UNIVERSITY OF CALIFORNIA SAN DIEGO

# Comparison of methods for isolating extracellular vesicles from human breast milk for analyzing miRNA variation

A Thesis submitted in partial satisfaction of the requirements
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in

Biology

by

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The Thesis of Katrina Rene Myers is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Comparison of methods for isolating extracellular vesicles from human breast milk for analyzing miRNA variation

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Katrina Rene Myers

Master of Science in Biology

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Professor Amy Non, Chair

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Human milk is an important biofluid for nutrition and regulatory signaling transmitted from mother to infant and is also known to be rich in microRNA (miRNA), a small noncoding RNA that can prevent translation or promote mRNA degradation. miRNA can be found inside extracellular vesicles (EVs), that likely protect them from digestion and RNase degradation in an infant's stomach. Thus, these miRNAs represent a potential for intergenerational transmission of epigenetic signals from mother to infant. Little research has investigated

miRNA activity in human milk, and no consensus exists on the best technique for isolating EVs in milk for the eventual goal of small RNA sequencing. The objective of this study is to compare four techniques for isolating EVs from human milk for miRNA analyses: 1) Exoquick, 2) ultra-centrifugation, 3) ExoRNeasy and 4) Size Exclusion Chromatography (SEC). An additional goal for the SEC approach is to determine which fractions of the milk are enriched in EVs to guide the selection and pooling of fractions for downstream miRNA sequencing. Our findings confirm the presence and successful detection of EVs isolated using the Exoquick, ultra-centrifugation and SEC methods. Also, the results identify which SEC fractions likely contain the most EVs. Finally, the study displays the variability of amount and size of small RNA extracted across the methodologies of interest. These results provide a significant foundation to understanding variation among EV-enriched isolates across four distinct methodologies.

# INTRODUCTION

Human milk is an important source of nutrition, antibodies, hormones, and other biomolecules that can be transmitted from mother to infant during breastfeeding. Breastfeeding can transmit maternal signals of psychosocial stress across generations, and epigenetic mechanisms may play a role in this transmission process. Compared to other bodily fluids, human milk is rich in microRNAs (Weber et al., 2010), which are short non-coding RNA sequences that can function as post-transcriptional regulators of gene expression. These miRNAs can be found throughout the lipid, skim, and cellular portions of the milk as well as inside of extracellular vesicles (EVs) secreted from mammary epithelial cells (Alsaweed et al., 2015).

Mature miRNAs, defined as single stranded short (21-25 nt long) non-coding RNAs, are the result of multiple levels of RNA processing. They generally originate from primary miRNA (pri-miRNA) which are several hundred nucleotide long transcripts containing stem-loop structures (Lee et al., 2002). These pri-miRNAs are cleaved within the nucleus by a protein complex containing Drosha and DiGeorge syndrome critical region in gene 8 (DGCR8) into shorter (~70 nt) hairpin fragments termed precursor-miRNA (pre-miRNA) (Han et al, 2004; Lee et al, 2003). The next step of processing occurs after protein mediated transfer of the pre-miRNA from the nucleus to the cytoplasm of the cell. In the cytoplasm, Dicer enzymes further cleave the ~70 nt pre-miRNAs into miRNA duplexes which are generally about 20-25 nt long (Hutvagner et al, 2001; Wahid et al, 2010). Finally, one of the two duplexed strands of miRNA associates with an Argonaute 2 (Ago2) protein along with several other factors to form an RNA-induced silencing complex (RISC) (Hammond et al.; Kim et al.). The miRNA acts as a guide within the RISC to target complementary strands of messenger RNA (mRNA), most often in the 3’ UTR of the mRNA. Depending on the level of complementarity between the miRNA and mRNA, the

RISC will either cause mRNA cleavage or prevention of mRNA translation (Carthew and Sontheimer, 2009). Thus, miRNAs provide an epigenetic mechanism for rapid regulation of gene expression at the post-transcriptional level.

Extracellular vesicles are small membrane bound vesicles (about 30 to 1000 nm in diameter) that can contain and transport a variety of biomolecules such as proteins, lipids, and nucleic acids. They are formed from the inward budding of the lipid bilayer of endosomes and released from the outer cell membrane through multivesicular bodies (Abels and Breakefield, 2016). The membranes of EVs tend to be rich in specific tetraspanin proteins such as CD81, CD63, and CD9 which can be used as biomarkers to detect their presence in a sample (Bobrie et al., 2011). Importantly, EVs are regularly exchanged between donor and recipient cells and are an important source of inter-cell communication through delivery and uptake of their contents. Human milk miRNAs contained within EVs have shown to be more resistant to degradation than synthetic non-EV contained miRNAs in response to harsh conditions such as 100˚C incubation, freeze-thaw cycles, and RNases (Zhou et al, 2012). Additionally, human milk miRNAs have been found to be resistant to low pH conditions (pH of 1) (Kosaka et al., 2010). These studies suggest the potential for human milk EV-miRNAs to survive the digestive tract of an infant. Once in the gut, the EVs can be absorbed through the infant's intestinal cells. From there, the miRNAs that originated from the mother's mammary epithelial cells have the potential to repress translation of target mRNAs throughout the infant's body, potentially altering developmental programming of infant metabolism and growth (Alsaweed et al., 2015).

