Spring 5-2022

Characterization of Gangliosides and Fatty Acids in Extracellular Vesicles from Human Milk

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A Thesis by

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Submitted in partial fulfillment of the requirements for the degree of

Master of Science in Food Science

May 2022

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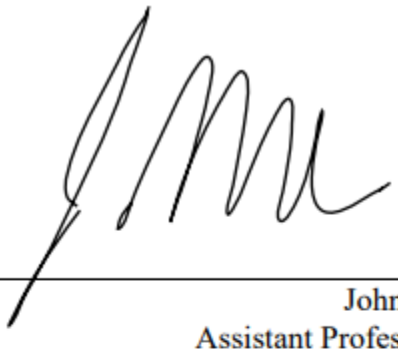

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ACKNOWLEDGEMENTS

Thank you, Dr. Miklavcic, Dr. Were, and Dr. Bisoffi for your time and for providing support in every way throughout this thesis project. I would also like to thank the study participants from UCI clinics who provided human milk samples, the Glynn Lab (Chapman University), the Curtis Lab (Univ. Alberta, Canada), and UC San Diego Lipidomics Core for providing data analysis, and my peers and colleagues who have provided valuable knowledge, peer-reviewed this paper, and helped me improve my laboratory research skills. This research was funded by the United States Department of Agriculture, award number 2020-67018-33315

DEDICATION

I dedicate this work to my parents who have continuously supported my siblings and my desire to pursue higher education with the hopes that it will enable us to achieve amazing opportunities and career goals.

ABSTRACT

Characterization of gangliosides and fatty acids in extracellular vesicles from human milk

by Aliya Amin

Extracellular vesicles (EVs) are bioactive components of human milk that may impact functionality to regulate growth, cognitive development, metabolism, and immunity in newborns. The biogenesis of EVs and the mechanism by which EVs elicit biologic effects *in vivo* have not been characterized entirely. Gangliosides and fatty acids are integral in the formation, release, stability, and functionalities of EVs. Presumably, EV function is related to EV composition and lipids in EVs influence the bioavailability of EV cargo and downstream functions of EVs. The objective of this research was to characterize the fatty acid and ganglioside composition of EVs in human milk. Human milk (n=31) was obtained 2-4 weeks postpartum and EVs were isolated using a precipitation method. Gangliosides and fatty acids were extracted from the milk EVs using a modified Folch method. Relative (%w/w) fatty acids and ganglioside content was determined by GC-MS and HILIC-HPLC/MS-MS. The relative content of fatty acids and gangliosides within human milk EVs was assessed in relation to the relative content in the whole human milk. The EV isolation procedure yielded 2.08×10^{10} EV particles/mL of human milk and 4.71 (± 2.9) μg protein/μL of human milk. The relative content of 18:2, ω-6 fatty acids, and PUFAs were not correlated between human milk and human milk EVs ($p > 0.05$). 22:6, 20:4, and 18:0 are enriched in EVs (1.5 – 2.5-fold); while 18:1 content is 50% lower in EVs than whole human milk. In contrast, 13/15 molecular species of GM3 and GD3 assessed were not positively correlated between human milk EVs and human milk. Total saturated species of GM3 are enriched 2-fold in human

milk. GM3 d40:1, GM3 d34:1, and GD3 d36:1 are significantly enriched in human milk EVs. 18:2 and 20:4 contents are not correlated between human milk EVs and the whole human milk, indicating discriminate partitioning of lipids species containing these fatty acids into EVs in EV biogenesis, or the conversion of these fatty acids into mediators which aid in permeability of EVs and influence functionality. Further understanding of EV composition may offer insight into the roles of human milk EVs in infant growth and development.

Keywords: exosome, lipids, nanoparticle tracking analysis, nutrition

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CM: Chloroform-methanol

DLS: Dynamic light scattering

exRNA: Exosomal RNA

EV(s): Extracellular vesicle(s)

HPLC: High-performance liquid chromatography

IEC: Intestinal epithelial cells

ILV: Intralumenal vesicle

LCPUFA: Long-chain polyunsaturated FAs

MFGM: Milk fat globule membrane

MVB: Multivesicular body

NEC: Necrotizing enterocolitis

NTA: Nanoparticle tracking analysis

PBS: Phosphate-buffered saline

1 EXTRACELLULAR VESICLES IN HUMAN MILK: A LITERATURE REVIEW AND INTRODUCTION

1.1 Introduction

Extracellular vesicles are 40-1,000 nm-sized, lipid membrane-bound, cell-derived structures known as exosomes, microvesicles, and apoptotic bodies. EVs are released by most cells and are present in tissues and biological fluids such as blood, tears, saliva, urine, cerebrospinal fluid, and milk (Wiklander et al., 2019). Extracellular vesicles carry biological cargo such as lipids, protein, RNA, and DNA and are involved in intercellular communication (Chuo et al., 2018). (Zonneveld et al., 2014) have identified the roles of EVs in infant health and development and in the treatment of neuronal, coronary, and intestinal diseases. The lipid fraction of milk EVs contains lipid-containing compounds such as gangliosides and fatty acids (Yu et al., 2011). Gangliosides are sialic acid-containing glycosphingolipids, and are found in milk and blood, nerve, muscle and brain tissues of humans. Gangliosides in milk occur as free, protein-bound part of the milk fat globule membrane (MFGM) and EVs (Wiklander et al., 2019). Human milk-derived gangliosides may impart various health benefits to the immune system, gastrointestinal tract, and nervous system (Chuo et al., 2018).

However, the relative content and composition of gangliosides and fatty acids have not been investigated in human milk EVs. EV function may depend on EV compositions since structure and function are interrelated in biology. Additionally, characterizing the composition of EVs may be a starting point to understanding the biogenesis and functions of EVs specific to human milk. Understanding the lipid content and composition of EVs in milk may inform the food industry on how to optimize infant formulas and milk fortifier products to better match the composition of human milk, thereby narrowing disparities in health outcomes between

consumers of infant formulas and human milk. Additionally, characterizing the composition of EVs may help identify the mechanisms involved in EV biogenesis, which is necessary to understand before EVs can be used as food ingredients or clinically as biomarkers.

1.2 Overview of Human Milk in Infant Health

Human milk is the optimal nutritional source for neonatal and infant growth and development and its benefits are attributed to its biological composition such as lactoferrin, immunoglobulin A and cytokines which are involved in immunological functions (Dufton & Perretti, 2010). Oligosaccharides strengthen gut barrier integrity in infants and are protective against lower respiratory infections (Caplan et al., 2001; Ohtsuka et al., 2011). Human milk lipids and fatty acids serve as the primary energy source for infants and regulate growth and cognitive development, metabolism, and the immune system (Ramiro-Cortijo et al., 2020). In addition to these bioactive components, human milk contains a diverse microbiome consisting of *Lactobacillus* and *Bifidobacterium* that influence the infant microbiota (Camuesco et al., 2006). The bacteria participate in immunological functions such as T-helper cell balance, mucus production, and tight junction regulation (Witkowska-Zimny & Kamiska-El-Hassan, 2017). Human milk and its complex composition are being investigated as potential therapeutic fluids (Galley & Besner, 2020).

1.3 Description and Significance of Extracellular Vesicles

EVs can be classified by size and distinct population subtypes include apoptotic bodies, microvesicles, and exosomes. Cells that undergo apoptosis release apoptotic bodies 1-5 μm in diameter and contain intracellular fragments, cell organelles, and fragmented DNA (Gyorgy et al., 2011). Microvesicles range between 100-1000 nm in diameter and are produced by outward

budding of the plasma membrane. The best characterized extracellular vesicles are Exosomes which range from 30-100 nm in diameter, and originate from endosomes (Fernando et al., 2017).

The type of cell and physiological state of the cell controls the content of exosomes and can be differentiated from other extracellular vesicles by testing for tetraspanins and specific biomarkers (CD81, CD63, and CD9) (Wassmer & Hoflack, 2010). Exosomes modulate immune functions (Niel et al., 2018), transfer genetic material (Valadi et al., 2007), and mediate intercellular communication using various biomarkers (Raposo & Stoorvogel, 2013).

EVs are present and detectable in nearly all bodily fluids. Based on their different cellular origins, EVs play diverse roles in physiological functions such as inflammation, neuronal function, cell proliferation (Zhou et al., 2020), and EVs are thus essential vehicles in local and distant intercellular communication. One of the first studies on the functional activities of EVs reported that vesicles secreted from prostate epithelial cells promoted sperm cell motility (Stegmayr & Ronquist, 1982). Since then, the antigen-presenting activity of exosomes has been investigated in stimulating antitumoral immune responses (Bobrie et al., 2011; Chaput & Thery). Tumor cells secrete EVs, which may promote angiogenesis and tumor cell migration contributing to tumor progression (Hood et al., 2011). In addition, mRNA, microRNA, and exosomal RNA isolated in exosomes from mouse and human mast cells can be delivered to target cells and be biologically active (Valadi et al., 2007). Valadi et al., (2007) also revealed that mRNA distribution in exosomes differed from the cytoplasm of donor cells, suggesting that nucleic acids are selectively loaded into exosomes. Exosomes carry cell and cell-specific proteins, lipids, nucleic acids (miRNA and mRNA) that are being investigated in their roles as biomarkers with potential applications in disease characterizations (**Table 1**).

Table 1. EVs from various human biological fluids as biomarkers for disease

Origin of EVs	a) Focus of Study and b) Biomarker	Key Findings	Citations
Saliva	a) EV composition as indicators for oral and non-oral cancers b) miRNA	Most of the miRNA in saliva is packaged in exosomes EVs are concentrated in saliva Salivary EVs are enriched in exosomal RNAs (exRNA)	(Chiabotto et al., 2019)
Neuronal exosomes from blood, cerebral spinal fluid	a) Potential fluid biomarkers for the diagnosis of neurodegenerative disease b) Protein	Exosomes have protective roles in Alzheimer's disease pathogenesis	(Hornung et al., 2020)
Urine	a) Potential biomarkers for diagnosis of kidney disease b)RNA	The integrity of RNA in urinary EVs is similar to that of kidney tissue RNA in EVs is better preserved than in whole urine because EV membranes protect against RNase	(Salih et al., 2014)

Standardized EV characterization methods are necessary to understand EVs' specific functions and characteristics from different biological fluids. The present study investigated and characterized the lipid-enriched fractions of human milk-derived EVs.

1.3.1 Extracellular Vesicles in Human Milk

Extracellular vesicles in human milk are bioavailable and contain bioactive lipids, proteins, noncoding RNAs, and mRNAs (Zempleni et al., 2017). More importantly, exosomes in milk protect labile cargos (miRNA, mRNA) against degradation in the human body and serve as vehicles to uptake cargo into tissues (Benmoussa et al., 2016; Izumi et al., 2012). The intestinal uptake of milk exosomes is facilitated by endocytosis and has been studied in human colon carcinoma Caco-2 cells and rat primary small intestine IEC-6 cell cultures (Wolf et al., 2015).

Multiple mechanisms of formation, release and uptake of extracellular vesicles have been identified.

the content of miRNAs in various infant formulas is <5% of that in "mature" human milk that is produced 2 weeks after lactation (Zempleni et al., 2017; Zhou et al., 2012). Little is known about the importance of human milk-derived exosomes and respective cargos in infant health. The difference in miRNA amounts between infant formulas and human milk is important because miRNAs regulate human genes and develop the immune system (Zhou et al., 2012). A longitudinal study compared the effects of infants fed human milk, milk formulas, and soy formulas regarding body composition development. Infants fed human milk had the highest percentage of fat mass (27.84% fat mass, FM) during the first three months of life as compared to infants fed milk formulas (23.47% FM) and soy formulas (21.37% FM) (Andres et al., 2013). The differences in body composition were caused by different feeding treatments; however, it is not clear whether the exosomes and their cargoes found in the different treatments may have also influenced body composition.

Most studies on exosomes have been on bovine milk and have focused on characterizing mRNAs and miRNAs functions in the human body. In contrast, characterization of lipids from human milk EVs is limited, and lipid characterization has focused on human milk as a whole (**Table 2**).

Table 2. Characterization of gangliosides and fatty acids in human milk

(A) Lipid **(B) Study Design**	**Key Findings**	**Citations**
(A) Ganglioside (GA) (B) Longitudinal study on breast milk GA concentrations in Malaysian mothers using HPLC-MS[a], results compared to a larger cross-sectional study	GD_3:GM_3 ratio decreased through lactation period from 3:1 in transitional milk to 0.17:1 in 6-12 month mature milk GM_3 was the dominant GA class (86–89% of the total GAs measured)	(Lin et al., 2015)

	GA concentration 0.25 mL samples of transitional human milk: 7.1 ± 7.7 mg L^{-1} GM_3, 20.2 ± 13.0 mg L^{-1} GD_3, and 27.3 ± 16.7 mg L^{-1} total GAs	
(A) Fatty Acids (FA) (B) Analyze FA composition of GM3 and GD3 in pooled human and bovine milk using MALDI-TOF-MS[b]	Long-chain fatty acids higher in bovine milk GA (GM3: 73.71 ± 3.39%, GD3: 79.19 ± 2.79%) than human milk GA (GM3: 51.25 ± 0.65%, GD3: 34.04 ± 1.80%) Fatty acid concentration (g/100 g FA) in human milk GM3 vs. GD3: Palmitic acid (16:0): 11.65 ± 0.73, 18.42 ± 0.13; Stearic acid (18:0): 16.59 ± 0.40, 18.67 ± 0.31; Behenic acid (22:0): 18.57 ± 0.47, 10.87 ± 0.98; SFA: 78.11 ± 0.21, 82.24 ± 2.14; MUFA: 16.92 ± 0.24, 13.92 ± 0.63; PUFA: 4.97 ± 27, 3.84 ± 0.31	(Bode et al., 2014)

[a]High performance liquid chromatography-mass spectrometry (HPLC-MS)
[b]Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS)

Glycoproteins on the surface of exosomes and lipids in the membrane bilayers of exosomes are involved in the stability of exosomes, the uptake of exosomes by recipient cells, and the regulation of genes and metabolism in the human body (Escrevente et al., 2011). Two main databases, ExoCarta and Vesiclepeida (Mathivanan et al., 2012; Vesiclepedia, 2013), catalog the composition and cargos of EVs in various species. ExoCarta focuses on the cargo found in exosomes, while Vesiclepedia catalogs extracellular vesicles aside from exosomes. The ExoCarta database has identified roughly 81 proteins in human milk exosomes, but the specific lipid composition in exosomes is unclear and is estimated to contain around 1000 different lipid species (Zempleni et al., 2017). Therefore, further studies are needed to characterize the content and composition of human milk extracellular vesicles, particularly EV lipid composition, and better understand the roles of exosomes in infant health and development can be assessed.

