Exosome protein diversity is greater in preterm milk than term milk

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This thesis is submitted to the

University of Ottawa

in partial fulfillment of the requirements for the

M.Sc. degree in Biochemistry (Specialization Bioinformatics)

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Abstract

Infants born prematurely are a vulnerable population with diverse nutritional needs to battle their increased risk of gastrointestinal (GI) diseases. Human milk is considered the 'gold standard' of infant nutrition. Human milk not only provides nutrition for newborn growth, but contains bioactive components which contribute to GI maturation, immune protection and neurological development. Among these bioactive components are extracellular vesicles known as exosomes. Exosomes are double-lipid membrane vesicles containing mRNA, microRNA and proteins, secreted by cells as a form of cell-to-cell communication. Human milk exosomes contain immune-related microRNA and proteins that withstand *in vitro* simulated human digestion, suggesting that signals are being delivered to the cells residing in the GI tract of a newborn. In premature birth, disruption of GI tract maturation predisposes the infant to increased susceptibility of GI inflammatory diseases. To prevent inflammation, immune tolerance in the GI tract of premature infants should be promoted and I hypothesized that exosomes differ between preterm and term milk, and may contribute to the anti-inflammatory effects of human milk. Human milk exosomes from mothers who gave birth to term or preterm infants were characterized based on size, surface protein markers and total protein. Preterm milk exosomes contained a more diverse protein profile. The effects of milk exosomes on intestinal epithelial cells were observed in an *in vitro* model using Caco-2/15 cells. Milk exosomes were able to attenuate the inflammatory response induced by heat-killed bacteria as measured by the transcription of pro-inflammatory cytokines.

Acknowledgements

First, I would like to thank my supervisor, Professor Illimar Altosaar, for the incredible opportunities I have had throughout my Master's that have shaped my future. I am grateful for the support and encouragement that Illimar provided to play the game and go for the goal. My experience in the Altosaar lab has undoubtedly set the stage for my career in research. I would like to give a special thanks to my co-supervisors Dr. Emanuela Ferretti and Professor Ilya Ioshikhes for their advice, guidance and support the past couple of years.

I would also like to thank my friends and family for their enthusiasm and patience when I get too excited talking about milk. A special thanks to both of my parents and Ryan for being my cheerleaders and source of motivation. Mom, you were always there when challenges arose, and you provided me with the resilience to keep going. Dad, you have always provided me with support and you are my source of inspiration, "if you're not falling, you're not trying hard enough."

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Frontispiece – Mother and child, Ed. Wiiralt, Paris, 1931

List of Abbreviations

APC	Allophycocyanin
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CD	Cluster of differentiation protein
CXCL2	C-X-C motif chemokine 2
DDM	n-dodecyl β-D-maltoside
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylene-diamine-tetra-acetic acid
EE	Early endosome
ESCRT	Endosomal sorting complexes required for transport
FITC	Fluorescein isothiocyanate
GI	Gastrointestinal
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ILV	Intraluminal vesicles
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MHC	Major histocompatibility complex
MVB	Multivesicular body
NEC	Necrotizing enterocolitis
OHSN-REB	Ottawa Health Science Network Research Ethics Board
PANTHER	Protein ANalysis THrough Evolutionary Relationships
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PTM	Preterm milk – milk from females who gave birth to preterm infants
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
TCEP	Tris (2-carboxyethyl) phosphine
TM	Term milk – milk from females who gave birth to term infants

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1. Introduction

Postpartum, the naïve gastrointestinal (GI) tract of the newborn infant is exposed to a plethora of novel microbes [1, 2]. In healthy, term births, the GI tract continues maturation with environmental exposure and enteral feeds, initiating commensal bacteria colonization [3]. In preterm infants, organ maturation that was well underway *in utero* is suddenly disturbed leading to GI and immune complications upon exposure to novel microbial species. Because the gut has increased permeability in preterm infants, the immune system may encounter commensal bacteria and unnecessarily launch an inflammatory response [1, 4, 5]. The lack of immune tolerance may lead to sepsis and potentially necrotizing enterocolitis (NEC) [4, 6-10].

1.1. Necrotizing enterocolitis

NEC is a severe gut disorder in newborns that predominantly affects premature infants. It is the leading cause of death from GI-related diseases among preterm infants in North America [9]. There are multiple factors contributing to the pathogenesis of NEC, commencing with a lack of immune tolerance to bacteria. The lack of immune tolerance induces an inflammatory response that may lead to intestinal necrosis [4, 7-11]. NEC incidence is approximately 12% in very low birth weight infants (<1500 grams), where overall mortality is estimated to be 30% [6]. This disease poses a large burden on the healthcare system. The National Institutes of Health (2012) reported that an estimated $5 billion per year is spent on hospitalizing NEC patients in the United States, accounting for 19% of neonatal expenses. NEC survivors are hospitalized, on average, 22 days longer than preterm infants that are healthy [6]. Furthermore, NEC survivors are subject

to long-term major complications, including dysmotility disorders, short bowel syndrome and a variety of neurodevelopmental issues [11].

NEC pathophysiology remains largely unknown. However, previous studies have suggested that the interaction between prematurity, enteral nutrition and the imbalance of intraluminal bacteria in the immature intestine, combined with underdeveloped microbial tolerance, all contribute to NEC [4-9, 11-13].

Human milk is thought to help prevent NEC [9, 10, 14-18]. While specific therapies for NEC are currently unavailable, the presence of many protective bioactive factors in human milk implicates and supports its advantages [10, 14, 15, 18]. Studies conducted on cohorts of newborns show that human milk alone is highly effective in decreasing NEC risk, whereas the incidence of NEC is increased six to ten fold in very low birth weight infants fed formula [19]. A meta-analysis of four clinical trials concluded that infants who received human donor milk were three times less likely to develop NEC and four times less likely to have confirmed NEC [15]. Feeding human milk promotes an improved host defense and GI maturation [2, 16-18], indicating the importance of human milk in the prevention of NEC development in preterm infants.

Immune tolerance in newborns is essential to prevent such GI inflammatory diseases. Multiple studies have demonstrated that human milk prevents and protects against GI inflammatory-related diseases [14, 17, 20].

1.2. Human milk

For best health outcomes for newborn infants, the World Health Organization recommends exclusive human milk feeding for the first six months of life, along with

continued breastfeeding up to two years and beyond, based on the Cochrane Review by Kramer and Kakuma [21, 22]. Infants that are fed formula have an increased frequency of digestive issues and are more prone to GI and respiratory diseases [15, 17, 18, 20]. Human milk not only contains nutrients necessary for infant growth and development, but also numerous bioactive components that provide protection and contribute to immune and GI development and maturation [14, 20, 23-43].

1.2.1. Human milk production

Human milk is a dynamic fluid with its composition varying dependent on the time of day, gender of infant, time postpartum, feeding, between mothers and ethnic backgrounds [23, 44-46]. Mammary epithelial cells, also known as lactocytes, differentiate from alveolar cells in the mammary gland during pregnancy [47-49]. At parturition, prolactin is released and progesterone and estrogen decrease, resulting in the lactocytes synthesizing and transporting components for secretory activation into milk [48]. Normally, secretory activation occurs upon giving birth to full-term infants [47, 50]. Milk ejection is controlled by oxytocin, and milk secretion is regulated by metabolic and reproductive hormones. The infant suckling is the leading stimulus for milk ejection [47, 48]. Galactopoesis, ongoing milk production, occurs once prolactin secretion decreases and lactation has been established through continuous milk removal [47].

Colostrum is produced immediately postpartum for two to three days [23, 48, 51]. Immunological components such as lactoferrin, leukocytes and secretory IgA are higher in colostrum than other milks to provide protection to the newborn after it is exposed to novel microbes upon birth [23, 52-54]. Because colostrum contains lower concentrations

of lactose and higher concentrations of immune-related components compared to mature milk, it is thought to play a more immunological role, as opposed to a nutritional role [23, 54]. The milk produced from anywhere between five days to two weeks after birth is called transitional milk. Transitional milk production begins with secretory activation, when the tight junctions of the mammary epithelium begin to close [48]. This closure leads to increased lactose and decreased sodium-to-potassium ratio. Secretory activation usually occurs within the first few days of giving birth, however, preterm delivery and maternal obesity may cause delayed secretory activation, where a delay is considered to be greater than 72 hours [23, 55]. During the transitional milk period, the amount of milk production increases to provide the necessary nutritional content to support the rapid development of the infant. After transitional milk, mature milk follows and flows. Fully mature milk is thought to be produced by four to six weeks. At this point, human milk remains relatively constant in composition [23, 56].

1.2.2. Human milk composition

Macronutrients in milk remain conserved across populations regardless of maternal diet. The protein in human milk is in the whey or the casein fraction [23, 57]. Lactoferrin, casein, serum albumin, lysozyme, α-lactalbumin and secretory IgA are the most abundant proteins in human milk [23, 33, 57, 58]. Milk from mothers that delivered preterm infants (PTM) have increased amounts of protein, fat, energy and carbohydrate than milk from mothers that gave birth to term infants (TM) [59]. Fat content in milk varies depending on the time of day, with an increase in afternoon and evening feeding compared to morning and night feeds. The concentration of fat in hindmilk (milk at the

end of feeding) can be double or triple that of foremilk (milk at the initiation of feeding) [23, 33]. The main macronutrients of milk changing in response to maternal diet are the fatty acids, specifically, long chain polyunsaturated fatty acids [60-62]. In addition to macronutrients in milk, there are bioactive components.

The bioactive components in human milk provide protection to the newborn. These components originate from lactocytes, cells within milk or maternal serum; transported across the mammary epithelium [23, 63, 64]. The cells found in human milk are either from the mother's breast or blood (Figure 1). The cells from the breast include epithelial cells (lactocytes and myoepithelial cells), progenitor cells and stem cells [65]. The stem cells in human milk spontaneously differentiate into cells from the three germ layers [30, 39]. The stem cells in milk have been shown to incorporate into the brain of suckling pups [66]. Another study cross-fostering dams expressing TdTomato constitutively with wild-type pups, demonstrated the presence of TdTomato^{+} cells in blood, stomach cavity, gastric lining, thymus, pancreas, liver, spleen and brain of pups [67]. Some of the TdTomato^{+} cells incorporated into the pups' organs contained stem cell markers, OCT4, NANOG and CD49f, whereas others did not. This may suggest that the stem cells are differentiating and incorporating into the pups' tissues and/or that other cells in milk are also being incorporated or circulating in the blood stream [67]. This demonstrates the ability of milk cells to withstand degradation, migrate across the epithelium and functionally integrate into organs.

Other important cells in human milk are the immune-related cells from the mother's blood. These cells include B cells, T cells, NK cells, eosinophils, neutrophils, monocytes and immature granulocytes. Leukocytes from human milk have proved to

withstand degradation and reach the GI tract where they translocate into Peyer's patches, blood, lymph nodes, spleen and liver of pups [68]. The cells of human milk demonstrated the ability to phagocytose pathogens and produce other bioactive components to facilitate immune system development in the newborn [65, 69]. These findings demonstrate the importance of cells in human milk in providing active immunity to newborns.

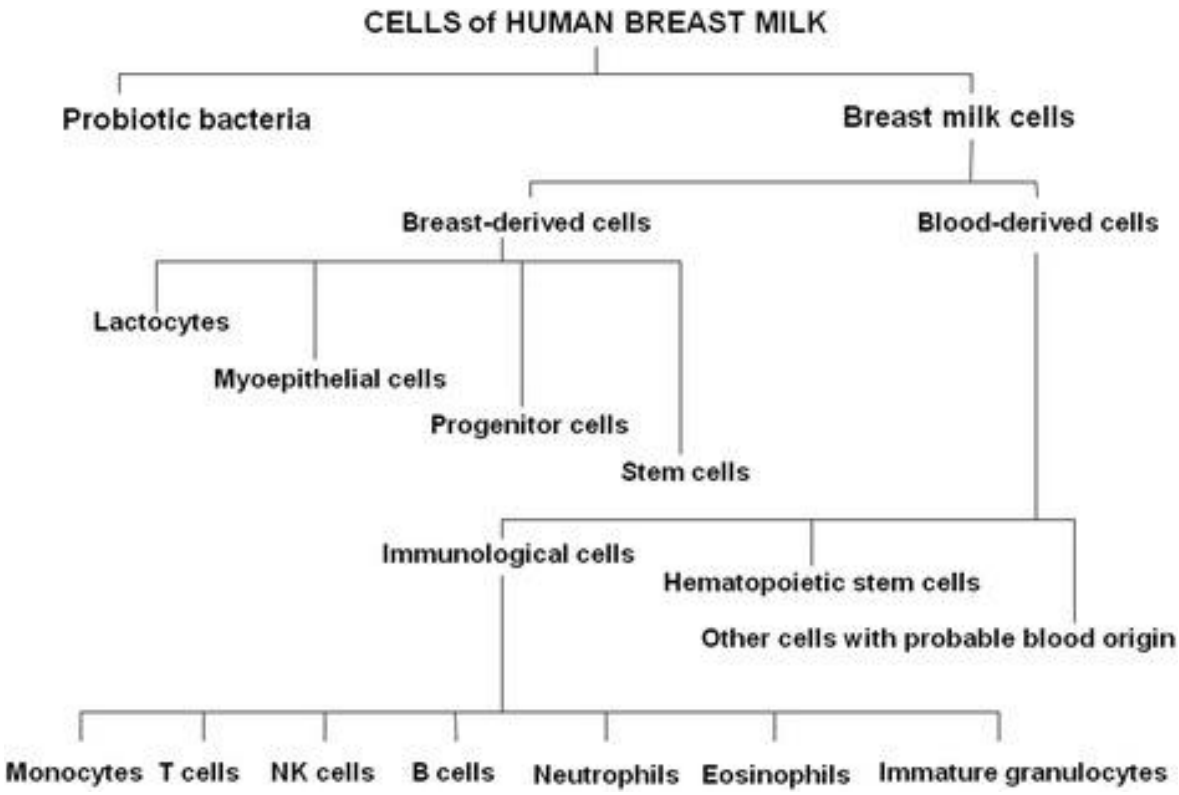

CELLS of HUMAN BREAST MILK
Probiotic bacteria
Breast milk cells
Breast-derived cells
Blood-derived cells
Lactocytes
Myoepithelial cells
Progenitor cells
Stem cells
Immunological cells
Hematopoietic stem cells
Other cells with probable blood origin
Monocytes
T cells
NK cells
B cells
Neutrophils
Eosinophils
Immature granulocytes

Figure 1. Cells present in human milk. Source: from Witkoska-Zimny and Kaminska-El-Hassan [65].

Other bioactive components found in human milk include immunoglobulins, chemokines, cytokines, growth factors, sugars and hormones [23, 34, 63, 64]. For example, secretory IgA is an important antibody found in human milk that binds pathogens to prevent their attachment and colonization in the infant GI tract [37, 70]. Human milk oligosaccharides also have an antimicrobial role by promoting intestinal colonization of beneficial bifidobacteria and preventing attachment of pathogens to mucosal surfaces [28, 36]. Additional milk components that have an anti-microbial/viral role include lactoferrin, lactadherin and mucins [71-73]. For instance, lactoferrin binds iron to prevent GI colonization of pathogens which require iron for growth [34, 71]. Bioactive lipids, proteins and RNA are delivered to the infant through milk. Milk fat globules, made up of lipids, are 'packages' that comprise beneficial proteins to be delivered to the infant [74]. Human milk exosomes are another form of packaging for beneficial immune-related components to be delivered to the GI tract of newborns [25].

1.3. Exosomes

Exosomes are extracellular vesicles that are approximately 20-200 nm in diameter, secreted by most cells and are found in all biological fluids. They are double-lipid membrane vesicles that contain functional microRNA, mRNA, and proteins [25, 75-79]. Exosomes play a role in cell-to-cell communication by delivering specific contents to target cells. Key surface markers of exosomes which distinguish them from other extracellular vesicles such as apoptotic bodies or blebs, are the tetraspanins: cluster of differentiation protein (CD) 9, CD63 and CD81. They also tend to have major histocompatibility complex (MHC) class I and class II protein markers on their surface (Figure 2) [76, 79, 80]. Exosomes have a similar topology on their surface to that of the cell they originated from, due to their biogenesis [80].

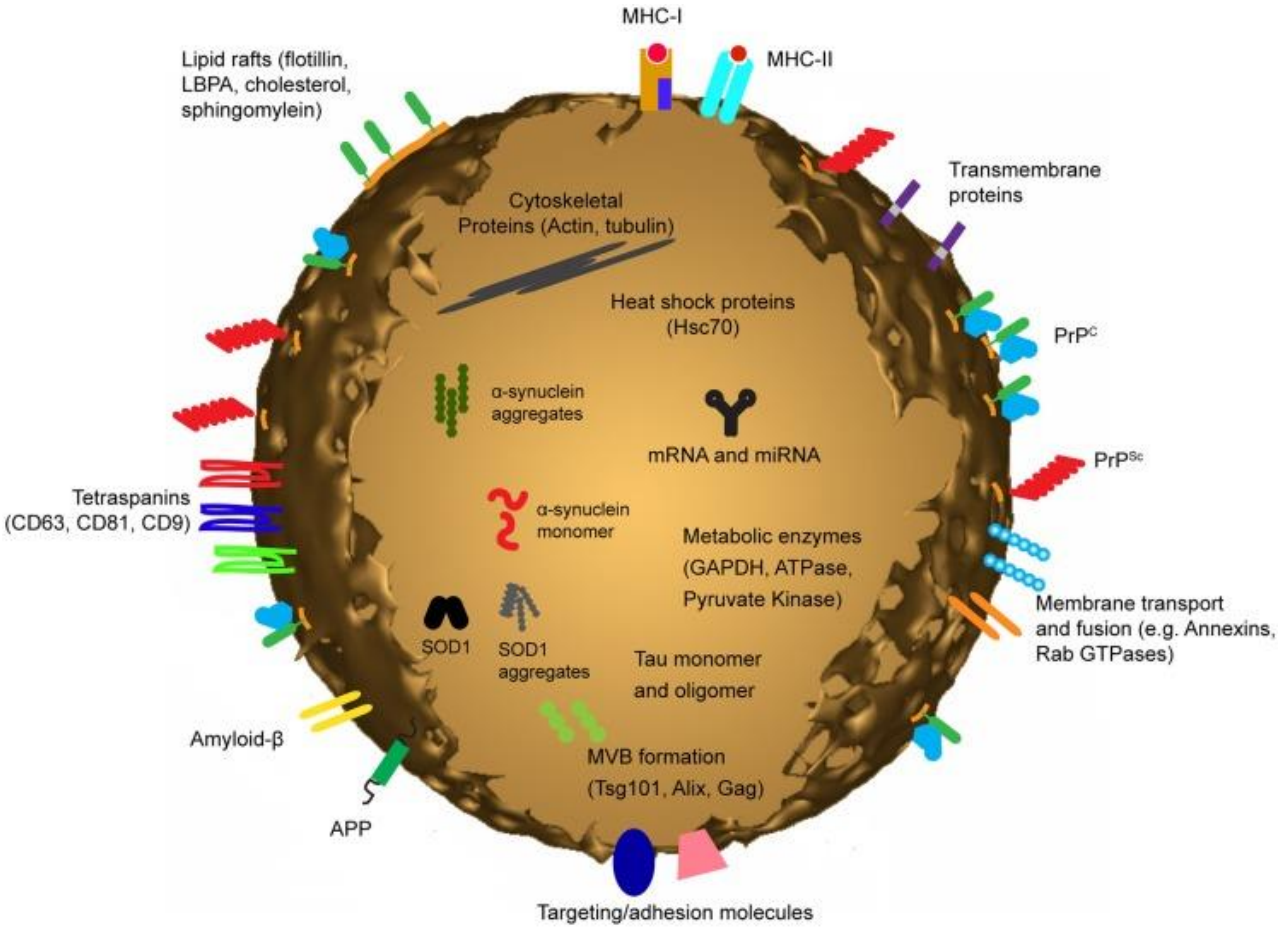

MHC-I
MHC-II
Lipid rafts (flotillin, LBPA, cholesterol, sphingomylein)
Transmembrane proteins
Cytoskeletal Proteins (Actin, tubulin)
Heat shock proteins (Hsc70)
PrP^C
α-synuclein aggregates
mRNA and miRNA
PrP^Sc
Tetraspanins (CD63, CD81, CD9)
α-synuclein monomer
Metabolic enzymes (GAPDH, ATPase, Pyruvate Kinase)
Membrane transport and fusion (e.g. Annexins, Rab GTPases)
SOD1
SOD1 aggregates
Tau monomer and oligomer
Amyloid-β
MVB formation (Tsg101, Alix, Gag)
APP
Targeting/adhesion molecules

Figure 2. Diagram of an exosome, Source: from Bellingham *et al.* [80].