Several studies have identified an abundance (up to 800 unique reads) of variable miRNA in human milk samples and many of the top reads are miRNAs associated with immune function, disease, and cellular regulation processes (Benmoussa and Provost, 2019). Because miRNAs can

have up to hundreds of potential target mRNAs, they often associate broadly with many types of gene regulation. One of the most abundant milk miRNAs, hsa-miR-148a, is known to interact with DNA methyltransferase 1, which is an essential regulator of methylation throughout the genome (Hong et al., 2018; Wang et al., 2019). Thus, levels of hsa-miR-148a in milk and their interactions with *DNMT1* can potentially lead to large scale epigenetic effects changes. Other highly expressed miRNAs in human milk include miR-30d-5p which has been proposed as a potential biomarker for human tumors (Zhao et al., 2022) and miR-200a-3p which is known to regulate apoptosis and cellular proliferation (Feng et al., 2014). Also, miR-148a-3p, miR-30b-5p, miR-182-5p, and miR-200a-3p all associate with immune related pathways (Zhou et a., 2012).

In the earlier stages of EV research, ultracentrifugation was considered the primary and 'gold standard' method of EV Isolation. Ultracentrifugation relies on the principle of sedimentation of particles based on their relative densities when under centrifugal force. Because larger particles are denser, they pellet at the bottom of the tube while smaller particles remain suspended in the supernatant. Initial sequential centrifuge spins, varying between hundreds to tens of thousands x g, are generally used to pellet cellular debris as well as any other particles larger than EVs in the biofluid. Then, much higher centrifugal speeds (100,000-200,000xg) are used to pellet the desired size of particles which is generally around 30-100 nm for EVs such as exosomes. While this method is widely used and studied, it has also been shown to result in relatively low yield of EVs, can potentially damage or cause aggregation of the vesicles, and can favor isolation of certain sized vesicles (Linares et al., 2015; Livshits et al, 2015; Nigro et al., 2021, Vaswani et al., 2017).

As the field of EV research expanded, more methodologies have been created and tested for effectiveness and purity of EV isolation. ExoQuick is an EV isolation method that utilizes a

"net-like" polymer precipitation solution that captures EVs and allows for them to be pelleted at normal centrifugation speeds (5000 x g). An advantage to this methodology is the simplicity and quickness of the protocol which only requires addition of ExoQuick solution, incubation at 4˚C, and a 30-minute centrifuge spin. However, because the polymer compound is not binding any markers of EVs and relies on capturing particles of a certain size, there is a high possibility for non-specificity and inclusion of non-EV particles in the resulting pellet. A study comparing several EV isolation methods including UC and ExoQuick in human breast cancer cell media found the presence of several EV markers in western blots for both methods with stronger band intensities in UC samples. Conversely, Exoquick had stronger band intensities for Ago2 than UC, potentially indicating higher levels of non-EV contaminating RNAs associated in ribonucleoproteins (RNPs). They also measured higher particle concentrations in ExoQuick when using nanoparticle tracking analyses but comparable amounts of total RNA between Exoquick and UC Bioanalyzer readings (Van Deun et al., 2014).

The exoRNeasy kit (Qiagen, Hilden, Germany) is a membrane affinity spin column-based approach to EV isolation that also includes small RNA extraction. The kit requires a biofluid to be mixed with an EV binding buffer, bound to a spin column, and washed. Then a Qiazol (phenol and guanidine thiocyanate) solution is added to the column to lyse and elute the vesicles from the column. The resulting lysate is mixed with chloroform, forming a phasic separation of RNA, DNA, and organic substances. The RNA phase is recovered, bound to an RNA spin column, treated to a series of washes, and then eluted as final EV-RNA product. This kit allows for a quick and streamlined process for EV-RNA isolation but does not allow for intermediate characterization of exosomes. However, the kit can be separated into two protocols, exoEasy and miRNeasy, which does allow for the elution of EVs before RNA isolation. One

study compares EV isolation from human plasma using UC, SEC, and the exoEasy kit. They detected presence of EV biomarkers such as CD63, CD 81, and Tsg101 in western blots of SEC samples, but failed to detect these same markers in the UC or exoEasy samples (Stranska et al., 2018). However, a different study did detect the presence of EV biomarker Tsg101 in a western blot for an exoEasy human plasma sample although the bands were less prominent in comparison to a UC sample (Enderle et al., 2015).

SEC is a method of particle separation that involves fractionation of a solution by passing it through a column filled with a gel matrix that is made of spherical beads with a specific pore size (75 nm for Sepharose CL-2B). Larger molecules pass through the interstitial space of the gel without entering the pores and therefore are eluted more quickly from the column and into earlier fractions. Smaller particles can enter the pores of the gel matrix and are retained in the column for longer as they pass through the pores, thus eluting more slowly from the column and into later fractions. (Aries-Barros and Azvedo, 2017). Some studies have found SEC to result in a higher yield of EVs in comparison to UC methods in bovine milk and human serum as measured by western blots (An et al., 2018; Vaswani et al, 2017). However, another study found SEC isolations to result in lower yields of EVs in western blots in comparison to various UC protocols and ExoQuick isolation in human serum (Brennan et al., 2020). Furthermore, a study of human and bovine milk samples using SEC and a high-speed UC method (340,000 x g) found presence of EV markers in western blots and similar RNA content in Bioanalyzer results from both methods (Blans et al., 2017).

Based on the previously mentioned studies, EV isolation techniques often lack consistency in results, contributing to the difficulty of comparing EV isolation methodologies. Despite being a rich source of EVs and miRNA, human milk is understudied and very few EV

studies have investigated how differences in EV isolation method relate to the types and variation of small RNAs. Furthermore, most of these studies only analyze the EV content of the biofluids qualitatively with western blots without full small RNA sequencing to determine presence and variability of miRNAs. Therefore, the goal of this study is to compare existing methods for enrichment of EVs derived from human milk in preparation for next generation small RNA sequencing. Four techniques were compared for isolating EVs from human milk for miRNA analyses: 1) Exoquick, 2) ultra-centrifugation, 3) ExoRNeasy and 4) Size Exclusion Chromatography. An additional goal for the SEC approach is to determine which fractions of the milk are enriched in EVs to guide the selection and pooling of fractions for downstream miRNA sequencing. After isolation, EVs were characterized and visualized, small RNAs were extracted and measured using a Bioanalyzer, and these RNA ultimately will be sequenced to determine which method is preferable for isolation of miRNA from EVs in human milk.