1.3.2 Biogenesis of Mammalian Extracellular Vesicles

The biogenesis of exosomes by exocytosis of multivesicular endosomes was discovered in 1983 (Harding et al., 2013). Extracellular vesicles are generated at two main cellular sites, the plasma membrane and the endosomal membrane. Ectosomes are vesicles that shed directly from the plasma membrane, while Exosomes are generated through the fusion of multivesicular bodies (MVBs), formed from the inward budding of the endosomal membrane with the plasma membrane (Hornung et al., 2020). Multivesicular bodies contain intralumenal vesicles (ILVs) that are released as exosomes into extracellular spaces following the fusion of MVBs with the plasma membrane (Paulaitis et al., 2018). The exact mechanisms involved in exosome uptake into recipient cells is unknown; however, non-specific endocytotic mechanisms (Hoshino et al., 2015), receptor-dependent pathways, and the direct fusion of exosomes with plasma membranes have been proposed (Montecalvo et al., 2012).

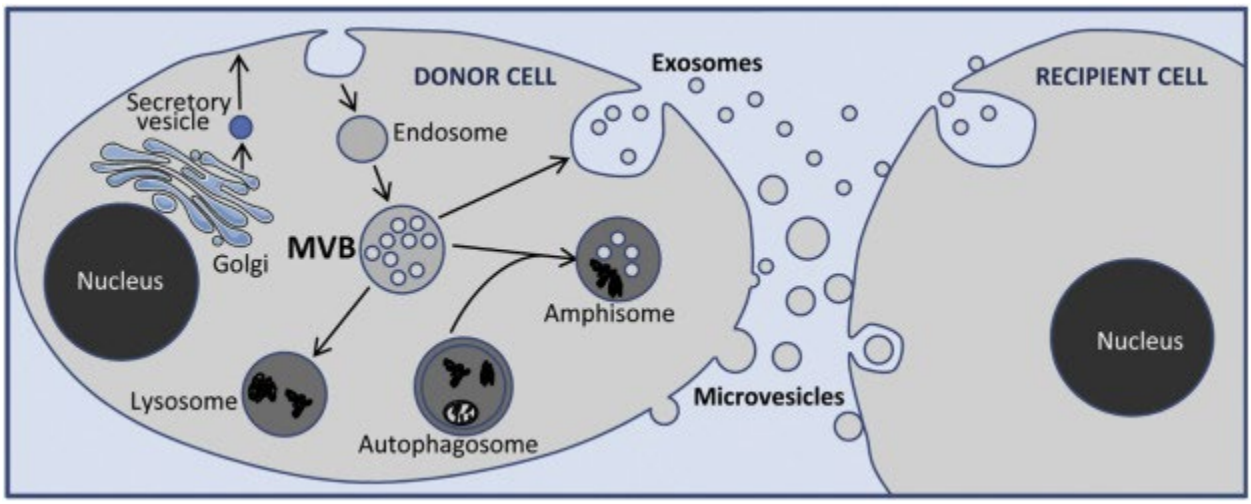


Figure 1. Formation and release of exosomes from mammalian cells. Retrieved from Skotland et al. (2020): Exosome release requires: (1) the formation of intralumenal vesicles (ILVs) in endosomes that fuse with multivesicular bodies (MVBs), (2) the transport of MVBs to the plasma membrane, and (3) the fusion of MVB membrane with the plasma membrane, resulting in the release of exosomes into the extracellular space. The exosome can participate in intercellular communication by binding to the plasma membrane of a cell or is taken up by recipient cells

The formation and release of EVs involve interactions with lipids and fatty acids (Hurley, 2010). The role of lipids in EV release includes the structural part of lipids in the EV membrane, the effects of lipid metabolism on EV membrane fluidity and structure, and the possible roles of lipids as signaling molecules (Skotland et al., 2020). the levels and activities of different classes of membrane lipids are essential for the formation and release of EVs through unique activities (**Table 3**). The lipid composition of EV membranes is suspected to affect the stability of EVs in different extracellular environments and the uptake of EVs into recipient cells (Skotland et al., 2020).

Table 3. The roles of lipids and fatty acids in the formation and release of exosomes from mammalian cells

Class of Lipid	Key Findings	Citation
Phospholipids	Phospholipase D2 is required for the formation of ILVs[a] and controls the budding of ILVs into MVBs[b]	(Jimenez & Zimmermann, 2018)
Sphingolipid (A) Ceramide	Ceramide transport protein with saturated fatty acid palmitate (C16:O) induced a lipotoxic stress leading to EV release in hepatocytes	(Kakazu, 2016)
Cholesterol	Cholesterol prevents ion leakage and affects membrane fusion When cholesterol levels were decreased by adding methyl-β-cyclodextrin, a cholesterol-sequestering agent, cellular levels of caveolin-1, a membrane protein associated with endocytosis increased	(Llorente et al., 2007)

[a]Intralumenal vesicles
[b]Multivesicular bodies

1.3.3 Functions of Bovine and Human Milk EVs

Milk is a unique source of exosomes since lactating mammals can produce larger volumes of milk per day (750-1,035 mL of human milk per day, 20 L for bovine milk),

compared to other fluids with exosomes such as blood plasma (400-1,500 mL per day). However, exosomes from human milk have been studied less than blood and culture-derived exosomes. The practical use of milk exosomes has been proposed in medicine and biology due to the lipids, nucleic acids, and proteins present; however, further characterizations of the composition of exosomes in milk are necessary to understand the functionalities (Sedykh et al., 2020). Additionally, the molecular composition of human milk exosomes is dependent on environmental factors, contact with allergens, and lactation stage (Torregrosa et al., 2014).

Human milk may confer protection from gastrointestinal (GI) diseases as found in studies on necrotizing enterocolitis (NEC) which affects premature infants. Treatments for NEC involve antibiotics and medical, and nutritional therapies; however, the provision of human milk saccharides and anti-microbial peptides were effective in the treatment of NEC (Good et al., 2016; Gunasekaran et al., 2019; Pammi & Suresh, 2017). Human milk oligosaccharides, leukocytes, and growth factors provide immunological defenses which reduce inflammatory responses, thereby reducing the incidence of NEC (Nolan et al., 2020).

In addition, milk exosomes are being investigated in GI treatment because human milk-derived exosomes are stable, resilient to digestion, can withstand stressors in systemic circulation (Liao et al., 2017), and can undergo endocytosis by intestinal epithelial cells (Galley & Besner, 2020). The milk-derived exosomes' activities on intestinal functions are summarized in (**Table 4**).

Table 4. Activities of milk extracellular vesicles in intestinal health and development

(A) Biological component and (B) Experimental model	**Key Findings**	**Citation**
(A) miRNA Lgr5 (B) Rat milk exosomes	Increased intestinal cell proliferation by activating stem cell marker, Lgr5	(Hock et al., 2017)

(A) miRNA let-7b (B) Human milk exosomes	Regulates inflammation by activating Toll-like receptor 4	(Vilella et al., 2015)
(A) Peptides (lactotransferrin and lactadherin) (B) Human milk exosomes	Peptides were upregulated when intestinal cells were treated with human milk exosomes	(Wang et al., 2019)
(A) Oligosaccharide 2'-fucosyllactose (B) Human milk exosomes	Increases endothelial nitric oxide synthase 3 (eNOS) production, protects against intestinal disease and necrotic pathology	(Good et al., 2016)

Additionally, when human milk EVs oral versus intraperitoneal (IP) injection methods in a rat NEC model were compared, the orally delivered method had higher efficacy. Two hundred eighty-eight mature miRNAs and 610 miRNAs at low abundance were found in the orally-delivered isolated exosomes, which may be related to the high stability of human milk EVs in the digestive tract (Liao et al., 2017). Human milk EVs also regulates proliferation and apoptosis demonstrated in rats (IEC-6) and humans (FHS74) (Hock et al., 2017; Pisano et al., 2019). While some functions of human milk EVs have been described, the structural composition of EVs requires further research and will help inform additional functions.

1.3.4 Isolation of EVs from Human Milk

The minimal information for studies of extracellular vesicles (MISEV) are guidelines for verifying the separation and isolation of EVs, characterization and functional studies to stimulate improved reliability and reproducibility of EV studies (Thery et al., 2018). MISEV2018 guidelines recommend that methods used to prepare EVs are described thoroughly. EVs are characterized quantitatively by total particle number or relative protein or lipid content and tested for the presence of non-vesicular co-isolated components or EV markers.

Differential ultracentrifugation and methods involving low buoyant densities and differences in flotation velocities of EVs are most used to isolate EVs from human milk but result in low purity and EV integrity (Aalberts et al., 2012; Gardiner, 2014; van Niel et al., 2003). Commercially available kits have also been developed to isolate EVs quickly from human milk. Bickmore (2020) isolated EVs using a small volume (1 ml) of human milk with a precipitation reagent (ExoQuick TC®; BI, Palo Alto, CA). EVs were successfully isolated with a yield of 8.9 × 109 ± 1.1 × 10^9 EV particles/mL of human milk. However, EV analysis resulting from commercial isolation kits often fails to differentiate between different classes and sizes of EVs, and additional confirmation tests such as immunoblotting, mass spectrometry, and imaging are needed (Gardiner, 2014; Raposo & Stoorvogel, 2013). Conventional electron microscopy can visualize both small and large EVs; however, the morphology of the EVs may be altered during this process from a rounded shape to a cup-shaped morphology (Raposo et al., 1996). Nanoparticle tracking analysis allows for a more accurate determination of the size distribution of isolated EVs in solution (Nolte-'t Hoen et al., 2012).

The isolation and purification of human milk EVs are affected by the storage temperature of human milk. Zonneveld et al., (2014) found that human milk spiked with cells of murine origin had higher amounts of EV markers when stored at -80 °C (CD9: 22-225% and murine MHC Class II: 186-2,222%) as compared to human milk that was analyzed at room temperature immediately after isolation. Low-temperature storage results in the formation of ice crystals that cause stress and cell death and induce the formation of new EVs; thereby altering the original isolated EV sample. It is unknown how the time after human milk expression affects EV content, composition, and stability. So, guidelines for a standardized collection, storage, and isolation protocol to minimize functional analysis of milk EVs if of utmost interest.

More accurate methods to discriminate between the different classes of EVs such as exosomes from microvesicles, are also necessary since distinctions based on general properties such as size, densities, morphologies, and protein composition are insufficient (Bobrie et al., 2011). More importantly, standardized isolation and purification methods must be established before human milk EVs can be used as biomarkers, carriers for vaccines, and as food ingredients (Raposo & Stoorvogel, 2013). Some procedures have been established to address the need for more accurate purification methods, such as protocols involving microfiltration of human milk to reduce bacterial contamination (Fournell et al.) and specific conditions to store human milk to preserve its chemical and nutritional content (McKendry, 2014). However, standardized methods for the isolation of EVs from human milk have not been established.

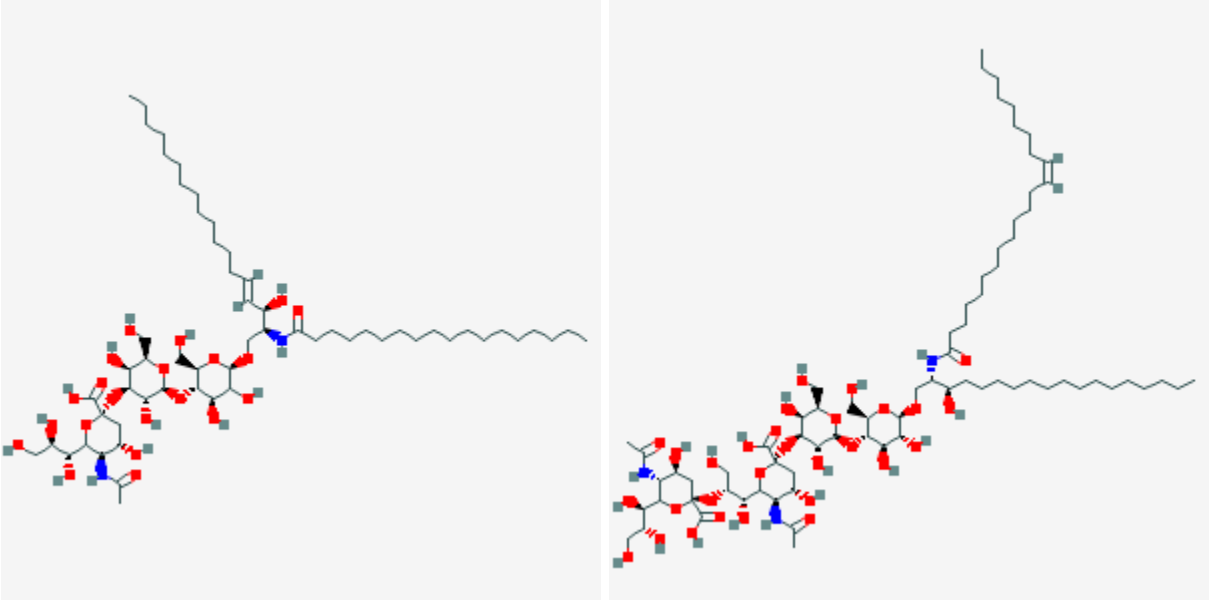
1.4 Lipid Composition of Extracellular Vesicles

1.4.1 Role of Gangliosides in Human Milk

GAs are sphingolipids distinguished by the type of ceramide attached to the sialic acid-containing oligosaccharides (Lin et al., 2015). The classes of gangliosides are characterized Svennerholm, (1963) using high-performance liquid chromatography (HPLC) are based on the variety of sialo-oligosaccharide groups found in GAs and the species and cell-dependent FAs found in the ceramide.

The dietary GAs from human milk are part of the milk fat globule membrane in milk (Jensen et al., 1990) and are involved in neurological development, memory formation, immune system regulation, and *Bifidobacterium* proliferation in the gut of infants (McJarrow et al., 2009). The main classes of GAs identified and studied in human milk are the mono-sialylated variant GM_3 (α-Neu5Ac(2–3)-β-D-Galp(1–4)-β-D-Glcp(1–1)-Cer) and the di-sialylated variant GD_3 (α-Neu5Ac(2–8)-α-Neu5Ac(2–3)-β-D-Galp(1–4)-β-D-Glcp(1–1)-Cer) (Lin et al., 2015)

(**Figure 2**). The reported sum concentration of GM_3 and GD_3 in milk varies from 2 mg L^{-1} (Martin-Sosa et al., 2004) to 25 mg L^{-1} (Pan & Izumi, 1999).



(a) Ganglioside GM_3 (D18:1/18:0) (b) Ganglioside GD_3 (d18:0/22:1(13Z))

Figure 2. structures of ganglioside classes GM_3 (a) and GD_3 (b) (PubChem, 2020a, 2020b). GM_3 is primarily located within the cell membrane and exosomes, while GD_3 is in the membrane, myelin sheath, and exosomes. Colors are used to identify common atoms: carbons are gray, oxygens are red, and nitrogens are blue.