Exosomes are double-lipid membrane extracellular vesicles that contain microRNA, mRNA and proteins. Exosomes are involved in cell-to-cell communication through delivery of bioactive molecules. The surface protein markers and the entire proteome of term and preterm milk exosomes are reported below in Section 4.1.

1.3.1. Exosome biogenesis

Exosomes are packaged in a non-stochastic manner for cell-to-cell communication. The contents of exosomes demonstrate upregulation of specific microRNA, RNA and proteins compared to those within the cytosol of a cell they are released from [75, 79]. Figure 3 demonstrates the formation of exosomes. Briefly, exosomes are formed by invagination of the cell's outer membrane into an early endosome (EE). The EE becomes enriched for CD63 and undergoes subsequent invagination forming intraluminal vesicles (ILVs) [80-82]. Multiple proteins have been shown to play a role in the formation and packaging of ILVs, e.g. endosomal sorting complexes required for transport protein (ESCRT)-0, -I, -II, -III and Alix. Each ESCRT protein plays a different role in ILV packaging. It is suggested that ESCRT-0 binds ubiquitinated proteins on the membrane of the EE, ESCRT-I and -II initiate budding of the ILVs and ESCRT-III aids in the final formation of the ILVs [82]. The proteins involved create purposefully 'packaged' vesicles within the EE turning it into a multivesicular body (MVB) [80, 82]. For example, Alix plays a role in selecting specific microRNA for packaging [83]. The MVB fuses with the outer membrane, facilitated by Rab GTPases, and releases the exosomes into the extracellular space as a form of intercellular communication (Figure 3) [75-77]. Microvesicle formation is distinguished from that of exosomes, as microvesicles bleb off the outer plasma membrane [76].

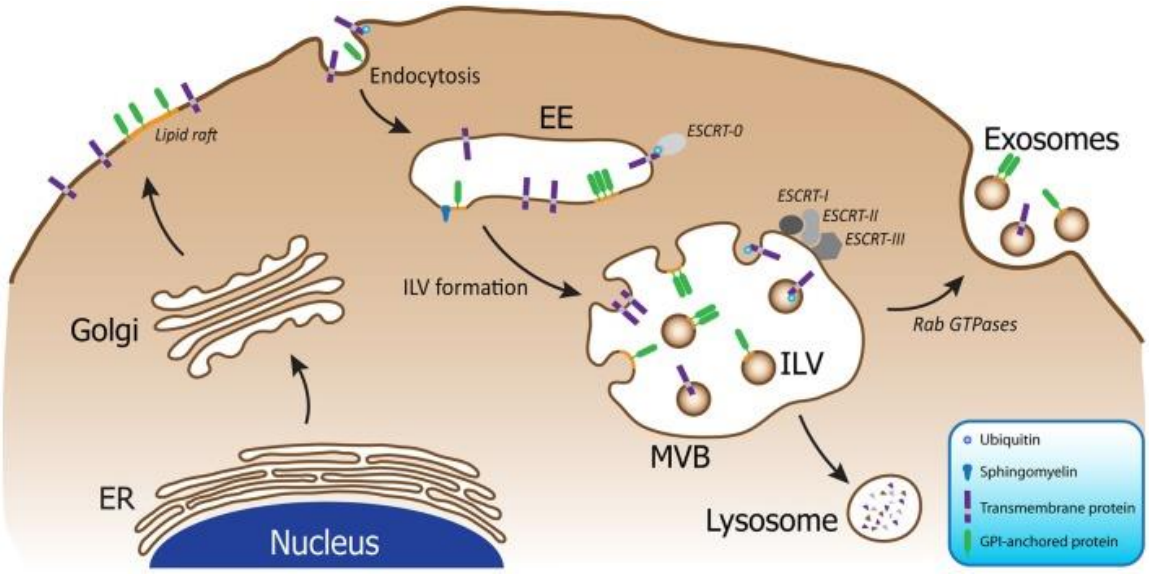

Endocytosis
EE
ESCRT-0
Lipid raft
Exosomes
ESCRT-I
ESCRT-II
ESCRT-III
ILV formation
Rab GTPases
Golgi
ILV
MVB
ER
Nucleus
Lysosome
Ubiquitin
Sphingomyelin
Transmembrane protein
GPI-anchored protein

Figure 3. Exosome biogenesis. Source: Cartoon adapted from Bellingham *et al.* [80]. Exosome biogenesis begins with the formation of an early endosome (EE) through endocytosis of the outer plasma membrane of a cell. The EE undergoes invagination forming intraluminal vesicles (ILV), with the help of endosomal sorting complexes required for transport (ESCRT) proteins, in a multivesicular body (MVB). Rab GTPases facilitate fusion of the MVB with the plasma membrane of the cell, releasing the exosomes into the extracellular matrix to be delivered to target cells.

1.3.2. Milk exosome composition

Exosomes were discovered in human milk in 2007 by Admyre *et al.* [25]. Human milk exosomes contain microRNA and proteins that may be involved in immune processes [25, 32, 41]. The most abundant microRNA found in human milk exosomes are immune-related, such as miR182-5p, miR30b-5p, miR29a-3p and miR-146b-5p. miR182-5p promotes immune response by T cells. miR30b-5p enhances immunosuppression and cellular invasion. miR29a-3p targets IFNγ and suppresses the immune response to intercellular pathogens. miR-146b-5p targets the NF-κβ pathway [41]. Exosomal microRNA identified in milk have distinct patterns throughout the lactation period, suggesting that they might play an important role in postnatal development and GI maturation [44, 45, 84-86]. For example, immune-related microRNA are more abundant in colostrum than mature milk [86, 87].

Immune cell regulatory proteins are found in milk exosomes [25, 88]. For example, lactadherin plays a role in clearing apoptotic cells and is abundant in the milk exosomal fraction. Further, Admyre *et al.* discovered the presence of the CD36 protein in milk exosomes. CD36 plays a role in binding oxidized low-density lipoproteins, cell adhesion and mediating phagocytosis. Notably, immunoglobulins, such as IgA, are also found in human milk exosomes [25]. These immunomodulatory components packaged within milk exosomes are thought to be delivered to the GI tract of the newborn [42, 89].

1.3.3. Human milk exosome stability and effects

Human milk exosomes have proven to be durable *in vitro*. They withstand degradation after being subjected to freeze-thaw cycles, RNase treatment, high

temperatures, low pH or simulated *in vitro* digestion. The milk exosomes and their encapsulated cargo - microRNA, proteins - remain intact despite harsh condition treatment [25, 41, 42, 89]. These findings indicate the stability of exosomes upon ingestion by the infant, suggesting that they survive gastric passage, allowing their contents to be delivered to the GI tract.

Human milk exosomes are taken up by intestinal epithelial cells [89]. After *in vitro* simulated digestion, human milk exosomes labelled with fluorescent dye were incubated with human intestinal epithelial crypt-like cells and exosomal uptake was visualized. This particular study reported some degree of exosome localization to the nucleus in human intestinal epithelial crypt-like cells, suggesting that milk exosomes may be regulating nuclear-encoded genes [89].

Human milk exosomes have been shown to regulate cytokine release in cells *in vitro*. Upon activation of peripheral blood mononuclear cells, human milk exosomes altered cytokine release by inhibiting IL2, IFNγ and TNFα and by promoting IL5 cytokine production. This demonstrates that human milk exosomes have the ability to regulate cytokine expression in specific cell types [25].

1.3.4. Mammalian milk exosome stability and effects

Milk exosomes from other mammals are also taken up by intestinal epithelial cells. They have been shown to cross confluent intestinal epithelial cells *in vitro*. They also demonstrate beneficial effects on intestinal epithelial cells by promoting growth and proliferation and attenuating cell death [31, 35, 42, 90]. To assess exosome uptake in an intestinal model, fluorescently labelled bovine milk exosomes were subjected to *in vitro*

simulated digestion and incubated with human Caco-2 cells [42]. Bovine milk exosomes were added to the apical compartment of post-confluent Caco-2 cells grown on Transwell plates. After two hours of incubation, the medium from the basolateral compartment was collected and RNA was extracted. Bovine specific microRNA analysis was performed to compare with microRNA analysis previously performed on bovine milk exosomes alone. This study found that the microRNA in bovine milk exosomes remained stable after crossing the post-confluent Caco-2 cell layer [42]. To analyze the effects of exosomes on intestinal epithelial cells, porcine milk-derived exosomes were incubated with porcine intestinal epithelial cells (IPEC-J2). The porcine milk exosomes promoted cell proliferation. Furthermore, porcine milk exosomes, with a concentration of RNA between 0.125 μg and 0.25 μg, administered to mice daily resulted in a significant increase of the depth of crypts and the height of villi in the intestine [90]. In addition, rat milk exosomes incubated with rat intestinal epithelial cells (IEC-18) resulted in enhanced cell proliferation, viability and stimulation of stem cell activity [31]. These results demonstrate the ability of milk exosomes to drive intestinal growth and development through cell proliferation. This GI maturation is necessary for newborns to develop tolerance to the *ex utero* environment.

1.4. Rationale

Milk exosomes have demonstrated the ability to alter intestinal growth and proliferation [31, 35, 89, 90]. They also contain immune-related components that may be promoting GI development and maturation [25, 41]. TM and PTM contain different components [23, 91], however, previous studies have not analyzed the difference in

exosome populations within the milks from these cohorts. Premature infants are a vulnerable population and require optimized nutrition to promote GI development and immune maturation to avoid inflammatory diseases such as NEC. NEC begins with bacteria reaching the epithelium and intensifying the inflammatory cascade in the immature intestine [4-9, 11-13] and human milk exosomes may be able to prevent this.

Human milk exosomes may differ between mothers that gave birth to term infants or preterm infants. Furthermore, TM exosomes may have the potential to subdue and contain the inflammatory response and promote immune tolerance in intestinal epithelial cells, two functions that appear vital in preventing the occurrence of NEC [11].

Despite human milk being the 'gold standard' of infant feeding [40], in neonatal intensive care unit practice, there are circumstances where human milk is not available. Therefore, it is imperative to continue to study the mechanisms by which human milk modulates neonatal growth and development, to allow for further, more targeted research in preterm infant feeding strategies.

2. Hypothesis & Aims

2.1. Hypothesis 1

Human milk exosomal size, concentration, surface proteins and total protein content will differ between milks of mothers that gave birth to term or preterm infants.

2.1.1. Aims 1-3

1. Compare size and concentration of milk exosomes from TM or PTM.
2. Differentiate protein markers on the surface of TM or PTM exosomes to identify their cellular origin.
3. Analyze and compare total protein content of TM and PTM exosomes.

2.2. Hypothesis 2

TM exosomes will influence the inflammatory response in an *in vitro* intestinal epithelial cell model.

2.2.1. Aims 1-3

1. Demonstrate an inflammatory response in the Caco-2/15 intestinal epithelial cell model.
2. Asses the ability of TM exosomes to alter the inflammatory response in the Caco-2/15 intestinal epithelial cell model.

3. Methods

3.1. Collection of human milk

Sample collection was approved by the Ottawa Health Science Network Research Ethics Board (OHSN-REB) to collect milk from lactating females (OHSN-REB Protocol# 20170586-01H). Informed consent for collection of human milk was obtained from lactating females. Milk from five mothers that gave birth to term or preterm infants was collected using a manual pump or by hand expression. Milk was processed as described below within an hour of collection. Maternal and infant age, gender and parity was recorded when possible (Supplementary Table 1).

3.2. Human milk exosome purification

Exosomes were isolated through a series of centrifugations and ultracentrifugations. Briefly, whole milk was centrifuged twice at 4,600 x *g* for 30 minutes, at room temperature initially and 4°C the second centrifugation, to remove fat and cellular debris. The remaining skim layer was centrifuged at 20,000 x *g* for 30 minutes at 4°C to pellet vesicles larger than 200 nm. The supernatant was either stored at 4°C overnight or frozen at -80°C for longer term prior to ultracentrifugation. The supernatant was ultracentrifuged twice at 100,000 x *g* for 1.5 hours at 4°C. The pellet was resuspended in phosphate-buffered saline (PBS).

Exosomes isolated from five term mothers were pooled and exosomes isolated from five preterm mothers were pooled and run in triplicate for characterization of exosomes (section 3.3, 3.4, 3.5).

3.3. Nanoparticle tracking analysis of human milk exosomes

To determine particle size and concentration of TM and PTM exosomes the ZetaView instrument (Particle Metrix, Meerbusch, Germany) was used. Exosomes were diluted to 1:100,000 in PBS and inserted into the ZetaView sample compartment. Each pool of TM or PTM exosomes was measured in triplicate.

3.4. Flow cytometry analysis of human milk exosomes

Exosomal surface proteins on TM and PTM exosomes were identified using the MACSPlex human exosome kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as per the manufacturer's instructions. Briefly, total exosomal protein concentration was measured with Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States) using bovine serum albumin (BSA) as a standard. Exosome preparations with 4-20 μg of protein were incubated with 37 different antibody-coated capture beads and subsequently Exosome Detection Reagent cocktail of Allophycocyanin (APC)-conjugated CD9, CD63, and CD81. The flow cytometer BD LSRFortessa (BD Biosciences, San Jose, California, United States) located at the UOttawa Flow Cytometry and Virometry Core was used along with Flowjo VX (FlowJo, LLC, Ashland, Oregon, United States) software for analyses. A minimum of 10,000 events was measured per sample. The side scatter and forward scatter was selected for the multiplex beads. The single bead populations which were APC-positive were separated from those that were not to identify those bound to exosomes. The beads coated with different antibodies that were bound to the milk exosomes were identified, as per the manufacturer's instructions.

3.5. Human milk exosomal protein preparation for mass spectrometry analysis

The exosomes from each cohort, TM and PTM, were vortexed at a low speed for five seconds. The sodium dodecyl sulfate (SDS) solubilization solution (8 M urea, 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8, 0.05% SDS, 35 mM ethylene-diamine-tetra-acetic acid (EDTA), 5% glycerol) was added at a 1:4 ratio with sample and vortexed for five seconds. The concentration of protein was quantified by a Bradford Assay. Tris (2-carboxyethyl) phosphine (TCEP) was added to 30 µg of protein from each sample at a ratio of 1:25 and incubated at room temperature for 55 minutes on a shaker (450 rpm) in the dark. A ratio of 1:25 iodoacetamide was added and incubated at room temperature for 55 minutes on a shaker (450 rpm) in the dark. Trypsin/Lys-C solution (Promega, Madison, Wisconsin, United States) was added to the samples and incubated overnight at room temperature on a shaker (450 rpm) in the dark. Formic acid was added to the sample and vortexed to mix well. The sample was centrifuged at 10,000 x *g* for 1 minute and desalted using TopTips (Fisher Scientific, Waltham Massachusetts, United States). To desalt, the TopTips were washed three times with 50 µL of 70% acetonitrile, 30% water and 0.1% formic acid and washed three times with 50 µL of 100% water and 0.1% formic acid prior to adding the sample. After adding the sample, the TopTips were washed three times with 50 µL of 100% water and 0.1% formic acid. The sample was eluted three times with 50 µL of 70% acetonitrile, 30% water and 0.1% formic acid and placed in a speed vacuum to dry. The samples were run in triplicate on the Thermo Scientific™ Orbitrap Fusion™ Tribrid™ (Waltham, Massachusetts, United States).

Gene Ontology data analyses were performed using Protein ANalysis THrough Evolutionary Relationships (PANTHER) [92, 93].

3.6. Caco-2/15 intestinal epithelial cell model culture

The human intestinal epithelial cell line Caco-2/15, a clone of the parental Caco-2 cell line was used as a model of the small intestine. Cells were cultured as per the standard protocol developed in the Beaulieu lab at the University of Sherbrooke [94-96]. Briefly, they were cultured in 6-well tissue culture dishes (Falcon Plastics, Los Angeles, California, United States) at 37°C, in an atmosphere of 5% $C0_2$-95% air, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM glutamine, 10% fetal bovine serum (Gibco, Burlington, Ontario, Canada) and 20 mM HEPES. Caco-2/15 cells underwent a full enterocytic differentiation process that occurred spontaneously once confluency was reached and was completed, approximately 25-30 days post-seeding [94-96].

3.7. Caco-2/15 cell response to heat-killed bacteria

Heat-killed bacteria (*Escherichia coli* and *Salmonella typhimurium*) at a concentration of 10^{10} CFU/mL was added to the media of post-confluent Caco-2/15 cells. The cells were harvested at different timepoints (0, 1.5, 3, 4.5, 6, 12 and 24 h) after exposure to the heat-killed bacteria and compared to unstimulated controls. Total RNA was extracted with TRIzol (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's protocol. Quality control assessment of the RNA was performed using the Agilent Bioanalyzer (Agilent, Santa Clara, California, United States). Expression of pro-

inflammatory cytokines CXCL8, CXCL10 and CCL2 mRNA was measured by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA (1 µg) was used for first-strand cDNA synthesis using Superscript II (Invitrogen). The Mx3000P real-time PCR system (Stratagene, Cedar Creek, Texas, United States) was used to thermo-incubate reactions beginning with 10 minutes of Taq activation (95°C), 40 cycles of melting, primer annealing and extension. Measurements were performed in duplicates. The primer sequences were generated using Primer3 software: CXCL8 sense, GTGCAGTTTTGCCAAGGAGT; CXCL8 antisense, CTCTGCACCCAGTTTTCCTT; CXCL10 sense, CTGTACGCTGTACCTGCATCA; CXCL10 antisense, TTCTTGATGGCCTTCGATTC; CCL2 sense, CCCCAGTCACCTGCTGTTAT and CCL2 antisense, AGATCTCCTTGGCCACAATG.

Differences in gene expression were calculated by comparing ΔCt ($r\Delta Ct = Ct_{reference\ gene} - Ct_{target\ gene}$) of the heat-killed bacteria stimulated versus the unstimulated, normalizing with ribosomal protein lateral stalk subunit, *RPLP0*, as the validated reference gene.

3.8. Pre-treatment of Caco-2/15 cells with human milk exosomes

Post-confluent Caco-2/15 cells were incubated with culture medium or medium containing purified human milk exosomes (20-30 µg/ml of protein, three biological replicates, each in triplicate) for 22 hours. Heat-killed bacteria was added for 2 hours. The cells were harvested, and RNA was extracted as previously described. Cytokine expression of IL8, IL6, CXCL10, TNFα, CCL2, IL1b and IL10 was evaluated by real-time qRT-PCR.

3.9. Statistics

Two-way ANOVA test was used to compare cytokine expression in cells pre-treated with human milk exosomes and those not treated. Statistical significance was set at $p < 0.05$.

4. Results

4.1. Characterization of milk exosomes

4.1.1. Size and concentration of vesicles in the exosomal fraction from human milk

The exosomal fraction isolated from human milk through a series of centrifugations and ultracentrifugations was within the expected size range for exosomes: between 20 to 200 nm [76]. However, there were vesicles larger than 200 nm in both TM and PTM, these vesicles may be apoptotic bodies or blebs. The median size of the particles in the exosomal fraction isolated from TM was 94.07 nm, and the average was 100.00 ± 3.35 nm. The median size of the particles in the exosomal fraction isolated from PTM was 112.73 nm, and the average was 121.67 ± 2.10 nm (Figure 4). The size of the vesicles in the exosomal fraction from TM and PTM was not significantly different ($p < 0.05$). The concentration of particles as measured by the ZetaView in the exosomal fraction from TM and PTM was $1.50 \times 10^7 \pm 2.16 \times 10^6$ particles/mL and $5.63 \times 10^7 \pm 1.70 \times 10^6$ nm, respectively. The concentration was normalized to the volume of milk. PTM contained a significantly higher concentration of particles in the exosomal fraction compared to TM ($p < 0.05$).