One benefit of the study of miRNA in human milk is to gain an understanding of how maternal experiences or characteristics may influence frequency of miRNAs in the milk. While some studies have investigated miRNA variation in relation to maternal or infant health (Floris et al.,2016), preterm birth, and oxytocin exposure, (Gutman-Ido et al., 2022), less research has investigated effects of maternal stress or mood. Early childhood is a sensitive window of development during which stressful experiences of a mother may have long-term epigenetic consequences for the developing infant. Although it is beyond the scope of this thesis, one eventual goal of investigating these methodological options for EV isolation is to apply them to a larger sample of human milk to explore potential associations between maternal mood and stress and the miRNA profiles of their milk.

# METHODS

## Samples

Samples were obtained from three distinct lactating mothers recruited from a San Diego maternal support group. In order to compare the extracellular vesicle isolation methodologies, milk was processed from each of the three mothers, and each milk sample was processed in triplicate for a total of nine samples (i.e., 3 technical replicates of 3 biological samples). The three mothers were labelled as J, K, and L and the triplicates were noted as 1, 2, 3. At the time of milk donation, the infant from sample J was one year old, the infant from Sample K was 11 months old, and the infant from sample L was 2 months old. The infants from samples J and K are female and the infant from sample L is male. All the infants were healthy and self-identified as white race/ethnicity. These nine samples were processed through each of the four EV isolation methodologies. As only deidentified samples were used in the study, UCSD IRB exempted the study from review. Written consent was given by mothers for use of their milk for this study's purpose.

## Milk Preparation

First, whole milk was thawed, and the lipid portion was separated through centrifugation at 2000xg for 10 minutes. The lipid portion was removed, and the remaining skim liquid was further centrifuged at 12,000xg for 30 minutes to pellet and then discard cellular debris (Wang, 2017). Samples used for Exoquick and ultracentrifugation were also filtered through 0.45 um PVDF filters to further remove cellular debris and large particles. An overview of all four methods used to isolate EVs is illustrated in Figure 1.

**EV Isolation**

*Exoquick:* 500 μL of filtered skim milk was combined with 250μL of ExoQuick-TC solution (SBI, Palo Alto, CA, USA) and refrigerated for at least 12 hours at 4℃. Then the mixture was centrifuged at 1,500xg for 30 minutes to pellet the extracellular vesicles. The supernatant was removed, and the pellet was resuspended in 1x PBS and stored at -80˚C.

*Ultracentrifugation*: 500 μL of filtered skim milk was centrifuged for 70 minutes at 120,000xg using a 50.4 Ti Fixed-Angle Titanium Rotor for the ultracentrifuge (Beckman Coulter, Indianapolis, IN, USA). The supernatant was removed, and the pellet was resuspended in 1x PBS and stored at -80˚C.

*Size Exclusion Chromatography*: SEC columns were prepared by adding degassed Sepharose CL-2B resin to a 10 mL plastic column stored at 4˚C. 500 μL of skim milk was added to the column and fractionated with 1x PBS as a running buffer. A total of 35 fractions of 500 μL each were collected for each sample. Fractions were stored at -80˚C.

*ExoRNeasy*: RNA was extracted from 500 μL of skim milk following the standard protocol provided by the exoRNeasy Midi kit (Qiagen, Hilden, Germany). In brief, the milk was mixed with an EV binding buffer, added to a spin column, and washed. Then a Qiazol (phenol and guanidine thiocyanate) solution was added to the column and the resulting lysate was mixed with chloroform, forming a phasic separation of RNA, DNA, and organic substances. The RNA phase was removed, added to a new spin column, treated to a series of washes, and then eluted as final EV-RNA product.

**Characterization/Visualization:**

For EVs isolated using ExoQuick and ultracentrifugation: Nanoparticle tracking analysis was performed with a Nanosight (NS300) to quantify the concentration and size of EVs. In

preparation for transmission election microscopy (TEM), samples were diluted 1:50 with ultrapure water. Samples were then stained with uranyl acetate, fixed to holey carbon coated gold grids, and imaged using a JEM-1400 Plus TEM (JEOL, Tokyo, Japan).

For protein gels and western blots: 10 μL of EV isolate (from ultracentrifuge or ExoQuick), SEC fractions, or control milk was mixed in 1:1 ratio with Laemmli Buffer and heated at 95°C for 10 minutes. Then, the solutions were run on mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA) in Tris Glycine SDS solution. Stain-free protein gels were imaged using a Gel Doc™ EZ System (Bio-Rad, Hercules, CA). Gels were transferred to PVDF membranes using Tris Glycine with 10% methanol as transfer buffer. Blots were blocked in 2% milk in Tris-Buffered Saline and 0.1% Tween solution. Blots for CD81 were primarily probed with mouse derived anti-human CD81 antibody (Biolegend, Clone: 5A6, San Diego, CA) at a 1:1000 dilution in 2% milk overnight at 4˚C. Blots for CD63 were primarily probed with mouse derived anti-human CD63 antibody (BD biosciences, Cat:556019, Franklin Lakes, NJ) at a 1:1000 dilution in 2% milk overnight at 4˚C. Then, blots were washed and probed with goat anti-mouse secondary antibody in a 1:20,000 for ~1 hour (MilliporeSigma, Cat:12-349, Burlington, MA). Blots were imaged using a ChemiDoc XRS+ System (Bio-Rad, Hercules, CA).