The variations in ganglioside content are attributed to different extraction protocols (Jensen et al., 1990), different stages of lactation, and differences in maternal diet (Lin et al., 2015). The ganglioside concentrations at 2, 6, and 12 months of lactation are 14.8, 25.3, and 16.6 mg L^{-1}, respectively, with average total ganglioside concentrations increasing by 5 mg L^{-1} from the second to the third trimester (Lin et al., 2015). The composition of gangliosides compared to human milk colostrum (the first form of milk produced) and later human milk, cow's milk, and infant formulas found that the total lipid-bound sialic acid levels (an indirect measure of ganglioside concentration) were two times higher in human milk than in cow's milk or infant formulas (Pan & Izumi, 2000). The most abundant ganglioside in human colostrum (early human milk) was GD3 (49%). In later human milk, the amount of GD3 decreases to

31.8%. Although gangliosides in human milk have been studied, the relative amount and compositions have not been specifically characterized in human milk exosomes.

1.4.2 Role of Fatty Acids in Human Milk

Fatty acids provide half of the energy intake for breastfed infants. Human milk fatty acids are also important regulators of development, metabolism, immune system functions and contribute to the lipid composition of cells; therefore, the content and composition of fatty acids present in human milk exosomes, which are comprised of a lipid bilayer, are of interest (Skotland et al., 2020). The levels of unsaturated and saturated fatty acids in human milk slightly differ depending on maternal diet and disease prevalence (Galley & Besner, 2020).

The nutritional and physiological properties of human milk fat are characterized by lipid and fatty acid composition (Ramiro-Cortijo et al., 2020). The saturated fatty acid, palmitic acid (C16:0) accounts for 25% of human milk fatty acids (Koletzo, 2016). Monounsaturated fatty acids are more stable than saturated fatty acids and account for 45-50% of human milk fat, with 36% present as oleic acid (C18:ln-9). Oleic acid lowers the melting point of triglycerides and the added liquidity enables the formation and transport of the milk fat globule (Jensen, 1999). Long-chain polyunsaturated fatty acids (LCPUFAs), such as linoleic acid and its derivatives, arachidonic acid (ARA), and docosahexaenoic acid (DHA), have anti-cancer and anti-inflammatory activities (Aglago et al., 2019; Jensen et al., 1990).

LCPUFAs have protective effects on intestinal diseases and necrotizing enterocolitis (Ramiro-Cortijo et al., 2020). For example, the addition of ARA and DHA to human adult and fetal intestinal epithelial cells resulted in a decrease in pro-inflammatory responses in intestinal inflammatory diseases(Wijendran et al., 2015) and improved the intestinal barrier by inducing the release of cytokines (Li et al., 2008).

The content and composition of fatty acids in human milk EVs have not been characterized and may provide clarity on EV functionality.

2 RATIONALE AND SIGNIFICANCE

Interest in EVs has increased since the discoveries that EVs are abundantly present in body fluids (Raposo & Stoorvogel, 2013). Investigation into the structure and function of EVs in human milk is at an early stage. Increased understanding of the composition of EVs in human milk will allow researchers manipulate EV functionalities and open a gateway to understanding potential equivalence of bovine milk EVs (Sedykh et al., 2020).

The ability of cells to produce lipid-bound vesicles appears to be universal, as this process has been observed in plants, bacteria, and animals (Skotland et al., 2020). However, the discovery of abundant amounts of extracellular vesicles in human body fluids such as human milk and the finding that healthy human cells, in addition to apoptotic cells, shed vesicles from the plasma membrane has expanded research interest in this field; particularly for the roles of EVs in intracellular communication, infant development, and in intestinal diseases (Raposo & Stoorvogel, 2013). The biological cargo of EVs determines the functional roles of EVs in the human body. Nucleic acids and proteins from human milk EVs have been characterized and studied in more detail than the lipid fraction of EVs, as they are involved in gene regulation. Characterization of EV lipids such as gangliosides and fatty acids is necessary since lipids are involved in EV release and formation and form the lipid bilayer (Skotland et al., 2020). Alterations in lipid composition may impact the transportation of the molecular cargo transported by EVs, thereby affecting the functionality of human milk EVs. Interest in human milk as a therapeutic bio-fluid is partially attributed to the resilience of human milk EVs to gastrointestinal

stresses and their ability to be taken up by intestinal epithelial cells through endocytosis (Galley & Besner, 2020).

This research characterized the content and composition of fatty acids and gangliosides in EVs from human milk. The rationale is lipid content between individuals and within human milk (ex. time of day, etc) varies; therefore, EV composition is also expected to vary. The central hypothesis is that specific fatty acids and gangliosides are present in different proportions in human milk EVs relative to the whole human milk. Differential partitioning of lipid species into or within EVs may have a functional consequence to EVs in human milk. In previous research, EVs in milk was shown to be enriched two-fold in essential fatty acid precursors relative to human milk as a whole and omega-3 fatty acids products (EPA and DHA) are increased in the milk (Miklavcic et al., 2020). Polyunsaturated fatty acids are partitioned in human milk EVs in a structured and organized manner that presumably impacts EV function.

The research is innovative because EV research so far has largely been on miRNA, and little attention has been given to the lipid profile of EVs and the presumed effects of EV lipids on infant health. Lipids are dynamic and participate in the formation, release, stability, and functionalities of EVs. EVs contain lipid bilayers, and the composition of membranes varies and affects EV bioavailability, digestion and absorption, uptake and transport, and the downstream effects of recipient cells . Further understanding of human milk EV composition and bio-availabilities, particularly through the characterization of EV-derived lipids, will offer insight into the formation, release, and functionalities of EVs. Characterizing the amounts and types of lipids in human milk EVs may inform the food industry on how to optimize infant formulas and milk fortifier products to better match the composition of human milk, thereby increasing the effectiveness of infant formula as an alternative when human milk is not available.

3 METHODS

3.1 Isolation of EVs from Human Milk

Human milk (n=37) were obtained 2-4 weeks postpartum from mothers receiving care at the University of California, Irvine clinics. Inclusion criteria was over 18 years old, English-speaking, absence of alcohol or drug use during pregnancy, lack of endocrine, hepatic, or renal disorders, a normal uterus and cervix, and a singleton pregnancy. The milk samples used in the study. A modified protocol from Bickmore (2020) was followed to isolate EVs from human milk (n=31) using a commercial EV isolation kit (**Figure 3**), ExoQuick TC® (SBI, Palo Alto, CA).

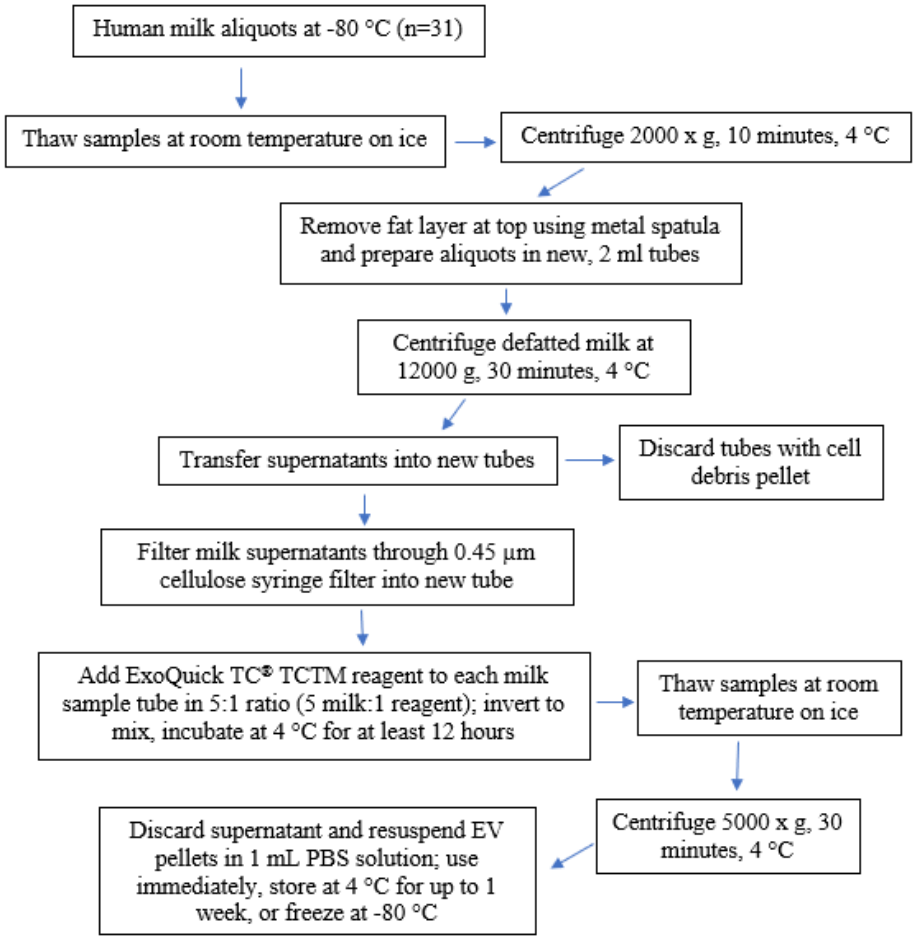


Figure 3. Isolation of EVs from human milk

Modifications to the ExoQuick protocol (SBI, 2018) include that isolated EVs were not purified with the ExoQuick® ULTRA columns Bickmore (2020), but instead were resuspended in 1 mL of PBS solution and immediately stored at -80 °C until further analyses. Isolated EVs were verified and quantified using Qubit™ 4 fluorometer and nanoparticle tracking analysis.

3.2 EV Isolation Confirmation Tests

3.2.1 Protein Quantification

Qubit™ 4 Fluorometer (Thermofisher; Waltham, Massachusetts) was used to measure the protein concentration of EVs isolated from human milk. The instrument was calibrated with three Qubit protein standards to generate a standard curve. The samples were prepared following the manufacturer's instructions and diluted to 1:100 with PBS solution prior to analysis.

3.2.2 Nanoparticle Tracking Analysis (NTA)

ZetaView® Nanoparticle Tracking Analyzers PMX120 (Particle Matrix, Munich, Germany) with ZetaView Software Version 8.05.12 SP1 (Particle Metrix GmbH, Inning, Germany) was used following the manufacturer's instructions (PMX, 2020) to measure the size (nm) and concentration (particles/ml of sample) of EVs. EVs were freshly isolated (<2 hours) and serially diluted to 1/1,000 with PBS prior to analysis. A 100-250 particle per frame rate optimizes instrument sensitivity; therefore, the dilution factor depends on the concentration of EVs in the sample being tested. Prior to sample analyses, a diluted (1:250,000) 100 nm polystyrene solution was prepared to auto-align the lasers in the instrument. Analysis parameters were set to a minimum brightness of 20, a maximum area of 1000 pixels, and a minimum area of 20 pixels. Camera sensitivity was set to 80 with a framerate of 30 frames per sec, and a shutter speed of 100. Measurement parameters were set at 11 positions with 2 cycles of reading per position. A minimum of 8 out of 11 valid positions are required to accept data and generate

analyses. Statistical outliers are automatically detected by the instrument and removed from the final data set.

3.3 Total Fatty Acid Analysis

Fatty acid analysis was performed at the University of California, San Diego Lipidomics Core (Quehenberger et al., 2010) using gas chromatography-mass spectrometry (Agilent 5975 GC/Mass Spectrometer).

3.4 Isolation of Gangliosides from Human Milk

Gangliosides were extracted through liquid phase separation using a 2:1 solution of chloroform-methanol (CM). Samples were thawed on ice at room temperature, transferred to 15 mL conical tubes, followed with the addition of 2:1 CM, and then vortexed for 15 seconds. 2 mL of CM was added to the human milk samples, while 600 µL of CM were added to the human milk EV samples. The samples were centrifuged at 241 x g for 10 minutes at 4 °C to induce phase separation into three layers. The supernatants were transferred to 2 mL glass vials with Teflon-coated screw-cap lids (Thermo Fischer). An additional 1 mL CM was added to the original 15 mL conical tubes and processed again to collect any additional supernatant. Samples were dried using a heating module (Thermo Scientific, Reacti-Therm III #TS-18824) at 45 °C under nitrogen gas.

3.5 Total Ganglioside Analysis

Gangliosides were stored in individual 2 mL glass vials flushed with nitrogen gas and sent to the University of Alberta, Canada for total GA analysis. Hydrophilic interaction chromatography and high-performance liquid chromatography coupled with mass spectrometry (HILIC

HPLC/MS-MS) were used to separate and measure seven different classes of GAs (GD3, GM3, GM2, GM1, GD1a, GD1b, and GT1b).

An Agilent 1200 series system coupled to a 3200 QTRAP mass spectrometer (AB Sciex, Concord, ON, Canada) and Analyst 1.4.2 software was used to analyze and acquire data for standards in solvent and sample solutions. Xbridge HILIC (150x2.1mm, 3.5um, Waters) was used for HPLC separation. mobile phase, **A** was 50/50 acetonitrile/buffer (50mM ammonium acetate) and **B** 100% acetonitrile, with the following gradient: 0.1min 5% **A**, increased to 80% **A** over 20 min, hold at 80% **A** for 2 min, then back to 5% **A** at 22.1 min for 8 min to re-equilibrate the column. A 10 µl injection volume with a flow rate of 300 µl/min was used, and the cycle time was 30 min/injection. A turbo ion spray source was employed under negative mode and nitrogen was used as curtain gas, drying gas, and nebulizing gas. Curtain gas, gas 1 and gas 2 were set at 20, 40 and 50, the ion spray voltage was at -4500V, and the ion source temperature was at 400ºC. The pooled samples were analyzed on Exactive Orbitrap at the full scan from 100 to 2000 amu under the same HPLC conditions.

4 RESULTS

4.1 Isolation and Confirmation of Human Milk EVs

The isolation of EVs was confirmed in accordance with MISEV criteria (Thery et al., 2018). Protein concentration was assessed by Qubit™ 4 fluorometer, and particle size and concentration were assessed by Nanoparticle Tracking Analysis. The average protein concentration of the EVs (n=31) was 4.71 (± 2.9) µg/µl of human milk, with minimum and maximum values of 2.65 and 7.52 µg EV protein/µl of human milk.

Human milk (n=2) EVs were assessed using NTA (**Figure 4**).