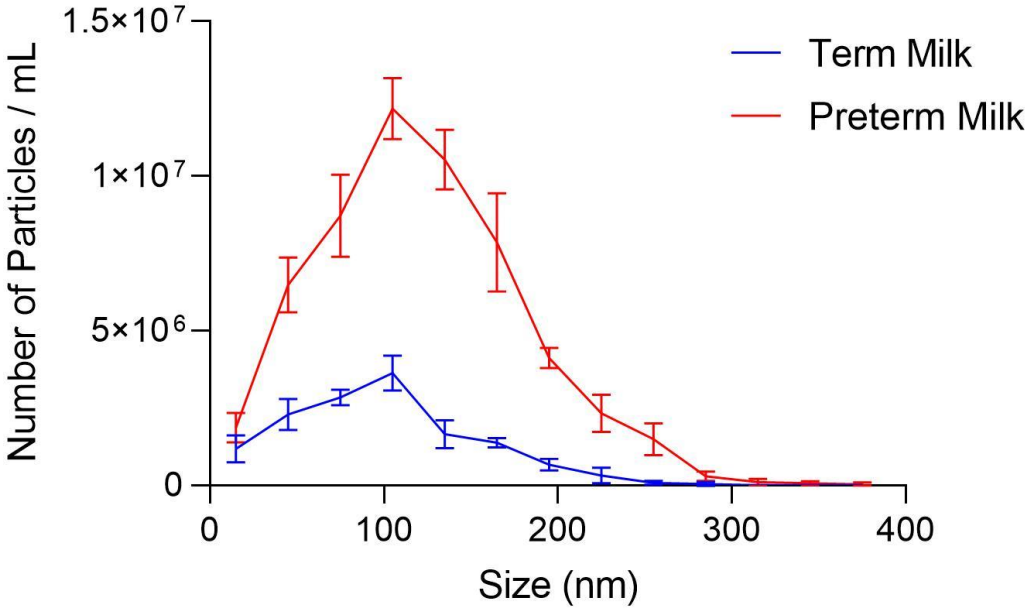

1.5×10^7
1×10^7
5×10^6
0
Number of Particles / mL
0
100
200
300
400
Size (nm)
Term Milk
Preterm Milk

Figure 4. Nanoparticle tracking analysis of particles in the exosomal fraction isolated from term milk and preterm milk. Milk exosomes were isolated from term milk or preterm milk through a series of centrifugations and ultracentrifugations. The size distribution and concentration of the exosomal fraction from the milk were measured by the ZetaView instrument. There was a lower concentration of exosomes isolated from term milk (blue, n=3) than from preterm milk (red, n=3).

4.1.2. Surface protein identification on human milk exosomes

The exosomal fraction isolated from TM and PTM through a series of centrifugations and ultracentrifugations was analyzed for exosomal surface markers to validate that exosomes were present. The MACSPlex human exosome kit (Miltenyi Biotec, Bergisch Gladbach, Germany), was used to identify key exosomal surface protein markers, as well as other surface proteins to predict the cellular origin of milk exosomes. There were vesicles in the exosomal fraction from milk that were positive for all or either CD9, CD63 or CD81, known exosomal tetraspanins [25, 75-77, 79, 81]. The vesicles that were positive for all or either of these three known exosomal tetraspanins were gated, separating exosomes from other potential contaminating extracellular vesicles. The gated population was used to identify other exosomal surface proteins that may provide insight into the cellular origin of exosomes in human milk (Table 1, 2). The kit allowed for identification of 37 different potential surface exosomal proteins (Supplementary Figure 5). The expression of surface proteins differed between TM and PTM exosomes. PTM exosomes presented unique surface proteins: CD29, CD146, CD105 and ROR1 (Table 2, Figure 5). PTM exosomes contained surface proteins from endothelial cells, while TM exosomes did not. TM and PTM exosomes had 12 common surface proteins, and of those 12, three were the known exosomal marker proteins: CD9, CD63 and CD81. The other nine surface proteins common to both groups are known to be expressed on a variety of immune cells and stem cells (Table 1). It is possible that the exosomes in milk originate from the range of immune cells present in milk (Figure 1) [68]. The endothelial protein markers may indicate that exosomes present in milk may be issuing from the mothers' endothelial cells, thus crossing the mammary epithelium.

Surface Protein	Distribution	Miltenyi MACSPlex human exosome kit identification[1]	MS^2 Confirmation PTM	MS^2 Confirmation TM
CD3	T cells [97]	Yes	No	No
CD14	Monocytes, macrophages and Langerhans cells [98]	Yes	Yes	Yes
CD24	Stem cells, mature granulocytes and B cells [99-101]	Yes	No	No
CD45	Haematopoietic stem cells (except for erythrocytes) [102]	Yes	Yes	Yes
CD56	Natural killer cells mainly but can also be expressed by alpha T cells, dendritic cells and monocytes [103]	Yes	No	No
CD133/31	Stem cells [104]	Yes	Yes	Yes
CD326	Most epithelial cells [105]	Yes	Yes	Yes
MHC class I	Nucleated cells [106]	Yes	Yes	Yes
MHC class II	B cells, tissue associated macrophages, Lymphoid progenitor stem cells, T cells [107]	Yes	Yes	Yes

[1]Flow cytometry identification using the Miltenyi MACSPlex human exosome kit

[2]MS mass spectrometry, Thermo Scientific™ Orbitrap Fusion™ Tribrid™

Table 1. Surface proteins common to exosomes isolated from term and preterm milk. Exosomes were isolated from term milk and preterm milk and surface proteins were identified through flow cytometry with the Miltenyi MACSPlex human exosome kit. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses of the total proteins in human milk exosomes was used to validate the surface proteins identified through flow cytometry with the Miltenyi MACSPlex human exosome kit. Legend: TM, exosomes isolated from term milk; PTM, exosomes isolated from preterm milk.

Surface Protein	Distribution	Miltenyi MACSPlex human exosome kit identification[1]	MS[2] confirmation
CD29	T cells, epithelial cells, myoepithelial cells and mesenchymal stromal cells [108, 109]	Yes	Yes
CD146	Mesenchymal stem cells, endothelial cells, smooth muscle cells and pericytes [110]	Yes	Yes
CD105	Endothelial cells [111]	Yes	Yes
ROR1	Cephalic mesenchymal cells and neural crest cells [112]	Yes	No

[1]Flow cytometry identification using the Miltenyi MACSPlex human exosome kit

[2]MS mass spectrometry, Thermo Scientific™ Orbitrap Fusion™ Tribrid™

Table 2. Surface proteins unique to exosomes isolated from preterm milk. Exosomes were isolated from term milk and preterm milk and surface proteins were identified through flow cytometry with the Miltenyi MACSPlex human exosome kit. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses of the total proteins in human milk exosomes was used to validate the surface proteins identified through flow cytometry with the Miltenyi MACSPlex human exosome kit. This table contains surface proteins unique to preterm milk exosomes.

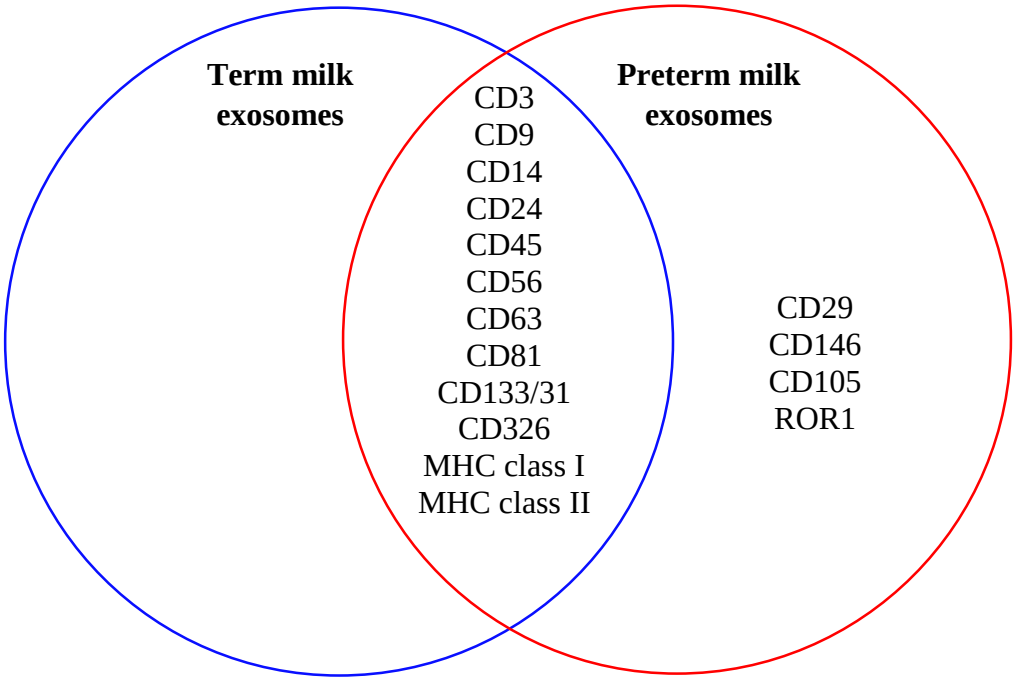

Term milk exosomes
CD3
CD9
CD14
CD24
CD45
CD56
CD63
CD81
CD133/31
CD326
MHC class I
MHC class II
Preterm milk exosomes
CD29
CD146
CD105
ROR1

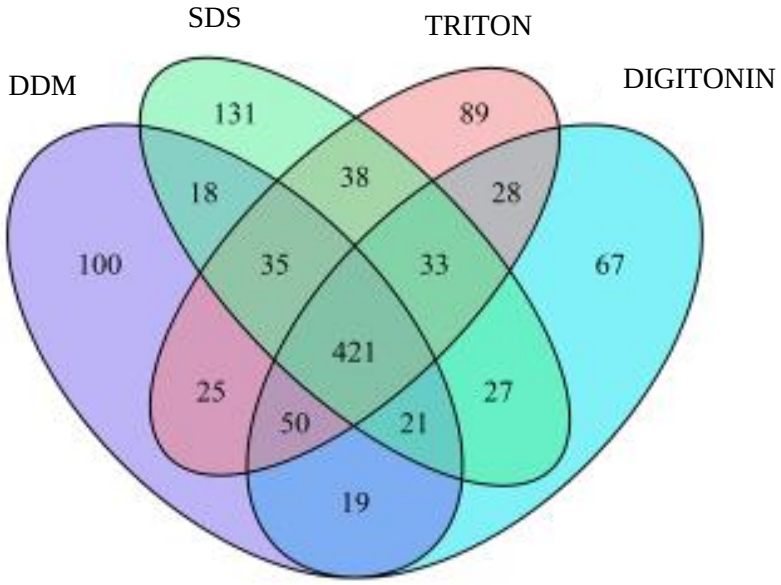
Figure 5. Venn diagram of surface proteins common to exosomes isolated from term milk and preterm milk. Exosomes isolated from term milk (blue circle) or preterm milk (red circle) contain surface proteins that were identified through flow cytometry with the Miltenyi MACSPlex human exosome kit.

4.1.3. Optimization of human milk exosomal protein preparation for LC-MS/MS

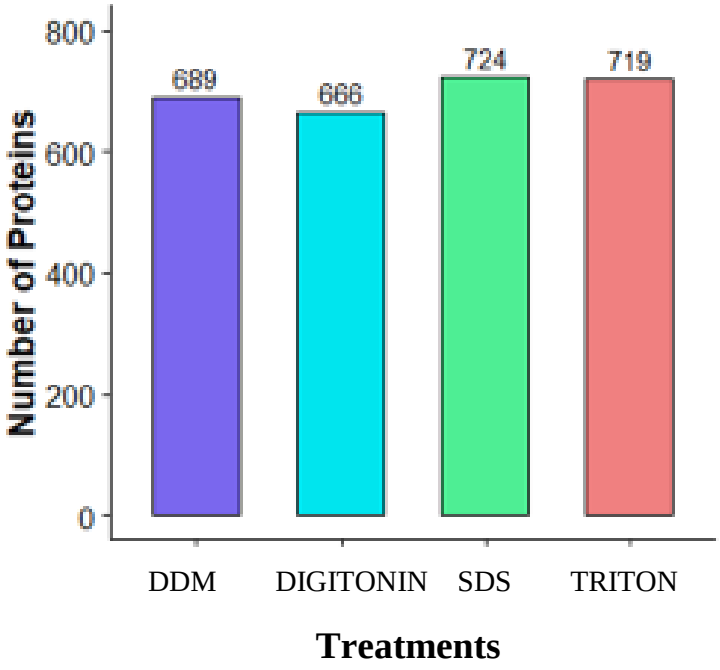
Few studies have performed liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis on human milk exosomes, therefore digestion optimization was required [25, 113]. Four detergents were tested to determine the optimal milieu allowing maximal digestion of arginine- and lysine-involving peptide bonds in human milk exosome proteins. SDS, Digitonin, n-dodecyl β-D-maltoside (DDM), and Triton X-100 were tested as detergents to allow Trypsin/Lys-C to cleave peptides. The number of protein IDs identified with high and medium confidence using different detergents, SDS, Digitonin, DDM and Triton X-100 was 724, 666, 689 and 716, respectively. Digesting the exosomes with SDS as the detergent provided the highest number of protein IDs (724), as well as 131 unique proteins (Figure 6).

Gene ontology analyses performed on the proteins identified using different detergents revealed that their molecular functions, biological processes and cellular components did not vary depending on detergent (Supplementary Figure 1, 2, 3). For this reason, SDS was tested with different concentrations of EDTA and the mild denaturant RapiGest SF (Waters, Sweden) to minimize the damage to the mass spectrometer instrument that SDS causes. SDS with 0, 5 or 7 mM of EDTA or 10 mM of RapiGest was used to disrupt the milk exosomes while unfolding the proteins present, and resulted in 678, 691, 720 and 628 identified high and medium confidence proteins, respectively. SDS with 7 mM of EDTA resulted in 720 identified proteins, the highest number, and 157 unique proteins (Figure 7). Mass spectrometry analysis of each of the four detergents revealed SDS with 7 mM of EDTA to be the optimal detergent based on the highest

number of protein IDs with high and medium confidence identified. Thus, the five pooled biological replicates from both TM and PTM were digested following this protocol.

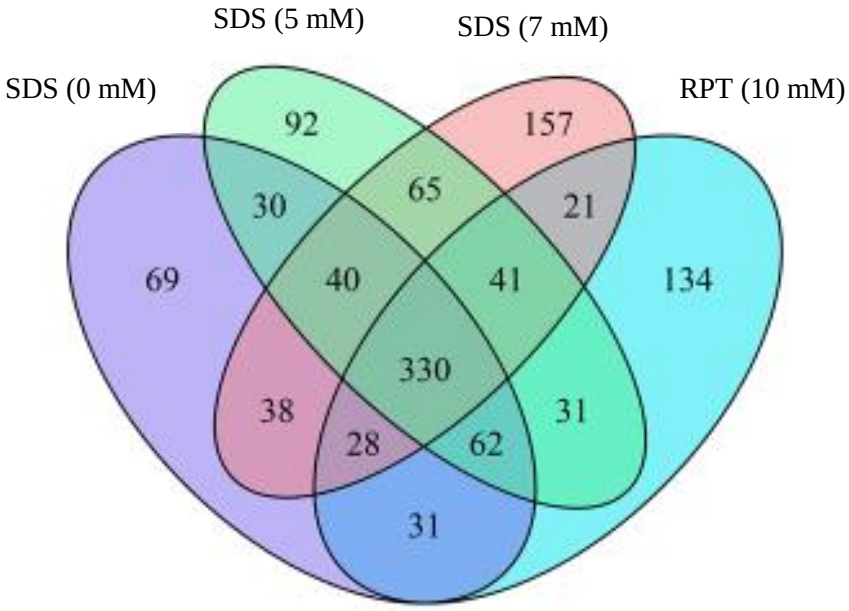

SDS
TRITON
DDM
DIGITONIN
131
89
18
38
28
100
35
33
67
421
25
27
50
21
19

A)

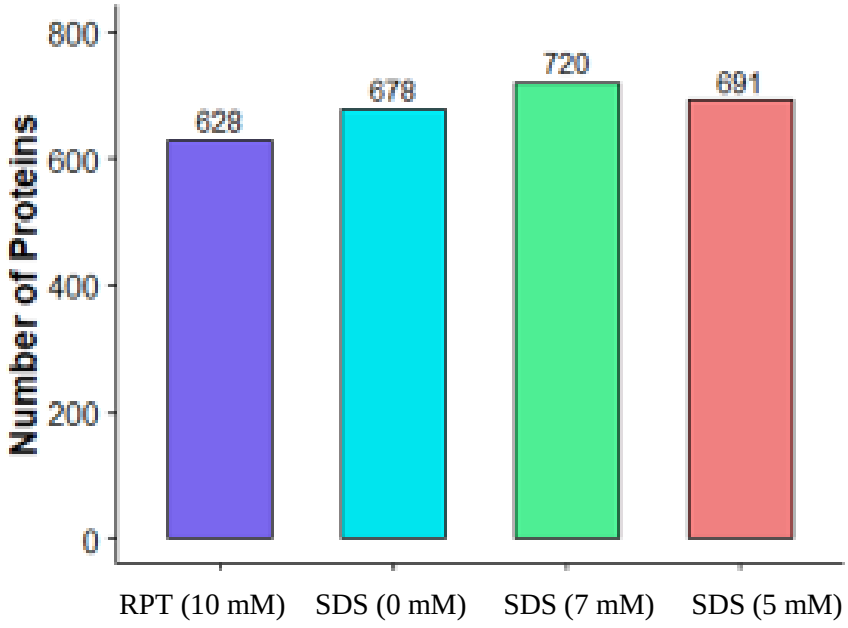

800
600
400
200
0
Number of Proteins
689
666
724
719
DDM
DIGITONIN
SDS
TRITON
Treatments

B)

Figure 6. The number of proteins identified using different detergents to allow trypsin to digest proteins from human milk exosomes. Sodium dodecyl sulfate (SDS), Digitonin, n-dodecyl β-D-maltoside (DDM), and Triton X-100 were used as detergents to disrupt human milk exosomes to allow for Trypsin/Lys-C to cleave peptides. The peptides were identified with liquid chromatography tandem mass spectrometry (LC-MS/MS). A) Venn diagram representing the number of unique and common proteins from milk exosomes identified with LC-MS/MS using SDS, DDM, Triton X-100 or Digitonin as detergents with Trypsin/Lys-C. B) Bar chart comparing the number of identified proteins from milk exosomes with high and medium confidence from LC-MS/MS using SDS, DDM, Triton X-100 or Digitonin as detergents with Trypsin/Lys-C (n=1).


SDS (5 mM)
SDS (7 mM)
SDS (0 mM)
RPT (10 mM)
92
157
30
65
21
69
40
41
134
330
38
31
28
62
31

A)


800
600
400
200
0
Number of Proteins
628
678
720
691
RPT (10 mM)
SDS (0 mM)
SDS (7 mM)
SDS (5 mM)
Treatments

B)

Figure 7. The number of proteins identified using sodium dodecyl sulfate (SDS) with different concentrations of ethylene-diamine-tetra-acetic acid (EDTA) or RapiGest to allow trypsin to digest proteins from human milk exosomes. SDS with 0, 5 or 7 mM of EDTA and 10 mM of RapiGest (RPT) was used to disrupt human milk exosomes to allow for Trypsin/Lys-C to cleave peptides to be identified with liquid chromatography tandem mass spectrometry (LC-MS/MS). A) Venn diagram representation of the number of unique and common proteins from human milk exosomes identified with LC-MS/MS using SDS with 0, 5 or 7 mM of EDTA or 10 mM of RapiGest with Trypsin/Lys-C. B) The number of proteins from human milk exosomes identified with high and medium confidence in LC-MS/MS using SDS with 0, 5 or 7 mM EDTA or 10 mM of RapiGest with Trypsin/Lys-C (n=1).

