach is more stringent and takes into account the differences between the biological replicates, the biological background and the variability of the samples. These differences might lead to discrepancies between our work and previous ones on milk EVs, but not without convergence when looking at the most expressed proteins [21, 34].

Finally, the experiments reported in this study used commercial skimmed, filtered and pasteurized cow’s milk of the same brand and type as our previous studies [9, 14]. We chose to keep the same cow’s milk type and associated experimental protocols so to allow comparison of our data and reproducibility in our experiments and between studies. It is possible that other cow’s milk brands or types (raw or processed [homogenized], ultra-high temperature [UHT] sterilized or pasteurized, filtered or not filtered, skimmed, half and half or whole and any combination of the theses possibilities) exhibit different protein profiles the same way as milk processing impacted milk EV and microRNA profiles [9, 71].
5. CONCLUSION

Milk EVs are emerging as a novel research arena of interest that focuses increasingly on their characterization, biological role, function and use as therapeutic tools and vehicle [41, 42]. The EV isolation protocols, protein markers available and the current trends in the field have led researchers to focus almost exclusively on milk exosomes, while ignoring numerous types of EV subsets whose characteristics, content and function may also be of interest [21]. By documenting the resistance of milk EVs to digestion [14], their enrichment in immunity-modulating microRNAs and immunity-associated proteins [9], our previous work unveiled the potential importance of milk EVs sedimenting at ultracentrifugations speeds lower than 100K. The current study unveiled major protein markers that are differentially enriched between the EVs sedimenting at 35K and those sedimenting at 100K, and provides specific markers that may be used to ensure reproducibility in milk EV research. The suitability and reliability of these markers remain to be determined and is limited by the availability of antibodies recognizing these bovine proteins. Although our results may be transposed to other biological fluids, one has to recognize and appropriately consider the several features that may be unique to milk EVs.

ACKNOWLEDGMENTS AND FUNDINGS

This work was supported by the Canadian Institutes of Health Research (CIHR) [Grants No. 319618 and 327522] through the Institute of Genetics (to P.P.).

DISCLOSURE OF CONFLICT OF INTERESTS

The authors state that they have no conflict of interests.
REFERENCES


[61] Y.S. Yi, Functional Role of Milk Fat Globule-Epidermal Growth Factor VIII in Macrophage-Mediated Inflammatory Responses and Inflammatory/Autoimmune Diseases, Mediators of inflammation 2016 (2016) 5628486.


FIGURE LEGENDS

Figure 1. Quantitative proteomic identification of milk proteins that can be sedimented by differential ultracentrifugation. (A) Milk was subjected to differential ultracentrifugation, and the 35K and 100K pellets were lysed with Trizol LS to recover all proteins. After performing mass-spectrometry analysis, the quantitative analysis of protein enrichment was performed using the MaxQuant Andromeda algorithm. Panel adapted from previous work [9]. (B) Venn diagram comparing the 35K and 100K pellets, in terms of protein enrichment. Proteins with a z-score higher than 1.96 and a Benjamini-Hochberg adjusted Welch p-value below 0.05 were considered specific to each subset. Panther GeneOntology (GO)-slim cellular component analysis was used to predict the cellular origin of the proteins in each pellet, as indicated in the circles. The complete list of the specific proteins and their respective enrichment in each pellets are provided in Supplementary File S1. (C) Top 15 of the most enriched proteins found in both 35K and 100K pellets based on the Exponentially Modified Protein Abundance Index (emPAI) normalized for each sample. The levels for the emPAI in each pellets are provided in Supplementary File S2.

Figure 2. Comparative enrichment of canonical EV protein markers in the 35K and 100K pellets. Comparison of the 35K and 100K pellets for their enrichment (ratio intensities pellet/total) in different EV subset protein markers (light EVs, dense EVs, large EVs or multiple EVs markers), for multivesicular body (MVB) markers/exosome-enriched proteins (tetraspanins, ESCRT complex proteins, Rab family) or proteins necessary for docking and internalization of EVs (Integrins), as defined previously [21, 34]. From inside to outside: 100K n1; 100K n2; 100K n3; 35K n1, 35K n2, 35K n3. The entire dataset and associated statistical analysis are provided as Supplementary File S3.

Figure 3. Identification of the proteins differentially enriched between 35K and 100K pellets. (A) Volcano plot representing the differential enrichment in milk proteins between the two pellets.
as well as the most significantly enriched proteins for each of them (z-score > 1.96 and adjusted p-value < 0.05). x axis = log2(35K/100K). y axis = −log10 (adjusted p-value). The horizontal line indicates p-value = 0.05, vertical blue lines indicate log2(35K/100K) threshold limits as defined through the z-score. Green and red dots correspond to the proteins above Z-score and below p-value thresholds for each pellet. The blue dots identify the proteins common to both pellets. The name of the most promising specific markers are displayed. (B) Heatmap and clustering of milk pellets based on the ratio [log2(35K/100K)] of the most specific proteins for each pellets. The complete list of the specific proteins and their respective enrichment in each pellets are provided in Supplementary File S1.

Figure 4. The predicted molecular function of the proteins associated to the milk pellets.

Panther GeneOntology (GO)-slim molecular function analysis was used to predict the molecular function of the proteins common to the two pellets (A) or specific to the 100K pellet (B). Functions associated to the milk 100K pellet, for which fold change versus database > 2 and p-value < 0.05, are displayed. No specific molecular function could be defined for 35K pellet-specific proteins.
FIGURES

Figure 1

A

Skim Milk + 2% Sodium Citrate

35,000 g pellet

100,000 g pellet

B

35K

Axon

Plastid

Vacuole

Golgi

Lysosome

Ves. Coat

Vacuole

Ribo.

20 Postsynaptic memb.

Actin

cytoskeleton

20 Postsynaptic memb.

Actin

cytoskeleton

1,838 Membrane Cytoplasm Mito PM ER

41 Extracellular region

100K

C

Quantitative Value (Normalized emPAI)

Figure 1
Figure 2
Figure 4
TABLES

Table 1. Pathways involving the proteins common to both 35K and 100K pellets. The proteins found in both pellets were submitted to the Reactome pathways analysis tool, and the top 20 of the pathways most likely to involve 35K proteins are displayed in this table.

Table 2. Pathways involving 35K proteins. The proteins found in the 35K pellets were analyzed with Reactome pathways analysis tool, and the top 20 of the pathways involving specific 35K proteins are displayed in this table.

Table 3. Pathways involving 100K proteins. The proteins found in the 100K pellets were analyzed with Reactome pathways analysis tool, and the top 20 of the pathways involving specific 100K proteins are displayed in this table.
<table>
<thead>
<tr>
<th>Pathway name</th>
<th>Entities</th>
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<th>Reactions</th>
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FDR, False Discovery Rate.

Table 1.
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FDR, False Discovery Rate.

Table 2.
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</table>

FDR, false discovery rate.

Table 3.
SUPPLEMENTARY DATA

Supplementary File S1. Identification and specific enrichment analysis of extracellular vesicle (EV)-associated proteins in milk pellets by LC-MS/MS.

Supplementary File S2. Quantification of the proteins found in each pellet based on the Exponentially Modified Protein Abundance Index (emPAI) normalized for each sample.

Supplementary File S3. Relative enrichment of extracellular vesicle (EV)-associated proteins in milk pellets.