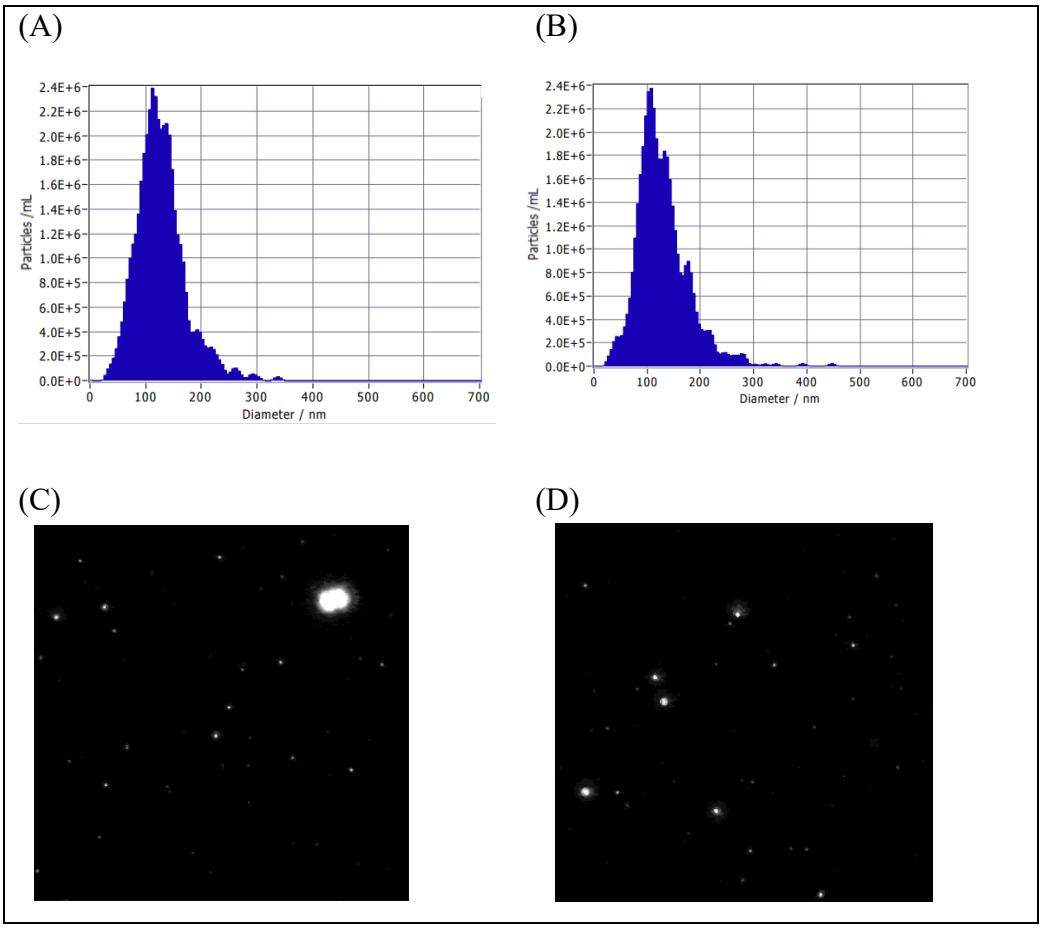


Figure 4. Size distribution and concentration of human milk EVs. Two different EV samples were tested: A: Sample 1 and B: Sample 2. EV samples were diluted to 1/1,000 with PBS. A still screen was captured from video for each sample (C and D, respectively) during NTA analysis, shown in the images on the right. White dots represent EVs.

Samples with higher amounts of EVs require further dilution to obtain readings of size and concentration during NTA analysis. Diameter, particle concentration, and average protein: EV particle ratio of human milk-isolated EV samples are summarized in **Table 5**.

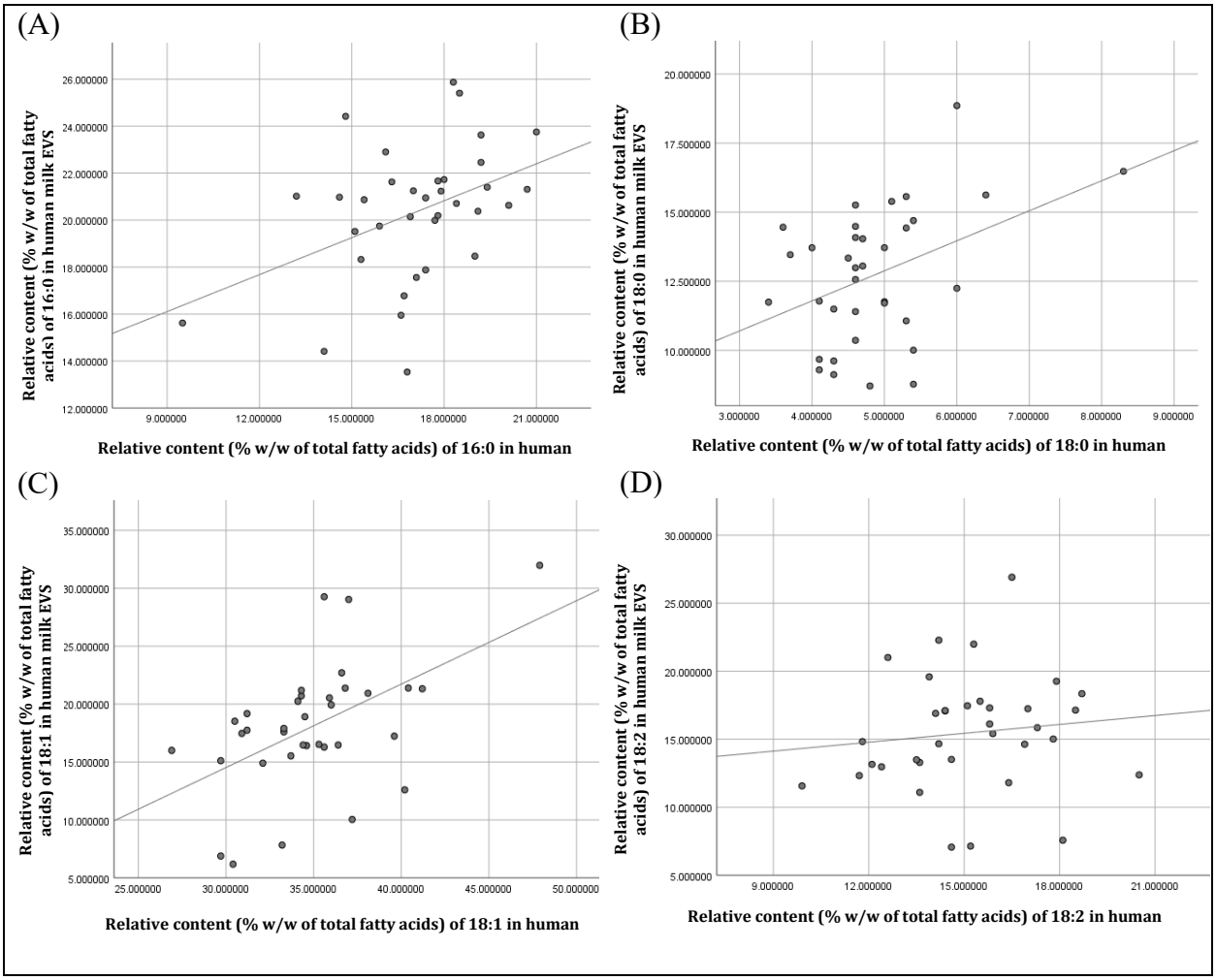
Table 5. Human milk-derived EV measurements

	Diameter (nm)	EV Concentration (particles/mL milk)	Protein:Particle (µg protein/10^{10} EV particles)
Sample 1	127.2 ± 44.0	2.1 x 10^{10}	2.06 x 10
Sample 2	129.1 ± 49.2	2.05 x 10^{10}	2.56 x 10
Average	128.2 ± 93.2	2.08 x 10^{10}	2.31x 10

The average diameter of EVs is 128.2 ± 93.2 nm, with an average concentration of 2.08 x 10^{10} EV particles/mL of milk and an average protein:particle ratio of 2.31x 10 µg protein/10^{10} particle.

4.2 Fatty Acids in Human Milk and EVs

Fatty acid relative content (%w/w) relative to the total content of fatty acids (n = 35) was analyzed. Linear regression assessed the relation between the relative content (% w/w) of fatty acids in human milk and in EVs isolated from respective human milk (**Figure 5-6, Table 6**).



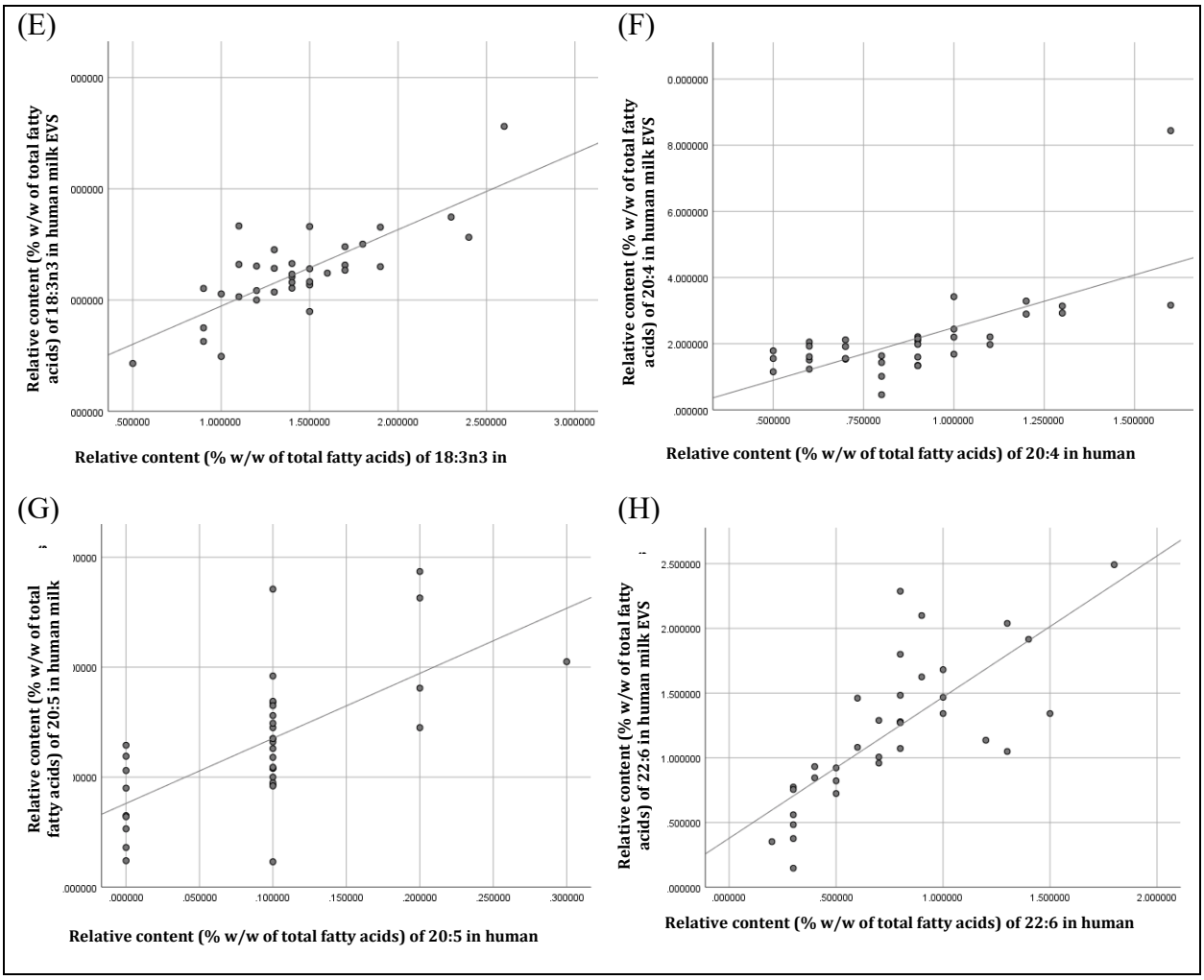


Figure 5. Correlation of fatty acid content between human milk EVs and the whole human milk. Fatty acids (n=35) were analyzed by GC-MS. Each data point represents the amount of fatty acid (% w/w of total fatty acids) for a participant. A: Palmitic acid, 16:0, B: Stearic acid 18:0, C: Oleic acid, 18:1, D: Linoleic acid, 18:2, E: Alpha-linolenic acid, ALA, 18:3n3, F: Arachidonic acid, 20:4, G: Eicosapentaenoic acid, EPA, 20:5, H: Docosahexaenoic acid, DHA, 22:6.

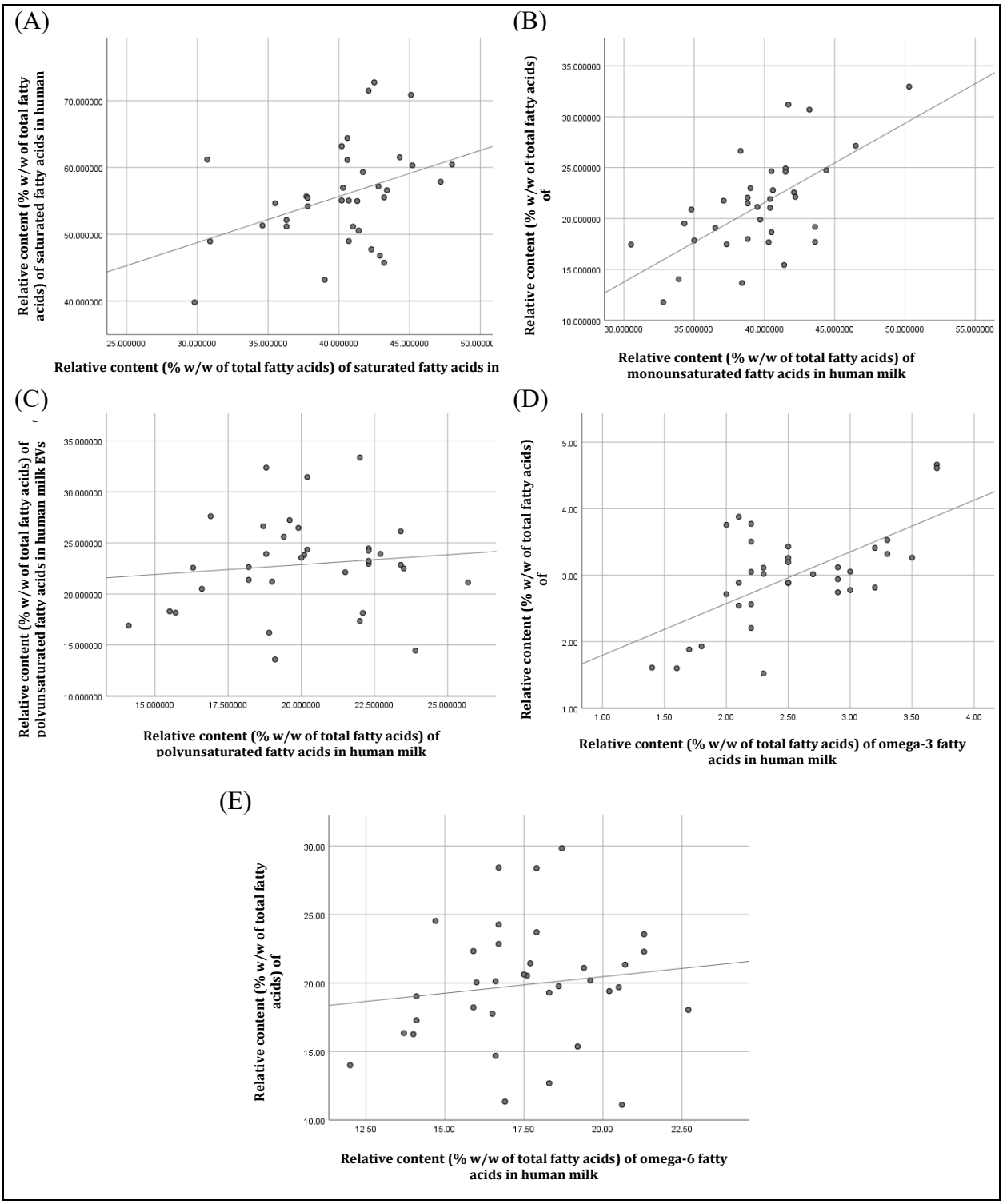


Figure 6. Correlation of fatty acid categories in human milk EVs relative to the whole human milk. Fatty acids (n=35) were analyzed by the GC-MS. Each data point represents the amount of fatty acid (%w/w of total fatty acids) for a participant. A: Saturated, B: Monounsaturated, C: Polyunsaturated, D: Omega-3, E: Omega-6.