**RNA Extraction/Quantification**

RNA was isolated from 500 μL of all EV isolates derived using ExoQuick, Ultracentrifugation, and SEC methods using the Norgen Plasma/Serum Circulating and Exosomal RNA Purification Kit (Norgen Biotek, ON, Canada). The ExoRNeasy kit includes a trizol based RNA extraction as part of the protocol, so no additional RNA extraction was necessary for that EV isolation method. All RNA was stored on ice during experimental procedures and stored at -80˚C thereafter. One μL of RNA extracted from all four methodologies

was analyzed using a Bioanalyzer RNA 6000 Pico chip (Agilent, Santa Clara, CA). The RNA samples and input controls (milk not treated with any EV isolation method) are currently being used to build cDNA libraries for eventual miRNA sequencing. Overall, the end goal of analysis will be to compare average miRNA sequence diversity and frequency across the four EV isolation methods and input controls. We will also examine variation within the SEC method, across all the fractions/fraction pools.

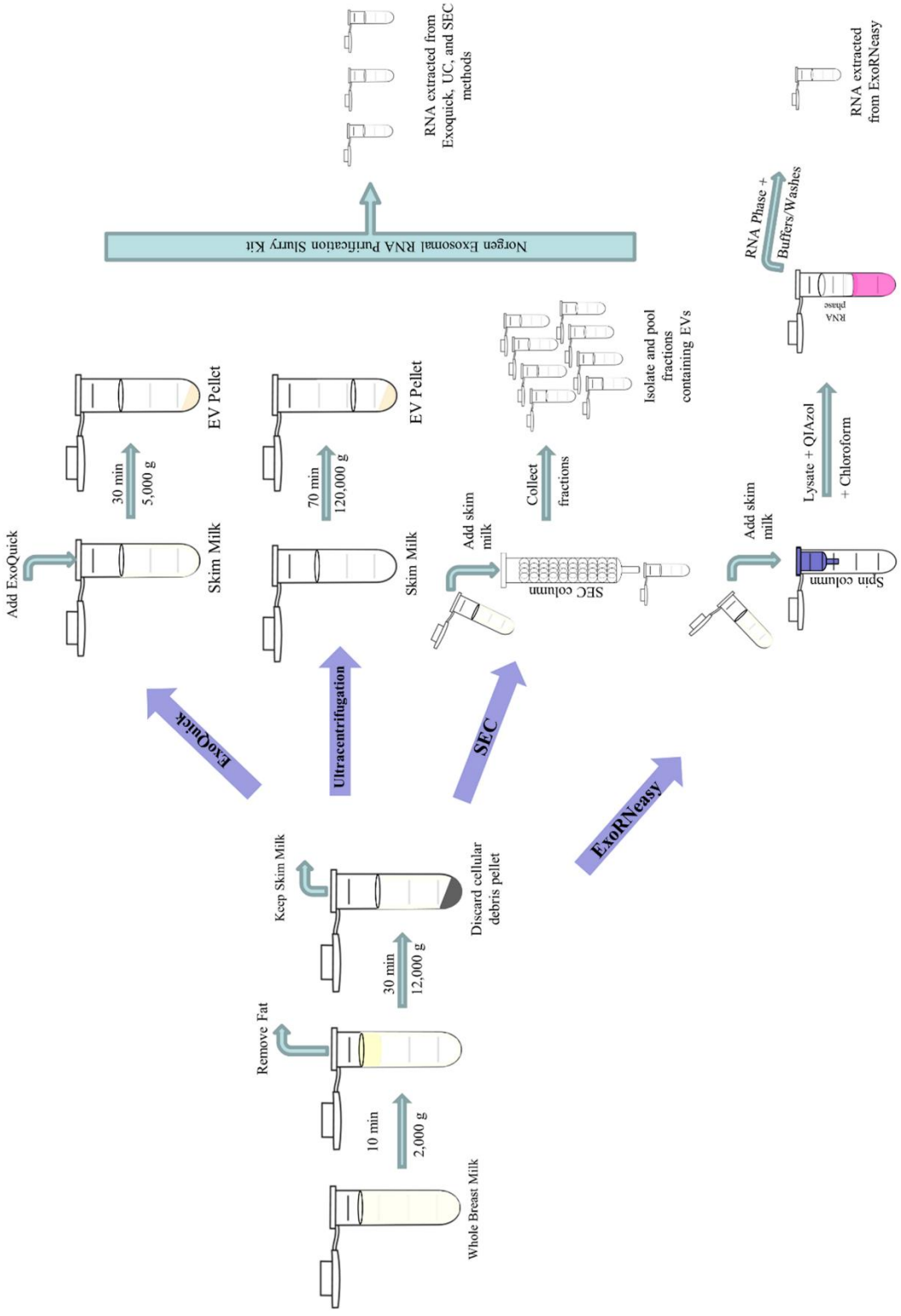


Figure 1: Diagram describing milk processing, the four EV isolation methodologies, and RNA extraction techniques.

# RESULTS

## Protein Gels comparing EV isolation methods

Protein gels were generated across the three different EV isolation methods in human milk to examine the presence, sizes, and amounts of proteins in the samples. (Figures 2.1 and 2.2). The fractions collected from a representative sample processed using the SEC EV isolation methodology are shown in Figure 2.1. The first clear bands appear in fraction 7 and continue to fraction 32, with some bands increasing in signal and others decreasing as the fractions progress. Larger proteins/complexes such as the bands seen around 70 kDa tend to exit the SEC column in earlier fractions as they are able to pass through the Sepharose gel matrix more easily. Conversely, smaller proteins such as the bands seen around 5-10 kDa tend to increase in intensity in later fractions because these smaller particles are hindered in the gel matrix and exit the column later.