4.1.4. Comparing proteins from TM and PTM exosomal fractions

LC-MS/MS was used to validate the surface protein markers identified on human milk exosomes with the MACSPlex human exosome kit and flow cytometry. Tables 1 and 2 indicate whether the surface protein markers identified through flow cytometry were confirmed with mass spectrometry. CD3, CD24, CD56 and ROR1 protein IDs were not identified through LC-MS/MS analyses in the exosomal samples from TM or PTM.

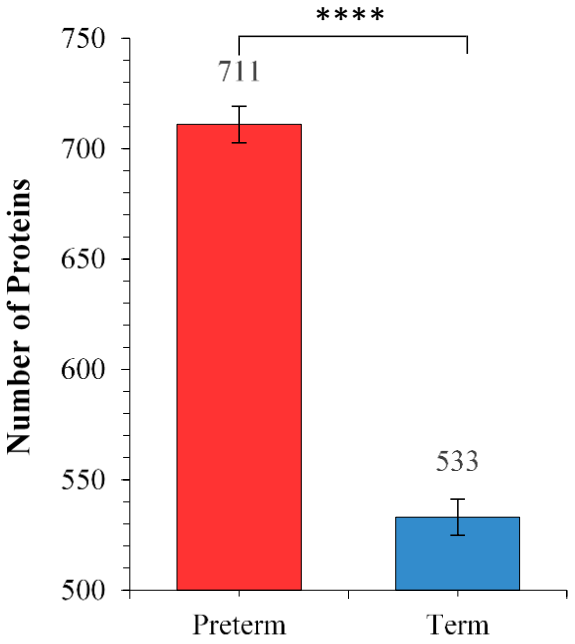
Mass spectrometry analysis of the two cohorts of mothers' milk exosomes demonstrated that PTM exosomes contain a significantly higher number of total proteins than TM exosomes (Figure 8A). PTM exosomes had as many as 321 unique proteins and TM exosomes contained 143 unique proteins (Figure 8B, C).

The biological processes of the total proteins isolated from TM and PTM exosomes did not vary with respect to the total number of proteins (Figure 9A). However, the unique proteins in PTM exosomes did contain a higher percentage of proteins related to metabolic and developmental processes. TM exosomes had a higher percentage of unique proteins related to biological adhesion, localization and cellular compartment organization/biogenesis (Figure 9B).

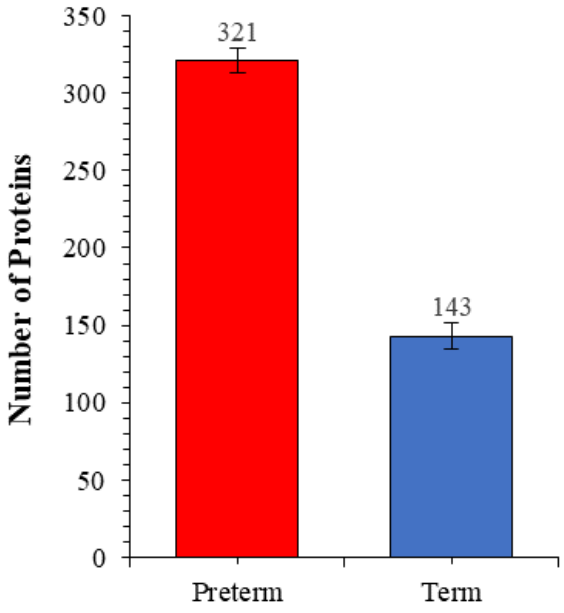
The molecular functions of the total proteins isolated from TM and PTM exosomes also did not vary with respect to the total number of proteins (Figure 10A). PTM exosomes contained unique proteins involved in transcription regulator activity, cargo receptor activity, and translation regulator activity. TM contained more unique proteins related to structural activity (Figure 10B).

The cellular components of the proteins from TM and PTM did not vary, even within the unique protein subsets (Figure 11A, B).

The protein classes of the total proteins did not vary with respect to the total number of proteins isolated from TM and PTM exosomes (Figure 12A). TM exosomes contained approximately quadruple the number of unique proteins related to membrane trafficking. Storage and transmembrane receptor regulatory/adaptor proteins were unique to TM exosomes. PTM exosomes contained unique proteins classified as extracellular matrix proteins (Figure 12B).

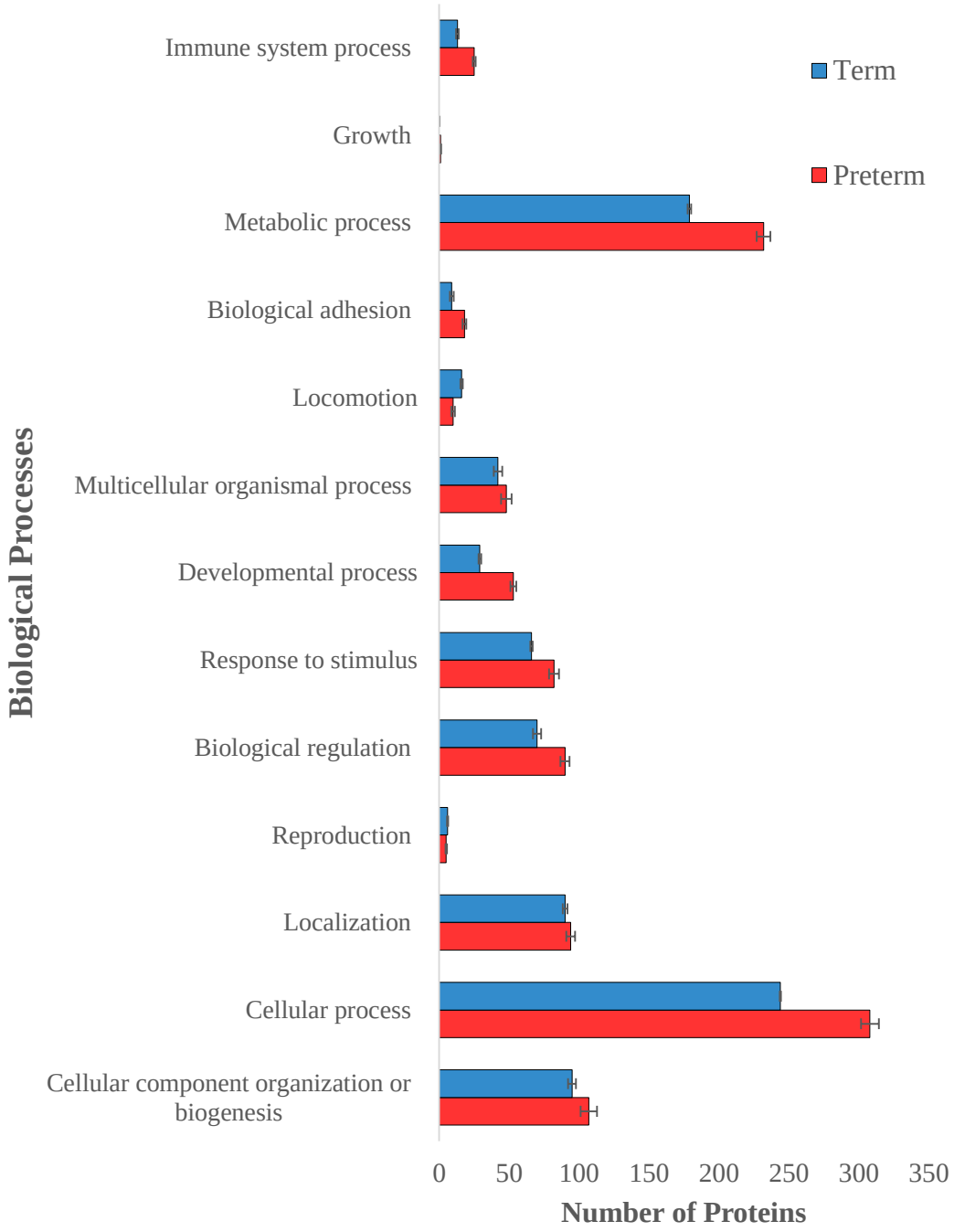

****
750
700
650
600
550
500
711
533
Number of Proteins
Preterm
Term

A)

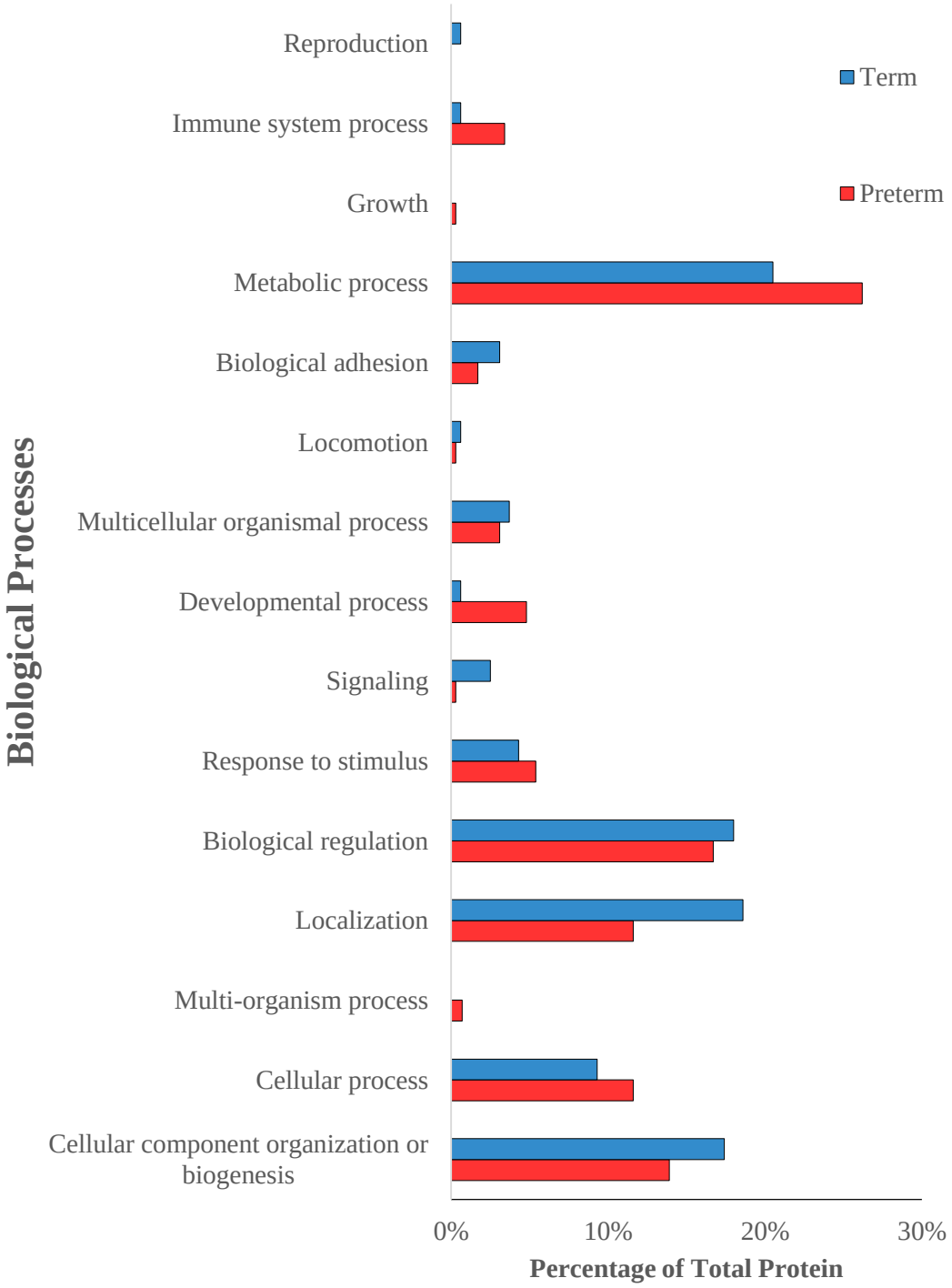

350
300
250
200
150
100
50
0
321
143
Number of Proteins
Preterm
Term

B)

Figure 8. Number of proteins identified through LC-MS/MS from exosomes isolated from term milk or preterm milk. Five biological replicates of milk exosomes from each group were pooled and the number of proteins identified through liquid chromatography tandem mass spectrometry (LC-MS/MS) were compared between the two cohorts (n=3). A) The total number of proteins with high and medium confidence common to replicate runs of pooled exosomal samples from term milk or preterm milk. B) The number of unique proteins identified in preterm milk exosomes or term milk exosomes. Legend: Term, exosomes isolated from term milk; Preterm, exosomes isolated from preterm milk; ****, $p < 0.0001$ using Welch's t-test.

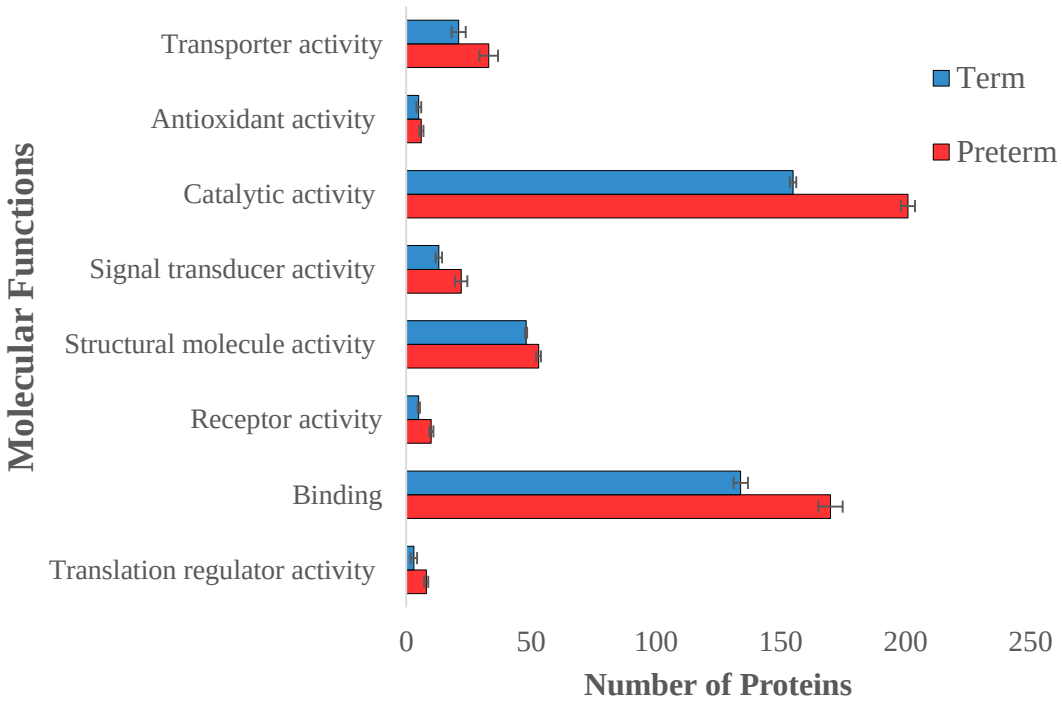

Immune system process
Growth
Metabolic process
Biological adhesion
Locomotion
Multicellular organismal process
Developmental process
Response to stimulus
Biological regulation
Reproduction
Localization
Cellular process
Cellular component organization or biogenesis
Biological Processes
Term
Preterm
0
50
100
150
200
250
300
350
Number of Proteins

A)

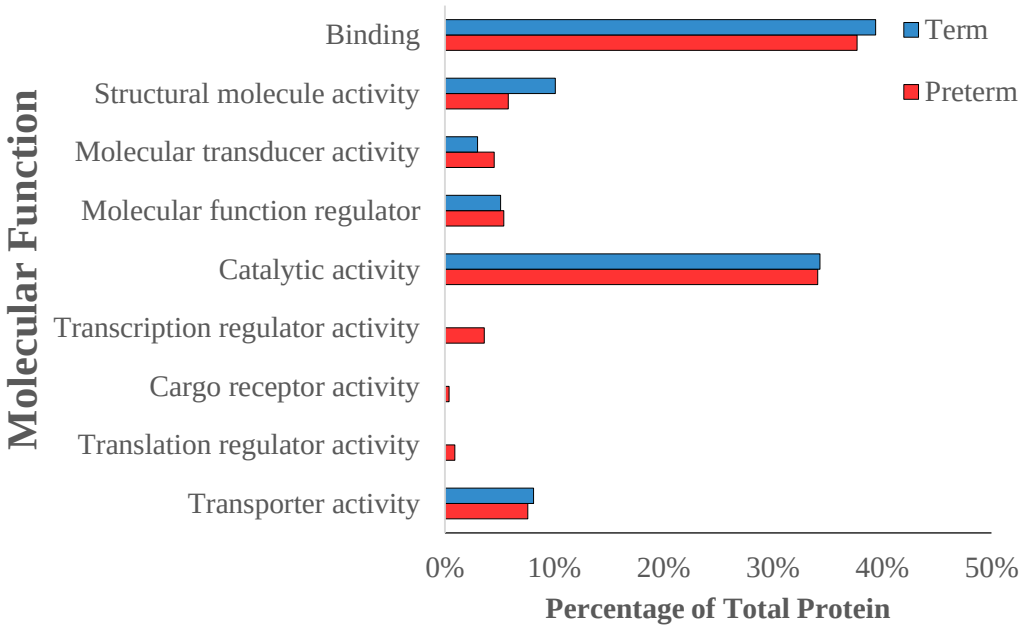

Biological Processes
Reproduction
Immune system process
Growth
Metabolic process
Biological adhesion
Locomotion
Multicellular organismal process
Developmental process
Signaling
Response to stimulus
Biological regulation
Localization
Multi-organism process
Cellular process
Cellular component organization or biogenesis
Term
Preterm
0%
10%
20%
30%
Percentage of Total Protein

B)

Figure 9. Biological related processes of proteins identified through LC-MS/MS from exosomes isolated from term milk or preterm milk. Five biological replicates of milk exosomes from each group were pooled and the proteins were identified through liquid chromatography tandem mass spectrometry (LC-MS/MS). The biological processes of the identified proteins were compared between term and preterm milk exosomes (n=3). The Protein ANalysis THrough Evolutionary Relationships (PANTHER) database was used to identify the biological processes of the proteins in milk exosomes [92]. A) The biological processes of the proteins found in human milk exosomes from term and preterm milk. B) The biological processes of the unique proteins from term milk exosomes or preterm milk exosomes expressed as percentage of total unique proteins. Legend: Term, exosomes isolated from term milk; Preterm, exosomes isolated from preterm milk.

