Supplementary tables

Table S1. Summary of published sample preparations for proteomics analyses of human milk extracellular vesicles.

Reference	Proteomics sample preparation	End-goal	Nr of proteins detected
(Admyre *et al.*, 2007)	1) 50 µg of exosomes in denaturing buffer (6 M urea, 0.05% SDS, 5 mM EDTA, and 50 mM Tris-HCl (pH 8.5)) 2) Digestion with 2 µg of trypsin overnight at 37°C. 3) Peptide elution using cation exchange columns. 4) Peptide purification using C18 Empore membrane (3M). *Tandem MS (MS/MS) analysis:* 1) Peptide separation using nano-HPLC with a linear gradient (2% ACN to 95% ACN). 2) Peptides sent to ESI-QqTOF mass spectrometer. 3) Data collected at MS1 (first 1s of data) with two most intense peaks for MS2 (first 3s of data).	Characterization of exosomes	73
(Van Herwijnen *et al.*, 2016)	1) 20 µl or 40 µl of sonicated EVs added to 10 µl 200 mm DTT and 10 µl sample buffer. 2) Samples separated on a 12% Tris-HCl gel. 3) In gel digestion using trypsin. *Tandem MS (MS/MS) analysis:* 1a) Peptide separation using Proxeon EASY-nLC 1000 with a C18 trap column. 2a) Peptides sent to LTQ-Orbitrap Elite. 3a) Data collected at MS1 at selected survey scan of 350 to 1500 mass/charge (m/z) with ten most abundant precursors for MS2 fragmentation. 1b) Peptide separation using Agilent 1290 Infinity LC system with a C18 trap column. 2b) Peptides sent to Q-Exactive instrument. 3b) Data collected at MS1 at selected survey scan of 350 to 1500 mass/charge (m/z) with ten most abundant precursors for MS2 fragmentation.	Proteomic characterization of vesicles	1963
(Yang *et al.*, 2017)	1) 200 µg of exosomes in denaturing buffer (4% SDS, 150 mM Tris-HCl pH 8.0, 100 mM DTT). 2) Exosomes mixed with UT buffer (8 M urea and 150 mM Tris-HCl at pH 8.0) and 0.05 M	Proteomic comparison of bovine and human milk samples	920

	iodoacetamide solution, washed several times with UT buffer. 3) Digestion with 40 µL trypsin at 37 °C for 24 h. 4) Obtained peptides labeled with iTRAQ reagents (Reagent-8plex Multiplex Kit) 5) Peptide separation using strong cation exchange (SCX) chromatography. 6) Sample desalted using C18 solid phase extraction column. *Tandem MS (MS/MS) analysis:* 1) Peptide separation using Easy-nLC. 2) Peptides sent to Q-Exactive mass spectrometer. 3) Data collected at MS1 at selected survey scan of 300–1800 mass/charge (m/z) with ten most abundant precursors for MS2 data at 2 m/z isolation window.		
(Wang *et al.*, 2019)	1) Exosomes in U2 buffer (8 m Urea,100 mm TEAB, pH 8.0) , then reduced with 10 mm DTT and alkylated with 50 mm iodoacetamide 2) Peptides larger than 10kDa removed by filtration. 3) Peptides purified by C18 columns. 4) Peptides labeled with iTRAQ reagents (iTRAQ 8-plex kit). 5) Sample fractionation using HPLC. *Tandem MS (MS/MS) analysis:* 1) Peptides separated using Eksigent nLC. 2) Peptides sent Triple TOF 5600 Plus mass spectrometer. 3) Data collected at MS1 selected range of 350-1500 m/z, MS2 spectra at 100-1500 m/z.	Characterization of human term and preterm milk exosomes	719

Table S2. Summary of advantages (+) and disadvantages (-) of methods recommended for human milk extracellular proteomics.

Isolation	**Differential ultra-centrifugation**		**Sucrose gradient**	
	+ Separation of EVs without added solutions + Increased yield	- Time-consuming - Multiple steps - Specialized equipment	+ Increased EV purity + Size separation in EV populations	- Additional reagents - Time-consuming
Protein solubilization and peptide cleavage	**SDS with/out EDTA**		**Trypsin/LysC**	
	+ Effective protein solubilization + Addition of EDTA minimizes damage to MS equipment	- Reduced compatibility with alternative peptide fractionations, such as in-gel peptide separation	+ Cleaves lysine and arginine bonds + Increases peptide detection in MS/MS	- No cleavage of serine or threonine - Time-consuming
Peptide fractionation	**Reverse phase nano-liquid chromatography**			
	+ Less sample, consumables required + Increased peptide selectivity and sensitivity + Easy coupling to MS/MS equipment + Faster analysis		- Cost - Equipment accessibility	

Peptide detection	**Tandem mass spectrometry (MS/MS)**			
	+ Increased protein sensitivity, identification + Moderate to fast analysis + Easy coupling to nano-LC equipment		- Difficulty resolving complex samples - Sensitive to buffer salts, contaminants - Incompatible with certain solvents - Cost	
Data acquisition	**Data dependent acquisition**		**Data independent acquisition**	
	+ Straight-forward analysis + Cost + Increased sensitivity for quantification	- Reduced identification of low abundance peptides - Reduced fragmentation precision	+ Increased peptide detection/identification +Identification of low abundance peptides	- Increased peptide fragmentation - Increased MS/MS spectra complexity
Data quantification	**Label-free**		**Isobaric label**	
	+ Automation of analysis + Unlimited number of quantitative comparisons	- Reduced accuracy for quantification	+ Up to 100% labelling efficiency + Increased accuracy of quantification	- Limited number of biological samples - Cost