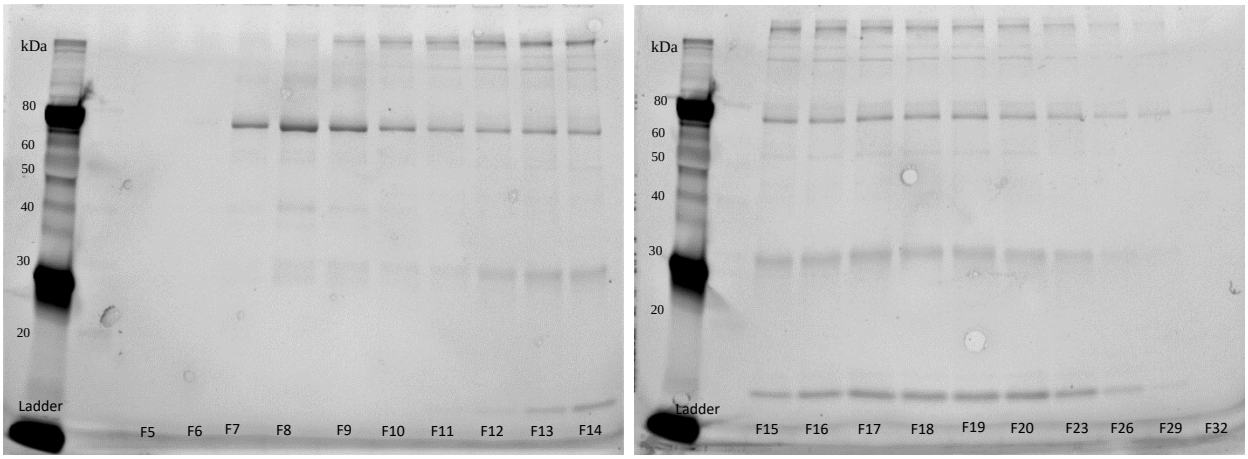


Figure 2.1: Protein Gel Image of Fractions 5-32 collected from SEC EV isolation method for sample J.

One sample from each mother in this study (labelled as J, K, and L) from the ultracentrifuge and ExoQuick EV isolation methods are included in the gel in Figure 2.2. Additionally, the final three lanes of the gel are control milk samples from mothers J, K, and L that have not undergone any EV isolation method. These control milk samples were only

defatted and centrifuged to remove cell debris, as were all milk samples pre-EV isolation. Isolates from all methodologies showed distinct protein bands at approximately 70 kDa, 30 kDa, and 5 kDa. However, the ultracentrifugation samples had much less total protein in the final EV isolate product than the ExoQuick samples. The ExoQuick samples appear to contain similar amounts of protein to the control milk. Additionally, across the 3 biological replicates of each method or control in Figure 2.2, there appears to be similar patterns and amounts of protein.

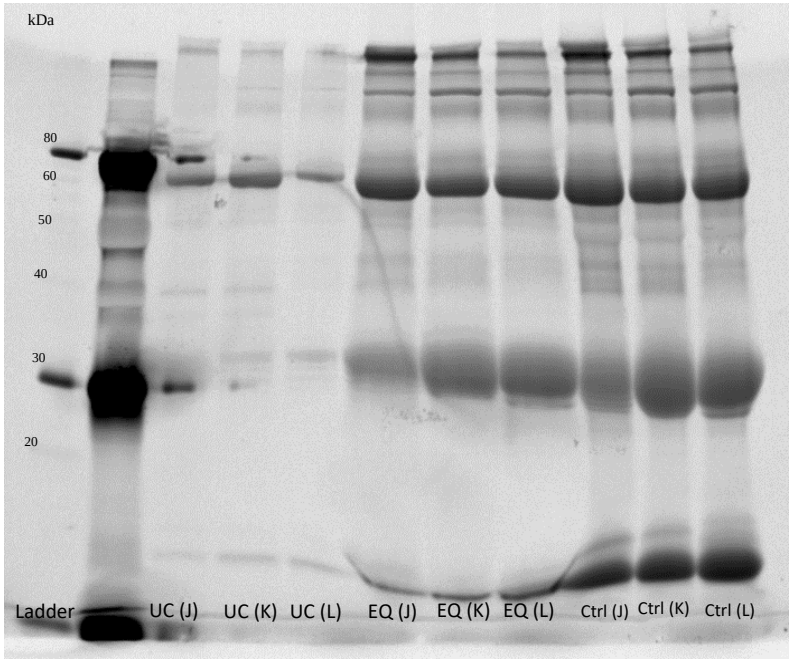


Figure 1.2: Protein Gel Image of milk processed through Ultracentrifuge and ExoQuick EV Isolation Methods and control (no EV isolation) milk. One biological replicate for each method was included (Samples J, K, L).

Western Blots to confirm presence of EVs

The western blots in Figures 3.1 and 3.2, support the presence of EVs in a representative sample processed using the SEC EV isolation methodology. In Figure 3.1, the two blots were probed with anti-CD81 primary antibody. CD81 proteins are present at the expected band size of 26 kDa from fractions 7-32, with the strongest signal in the earlier fractions 7-11.

In Figure 3.2, the same two blots were stripped and reprobed with anti-CD63 primary antibody. CD63 protein appears to be present in the same SEC fractions 7-32, again with the strongest signal in the earlier fractions 7-11. Some residual CD81 protein can still be seen in fractions 7-11 of the CD63 blots, likely due to incomplete stripping of the blot and the large amount of CD81 present in these fractions. Additionally, the CD63 blots tend to show a range of sizes from approximately 30-75 kDa and even higher molecular weights. This is common when probing with CD 63 as seen in other papers, likely due to multiple isoforms and glycosylation states of the protein. (Mathieu et al., 2021; van Niel et al., 2011). In Figure 3.3, the other two methods with an EV-enriched isolate product (UC and ExoQuick) along with control milk samples not treated to any EV isolation method are shown in blots probed with anti-CD81 antibody. All three biological replicates of each type (UC, ExoQuick, and controls) showed bands at the expected size for CD81, indicating the presence of EVs. The UC and control milk showed bands with higher intensity than the ExoQuick samples.