Table 6. Correlation of fatty acid (% w/w of total fatty acids) between human milk EVs and the whole human milk (n=35)

Fatty Acid	Slope	R	P-Value
Alpha-linoleic acid, ALA (18:3n3)	0.783	0.783	0.000
Arachidonic acid, ARA (20:4)	0.700	0.700	0.000
Docosahexaenoic acid, DHA (22:6)	0.762	0.762	0.000
Eicosapentaenoic acid, EPA (20:5)	0.659	0.660	0.000
Linoleic acid, LA (18:2)	0.118	0.118	0.499
Monounsaturated, MUFA	0.639	0.639	0.000
Oleic acid (18:1)	0.518	0.519	0.001
Omega-3, ω−3	0.625	0.625	0.000
Omega-6, ω−6	0.134	0.134	0.442
Palmitic acid (16:0)	0.417	0.417	0.013
Polyunsaturated, PUFA	0.114	0.395	0.372
Saturated, SAT	0.395	0.395	0.019
Stearic acid (18:0)	0.403	0.404	0.016

Ten of 13 fatty acids had a statistically significant linear positive correlation (**Figure 6**), indicating that the relative content of the fatty acids was similar between human milk and human milk EVs ($p < 0.05$). The linear correlations between the relative content of linoleic acid, total omega-6 fatty acids, and total polyunsaturated fatty acid in human milk and EVs isolated from human milk were not statistically significant ($p > 0.05$), indicating that the relative content was not similar between human milk and human milk EVs.

The mean ratios of the content of fatty acids in human milk EVs relative to the whole human milk were calculated and plotted with 95% confidence interval (**Figure 7**).

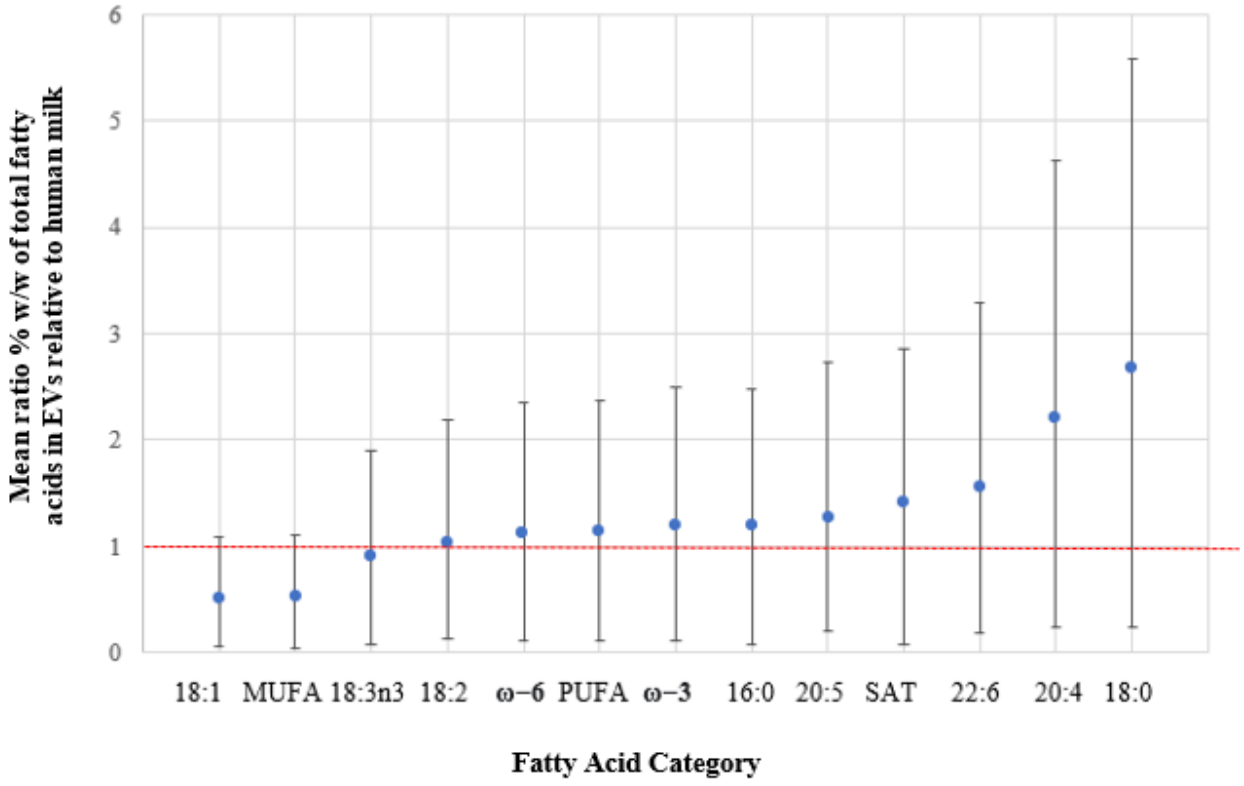
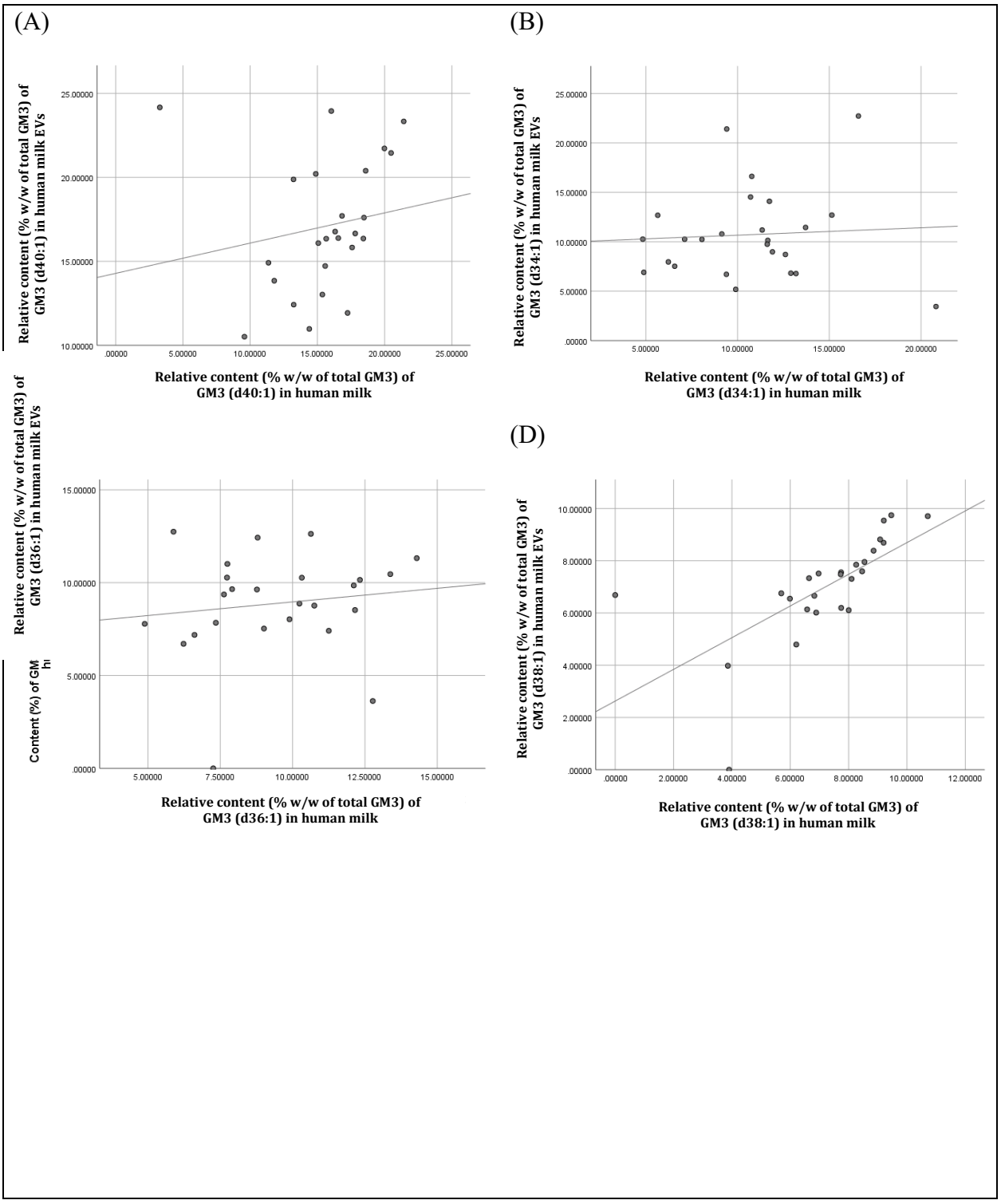


Figure 7. The enrichment of fatty acids in human milk EVs relative to the whole human milk. Fatty acids were analyzed by GC-MS. Circles represent the mean of the ratios of fatty acids (%w/w of total fatty acids) in EVs from human milk relative to the whole human milk within participant. Bars represent 95% confidence intervals. A value of 1 represents an equal relative percent composition of the fatty acid in EVs from human milk as in the whole human milk.

The relative content of fatty acids enriched in milk relative to EVs from low to high are linolenic acid (18:3n3), monounsaturated fatty acids (MUFA), and oleic acid (18:1). While the content of fatty acids enriched in EVs relative to milk (from low to high) are linoleic acid (18:2), omega-6, polyunsaturated fatty acid (PUFA), omega-3, palmitic acid (16:0), eicosapentaenoic acid (20:5), saturated fatty acid (SAT), docosahexaenoic acid (22:6), arachidonic acid (20:4), and stearic acid (18:0). Arachidonic and stearic acid are enriched 2-fold in EVs relative to human milk.

4.3 Gangliosides in Human Milk and EVs

Linear regression correlated the relative GM3 and GD3 gangliosides content (%w/w of total ganglioside species) in human milk and EVs isolated from human milk (**Figures 8-11**).



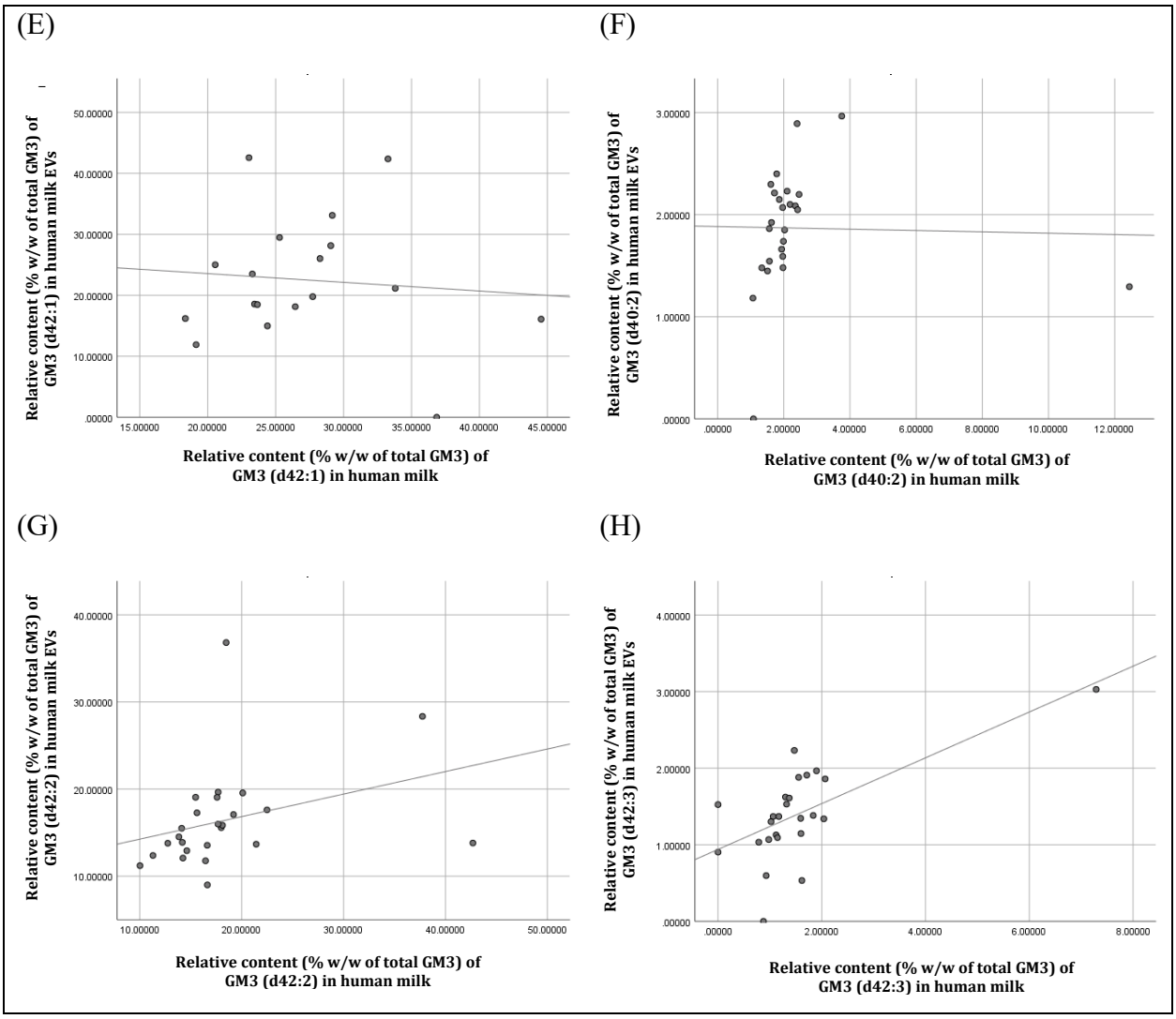


Figure 8. Correlation between the species of GM3 ganglioside in human milk EVs and the whole human milk. Gangliosides were analyzed by HILIC HPLC/MS-MS. Each data point represents the amount of the species of GM3 (%w/w of total GM3) for a participant. A: GM3d40:1, B: GM3d34:1, C: GM3d36:1, D: GM3d38:1, E: GM3d42:1, F: GM3d40:2, G: GM3d42:2, H: GM3d42:3.