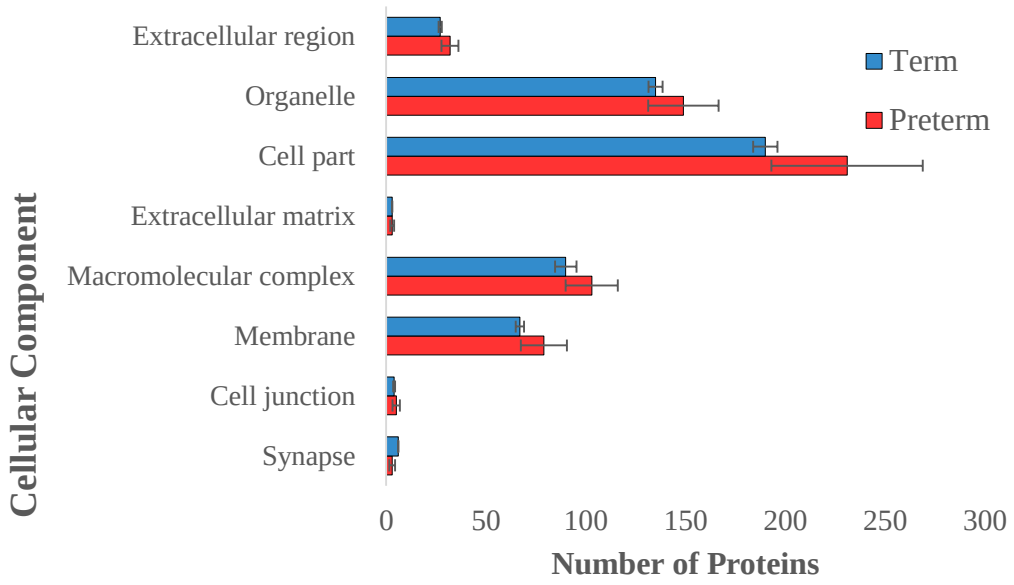

Molecular Functions
Transporter activity
Antioxidant activity
Catalytic activity
Signal transducer activity
Structural molecule activity
Receptor activity
Binding
Translation regulator activity
Term
Preterm
0
50
100
150
200
250
Number of Proteins

A)

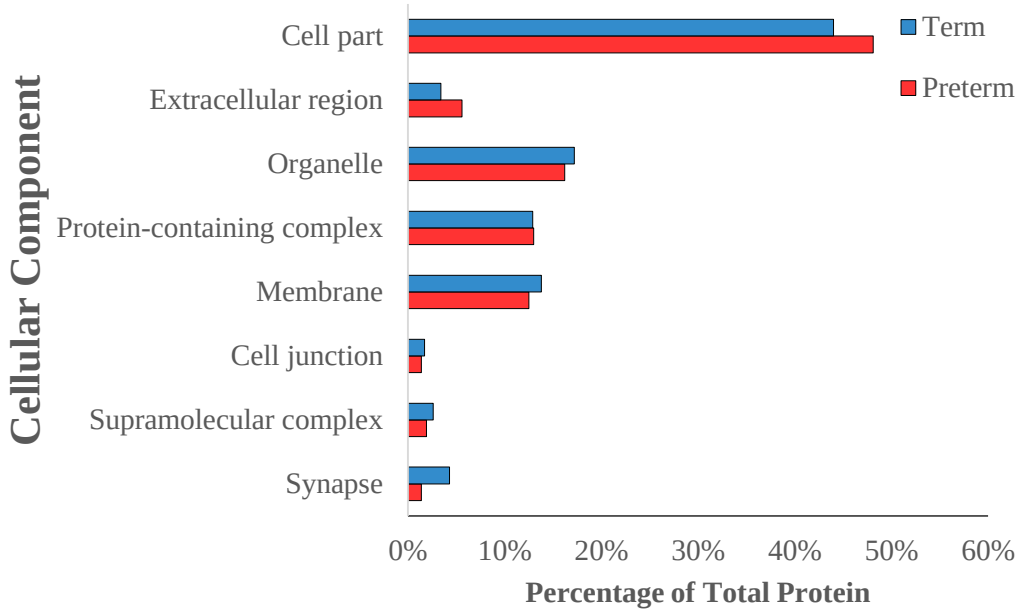

Molecular Function
Binding
Structural molecule activity
Molecular transducer activity
Molecular function regulator
Catalytic activity
Transcription regulator activity
Cargo receptor activity
Translation regulator activity
Transporter activity
Term
Preterm
0%
10%
20%
30%
40%
50%
Percentage of Total Protein

B)

Figure 10. Molecular functions of proteins identified through LC-MS/MS from exosomes isolated from term milk or preterm milk. Five biological replicates of milk exosomes from each group were pooled and the proteins were identified through liquid chromatography tandem mass spectrometry (LC-MS/MS). The molecular functions of the identified proteins were compared between term and preterm milk exosomes (n=3). The Protein ANalysis THrough Evolutionary Relationships (PANTHER) database was used to identify the molecular functions of the proteins in milk exosomes [92]. A) The molecular functions of the proteins found in human milk exosomes from term and preterm milk. B) The molecular functions of the unique proteins from term milk exosomes or preterm milk exosomes expressed as percentage of total unique proteins. Legend: Term, exosomes isolated from term milk; Preterm, exosomes isolated from preterm milk.

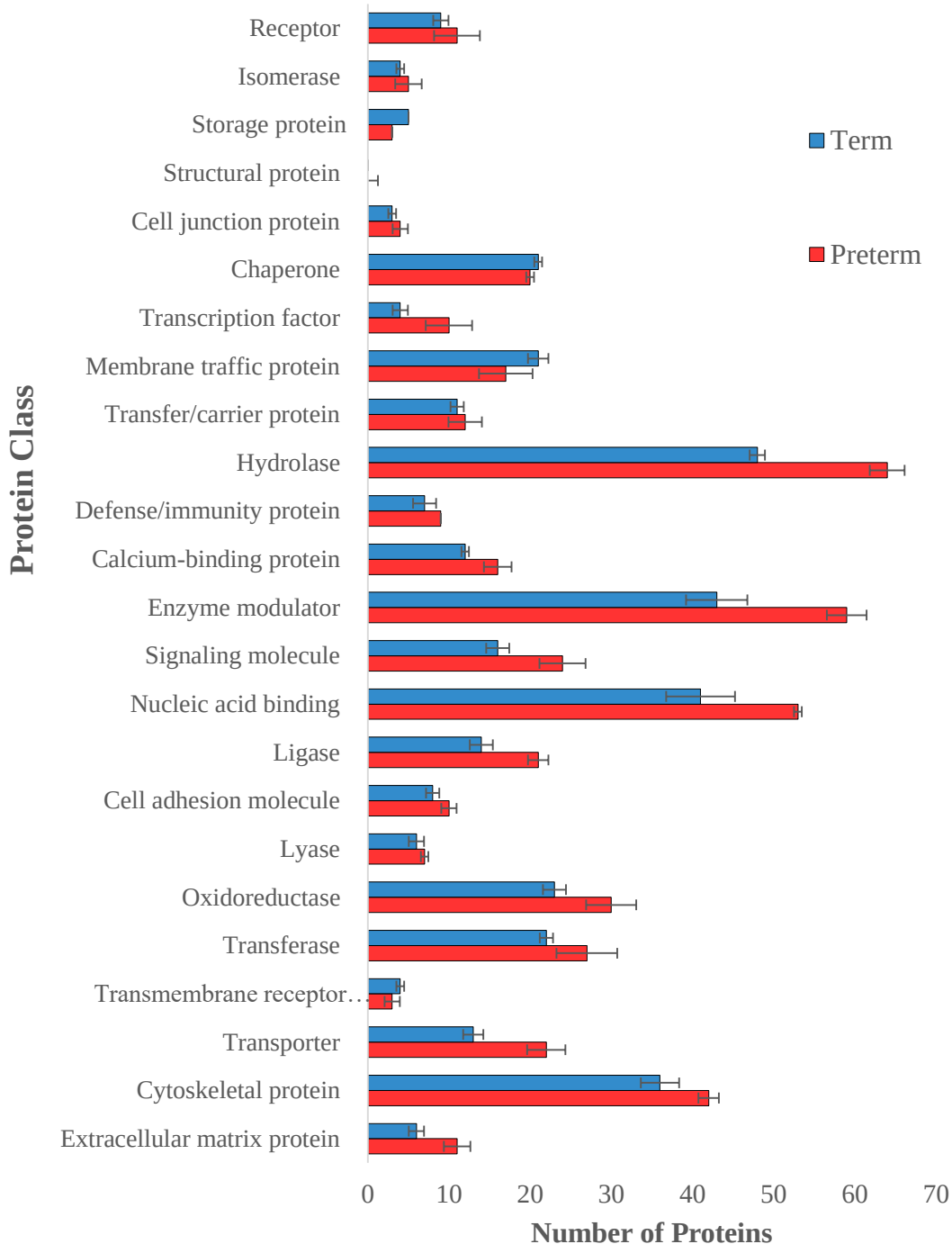

Extracellular region
Organelle
Cell part
Extracellular matrix
Macromolecular complex
Membrane
Cell junction
Synapse
Term
Preterm
Cellular Component
0
50
100
150
200
250
300
Number of Proteins

A)

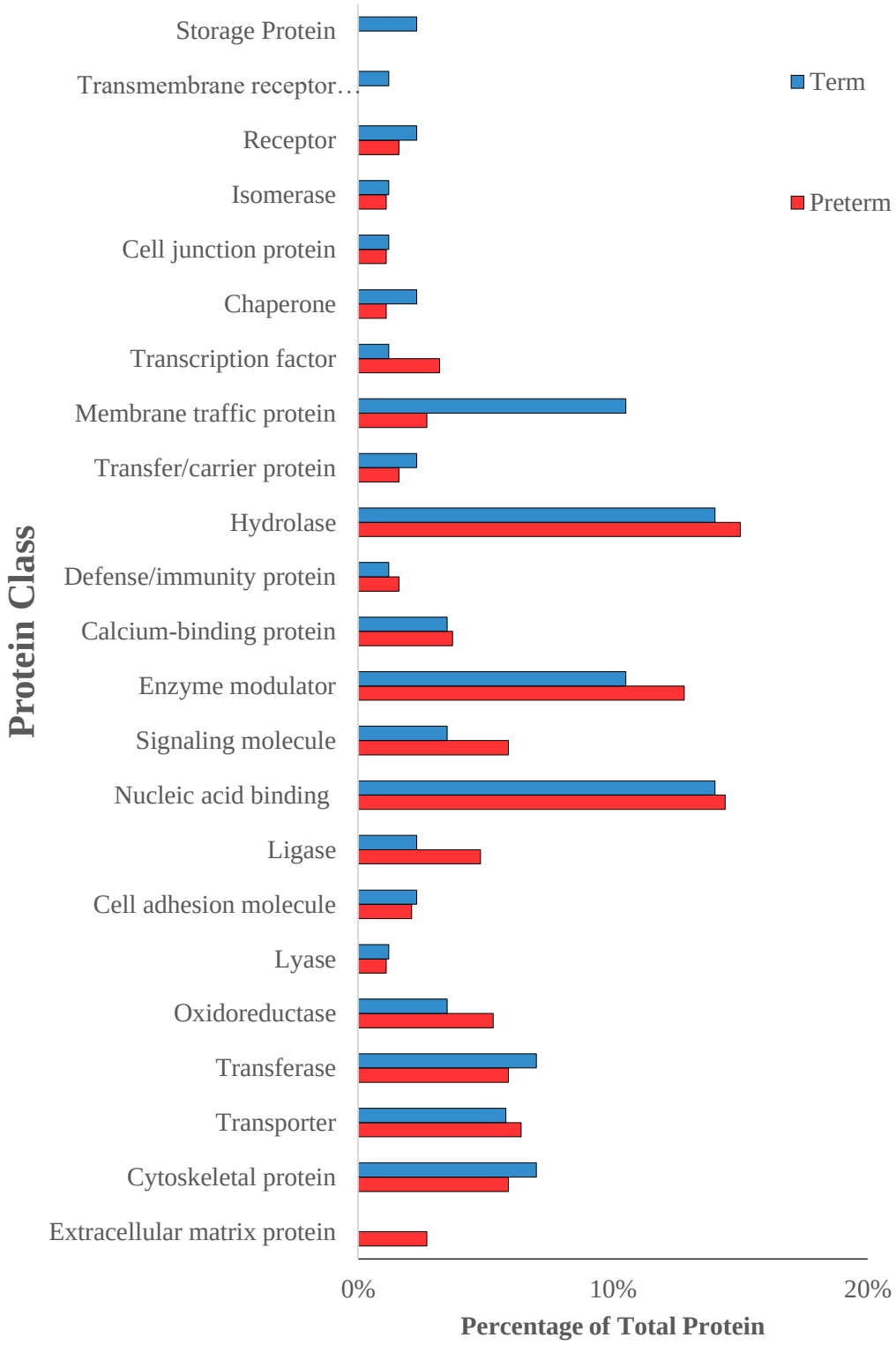

Cell part
Extracellular region
Organelle
Protein-containing complex
Membrane
Cell junction
Supramolecular complex
Synapse
Term
Preterm
Cellular Component
0%
10%
20%
30%
40%
50%
60%
Percentage of Total Protein

B)

Figure 11. Cellular components of proteins identified through LC-MS/MS from exosomes isolated from term milk or preterm milk. Five biological replicates of milk exosomes from each group were pooled and the proteins were identified through liquid chromatography tandem mass spectrometry (LC-MS/MS). The cellular components of the identified proteins were compared between term and preterm milk exosomes (n=3). The Protein ANalysis THrough Evolutionary Relationships (PANTHER) database was used to identify the cellular components of the proteins in milk exosomes [92]. A) The cellular components of the proteins found in human milk exosomes from term and preterm milk. B) The cellular components of the unique proteins from term milk exosomes or preterm milk exosomes expressed as percentage of total unique proteins. Legend: Term, exosomes isolated from term milk; Preterm, exosomes isolated from preterm milk.


Receptor
Isomerase
Storage protein
Structural protein
Cell junction protein
Chaperone
Transcription factor
Membrane traffic protein
Transfer/carrier protein
Hydrolase
Defense/immunity protein
Calcium-binding protein
Enzyme modulator
Signaling molecule
Nucleic acid binding
Ligase
Cell adhesion molecule
Lyase
Oxidoreductase
Transferase
Transmembrane receptor…
Transporter
Cytoskeletal protein
Extracellular matrix protein
Protein Class
Term
Preterm
0
10
20
30
40
50
60
70
Number of Proteins

A)


Storage Protein
Transmembrane receptor…
Receptor
Isomerase
Cell junction protein
Chaperone
Transcription factor
Membrane traffic protein
Transfer/carrier protein
Hydrolase
Defense/immunity protein
Calcium-binding protein
Enzyme modulator
Signaling molecule
Nucleic acid binding
Ligase
Cell adhesion molecule
Lyase
Oxidoreductase
Transferase
Transporter
Cytoskeletal protein
Extracellular matrix protein
Protein Class
Term
Preterm
0%
10%
20%
Percentage of Total Protein

B)

Figure 12. Protein class of proteins identified through LC-MS/MS from exosomes isolated from term milk or preterm milk. Five biological replicates of milk exosomes from each group were pooled and the proteins were identified through liquid chromatography tandem mass spectrometry (LC-MS/MS). The protein classes of the identified proteins were compared between term and preterm milk exosomes (n=3). The Protein ANalysis THrough Evolutionary Relationships (PANTHER) database was used to identify the protein classes of the proteins in milk exosomes [92]. A) The protein classes of the proteins found in human milk exosomes from term and preterm milk. B) The protein classes of the unique proteins from term milk exosomes or preterm milk exosomes expressed as percentage of total unique proteins. Legend: Term, exosomes isolated from term milk; Preterm, exosomes isolated from preterm milk; Transmembrane receptor…, Transmembrane receptor regulatory/adaptor protein.

4.2. Effect of human milk exosomes on Caco-2/15 inflammatory response

Post-confluent Caco-2/15 cells, prepared as described previously [96], were used for preliminary experiments to study the effects of TM exosomes on an intestinal model.

4.2.1. Inducing an inflammatory response in Caco-2/15 cells

Heat-killed *E. coli* and *S. typhimurium* was added to the media of post-confluent Caco-2/15 cells to demonstrate that the cells were capable of raising an inflammatory response. The inflammatory response was demonstrated by measuring the expression of pro-inflammatory cytokines: CXCL8, CCL2 and CXCL10. The heat-killed bacteria was added to the Caco-2/15 cell culture and incubated for various timepoints to determine when the inflammatory response was the strongest. The peak fold-change expression of these pro-inflammatory cytokines occurred between two to three hours after exposure of Caco-2/15 cells to heat-killed bacteria incubation. The expression of these cytokines was compared to that of post-confluent unstimulated Caco-2/15 cells (Figure 13).

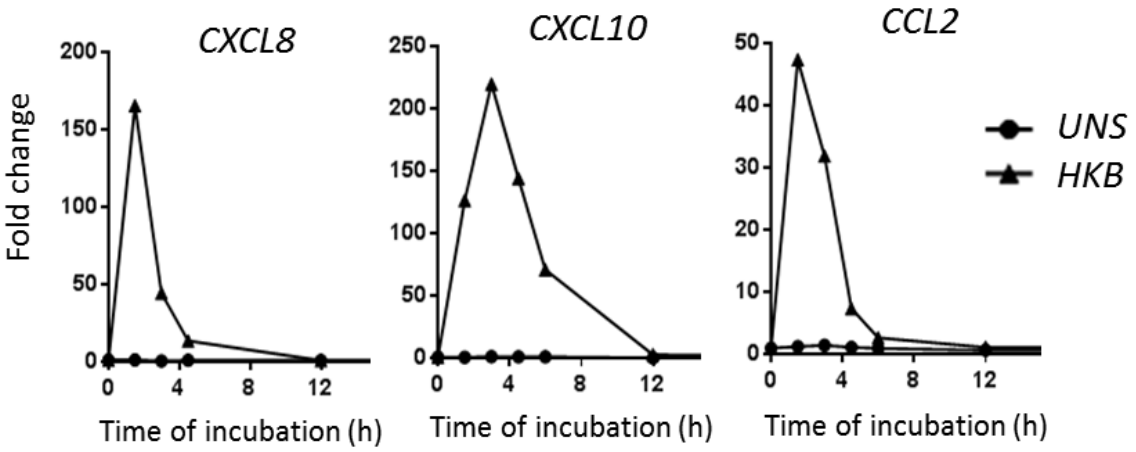

CXCL8
CXCL10
CCL2
Fold change
200
150
100
50
0
250
200
150
100
50
0
50
40
30
20
10
0
0
4
8
12
Time of incubation (h)
Time of incubation (h)
Time of incubation (h)
UNS
HKB

Figure 13. Pro-inflammatory cytokine, CXCL8, CXCL10 and CCL2 expression in Caco-2/15 cells is upregulated upon incubation with heat-killed bacteria. The fold-change in expression of pro-inflammatory cytokines CXCL8, CXCL10 and CCL2 between post-confluent Caco-2/15 cells incubated with heat-killed *E. coli* and *S. typhimurium* (HKB, triangles) and left unstimulated (UNS, circles). The expression of cytokine transcripts was measured by qRT-PCR and normalized to the reference gene, ribosomal protein lateral stalk subunit, *RPLP0* (n=1). Culture techniques and molecular methods followed standard protocols [12, 94, 96, 114, 115].

4.2.2. Bacteria-triggered inflammatory response in intestinal epithelial cells dampened when pretreated with human milk exosomes

To identify the potential effects of human milk exosomes on intestinal epithelial cells, TM exosomes were added to post-confluent Caco-2/15 cell culture medium prior to inducing an inflammatory response. Post-confluent Caco-2/15 cells were treated with TM exosomes or left untreated as a control. After 22 hours, an inflammatory response was induced by adding the heat-killed bacteria. After three hours of incubation with the heat-killed bacteria, the cells were harvested, and qRT-PCR was used to determine inflammatory cytokine expression levels of pro-inflammatory cytokines; IL8, CXCL10, TNF, CCL2, IL1b, IL6 and anti-inflammatory cytokine; IL10. The expression of the pro-inflammatory cytokines was significantly decreased in Caco-2/15 cells pretreated with human milk exosomes relative to the control (Figure 10) ($p < 0.05$). Anti-inflammatory cytokine IL10 expression was upregulated in cells pretreated with human milk exosomes, however, it was not significantly different ($p < 0.08$). These results suggest that human milk exosomes dampen an inflammatory response in Caco-2/15 cells in response to heat-killed bacteria.