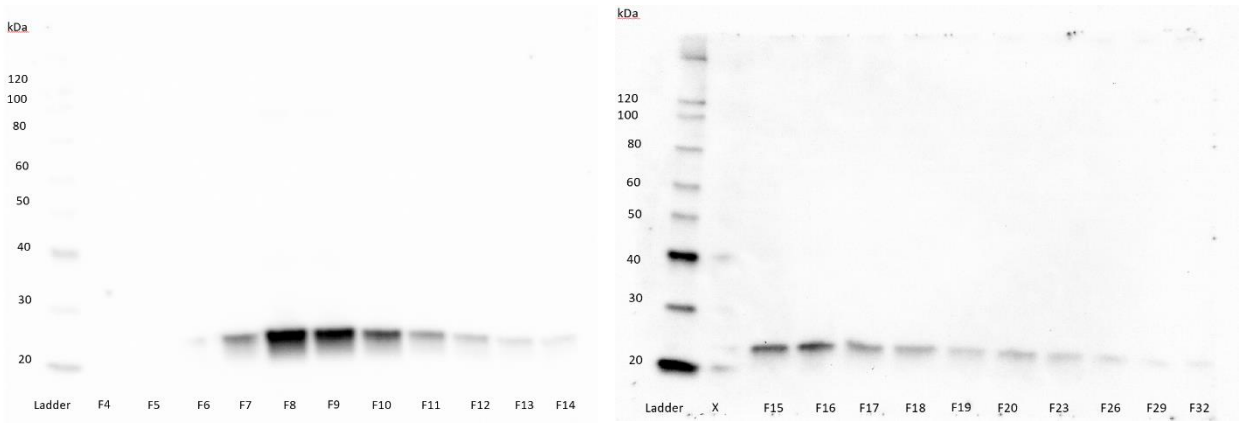


Figure 3.1: Western Blots of breast milk fractions collected using SEC method. Blots were blocked and probed in a 2% milk solution with CD81 primary antibody (1:1000) and Anti-Mouse secondary (1:20,000 dilution of EMD Millipore: 12-349). Expected Band: 26 kDa. The left blot was imaged at high resolution with an exposure time of 30 seconds and the right blot was imaged at high resolution settings with an exposure time of 300s.

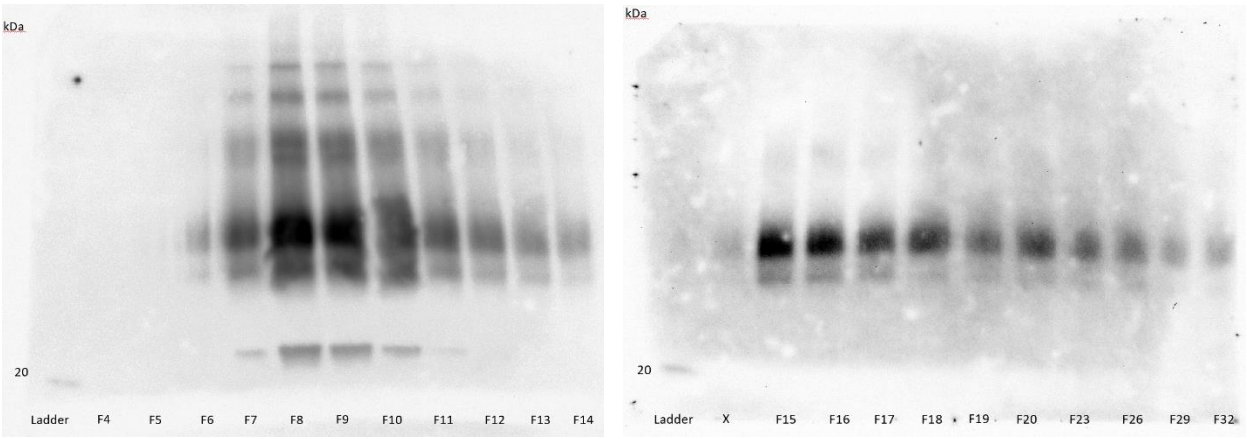


Figure 3.2: Western Blots of human milk fractions collected using SEC method. Blots were blocked and probed in a 2% milk solution with CD63 primary antibody (1:1000 dilution of BD biosciences: 556019) and Anti-Mouse secondary (1:20,000 dilution of EMD Millipore: 12-349). The left blot was imaged at high resolution with an exposure time of 75 seconds and the right blot was imaged at high resolution settings with an exposure time of 300s.