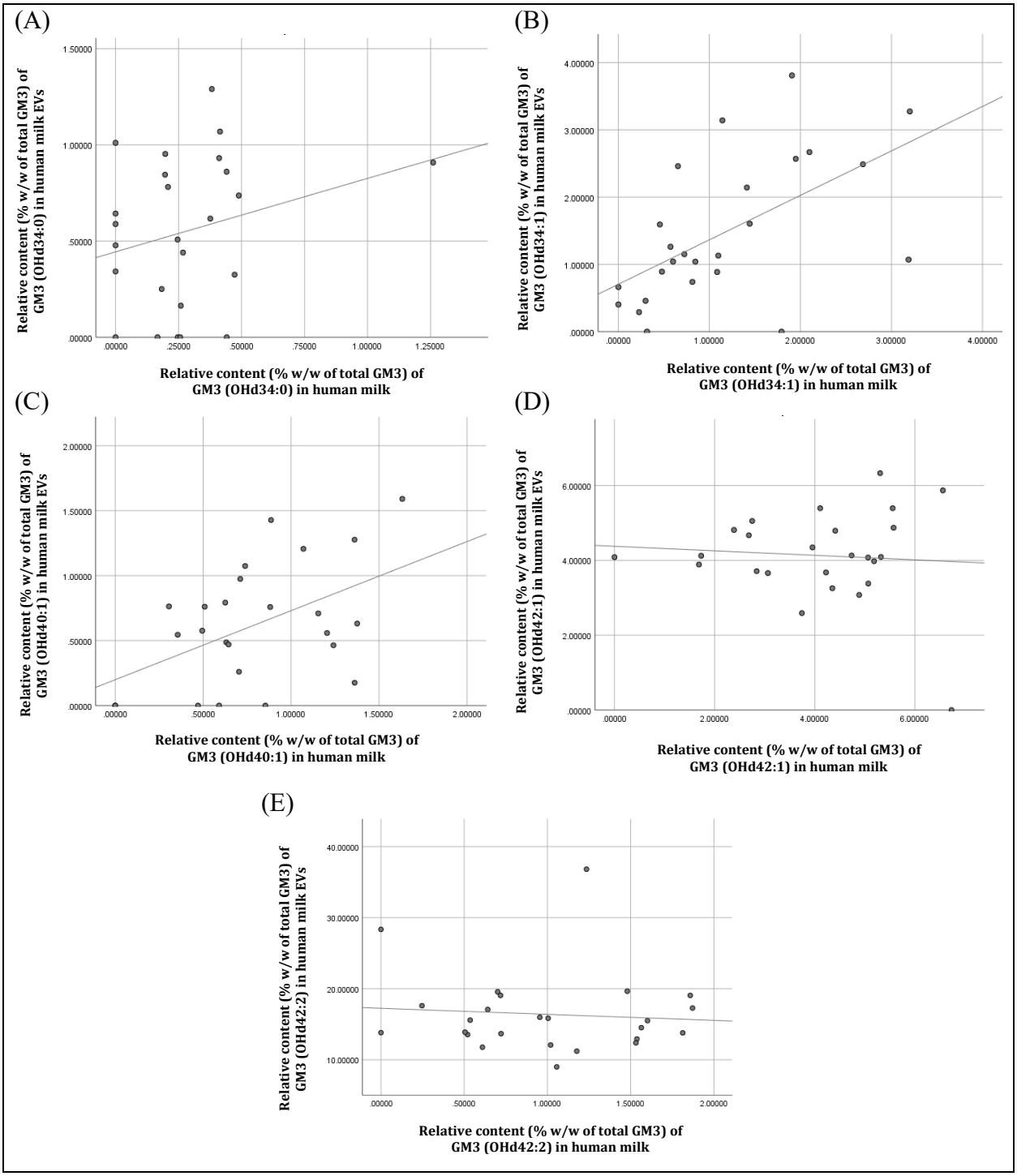
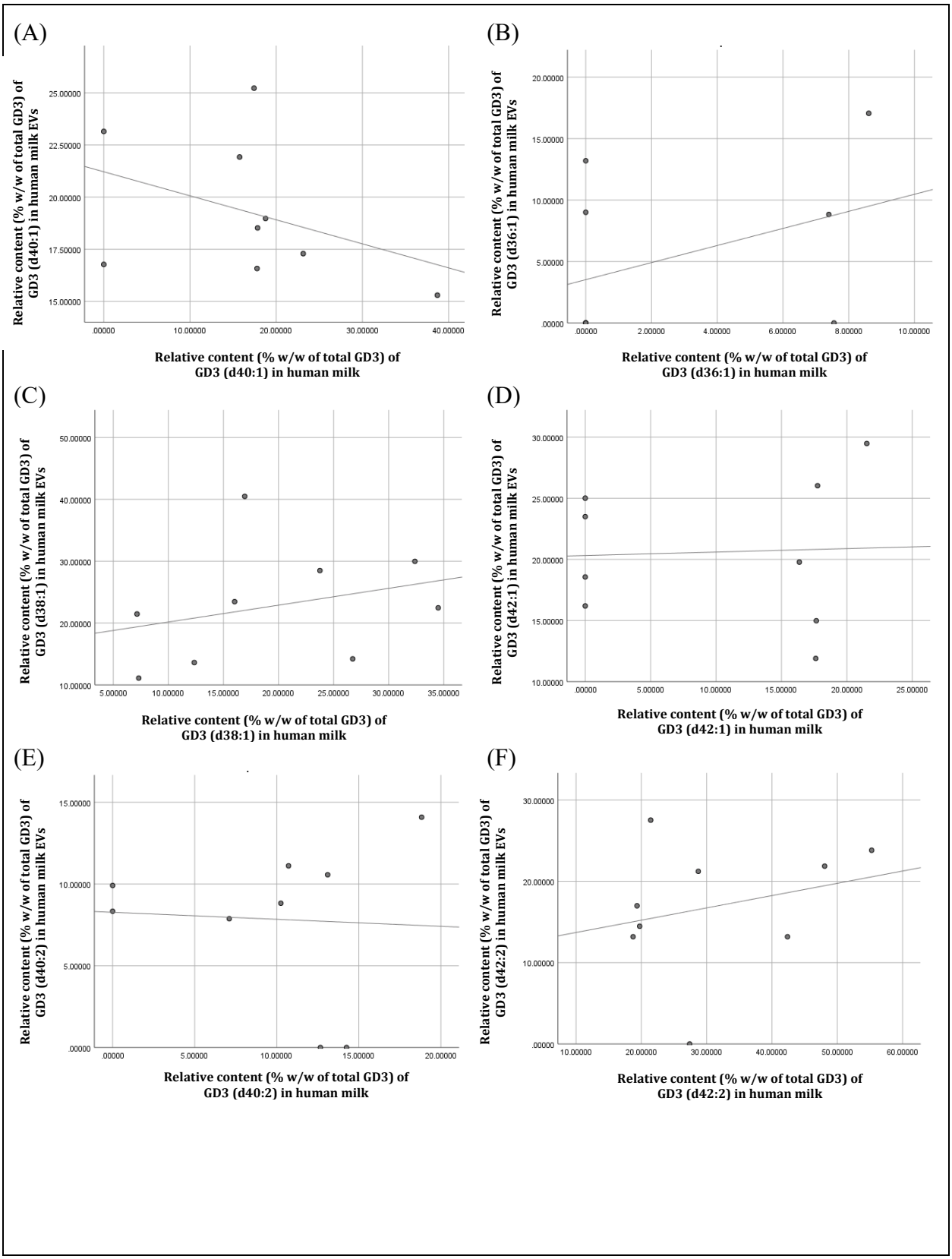


Figure 9. Correlation between the hydroxylated species of GM3 ganglioside in human milk EVs and the whole human milk. Gangliosides were analyzed by HILIC HPLC/MS-MS. Each data point represents the amount of the species of hydroxylated GM3 (%w/w of total GM3) for a participant. A: GM3OHd34:0, B: GM3OHd34:1, C: GM3OHd40:1, D: GM3OHd42:1, E: GM3OHd42:2.


(A)
Relative content (% w/w of total GD3) of GD3 (d40:1) in human milk EVs
25.00000
22.50000
20.00000
17.50000
15.00000
.00000
10.00000
20.00000
30.00000
40.00000
Relative content (% w/w of total GD3) of GD3 (d40:1) in human milk
(B)
Relative content (% w/w of total GD3) of GD3 (d36:1) in human milk EVs
20.00000
15.00000
10.00000
5.00000
.00000
.00000
2.00000
4.00000
6.00000
8.00000
10.00000
Relative content (% w/w of total GD3) of GD3 (d36:1) in human milk
(C)
Relative content (% w/w of total GD3) of GD3 (d38:1) in human milk EVs
50.00000
40.00000
30.00000
20.00000
10.00000
5.00000
10.00000
15.00000
20.00000
25.00000
30.00000
35.00000
Relative content (% w/w of total GD3) of GD3 (d38:1) in human milk
(D)
Relative content (% w/w of total GD3) of GD3 (d42:1) in human milk EVs
30.00000
25.00000
20.00000
15.00000
10.00000
.00000
5.00000
10.00000
15.00000
20.00000
25.00000
Relative content (% w/w of total GD3) of GD3 (d42:1) in human milk
(E)
Relative content (% w/w of total GD3) of GD3 (d40:2) in human milk EVs
15.00000
10.00000
5.00000
.00000
.00000
5.00000
10.00000
15.00000
20.00000
Relative content (% w/w of total GD3) of GD3 (d40:2) in human milk
(F)
Relative content (% w/w of total GD3) of GD3 (d42:2) in human milk EVs
30.00000
20.00000
10.00000
.00000
10.00000
20.00000
30.00000
40.00000
50.00000
60.00000
Relative content (% w/w of total GD3) of GD3 (d42:2) in human milk

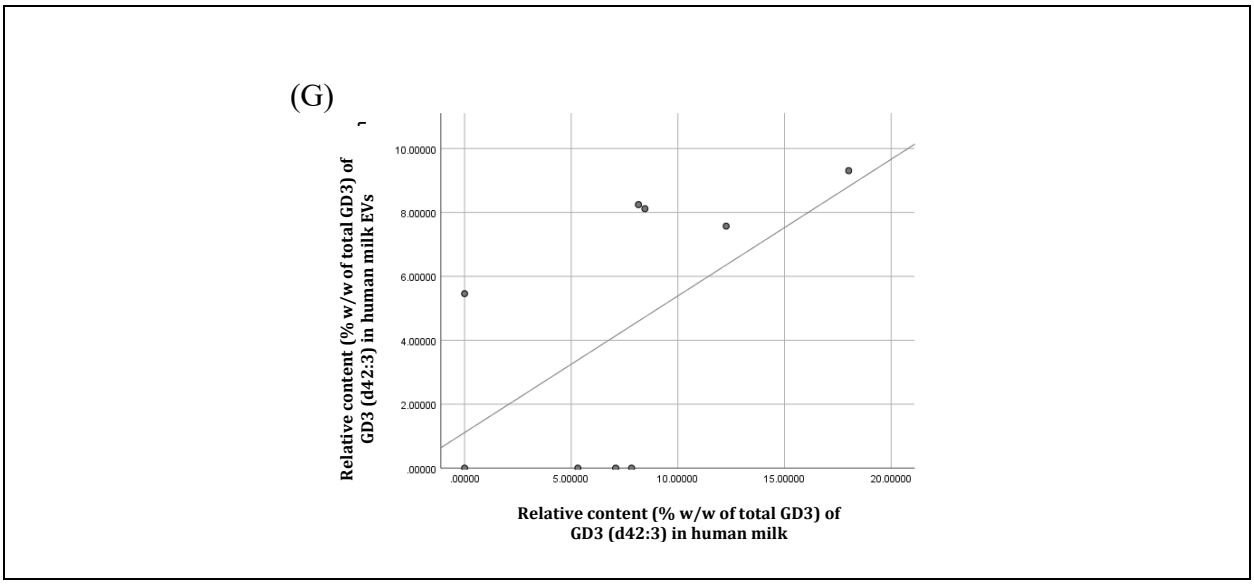
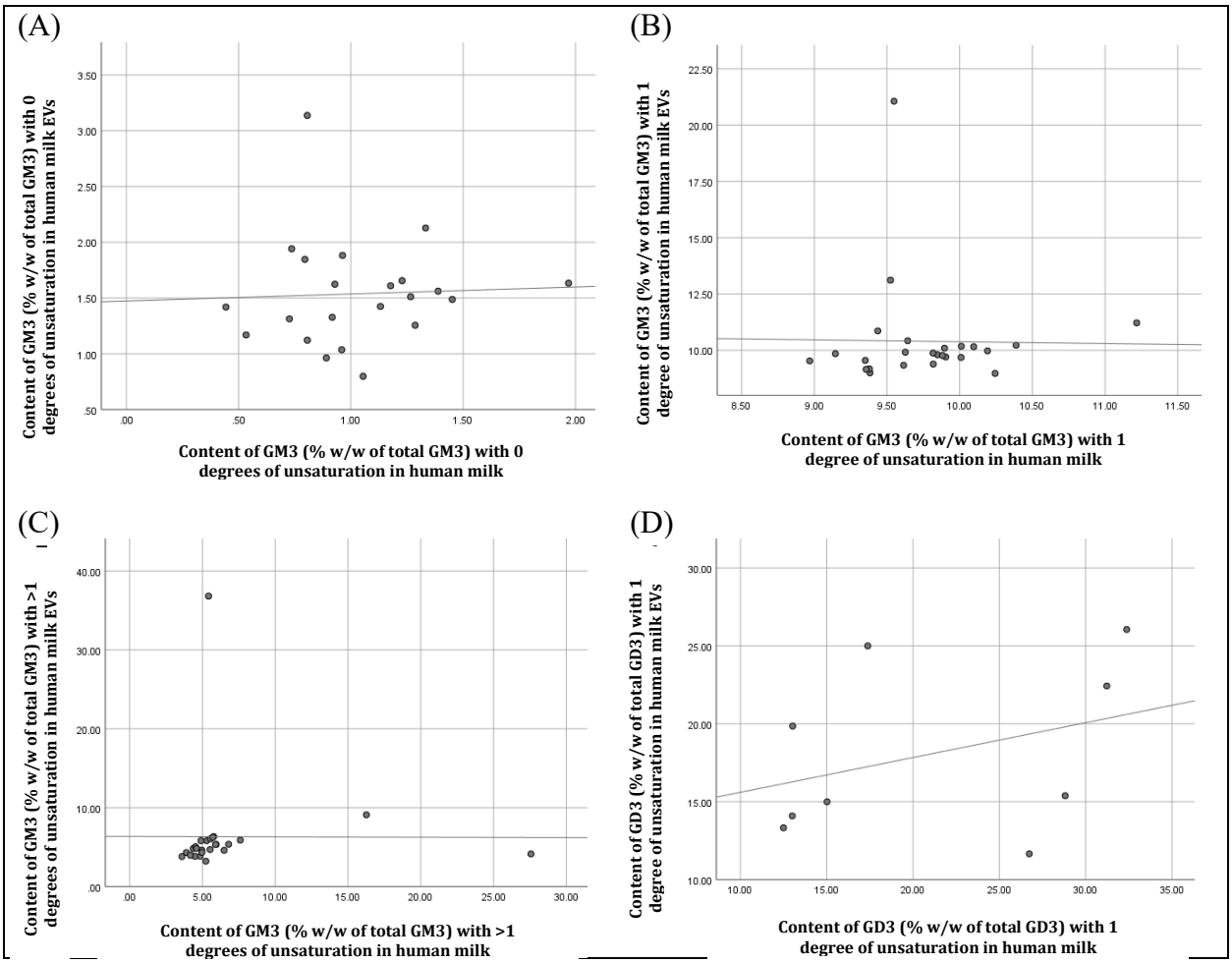