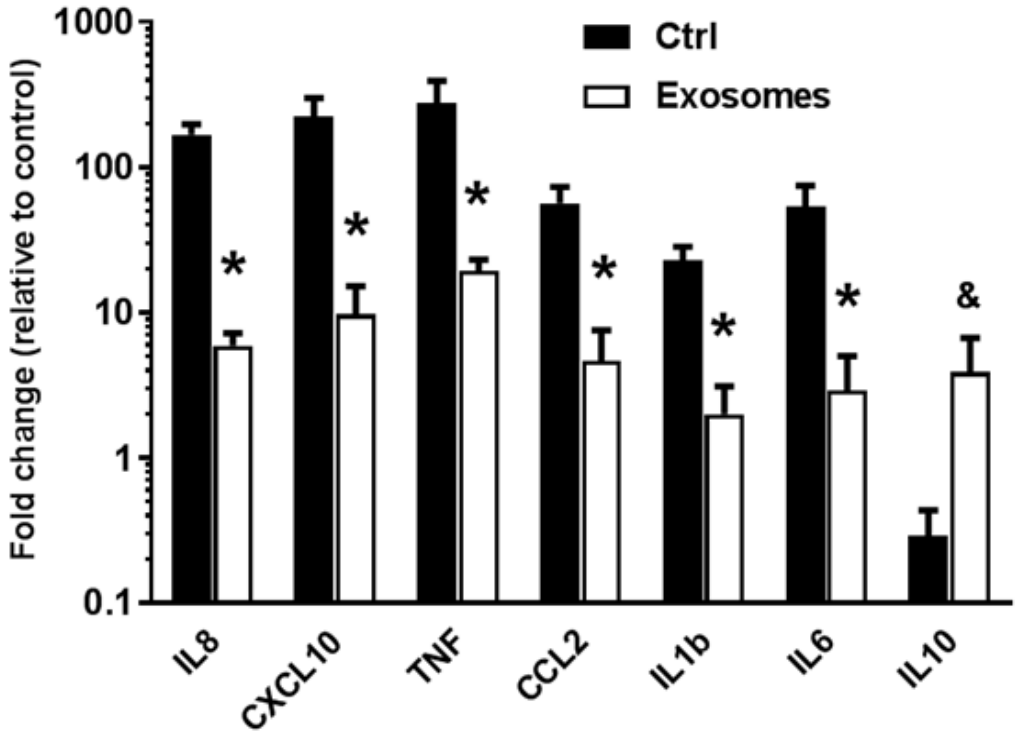

Ctrl
Exosomes
Fold change (relative to control)
1000
100
10
1
0.1
*
*
*
*
*
*
&
IL8
CXCL10
TNF
CCL2
IL1b
IL6
IL10

Figure 14. Attenuation of pro-inflammatory cytokine expression in Caco-2/15 cells pretreated for 22 hours with human milk exosomes after induced inflammation. Caco-2/15 cells incubated with term milk exosomes for 22 hours (Exosomes), prior to heat-killed *E. coli* and *S. typhimurium* exposure for three hours, expressed significantly ($p < 0.05$) less pro-inflammatory cytokines, IL8, CXCL10, TNF, CCL2, IL1b and IL6, compared to untreated cells (Ctrl). Expression of the anti-inflammatory cytokine, IL10, increased in cells pretreated with exosomes compared to those left untreated ($p < 0.08$). Values are the mean of three sets of Caco-2/15 cell culture with three biological replicates of exosome preparations. Legend: *, $p < 0.05$; &, $p < 0.08$ using 2-way ANOVA.

5. Discussion

Early enteral feeds set the stage for newborns with downstream effects lasting through adulthood. Human milk is the 'gold standard' of infant nutrition, however, sometimes it is not available or is insufficient for those born prematurely [40]. Premature infants are more susceptible to GI diseases and this propensity is thought to be due to the immaturity of the gut and lack of optimized nutrition for this population. To ensure the best possible health outcomes for all infants, it is necessary to understand how human milk has evolved to be the optimal source of nutrition for newborns. Human milk contains bioactive components that could be 'teased apart' from each other to identify their individual roles and effects on newborn growth and development. This study characterizes one of the components by focusing on human milk exosomes from TM and PTM and investigating their effects on human intestinal epithelial cells.

Exosomes are extracellular vesicles that are synthesized and secreted by most living cells as a method of cell-to-cell communication [75, 76, 78-80]. They are found in all biological fluids. Exosomes transfer RNA, microRNA, lipids and proteins to target cells to alter and influence gene expression [75-80]. There are immune-related microRNA and proteins in human milk exosomes that withstand degradation in harsh conditions [25, 31, 35, 41-43, 76, 78, 84, 89, 90]. Milk exosomes are taken up by intestinal epithelial cells *in vitro* and have been found to influence cell proliferation and prevent cell death [25, 42, 89, 116]. This present study focused on (1) characterizing exosomes from TM and PTM, and (2) understanding their potential to regulate inflammatory cytokine expression in an intestinal epithelial cell model.

5.1. Characterizing human milk exosomes based on size

Exosomes were isolated from TM and PTM employing methods from previous studies [117, 118]. The size range of the exosomal fraction isolated from milk through a series of centrifugations and ultracentrifugation contained particles that were within the known range of exosome diameter, 20-200 nm [76]. However, the isolates from both TM and PTM did contain particles larger than the known size range of exosomes, >200 nm (Figure 4). The larger particle contaminants may be other extracellular vesicles such as blebs and/or apoptotic bodies that were pelleted along with exosomes. For future experiments requiring purified milk exosome extracts, it would be beneficial to separate exosomes from other extracellular vesicles using more robust isolation methods that would minimize contaminating vesicles in the exosomal fraction. Sucrose gradient fractionation is another method to isolate exosomes, allowing for the separation of vesicles between 20-200 nm at a density of 1.15 to 1.19 g/mL [119]. However, other non-exosome vesicles or contaminating proteins may be isolated at the same density [76]. Bacteria release extracellular vesicles that range from 10 to 400 nm [120]. Bacterial extracellular vesicles are also isolated using traditional eukaryotic exosome isolation methods, such as ultracentrifugation and/or sucrose gradient fractionation [121]. Human milk contains over 360 prokaryotic genera [122], therefore, it is possible that with methods such as sucrose gradient fractionation and ultracentrifugation, bacterial extracellular vesicles are contaminating the eukaryotic-derived exosomal fraction in milk. Although immunoaffinity capture-based techniques are costly methods for large sample sizes, they separate exosomes based on known exosomal surface protein markers, such as CD9, CD63 and CD81, resulting in the most uniform population of exosomes [123].

Separating exosomes from other extracellular vesicles is required, as exosomal packaging is a selective process, unlike blebs or apoptotic body formation. Vesicles that are formed through zeiosis, blebbing off of the plasma membrane, are blebs and their packaging processes involve protein pathways that differ from those of exosomes [124].

The concentration of exosomes in TM and PTM may vary depending on factors that deal with the mother or her infant; e.g. secretory activation, mammary epithelial junction closure, ability of infant to suckle, etc. Particle size and concentration in the exosomal fraction isolated from TM and PTM was measured using laser microscopy. Based on nanoparticle tracking analysis, the concentration of vesicles significantly differed in TM or PTM exosomal fractions ($p < 0.0001$, Figure 4). This technique is based upon dynamic light scattering of particles in solution undergoing Brownian motion. The hydrodynamic radius calculated by the instrument and software determines the size and concentration of particles in the liquid sample [76, 125, 126]. The limitation of this technique is that it may not distinguish between distinct populations, such as vesicles that are 100 nm or 200 nm in diameter [76]. Another method that can be used to measure the concentration of vesicles derived from milk is a less utilized method based on tunable resistive pulse sensing, qNano. This is an additional method to measure and validate particle size and concentration, but has not been used previously on human milk exosomes [76]. Another method that has been used to characterize exosomes is transmission electron microscopy. There are also limitations to this technique due to the heterogeneity or lack of purity of the sample isolated from milk. The lack of purity may cause difficulties in visualizing and differentiating exosomes from proteins and other vesicles [76]. A study using urinary exosomes to compare methods that measure particle

size and concentration found that all methods: transmission electron microscopy, nanoparticle tracking analysis, tunable resistive pulse sensing and flow cytometry provided different results [127].

Based on the lack of research on methods to determine particle size and concentration in human milk exosomes, it is not yet possible to conclude if there is a significant difference between those isolated from TM or PTM. In the future, methods to quantify and measure size of milk exosomes should be compared and optimized for this novel line of research.

5.2. Characterizing human milk exosomes based on surface protein markers

Extracellular vesicle research requires a minimum of two forms of validation when working with exosomes due to the lack of optimized methods for isolation [128]. To further analyze the exosomal fraction isolated from TM and PTM the surface protein markers were identified using flow cytometry with the MACSPlex human exosome kit. While the exosomal fraction isolated from both TM and PTM may have been heterogeneous, with vesicles larger than 200 nm (Figure 4), it did contain vesicles with exosomal protein markers as identified through flow cytometry (Figure 5). Vesicles positive for CD9, CD63 and/or CD81 were detected by the MACSPlex human exosome kit detection antibodies through flow cytometry in the exosomal fraction isolated from TM and PTM (Figure 5). CD9, CD63 and CD81 are recognized as key tetraspanin markers of exosomes [75, 77, 79]. Other surface proteins were identified using the MACSPlex human exosome kit, containing beads with affinity for 37 exosomal surface proteins, and flow cytometry (Figure 5, Table 1, 2). The potential cellular origin of the

exosomes present in human milk may be predicted through identification of exosomal surface proteins. Exosomes are initially formed through invagination of the outer membrane of cells into an EE, which undergoes further invagination, creating a MVB containing exosomes. Due to the initial invagination of the outer plasma membrane, the exosomes will present with the same proteins found on the outer membrane of the cell. The MVB fuses with the outer membrane, releasing the exosomes with surface proteins similar to those of the cell, into extracellular space [75, 77, 79, 80]. Thus, identification of surface proteins on the exosomes in human milk provides insight into the cellular origin of the exosomes. Human milk contains cells (Figure 1) that may be secreting the exosomes or the exosomes may also be originating from mammary gland epithelial cells.

Preliminary analysis of exosomal surface proteins from three biological replicates (TM) (Supplementary Figure 4) indicated that surface protein markers vary between mothers. The differences in surface proteins between biological replicates may be attributed to the time of sample collection, gender, age or health of the infant and/or the variation between mothers. Based on this preliminary analysis, the samples were pooled from each cohort, to get a more comprehensive view of the potential differences between TM and PTM exosomes.

TM and PTM contained exosomes with surface protein markers: CD3, CD14, CD24, CD45, CD56, CD133, CD326, MHC class I and II (Figure 5, Table 1). These proteins are markers of T cells, monocytes, stem cells, granulocytes, NK cells, nucleated cells, B cells and epithelial cells. Thus, exosomes may be originating from immune cells in milk, as well as the mammary gland epithelium. Immune-cell derived exosomes have wide-ranging effects on the immune system by either promoting or dampening immune-

related activities [129]. Human milk exosomes may be altering the immune response in the newborn GI tract by either facilitating combat with pathogens or by promoting immune tolerance. Human milk exosomes have been shown to attenuate intestinal epithelial cell death and promote intestinal epithelial cell proliferation [31, 35]. Thus, we further hypothesized that milk exosomes may be altering inflammatory cytokine expression in intestinal epithelial cells, discussed further below.

PTM contained unique exosome surface proteins: CD29, CD146, CD105 and ROR1 (Figure 5, Table 2). The exosomes in PTM contained surface proteins that related to endothelial cells, distinct from TM exosomes. Endothelial cells line blood and lymphatic vessels, and there is a layer of endothelial cells between the blood-milk barrier. Upon premature birth, the mammary gland may not be fully prepared to begin lactation. Tight-junction closure and secretory activation may be delayed with premature births resulting in increased flux of molecules across the epithelium [23, 24, 48]. Thus, with PTM, increased amounts of exosomes from the blood and/or mammary endothelial cells may be exchanged across the mammary epithelium, into the milk, like macromolecules and solutes [130]. This may explain why PTM contains exosomes with endothelial protein markers and TM does not (Table 2).

5.3. Total protein analysis of human milk exosomes

Total protein analyses of exosomes derived from PTM has not been performed to date, to my knowledge. Due to the perturbation of gestation, mothers' milk varies, and the exosomal protein content was expected to differ between PTM and TM.

Due to the sparsity of studies performing mass spectrometry on human milk exosomes, optimization of protein preparation was required [25, 113]. Digestion of milk exosomes with Trypsin/Lys-C and SDS in 7 mM EDTA as a detergent resulted in the highest number of protein IDs with high and medium confidence (Figure 6, 7). Thus, this protocol was adopted to digest the pooled TM and pooled PTM exosomes.

Total protein analyses also enabled validation of the novel MACSPlex human exosome kit. The MACSPlex human exosome kit for flow cytometry was released in 2016 and has not previously been tested on human milk exosomes, to my knowledge. Mass spectrometry was used to validate the surface proteins identified on human milk exosomes using the kit with flow cytometry. CD3, CD24, CD56 and ROR1 surface proteins were identified using flow cytometry, however, the protein IDs were not identified through mass spectrometry analyses in the exosomal samples from TM or PTM (Table 1, 2). This may be due to antibody aspecificity, resulting in false-positive events. To determine if CD3, CD24, CD56 and ROR1 are false-positives, several negative controls should be used, such as exosomes derived from cells known to not present those surface proteins [131]. However, it is possible that these proteins were not identified in mass spectrometry due to the digestion protocol. To confirm if these proteins are present on human milk exosomes, larger sample sizes would be required with more technical replicates.

Mass spectrometry analyses of the isolated pooled milk exosomes demonstrated that PTM exosomes contain a significantly higher number of total proteins (711 proteins) than TM exosomes (533 proteins) (Figure 8A). These findings are consistent with those of flow cytometry, which identified more surface proteins on PTM exosomes than TM

exosomes. There was also a higher concentration of exosomes in PTM as identified by the ZetaView (Figure 4). This increase of exosomal concentration and proteins may be due to the delay of secretory activation, or secretory specialization, in the mammary gland of mothers who gave birth to preterm infants. The delayed secretory activation allows for increased passage of molecules across the mammary gland epithelium because the tight-junctions have not fully formed [23]. PTM exosomes also had a higher number of proteins that were unique (321 proteins) and TM exosomes contained 143 unique proteins (Figure 8B).

The classification of proteins from TM and PTM exosomes did not vary with respect to biological processes, molecular functions, cellular components and protein classes (Figure 9A, 10A, 11A, 12A). However, the roles of the unique proteins in PTM exosomes compared to the unique proteins in TM exosomes varied. The percentage of unique proteins involved in immune system processes and developmental processes with respect to total unique proteins in PTM exosomes was nearly double that found in TM exosomes (Figure 9B). The percentage of unique proteins from TM and PTM exosomes did not differ when their molecular functions were compared. PTM exosomes did contain unique proteins involved in cargo receptor activity, transcription and translation regulator activity, whereas TM exosomes did not (Figure 10B). Approximately 40% of the unique proteins identified in TM and PTM were involved in binding. These binding proteins are involved in exosome synthesis, such as GTPases, membrane trafficking regulator proteins, annexin and clathrin (Supplementary Table 2, 3). Further analyses of these unique proteins may provide insight into potential therapeutic targets to promote intestinal immunity in preterm infants.

Proteins unique to TM may be of use as potential therapeutics to supplement PTM. For example, lactoperoxidase is only found in TM exosomes. Lactoperoxidase catalyzes thiocyanate oxidation, yielding hypothiocyanate, which plays an antimicrobial role [38]. However, PTM exosomes do contain unique immune-regulatory proteins, such as, C-X-C motif chemokine 2 (CXCL2) (Supplementary Table 3). CXCL2 is a 73 residue antimicrobial chemokine that recruits neutrophils, thus promoting inflammation [132]. In preterm infants, it is necessary to promote immune tolerance to reduce inflammatory-related GI diseases, such as NEC. With further analyses into the distinct protein subsets in TM or PTM exosomes it may be possible to identify pathways involved in inflammation and NEC. These analyses would provide insight into whether PTM or TM contains more immune-regulatory proteins that may be preventing inflammation in the GI tract. Such analyses on immune-regulatory proteins in extracellular vesicles has been performed on bovine milk [133]. With this knowledge, feeding strategies for preterm infants may be optimized, whether it is feeding donor term milk or promoting and providing support for mothers breastfeeding preterms.

5.4. The effects of human milk exosomes on cytokine expression in intestinal epithelial cells

Upon treatment, milk exosomes have been shown to alter intestinal epithelial cell growth and proliferation and prevent intestinal epithelial cell death upon stressors [31, 35, 90]. Previous studies have demonstrated that human milk exosomes can modulate cytokine release in peripheral blood mononuclear cells [25]. Thus, this study investigated whether human milk exosomes can modulate cytokine expression in intestinal epithelial

cells. Caco-2/15 cell line was used as an *in vitro* intestinal epithelial cell model. The Caco-2/15 cell line is a clone derived from the parent line Caco-2, human epithelial colorectal adenocarcinoma cell line. Although the Caco-2 cell model has its limitations because it is a cancerous cell line, post-confluent cells become fully polarized exhibiting morphological and functional characteristics comparable to those of mid-gestation small intestinal villus enterocytes. The Beaulieu Lab, University of Sherbrooke, has demonstrated the similarity of the Caco-2 cell line to mid-gestation small intestinal villus enterocytes based on morphologic, functional, proliferative and transcriptomic analyses of the cell line [94-96, 114, 115, 134]. This *in vitro* intestinal epithelial cell model is a common model to study the effects of milk components on the intestine [27, 42, 116].

Post-confluent Caco-2/15 cells responded to heat-killed bacteria through upregulation of pro-inflammatory cytokine expression. Heat-killed bacteria was added to post-confluent Caco-2/15 cells and there was an increase of CXCL8, CXCL10 and CCL2 expression compared to unstimulated cells over a 24-hour period (Figure 13). The peak fold-change in expression of these pro-inflammatory cytokines occurred between two to three hours post incubation with the heat-killed bacteria. Post-confluent Caco-2/15 cells are an appropriate model to analyze the potential for milk exosomes to alter inflammatory cytokine expression in relevant responsive cells.

When Caco-2/15 cells were treated with human milk exosomes prior to exposure to heat-killed bacteria, inflammatory cytokine expression was altered. Expression of pro-inflammatory cytokines, IL8, CXCL10, TNF, CCL2, IL1b and IL6, was significantly decreased and anti-inflammatory cytokine IL10 expression was increased, but not significantly ($p < 0.08$) in cells pretreated with milk exosomes (Figure 14). This provides

evidence that human milk exosomes can regulate cytokine expression in an intestinal epithelial cell model.

The six observed pro-inflammatory cytokines are involved in the inflammatory cascade within the GI tract. The pleiotropic cytokine, IL6 is related to inflammation, playing a key role in the development of Th17 cells. Th17 cells are involved in tissue inflammation, destruction and multiple autoimmune diseases [135, 136]. CXCL10 is also strongly associated with autoimmunity [137-140]. IL8 is actively involved in the inflammatory cascade as it recruits and activates neutrophils to interstitial sites [29, 141-143]. TNF and IL1b are considered to be early response cytokines, up-regulating a broad range of the inflammatory cascade. They cause further synthesis of mediators and cytokines that can promote extracellular matrix production by fibroblasts and increase the expression of adhesion molecules on endothelial cells [144]. CCL2 modulates the inflammatory response by recruiting immune cells, such as T cells, dendritic cells and monocytes to the site of injury [145, 146]. Downregulation of these pro-inflammatory cytokines in the GI tract of premature infants may lead to the prevention of an unnecessary inflammatory reaction to novel microbial species.

As NEC is a severe GI disease that largely affects preterm infants and is the leading cause of GI-related deaths in the neonatal intensive care unit, it is of utmost importance to identify therapeutic agents which promote immune tolerance. Human milk is thought to prevent NEC [40], however, there are occasions where it is unavailable. Identifying components in human milk which promote immune tolerance will allow for their supplementation of donor milk or PTM to ensure improved outcomes of this vulnerable population. To my knowledge, this thesis is the first report of human milk

exosomes dampening the inflammatory response in an intestinal epithelial cell model. These findings may lead to identification of novel therapeutic agents for inflammatory-related GI diseases in newborns.

7. Future Directions

Significant differences were found between exosomes isolated from TM and PTM. As this study was performed on a small sample size, in the future it would be informative to increase the number of donors from both cohorts to demonstrate a more comprehensive and robust difference. Analyzing the samples separately, as opposed to pooling, may provide insight into the biological variations between individual mothers. More in-depth analyses on the pathways of the proteins derived from TM and PTM exosomes will be performed to identify proteins involved in inflammation and NEC.

The effects of TM exosomes on intestinal epithelial cells were observed at a transcriptional level, in the future, enzyme-linked immunosorbent assays will be performed to determine the effects of TM exosomes on cytokine production at the translational level. Human milk exosomes also contain microRNA. MicroRNA binds mRNA strands to inhibit translation through targeting for degradation or preventing binding of RNA polymerase. If the microRNA in exosomes are preventing translation by inhibiting RNA polymerase from binding to the mRNA strand, the strand will still be present. The mRNA will be quantified through qRT-PCR, even though it is not being translated. Therefore, quantifying inflammatory cytokines by measuring protein is the most robust way to observe the effects of exosomes. PTM exosomes will also be tested

for anti-inflammatory potential on the intestinal epithelial cell model to determine if there is a difference between TM and PTM exosomes.