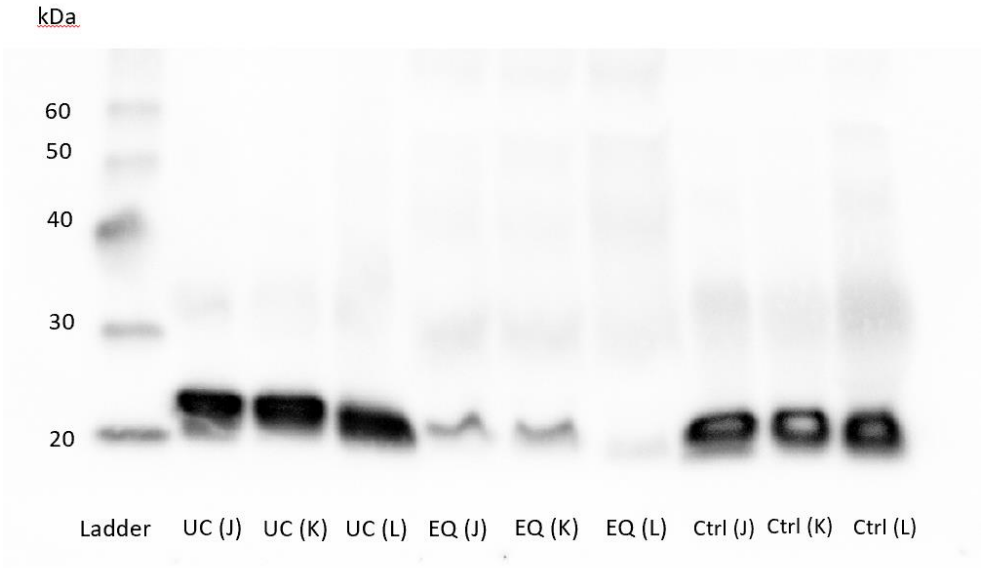


Figure 3.3: Western Blot of EV-enriched isolate collected using UC and ExoQuick (EQ) methods and control (no EV isolation) milk. One biological replicate for each method was included (Samples J, K, L). Blots were blocked and probed in a 2% milk solution with CD81 primary antibody (1:1000 dilution of BioLegend Clone: 5A6) and Anti-Mouse secondary (1:20,000 dilution of EMD Millipore: 12-349). The blot was imaged at high resolution with an exposure time of 22 seconds.

TEM and Nanosight results

TEM images of negatively stained samples of EVs are characterized by a round shape with a light halo or ring around the vesicle and a darker gray center. In the representative image for the ExoQuick EV sample (Figure 4 Panel A), there are a few particles that appear to be EVs. In the representative image for the ultracentrifuge EV sample (Figure 4 Panel B), there are several particles clustered together that appear to be EVs. Ultracentrifugation yielded an approximately 3 times higher concentration of particles/mL than Exoquick as measured with the Nanosight nanoparticle tracking analysis. The mean particle diameters of EVs derived from both methodologies were similar, but the EVs isolated in the ultracentrifugation protocol had a smaller mode particle diameter (Table 1).

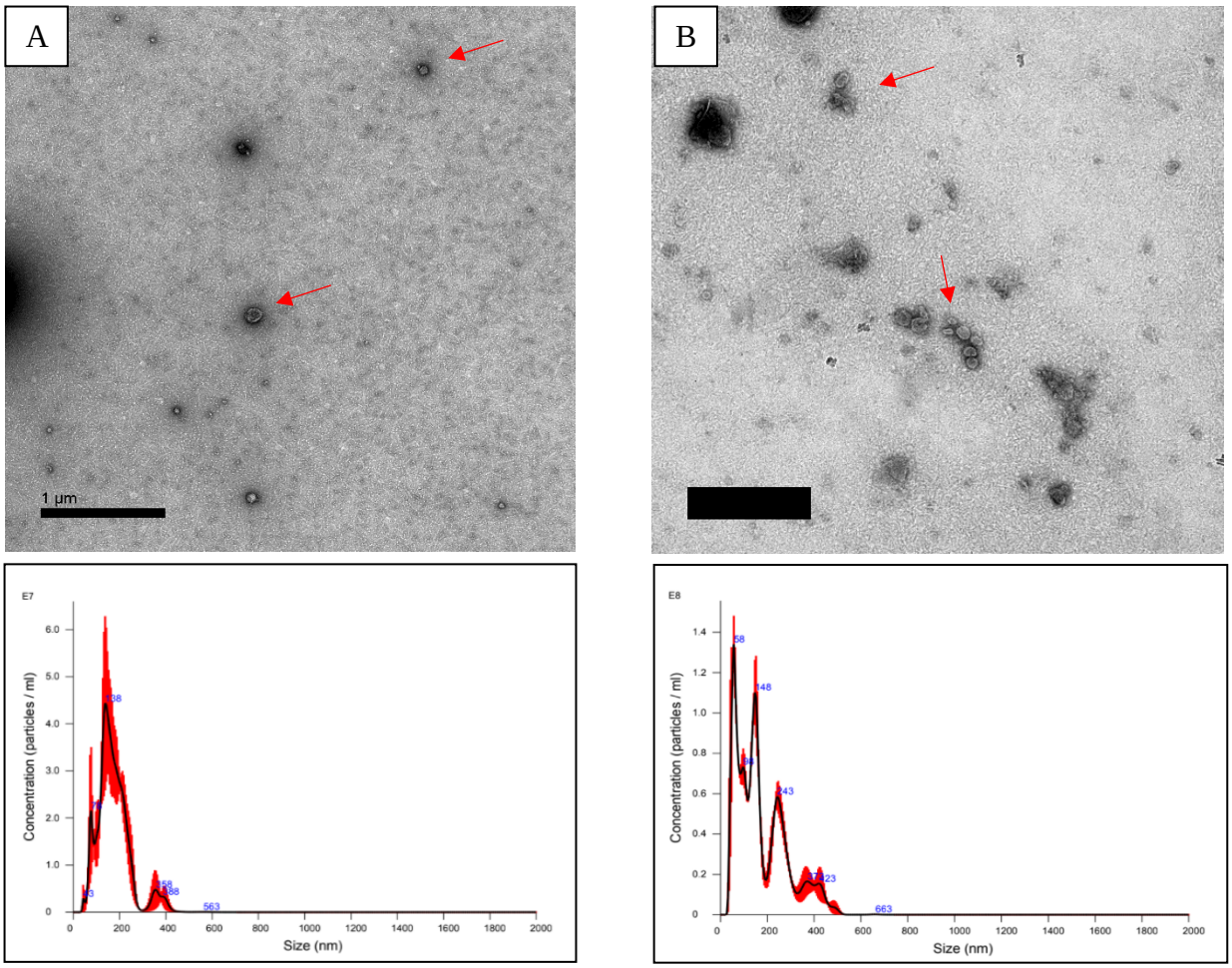


Figure 4: TEM (top) and Nanosight (bottom) images of EVs isolated from skim human milk using the ExoQuick method (Panel A) and the ultracentrifugation method (Panel B). Samples were diluted 1:50 in ultrapure water. Red arrows indicate representative EVs in the TEM images.