Figure 10. Correlation between the species of GD3 ganglioside in human milk EVs and the whole human milk. Gangliosides were analyzed by HILIC HPLC/MS-MS. Each data point represents the amount of the species of GD3 (%w/w of total GD3) for one participant. A: GD3d40:1, B: GD3d36:1, C: GD3d38:1, D: GD3d42:1, E: GD3d40:2, F: GD3d42:2, G: GD3d42:3.



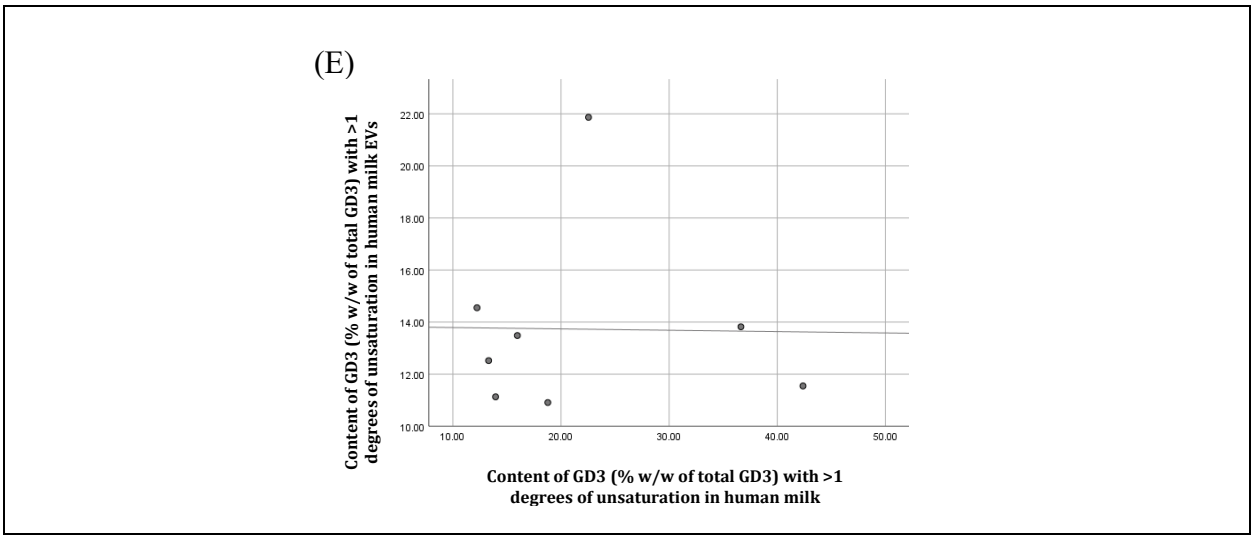


Figure 11. Correlation between the category of ganglioside in human milk EVs and the whole human milk. Gangliosides were analyzed by HILIC HPLC/MS-MS. Each data point represents the amount of the category of ganglioside (%w/w of the total ganglioside class) for a participant. Degrees of unsaturation in GM3: A = 0 (n = 25), B = 1 (n = 25), C = >1 (n = 25); degrees of unsaturation in GD3: D = 1 (n = 9), E = >1 (n = 8).

R and slope values from the linear regressions and calculated p-values are provided in the following tables (**Table 7, Table 8, Table 9**) and were used to analyze ganglioside content and composition of human milk and human milk EVs.

Table 7. Correlation of GM3 ganglioside (%w/w of the total GM3 ganglioside) content between human milk EVs and the whole human milk (n=25)

GM3 Species	R	Slope	P-Value
d40:1	0.173	0.180	0.406
d34:1	0.063	0.075	0.768
d36:1	0.134	0.417	0.521
d38:1	0.663	0.607	0.00
d42:1	0.089	0.130	0.410
d40:2	0.024	-0.006	0.910
d42:2	0.327	0.259	0.110
d42:3	0.657	0.299	0.00

Table 8. Correlation of GD3 ganglioside (%w/w of the total GD3) content between human milk EVs and the whole human milk (n=9)

Species of GD3	R	Slope	P-Value
d40:1	0.397	-0.115	0.289
d36:1	0.404	0.695	0.281
d38:1	0.298	-0.012	0.883
d42:1	0.045	1.388	0.165
d40:2	0.055	-0.043	0.886
d42:2	0.259	0.028	0.904
d42:3	0.571	0.428	0.108

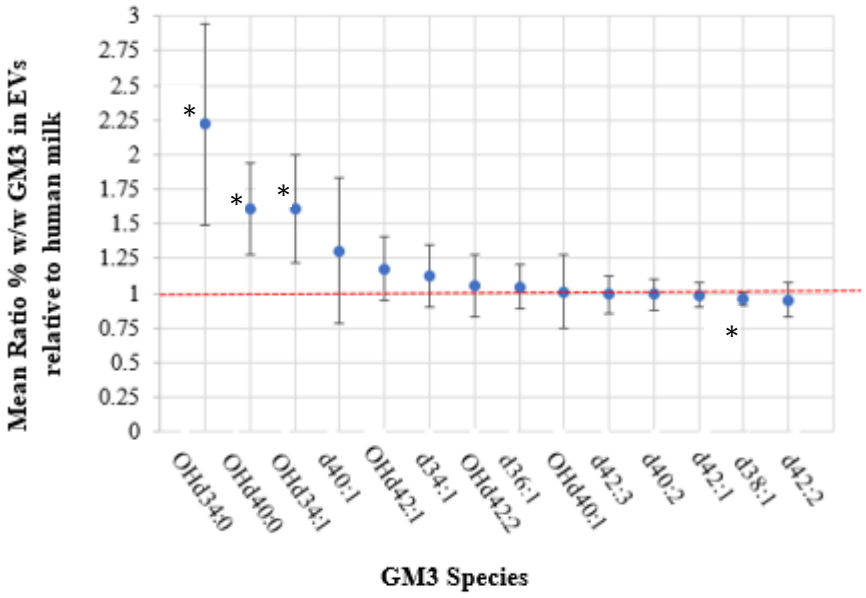
Table 9. Correlation of GM3 and GD3 gangliosides categories (%w/w of the total ganglioside class) between human milk EVs relative and the whole human milk

Ganglioside	R	Slope	P-Value
GM3 (0)	0.045	0.063	0.846
GM3 (1)	0.015	-0.082	0.940
GM3 (>1)	0.004	-0.005	0.986
GD3 (1)	0.354	0.223	0.033
GD3 (>1)	0.017	-0.005	0.969

Linear correlations between most GM3 species aside from OHd34:1, OHd40:1, OHd42:2, d38:1, d42:3 and all GD3 species were not statistically significant (**Figure 8-10),** were characterized by p-values >0.05, low R values, and indicate that the relative content of the ganglioside species or class was not correlated between human milk and human milk EVs (**Table 7** and **Table 8**). Only total monounsaturated species of GD3 displayed a significant positive correlation between the relative content in human milk and corresponding human milk EVs (**Figure 11**), p-value <0.05 (**Table 9**).

Mean ratios for the different species and categories of gangliosides were generated and plotted with 95% confidence intervals (**Figure 12** and **Figure 13**).

(A)



(B)

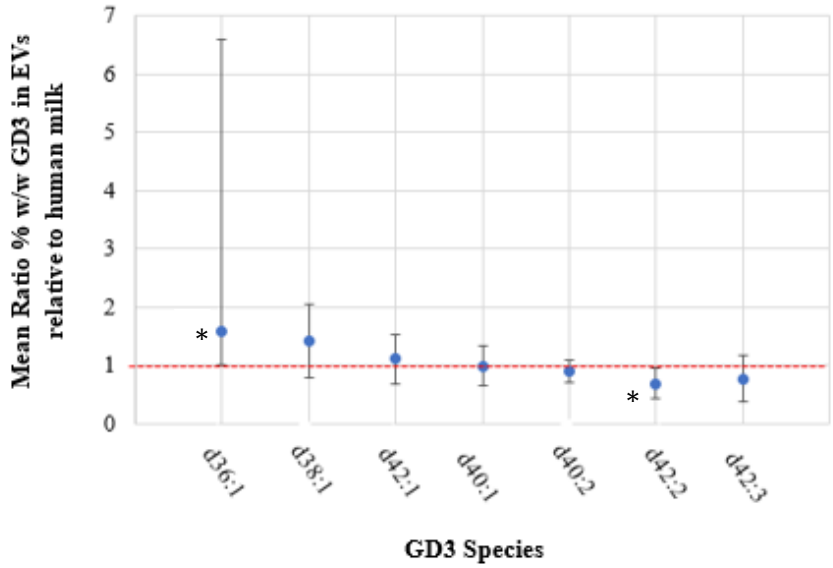
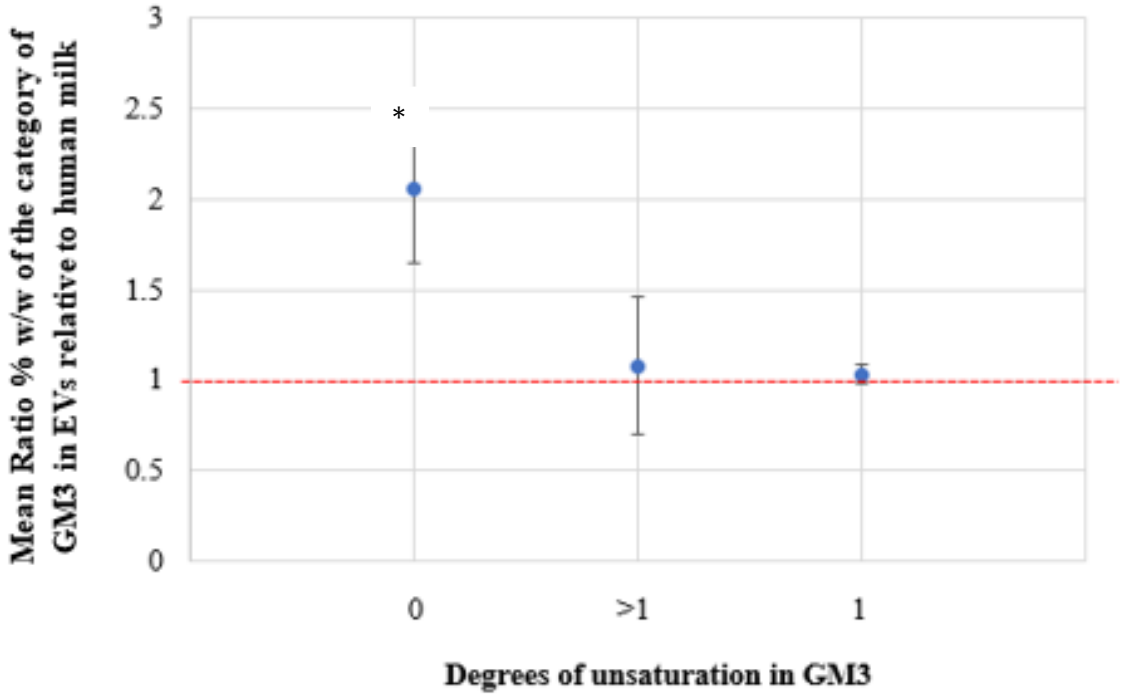


Figure 12. The enrichment of species of GM3 and GD3 gangliosides in human milk EVs relative to the whole human milk. Gangliosides [A: GM3 (n = 25); B: GD3 (n = 9)] were analyzed by HILIC HPLC/MS-MS. Circles represent the mean of the ratios of species of ganglioside (%w/w of the total ganglioside class) in EVs from human milk relative to the whole human milk. Bars represent 95% confidence intervals. A value of 1 represents an equal relative percent composition of species of ganglioside in EVs as in the whole human milk. An asterisk (*) indicates statistical significance.

While some GM3 and GD3 species ratios were around value 1 (**Figure 12**), indicating that similar amounts of GM3 and GD3 species in human milk and human milk EVs, some species were enriched in either human milk or in EVs. GM3 species enriched in EVs relative to human milk (from low to high) are d36:1, OHd42:2, d34:1, OHd42:1, d40:1, OHd34:1, OHd40:0, and OHd34:0. GM3 species OHd34:1, OHd40:0, and OHd34:0 were significantly enriched ~ 2-fold in human milk EVs. GD3 species enriched in EVs (from low to high) are d42:1, d38:1, and d36:1, with the latter enriched 1.5-fold in EVs.