The *in vitro* Caco-2/15 intestinal cell model experiments should be repeated with an increased sample size of biological replicates. If the results remain consistent upon increasing sample size, a more relevant *in vitro* model could then be used to validate these findings. A more physiologically relevant *in vitro* model would more closely mimic the GI tract of a newborn. For example, HIEC-6 cells from the American Tissue Culture Collection are derived from fetal intestinal epithelial cells. This model would be relevant, especially when looking for a treatment to prevent inflammation in the GI tract of preterm infants. Enteroids cultured from fetal intestinal tissue would be a preferred *in vitro* model. The GI tract contains many different cells that would be playing a role in the immune response. The complexity that an enteroid offers would allow for further investigation into the effects of human milk exosomes on other GI cells in a more representative environment.

8. Limitations

This study was performed on a small sample size of five biological replicates from each cohort. The information on the donors' age and gestational age was not available for all the donors. The milk sample collection from donors from each group varied with time postpartum. In the future, it would be valuable to have donors that donate milk within a specific range postpartum. For example, two of the term mothers donated milk within the first month postpartum, which is not fully mature milk, as was

the case for the other three mothers. This leads to increased variation that should be minimized as much as possible in this field of study.

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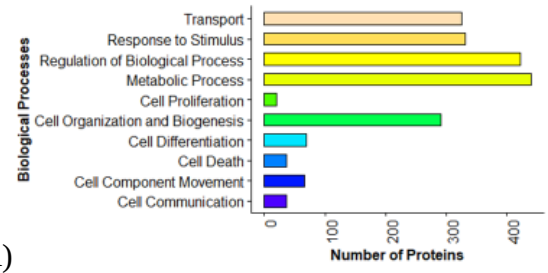
10. Appendix

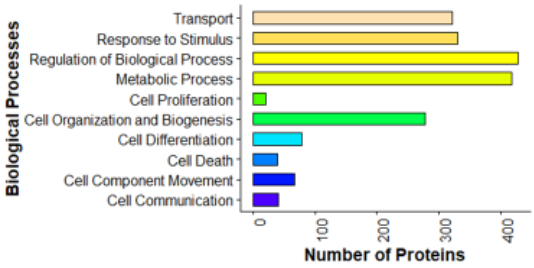
SAMPLE	DONOR'S AGE YEARS	TIME POSTPARTUM	INFANT GESTATIONAL AGE	INFANT GENDER	PARITY
1	27	5 d	33 w,6 d	Male	1
2	42	7 d	32 w,4 d	Male	1
3	33	8 d	34 w,1 d	Female	3
4	23	6 d	23 w,6 d	Male	1
5	25	4 d	34 w,2 d	Male	3
6		13 m, 15 d [1]	> 38 w	Female	2
7		14 m, 3 d	> 38 w	Male	2
8		3 m, 1 d	> 38 w	Male	2
9	48	9 d	39 w,4 d	Male	2
10	35	13 d	40 w,2 d	Male	1

[1]The donor was also feeding four-year-old female daughter.

Supplementary Table 1. Information regarding donor's age, stage of lactation (time postpartum), infant's gestational age, infant gender and number of pregnancies to a viable gestational age (parity).


Biological Processes
Transport
Response to Stimulus
Regulation of Biological Process
Metabolic Process
Cell Proliferation
Cell Organization and Biogenesis
Cell Differentiation
Cell Death
Cell Component Movement
Cell Communication
0
100
200
300
400
Number of Proteins

A)


Biological Processes
Transport
Response to Stimulus
Regulation of Biological Process
Metabolic Process
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Cell Organization and Biogenesis
Cell Differentiation
Cell Death
Cell Component Movement
Cell Communication
0
100
200
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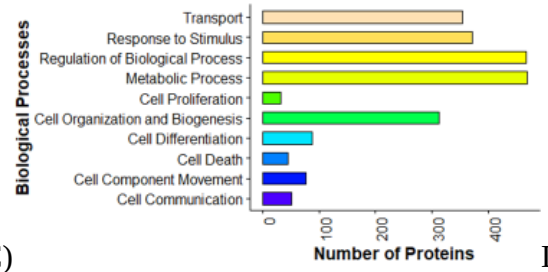
400
Number of Proteins

B)


Biological Processes
Transport
Response to Stimulus
Regulation of Biological Process
Metabolic Process
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Cell Organization and Biogenesis
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Cell Death
Cell Component Movement
Cell Communication
0
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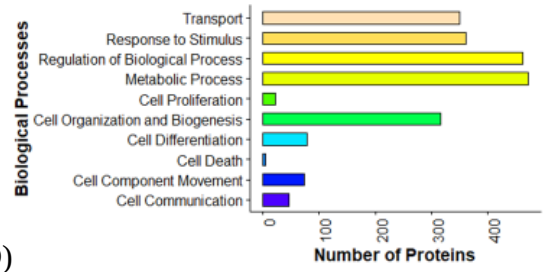
400
Number of Proteins

C)


Biological Processes
Transport
Response to Stimulus
Regulation of Biological Process
Metabolic Process
Cell Proliferation
Cell Organization and Biogenesis
Cell Differentiation
Cell Death
Cell Component Movement
Cell Communication
0
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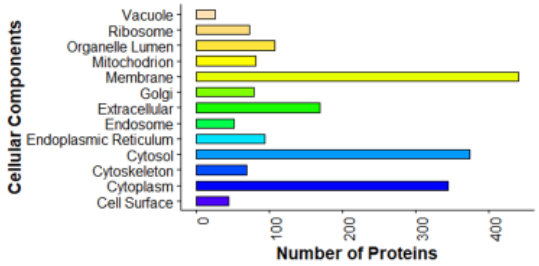
200
300
400
Number of Proteins

D)

Supplementary Figure 1. The biological processes of the proteins identified using different detergents to allow trypsin to digest proteins from human milk exosomes. The biological processes of the milk exosomal proteins identified after disruption using detergents Digitonin, n-dodecyl β-D-maltoside (DDM) (A), Digitonin (B), Triton X-100 (C) or Sodium dodecyl sulfate (SDS) (D) with Trypsin/Lys-C (Promega, Wisconsin, USA) to cleave peptides. The peptides were identified through liquid chromatography–tandem mass spectrometry (LC-MS/MS), (n=1).


Cellular Components
Vacuole
Ribosome
Organelle Lumen
Mitochodrion
Membrane
Golgi
Extracellular
Endosome
Endoplasmic Reticulum
Cytosol
Cytoskeleton
Cytoplasm
Cell Surface
0
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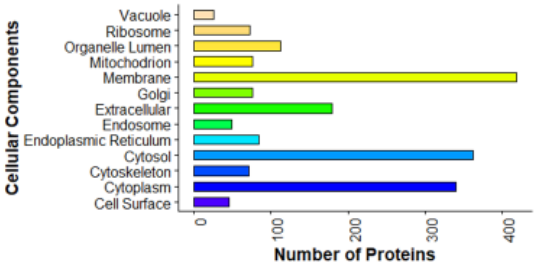
Number of Proteins

A)


Cellular Components
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Organelle Lumen
Mitochodrion
Membrane
Golgi
Extracellular
Endosome
Endoplasmic Reticulum
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Cytoskeleton
Cytoplasm
Cell Surface
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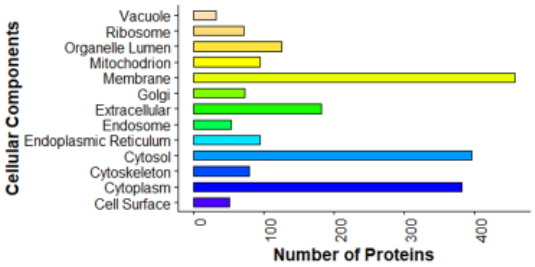
Number of Proteins

B)


Cellular Components
Vacuole
Ribosome
Organelle Lumen
Mitochodrion
Membrane
Golgi
Extracellular
Endosome
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Cytosol
Cytoskeleton
Cytoplasm
Cell Surface
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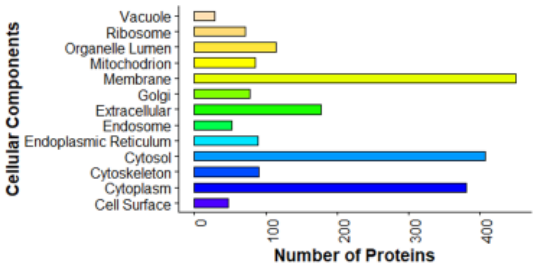
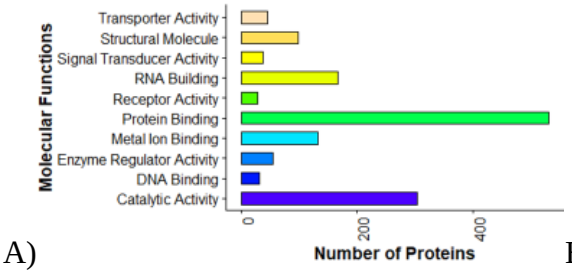
Number of Proteins

C)

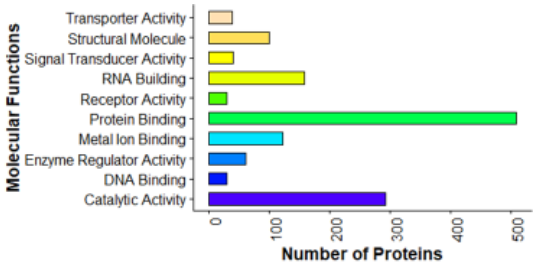

Cellular Components
Vacuole
Ribosome
Organelle Lumen
Mitochodrion
Membrane
Golgi
Extracellular
Endosome
Endoplasmic Reticulum
Cytosol
Cytoskeleton
Cytoplasm
Cell Surface
0
100
200
300
400
Number of Proteins

D)

Supplementary Figure 2. The cellular components of the proteins identified using different detergents to allow trypsin to digest proteins from human milk exosomes. The cellular components of the milk exosomal proteins identified after disruption using detergents Digitonin, n-dodecyl β-D-maltoside (DDM) (A), Digitonin (B), Triton X-100 (C) or Sodium dodecyl sulfate (SDS) (D) with Trypsin/Lys-C (Promega, Wisconsin, USA) to cleave peptides. The peptides were identified through liquid chromatography tandem mass spectrometry (LC-MS/MS) (n=1).


Molecular Functions
Transporter Activity
Structural Molecule
Signal Transducer Activity
RNA Building
Receptor Activity
Protein Binding
Metal Ion Binding
Enzyme Regulator Activity
DNA Binding
Catalytic Activity
0
200
400
Number of Proteins

A)


Molecular Functions
Transporter Activity
Structural Molecule
Signal Transducer Activity
RNA Building
Receptor Activity
Protein Binding
Metal Ion Binding
Enzyme Regulator Activity
DNA Binding
Catalytic Activity
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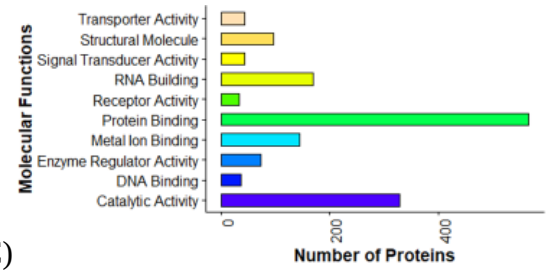
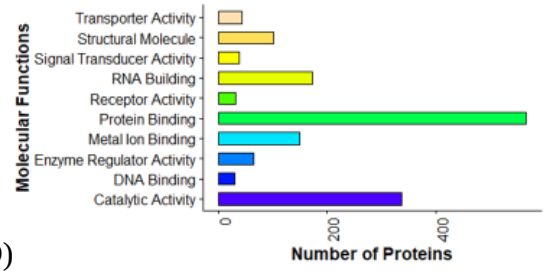
100
200
300
400
500
Number of Proteins

B)

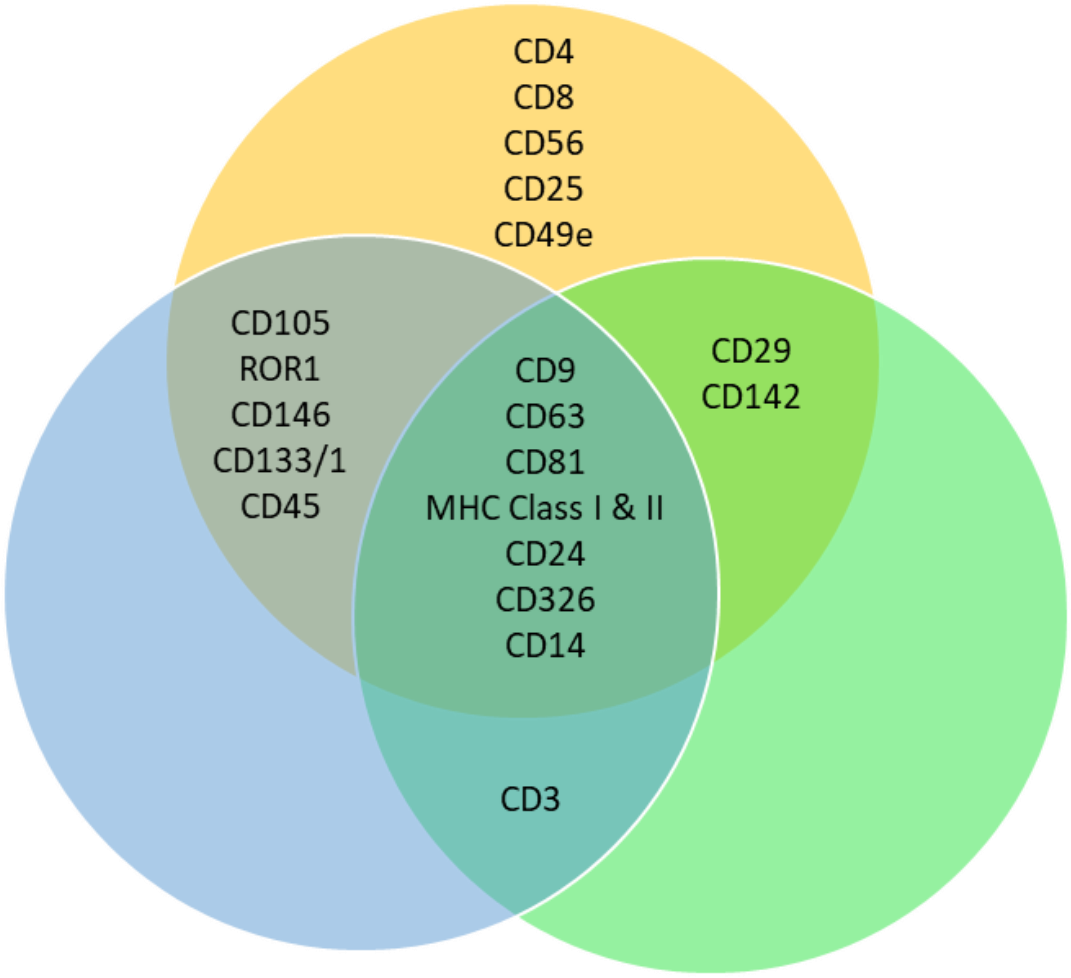

Molecular Functions
Transporter Activity
Structural Molecule
Signal Transducer Activity
RNA Building
Receptor Activity
Protein Binding
Metal Ion Binding
Enzyme Regulator Activity
DNA Binding
Catalytic Activity
0
200
400
Number of Proteins

C)


Molecular Functions
Transporter Activity
Structural Molecule
Signal Transducer Activity
RNA Building
Receptor Activity
Protein Binding
Metal Ion Binding
Enzyme Regulator Activity
DNA Binding
Catalytic Activity
0
200
400
Number of Proteins

D)

Supplementary Figure 3. The molecular functions of the proteins identified using different detergents to allow trypsin to digest proteins from human milk exosomes. The molecular functions of the milk exosomal proteins identified after disruption using detergents Digitonin, n-dodecyl β-D-maltoside (DDM) (A), Digitonin (B), Triton X-100 (C) or Sodium dodecyl sulfate (SDS) (D) with Trypsin/Lys-C (Promega, Wisconsin, USA) to cleave peptides. The peptides were identified through liquid chromatography tandem mass spectrometry (LC-MS/MS), (n=1).


CD4
CD8
CD56
CD25
CD49e
CD105
ROR1
CD146
CD133/1
CD45
CD9
CD63
CD81
MHC Class I & II
CD24
CD326
CD14
CD29
CD142
CD3

Supplementary Figure 4. Exosomal surface proteins identified with flow cytometry from 3 biological replicates. Each circle represents surface proteins from one biological replicate identified through flow cytometry with the Miltenyi MACSPlex human exosome kit (n=3).

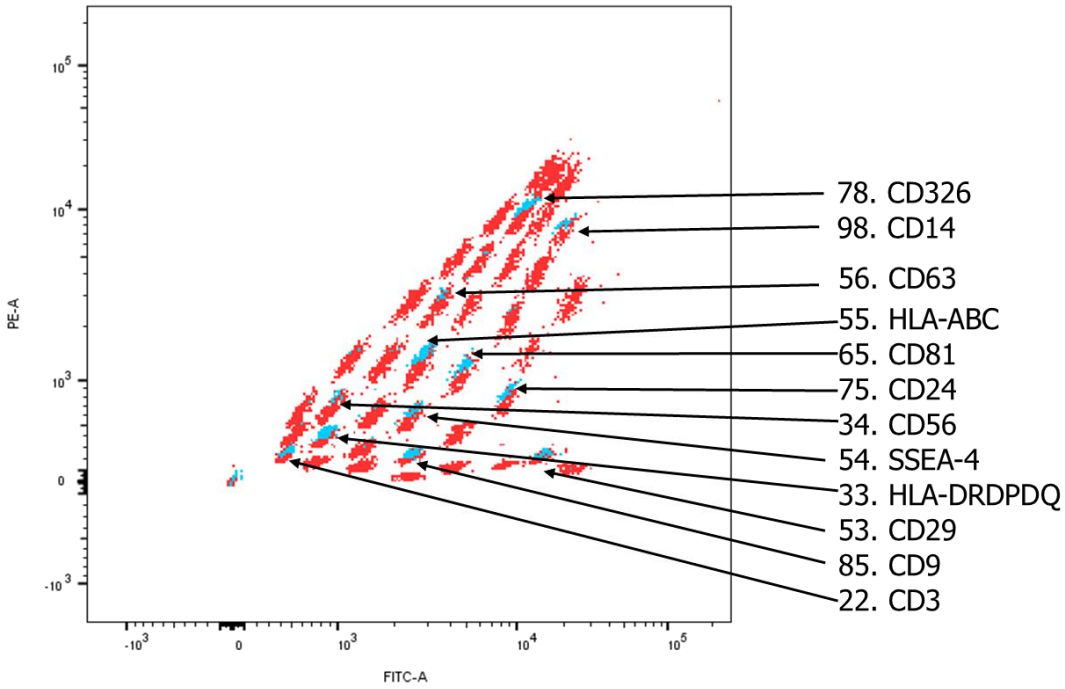
A)

B)

No.	Antibody	Isotype
22	CD3	mIgG2a
23	CD4	mIgG2a
24	CD19	mIgG1
32	CD8	mIgG2a
33	HLA-DRDPDQ	REA
34	CD56	REA
35	CD105	mIgG1
42	CD2	mIgG2b
43	CD1c	mIgG2a
44	CD25	mIgG1
45	CD49e	mIgG2b
46	ROR1	mIgG1κ
52	CD209	mIgG1
53	CD9	mIgG1
54	SSEA-4	REA
55	HLA-ABC	REA
56	CD63	mIgG1κ
57	CD40	mIgG1κ
63	CD62P	REA
64	CD11c	mIgG2b

No	Antibody	Isotype
65	CD81	REA
66	MCSP	mIgG1
67	CD146	mIgG1
68	CD41b	REA
74	CD42a	REA
75	CD24	mIgG1
76	CD86	mIgG1
77	CD44	mIgG1
78	CD326	mIgG1
79	CD133/1	mIgG1κ
85	CD29	mIgG1κ
86	CD69	mIgG1κ
87	CD142	mIgG1κ
88	CD45	mIgG2a
89	CD31	mIgG1
96	REA Control	REA
97	CD20	mIgG1
98	CD14	mIgG2a
99	mIgG1 control	mIgG1

C)



Supplementary Figure 5. MACSPlex human exosome kit (Miltenyi Biotec, Bergisch Gladbach, Germany) principle behind the identification of exosomal surface epitopes. A) Diagram demonstrating bead populations from the MACSPlex human exosome kit in a fluorescein isothiocyanate (FITC) versus phycoerythrin (PE) plot. B) Table linked to (A) to identify the antibodies corresponding to specific bead populations, (From Figure 2B, C Miltenyi Biotec MACSPlex human exosome kit datasheet). C) CD9, CD63 and CD81 (known exosomal tetraspanins) are APC-positive, meaning that human exosomes are present (blue) and the bead populations (red) that they are bound to in a FITC versus PE dot plot with a list of their corresponding surface antigens and numbers from (B).