(A)



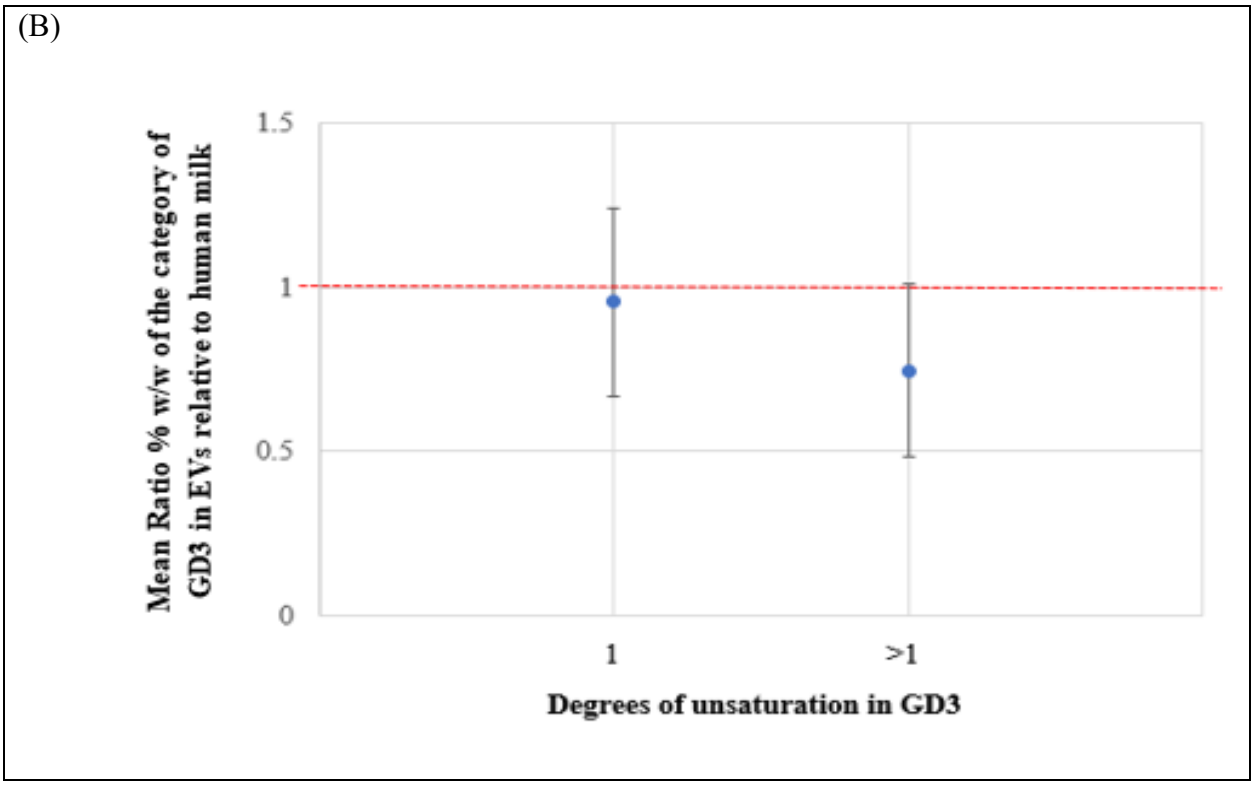


Figure 13. The enrichment of the category of GM3 and GD3 gangliosides in human milk EVs relative to the whole human milk. Gangliosides [A: GM3 (n = 25); B: GD3 (n = 9)] were analyzed by HILIC HPLC/MS-MS. Circles represent the mean of the ratios of the category of ganglioside (%w/w of the total ganglioside species class) in EVs from human milk relative to the whole human milk. Bars represent 95% confidence intervals. A value of 1 represents an equal relative percent composition of ganglioside species in EVs as in the whole human milk. An asterisk (*) indicates statistical significance.

All GM3 categories (saturated, monounsaturated, polyunsaturated) are enriched in human milk EVs (ratio value greater than 1.0), and the total saturated GM3 species were significantly enriched 2-fold in EVs. All GD3 categories (monounsaturated and polyunsaturated) are enriched in human milk (ratio value less than 1.0) with no significant difference between the relative content in human milk and human milk EVs.

5 DISCUSSION

EVs were isolated from human milk samples and analyzed for lipid composition and compared against the lipid composition of human milk as a whole. The formation and release of EVs during EV biogenesis is influenced by lipids present in the plasma membrane. The process

of EV secretion depends on the composition of lipids, as discussed in Section 1.2. The lipid composition of EVs was analyzed by characterizing fatty acids and gangliosides.

5.1 EV Isolation Confirmation

MISEV 2018 guidelines recommend quantifying isolated EVs using a minimum of 3 methods to confirm that isolation was properly executed. EV isolation was confirmed using by measuring protein concentration, particle size, and particle concentration. The mean protein concentration of the EV samples (n=31) was 4.71 (± 2.9) µg/µL human milk, with minimum and maximum values of 2.65 and 7.52 µg/µL human milk.

In a similar study conducted using the same original human milk samples, a mean protein concentration of 5.08 (±0.15) mg EV protein/dL human milk was obtained (Bickmore, 2020). However, protein quantification of biofluids is not always a consistent or a reliable method of quantification because of the presence of co-isolated molecules and low temperature storage-induced EV formation (Zonneveld et al., 2014). More extended storage of milk may result in the formation of more EV-like nanoparticles, as may be the case with the maximum value of 7.52 µg protein/µL milk obtained for one of the samples.

The average concentration of EVs was 2.08×10^{10} EV particles/mL of human milk with an average diameter of 128.2 ± 93.2 nm. The EV diameter value is lower than previously obtained for the same samples as reported by (Bickmore, 2020), where EV isolation yielded $8.9 \times 10^{9} \pm 1.1 \times 10^{9}$ EV particles/mL of human milk with 179.3 nm diameter. The decrease in yield may be attributed to increased storage time of EV samples, which has shown decreased recovery of EVs (Zonneveld et al., 2014). Additionally, the EV samples were subject to two occurrences of freeze-thaw, in addition to two freeze-thaw cycles in the Bickmore study (2020). There is a relation between storage and stability of EVs, where smaller EVs are more resistant to freeze-

thaw damage (Russell et al., 2019), as evidenced by the smaller average EV diameter obtained in the present study. Additionally, Bickmore (2020) used a different NTA instrument (Nanosight NS01) with lower accuracy, which also contributes to differences in EV particle yield in human milk.

5.2 Fatty Acids in Human Milk and EVs

Linear regression determined correlations between the mean relative amounts of fatty acids in milk and the mean relative amounts in milk EVs (**Table 6**). Regression analyses revealed varying levels of correlation (low-high) between the amounts of fatty acids found in milk and milk EVs. Mid to high correlations were found for most fatty acids and fatty acid categories, indicating that statistically significant and similar amounts were present in both milk and milk EVs. However, linoleic acid (LA), omega-6 fatty acids, and polyunsaturated fatty acids (PUFA) had very low correlations compared to the other fatty acids analyzed, evidenced by the slope values close to zero, low r values, and high p-values. The correlation of linoleic acid, total omega-6 fatty acids, and total polyunsaturated fatty acids are not statistically significant, and the relative contents differ between human milk and human milk EVs.

The relative amount of fatty acids in EVs from human milk was assessed using the whole human milk as a reference (Figure 7). A cut-off mean ratio value of 1 was used to determine the relative abundance of fatty acid in human milk as compared to milk EVs, where a mean value of 1 indicates that equal amounts of fatty acids were found in EVs and in milk. Mean ratio values less than 1, as observed for 18:1 (oleic acid), monounsaturated fatty acid (MUFAs), and 18:3n3 (alpha-lipoic acid, ALA), indicate that higher amounts of these fatty acids are found in human milk than in milk EVs. The other ten categories of fatty acids have mean ratio values higher than 1, indicating that higher amounts are found in milk EVs than in human milk.

In (Miklavcic et al., 2020)'s research, concentrations of long-chain omega-3 precursors, docosahexaenoic (22:6n-3) acid and eicosapentaenoic (20:5n-3) acid, were highly correlated in human milk and milk EVs. Human milk was enriched 1.5-fold in the content of long-chain PUFAs docosahexaenoic acid and eicosapentaenoic acid relative to EVs. However, milk EVs were enriched 3- and 2-fold in essential fatty acids, linoleic (18:2n-6) acid, and alpha-linolenic (18:3n-3) acid, relative to human milk. Differences in the amounts of these fatty acids may suggest selective partitioning of fatty acids into EVs in biogenesis for use in specific functions and processes.

Discriminate incorporation of fatty acids into EVs might have functional relevance. For instance, the relative content of omega-6 fatty acids and linoleic acid are not correlated (present in similar amounts) between human milk and human milk EVs (**Table 6**). Omega-6 fatty acids are important bioactive and structural components of plasma membranes. They are incorporated into phospholipids and affect membrane permeability and the activity of signaling pathways. LA is a precursor to arachidonic acid, a polyunsaturated omega-6 fatty acid that is enriched 2-fold in EVs. ARA forms prostaglandins are involved in vascular permeability, allowing molecules to move in and out of circulation (Dalvi et al., 2015). EVs require permeability to elicit functions in target tissues. Therefore, it is plausible that specific omega-6 fatty acids are present in higher amounts, specifically in human milk EVs, to aid in EV permeability of other functions (Jump et al., 2013). This claim is evidenced by the formation of prostaglandins by ARA, which is enriched in EVs and formed by LA.

While the 2-fold enrichment of ARA was not statistically significant, it is biologically significant. Small magnitudes changes in ARA relate to functional consequences in immune (B) cell markers (Miklavcic et al., 2017). For example, changes in [ARA] dietary intake result in

changes in tissue [ARA] and changes in B cell function. Changes in [ARA] tissues also influence changes in B cell function, and it is possible that ARA uses this same mechanism in EVs to alter functions.

5.3 Gangliosides in Human Milk and EVs

Linear correlations for the relative content of most GM3 species and all GD3 species were not statistically significant between human milk and human milk EVs; the relative contents of individual species of gangliosides differed in both types of samples. Similar to fatty acids, the relative abundances of species and classes of the two major gangliosides in human milk (GM3 and GD3) were characterized. Glycosphingolipids in EV membranes provide stability in extracellular environments (Skotland et al., 2020). Total saturated, monounsaturated, and polyunsaturated species of GM3 were enriched in human milk EVs, with a statistically significant ~2-fold enrichment of total saturated GM3. In contrast, total monounsaturated and polyunsaturated species of GD3 were enriched in human milk with no significant differences between the relative abundance of specific individual species of GD3 in human milk and human milk EVs. Differences in the enrichment of GM3 and GD3 species in EVs may be attributed to the relative abundance of the species in human milk. GD3 is present in higher concentrations than GM3 until 60 days postpartum, after which GD3 decreases and GM3 increases as the primary ganglioside in human milk (Eom et al., 1996).

The sphingolipid ceramide (the lipid tail of gangliosides) is required for the transfer of EV-associated domains (biological cargo) into the lumen of EV during EV synthesis (Trajkovic et al., 2008). Ceramidase, which transforms ceramide into sphingosine, regulates the interaction of MVBs and exosome release. Additionally, increasing the ceramide levels promotes exosome secretion (Skotland et al., 2020).

The requirement of ceramide implicates lipids in the formation, release, and transport of EVs and the communication functions of EVs; therefore, alterations in EV lipid composition may alter function. The ceramide component of individual human milk gangliosides may alter during storage and at different temperatures. A study on human donor milk analyzed the effects of storage temperature (4 °C and 23 °C) and heat treatments (63 °C/30 min, 72 °C/15 s, 127 °C/5 s, and 140 °C/6 s) on ganglioside stability over 3 months (Salcedo et al., 2018). Ganglioside species profile was stable at 4 °C but there was a reduction in GM3 d40:1 and GM3 41:1 at 23 °C. High-temperature treatments resulted in an increased formation of GD3 d35:1, GD3 d36:1, GM3 d38:1, and GM3 d43:1. Additionally, GD3 species were more stable than GM3 during 3 months of storage after being heat-treated. In contrast, GM3 d34:1 and GM4 d40:1 stability during storage after heat treatments were the most affected with high variability. In the present study, all human milk and isolated EV samples were stored at -80 °C when not used, processed at 4 °C (thaw, isolations, and extractions), and analyzed at room temperature (37° C), and stored for more than 1 year. Ganglioside ceramides enriched in human milk EVs were monounsaturated species: GM3 d40:1, GM3 d34:1, and GD3 d36:1. The monounsaturated species enriched in EVs investigated by Salcedo (2018) found variable stability at different temperature conditions and storage time, compared to most other ganglioside species. EVs are very stable because of their lipid membrane structure; therefore, GM3 d40:1, GM3 d34:1, and GD3 d36:1 may be selectively packaged into human milk EVs; therefore, they present in greater amounts in EVs as compared to free human milk to resist degradation from external conditions.

Significant differences in lipid composition of EVs compared to control cells (cells of origin) were observed in metastatic prostate cancer cell line PC-3 (Llorente et al., 2013). About 217 lipids were quantified in the PC-3 cell EVs with a total lipid concentration of 790.7 ± 68.5 in

nmol/mg protein, as compared to 94.2 ± 6.8 in PC-3 cells, indicating an 8.4 times higher ratio of lipids/protein in EVs than in parent cells. Additionally, Llorente et al. (2013) found that concentrations of glycosphingolipids, cholesterol, sphingomyelin (SM), and phosphatidylserine (PS) were 15 times higher in PC-3 EVs as compared to parent prostate cancer cells.

6 CONCLUSION

The present research characterized and compared the fatty acid and ganglioside composition of human milk and human milk derived EVs. Not enough evidence was obtained to refute the hypothesis that specific fatty acids and gangliosides are present in different proportions in human milk EVs relative to the whole human milk.

It was determined that EVs were enriched in distinct lipids (ARA, saturated GM3 species, GM3 d40:1, GM3 d34:1, and GD3 d36:1), indicating that there may be discrimination of lipids packaged into vesicles and that the vesicle membrane is highly ordered. The selective packaging of lipids is presumably involved in EV stability in external environments through effects on membrane structure and membrane fluidity, which are essential for EVs cell-to-cell communication. However, it is also possible that there is not discriminate packaging of lipids into EVs, and instead the relative abundances of lipids are influenced by metabolic activity occurring within EVs. In a previous study, EVs containing arginase-1 (ARG 1) were confirmed to be enzymatically active in patients with ovarian carcinomas (OvCa), contributing to tumor growth (Czystowska-Kuzmicz et al., 2019). EV-ARG1 suppressed the proliferation of T-cells in-vitro and in-vivo in OvCa mouse models with ARG1 using EVs as “vehicles” to illicit effects. Therefore, it is possible that metabolic activity such as elongation and desaturation of fatty acids and ceramides and/or sialyation of gangliosides are occurring within human milk EVs, resulting in different abundances of specific lipids in human milk EVs and whole human milk.

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