Accession Number	Gene Name; Gene Symbol
P26640	Valine--tRNA ligase;VARS;ortholog
Q9HAV0	Guanine nucleotide-binding protein subunit beta-4;GNB4;ortholog
Q9NUP9	Protein lin-7 homolog C;LIN7C;ortholog
P53621	Coatomer subunit alpha;COPA;ortholog
Q9Y5X9	Endothelial lipase;LIPG;ortholog
P17980	26S protease regulatory subunit 6A;PSMC3;ortholog
P62753	40S ribosomal protein S6;RPS6;ortholog
P61254	60S ribosomal protein L26;RPL26;ortholog
P02751	Fibronectin;FN1;ortholog
P54920	Alpha-soluble NSF attachment protein;NAPA;ortholog
P17858	ATP-dependent 6-phosphofructokinase, liver type;PFKL;ortholog
P30085	UMP-CMP kinase;CMPK1;ortholog
Q86Y82	Syntaxin-12;STX12;ortholog
P07225	Vitamin K-dependent protein S;PROS1;ortholog
P09211	Glutathione S-transferase P;GSTP1;ortholog
Q9BUF5	Tubulin beta-6 chain;TUBB6;ortholog
Q9NV23	S-acyl fatty acid synthase thioesterase, medium chain;OLAH;ortholog
P61026	Ras-related protein Rab-10;RAB10;ortholog
P35606	Coatomer subunit beta';COPB2;ortholog
P61764	Syntaxin-binding protein 1;STXBP1;ortholog
P04004	Vitronectin;VTN;ortholog
Q9NRJ3	C-C motif chemokine 28;CCL28;ortholog
Q92928	Putative Ras-related protein Rab-1C;RAB1C;ortholog
A0A096LPE2	SAA2-SAA4 readthrough;SAA2-SAA4;ortholog
P01033	Metalloproteinase inhibitor 1;TIMP1;ortholog
Q9NR31	GTP-binding protein SAR1a;SAR1A;ortholog
Q9H0E2	Toll-interacting protein;TOLLIP;ortholog
O75891	Cytosolic 10-formyltetrahydrofolate dehydrogenase;ALDH1L1;ortholog
Q14210	Lymphocyte antigen 6D;LY6D;ortholog
Q9BQ13	BTB/POZ domain-containing protein KCTD14;KCTD14;ortholog
O00560	Syntenin-1;SDCBP;ortholog
P29373	Cellular retinoic acid-binding protein 2;CRABP2;ortholog
P05386	60S acidic ribosomal protein P1;RPLP1;ortholog
P08708	40S ribosomal protein S17;RPS17;ortholog
Q8WVM8	Sec1 family domain-containing protein 1;SCFD1;ortholog
P02794	Ferritin heavy chain;FTH1;ortholog
P78539	Sushi repeat-containing protein SRPX;SRPX;ortholog
Q9NYL4	Peptidyl-prolyl cis-trans isomerase FKBP11;FKBP11;ortholog
P12429	Annexin A3;ANXA3;ortholog
P01111	GTPase NRas;NRAS;ortholog
Q96QK1	Vacuolar protein sorting-associated protein 35;VPS35;ortholog
P63244	Receptor of activated protein C kinase 1;RACK1;ortholog

P28838	Cytosol aminopeptidase;LAP3;ortholog
P68036	Ubiquitin-conjugating enzyme E2 L3;UBE2L3;ortholog
Q9Y3D6	Mitochondrial fission 1 protein;FIS1;ortholog
P62081	40S ribosomal protein S7;RPS7;ortholog
Q15084	Protein disulfide-isomerase A6;PDIA6;ortholog
P40227	T-complex protein 1 subunit zeta;CCT6A;ortholog
A0A0U1RRH7	Histone H2A;unassigned;ortholog
O60547	GDP-mannose 4,6 dehydratase;GMDS;ortholog
P45877	Peptidyl-prolyl cis-trans isomerase C;PPIC;ortholog
P60866	40S ribosomal protein S20;RPS20;ortholog
Q53EL6	Programmed cell death protein 4;PDCD4;ortholog
P08133	Annexin A6;ANXA6;ortholog
Q9BSG0	Protease-associated domain-containing protein 1;PRADC1;ortholog
P23284	Peptidyl-prolyl cis-trans isomerase B;PPIB;ortholog
P62854	40S ribosomal protein S26;RPS26;ortholog
O94760	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1;DDAH1;ortholog
P22090	40S ribosomal protein S4, Y isoform 1;RPS4Y1;ortholog
P22307	Non-specific lipid-transfer protein;SCP2;ortholog
P10809	60 kDa heat shock protein, mitochondrial;HSPD1;ortholog
P61960	Ubiquitin-fold modifier 1;UFM1;ortholog
P34096	Ribonuclease 4;RNASE4;ortholog
P62273	40S ribosomal protein S29;RPS29;ortholog
Q15019	Septin-2;SEPT2;ortholog
Q5JU69	Torsin-2A;TOR2A;ortholog
Q8NES3	Beta-1,3-N-acetylglucosaminyltransferase lunatic fringe;LFNG;ortholog
Q9H4M9	EH domain-containing protein 1;EHD1;ortholog
Q2I0M4	Leucine-rich repeat-containing protein 26;LRRC26;ortholog
O75340	Programmed cell death protein 6;PDCD6;ortholog
P62736	Actin, aortic smooth muscle;ACTA2;ortholog
P61019	Ras-related protein Rab-2A;RAB2A;ortholog
P27169	Serum paraoxonase/arylesterase 1;PON1;ortholog
P22676	Calretinin;CALB2;ortholog
Q15904	V-type proton ATPase subunit S1;ATP6AP1;ortholog
P49753	Acyl-coenzyme A thioesterase 2, mitochondrial;ACOT2;ortholog
P61224	Ras-related protein Rap-1b;RAP1B;ortholog
P61106	Ras-related protein Rab-14;RAB14;ortholog
P16144	Integrin beta-4;ITGB4;ortholog
O43633	Charged multivesicular body protein 2a;CHMP2A;ortholog
Q8N474	Secreted frizzled-related protein 1;SFRP1;ortholog
P33121	Long-chain-fatty-acid--CoA ligase 1;ACSL1;ortholog
P43686	26S protease regulatory subunit 6B;PSMC4;ortholog
O00231	26S proteasome non-ATPase regulatory subunit 11;PSMD11;ortholog
P07384	Calpain-1 catalytic subunit;CAPN1;ortholog

P07437	Tubulin beta chain;TUBB;ortholog
Q6YP21	Kynurenine--oxoglutarate transaminase 3;KYAT3;ortholog
P14174	Macrophage migration inhibitory factor;MIF;ortholog
Q9Y2V2	Calcium-regulated heat-stable protein 1;CARHSP1;ortholog
P22079	Lactoperoxidase;LPO;ortholog
P25325	3-mercaptopyruvate sulfurtransferase;MPST;ortholog
P05387	60S acidic ribosomal protein P2;RPLP2;ortholog
Q6UWU2	Beta-galactosidase-1-like protein;GLB1L;ortholog
Q6GMV3	Putative peptidyl-tRNA hydrolase PTRHD1;PTRHD1;ortholog
P42025	Beta-centractin;ACTR1B;ortholog
P25685	DnaJ homolog subfamily B member 1;DNAJB1;ortholog
Q9NQW7	Xaa-Pro aminopeptidase 1;XPNPEP1;ortholog
P35998	26S protease regulatory subunit 7;PSMC2;ortholog
O00232	26S proteasome non-ATPase regulatory subunit 12;PSMD12;ortholog
P20618	Proteasome subunit beta type-1;PSMB1;ortholog
P67936	Tropomyosin alpha-4 chain;TPM4;ortholog
P56537	Eukaryotic translation initiation factor 6;EIF6;ortholog
O60888	Protein CutA;CUTA;ortholog
A5D8V6	Vacuolar protein sorting-associated protein 37C;VPS37C;ortholog
Q8NG11	Tetraspanin-14;TSPAN14;ortholog
Q9Y2J8	Protein-arginine deiminase type-2;PADI2;ortholog
Q96FN4	Copine-2;CPNE2;ortholog
P27635	60S ribosomal protein L10;RPL10;ortholog
Q9Y6Y9	Lymphocyte antigen 96;LY96;ortholog
O60513	Beta-1,4-galactosyltransferase 4;B4GALT4;ortholog

Supplementary Table 2. Proteins unique to term milk exosomes identified with LC-MS/MS and Protein ANalysis THrough Evolutionary Relationships (PANTHER) database [92].

Accession Number	Gene Name/Gene Symbol
P62879	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2;GNB2;ortholog
Q99497	Protein DJ-1;PARK7;ortholog
Q16658	Fascin;FSCN1;ortholog
P78371	T-complex protein 1 subunit beta;CCT2;ortholog
Q9NS15	Latent-transforming growth factor beta-binding protein 3;LTBP3;ortholog
Q9BY76	Angiopoietin-related protein 4;ANGPTL4;ortholog
Q9Y6W5	Wiskott-Aldrich syndrome protein family member 2;WASF2;ortholog
P07948	Tyrosine-protein kinase Lyn;LYN;ortholog
A0A0B4J2B5	Immunoglobulin heavy variable 3/OR16-9 (non-functional) (Fragment);IGHV3OR16-9;ortholog
P11233	Ras-related protein Ral-A;RALA;ortholog
Q96I34	Protein phosphatase 1 regulatory subunit 16A;PPP1R16A;ortholog
Q9Y6E0	Serine/threonine-protein kinase 24;STK24;ortholog
Q9UNZ2	NSFL1 cofactor p47;NSFL1C;ortholog
Q9BR76	Coronin-1B;CORO1B;ortholog
Q8IZP0	Abl interactor 1;ABI1;ortholog
Q13410	Butyrophilin subfamily 1 member A1;BTN1A1;ortholog
P55072	Transitional endoplasmic reticulum ATPase;VCP;ortholog
O75083	WD repeat-containing protein 1;WDR1;ortholog
Q9NZT1	Calmodulin-like protein 5;CALML5;ortholog
P01591	Immunoglobulin J chain;JCHAIN;ortholog
P60953	Cell division control protein 42 homolog;CDC42;ortholog
Q9ULP9	TBC1 domain family member 24;TBC1D24;ortholog
P61586	Transforming protein RhoA;RHOA;ortholog
Q53SF7	Cordon-bleu protein-like 1;COBLL1;ortholog
Q96IJ6	Mannose-1-phosphate guanyltransferase alpha;GMPPA;ortholog
O00292	Left-right determination factor 2;LEFTY2;ortholog
P31948	Stress-induced-phosphoprotein 1;STIP1;ortholog
P62834	Ras-related protein Rap-1A;RAP1A;ortholog
P46940	Ras GTPase-activating-like protein IQGAP1;IQGAP1;ortholog
Q14766	Latent-transforming growth factor beta-binding protein 1;LTBP1;ortholog
P63096	Guanine nucleotide-binding protein G(i) subunit alpha-1;GNAI1;ortholog
P05556	Integrin beta-1;ITGB1;ortholog
P09497	Clathrin light chain B;CLTB;ortholog
Q9HCY8	Protein S100-A14;S100A14;ortholog
Q13277	Syntaxin-3;STX3;ortholog
Q96FQ6	Protein S100-A16;S100A16;ortholog
Q01518	Adenylyl cyclase-associated protein 1;CAP1;ortholog
P52907	F-actin-capping protein subunit alpha-1;CAPZA1;ortholog
P29353	SHC-transforming protein 1;SHC1;ortholog

Q6WKZ4	Rab11 family-interacting protein 1;RAB11FIP1;ortholog
P08134	Rho-related GTP-binding protein RhoC;RHOC;ortholog
Q7L7X3	Serine/threonine-protein kinase TAO1;TAOK1;ortholog
Q99733	Nucleosome assembly protein 1-like 4;NAP1L4;ortholog
P07737	Profilin-1;PFN1;ortholog
Q8NFH8	RalBP1-associated Eps domain-containing protein 2;REPS2;ortholog
P02675	Fibrinogen beta chain;FGB;ortholog
P13747	HLA class I histocompatibility antigen, alpha chain E;HLA-E;ortholog
O00182	Galectin-9;LGALS9;ortholog
Q92599	Septin-8;SEPT8;ortholog
O00159	Unconventional myosin-Ic;MYO1C;ortholog
P18065	Insulin-like growth factor-binding protein 2;IGFBP2;ortholog
Q5JWF2	Guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas;GNAS;ortholog
Q99584	Protein S100-A13;S100A13;ortholog
P62873	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1;GNB1;ortholog
Q12792	Twinfilin-1;TWF1;ortholog
Q02750	Dual specificity mitogen-activated protein kinase kinase 1;MAP2K1;ortholog
Q9BXI6	TBC1 domain family member 10A;TBC1D10A;ortholog
Q99747	Gamma-soluble NSF attachment protein;NAPG;ortholog
Q14247	Src substrate cortactin;CTTN;ortholog
O95967	EGF-containing fibulin-like extracellular matrix protein 2;EFEMP2;ortholog
P40121	Macrophage-capping protein;CAPG;ortholog
P11142	Heat shock cognate 71 kDa protein;HSPA8;ortholog
Q93045	Stathmin-2;STMN2;ortholog
Q9H2G2	STE20-like serine/threonine-protein kinase;SLK;ortholog
Q01970	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-3;PLCB3;ortholog
Q13501	Sequestosome-1;SQSTM1;ortholog
P98082	Disabled homolog 2;DAB2;ortholog
P33176	Kinesin-1 heavy chain;KIF5B;ortholog
Q9UJU6	Drebrin-like protein;DBNL;ortholog
Q14203	Dynactin subunit 1;DCTN1;ortholog
Q14512	Fibroblast growth factor-binding protein 1;FGFBP1;ortholog
P62745	Rho-related GTP-binding protein RhoB;RHOB;ortholog
Q9UK41	Vacuolar protein sorting-associated protein 28 homolog;VPS28;ortholog
Q13409	Cytoplasmic dynein 1 intermediate chain 2;DYNC1I2;ortholog
P35222	Catenin beta-1;CTNNB1;ortholog
P35221	Catenin alpha-1;CTNNA1;ortholog
P49368	T-complex protein 1 subunit gamma;CCT3;ortholog
P60981	Destrin;DSTN;ortholog

P50591	Tumor necrosis factor ligand superfamily member 10;TNFSF10;ortholog
Q9UPX8	SH3 and multiple ankyrin repeat domains protein 2;SHANK2;ortholog
Q14204	Cytoplasmic dynein 1 heavy chain 1;DYNC1H1;ortholog
P01024	Complement C3;C3;ortholog
Q14651	Plastin-1;PLS1;ortholog
P50990	T-complex protein 1 subunit theta;CCT8;ortholog
P43490	Nicotinamide phosphoribosyltransferase;NAMPT;ortholog
P12272	Parathyroid hormone-related protein;PTHLH;ortholog
P50148	Guanine nucleotide-binding protein G(q) subunit alpha;GNAQ;ortholog
O96013	Serine/threonine-protein kinase PAK 4;PAK4;ortholog
P11021	78 kDa glucose-regulated protein;HSPA5;ortholog
P84095	Rho-related GTP-binding protein RhoG;RHOG;ortholog
Q9H223	EH domain-containing protein 4;EHD4;ortholog
P06396	Gelsolin;GSN;ortholog
P0DOY2	Immunoglobulin lambda constant 2;IGLC2;ortholog
O94832	Unconventional myosin-Id;MYO1D;ortholog
P50395	Rab GDP dissociation inhibitor beta;GDI2;ortholog
O60437	Periplakin;PPL;ortholog
P08754	Guanine nucleotide-binding protein G(k) subunit alpha;GNAI3;ortholog
P36507	Dual specificity mitogen-activated protein kinase kinase 2;MAP2K2;ortholog
Q13576	Ras GTPase-activating-like protein IQGAP2;IQGAP2;ortholog
Q15365	Poly(rC)-binding protein 1;PCBP1;ortholog
Q16543	Hsp90 co-chaperone Cdc37;CDC37;ortholog
Q5VZK9	F-actin-uncapping protein LRRC16A;CARMIL1;ortholog
Q99832	T-complex protein 1 subunit eta;CCT7;ortholog
Q9Y5K6	CD2-associated protein;CD2AP;ortholog
Q9UBI6	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12;GNG12;ortholog
P62979	Ubiquitin-40S ribosomal protein S27a;RPS27A;ortholog
O00161	Synaptosomal-associated protein 23;SNAP23;ortholog
P50991	T-complex protein 1 subunit delta;CCT4;ortholog
P29992	Guanine nucleotide-binding protein subunit alpha-11;GNA11;ortholog
P11234	Ras-related protein Ral-B;RALB;ortholog
P17987	T-complex protein 1 subunit alpha;TCP1;ortholog
P13796	Plastin-2;LCP1;ortholog
P01871	Immunoglobulin heavy constant mu;IGHM;ortholog
P10909	Clusterin;CLU;ortholog
P04899	Guanine nucleotide-binding protein G(i) subunit alpha-2;GNAI2;ortholog
Q01814	Plasma membrane calcium-transporting ATPase 2;ATP2B2;ortholog
Q8N6N7	Acyl-CoA-binding domain-containing protein 7;ACBD7;ortholog
Q9HD15	Steroid receptor RNA activator 1;SRA1;ortholog
Q8N4C8	Misshapen-like kinase 1;MINK1;ortholog

P01834	Immunoglobulin kappa constant;IGKC;ortholog
P13489	Ribonuclease inhibitor;RNH1;ortholog
Q9H2K8	Serine/threonine-protein kinase TAO3;TAOK3;ortholog
O60716	Catenin delta-1;CTNND1;ortholog
Q02818	Nucleobindin-1;NUCB1;ortholog
Q8IZ21	Phosphatase and actin regulator 4;PHACTR4;ortholog
P55010	Eukaryotic translation initiation factor 5;EIF5;ortholog
P31949	Protein S100-A11;S100A11;ortholog
P48643	T-complex protein 1 subunit epsilon;CCT5;ortholog
Q96HC4	PDZ and LIM domain protein 5;PDLIM5;ortholog

Supplementary Table 3. Proteins unique to preterm milk exosomes identified with LC-MS/MS and Protein ANalysis THrough Evolutionary Relationships (PANTHER) database [92].

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Contributions of Collaborators

Professor Marc-André Langlois, Dr. Vera Tang & Anna Fritzsche – For the use of the UOttawa Flow Cytometry and Virometry Core and running samples on the LSRFortessa

Professor Maxim Berezovski & Dr. Zoran Minic– For the use and running samples on the Thermo Scientific™ Orbitrap Fusion™ Tribrid™

Yousef Risha – Preparing samples for mass spectrometry analyses and providing training on sample preparation

Ahmed Ibrahim – Gene Ontology analyses of mass spectrometry data and training on data analyses

Professor Jean François Beaulieu & Dr. Eric Tremblay – Cell culture work, sections:

3.6. Cell Culture

3.7. Caco-2/15 response to heat-killed bacteria

3.8. Pre-treatment of Caco-2/15 with human milk exosomes

3.9. Statistics

4.2. Effect of human milk exosomes on Caco-2/15 inflammatory response