Table 1: Nanosight quantification data of ExoQuick and ultracentrifuge methods. Samples were diluted 1:50 in ultrapure water.

| Methodology | Concentration (particles/mL) | Mean Particle Diameter (nm) | Mode Particle Diameter (nm) |
|---|---|---|---|
| ExoQuick | $1.09e^{9}$ +/- $1.58\ e^{8}$ | 172.8 +/- 5.2 | 160.7 +/- 13.8 |
| Ultracentrifugation | $3.66e^{9}$ +/- $1.09\ e^{8}$ | 173.6 +/- 5.7 | 54.2 +/- 4.0 |

Bioanalyzer Results

Results from the Bioanalyzer analyses show the presence, sizes, and relative amounts of small RNA in the samples prior to sequencing. As seen in some representative samples in Figure 5, the four methodologies of EV isolation show distinct profiles of RNA sizes and concentrations. The UC samples generally showed a smaller peak of RNA near the marker (25 nt) while the ExoQuick samples had larger amounts of RNA with a slightly broader range of sizes than the UC or SEC samples. The exoRNeasy samples had the largest amounts of RNA with an even broader range of sizes than the ExoQuick. Finally, the SEC samples tended to show tight peaks of small RNA with variable amounts of RNA depending on earlier versus later fractions (other fractions not pictured here).

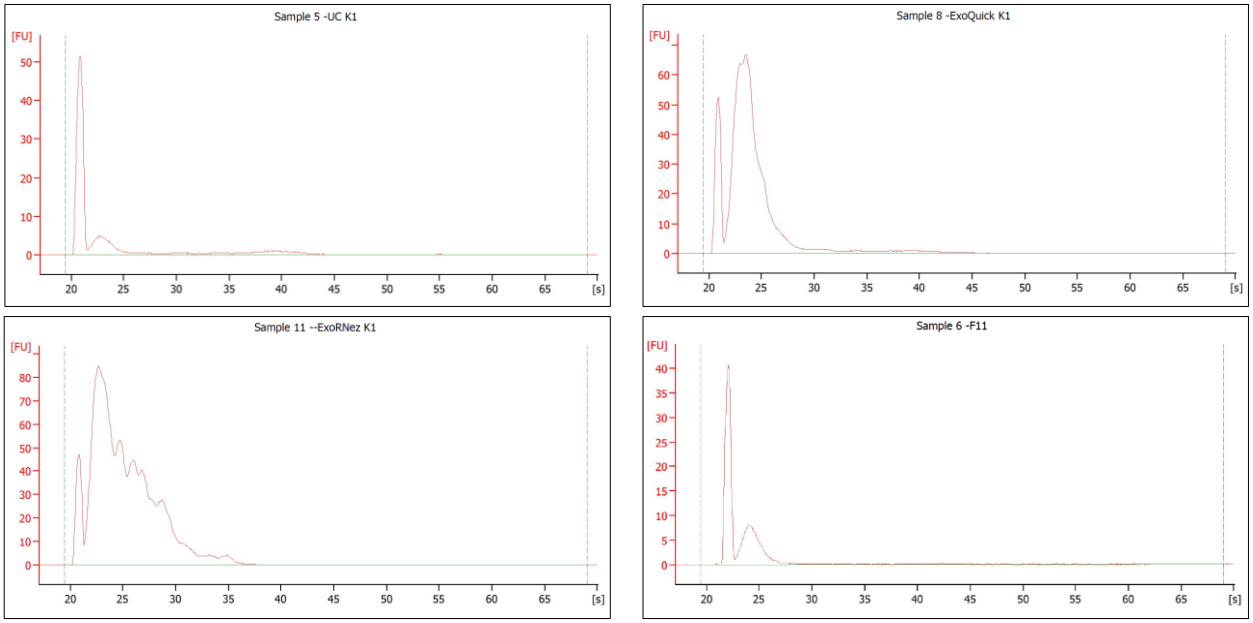


Figure 5: Selected Bioanalyzer traces for the four different EV isolation methodologies. UC (upper left), Exoquick (upper right), ExoRNeasy (bottom left), and one example fraction (fraction 11) from SEC (bottom right).

# DISCUSSION

The research presented here provides significant groundwork for the goal of determining the miRNA profiles and variation across EV isolation methodologies. This is the first study to compare EV isolation techniques in human milk across these four methods (Exoquick, UC, ExoRNeasy, and SEC) and will be the first to ultimately compare small RNA sequencing data from these samples.

One complication with isolating EVs is that the resulting isolate may be contaminated with proteins, especially ribonucleoproteins (RNPs). Some of the most abundant human milk proteins are serum albumin (~67 kDa), lactoferrin (~77 kDa), β and κ caseins (~20-25 kDa), and α-lactalbumin (~14 kDa) (Darragh and Lönnerdal, 2011; Jenness, 1979; Jovanovic et al., 2007; Spencer et al., 2010). These proteins may account for the main profiles of proteins seen across all EV-isolation methodologies, although more specific probing and western blots would be required to confirm their presence. Regardless, the ExoQuick methodology has the least amount of protein filtering as compared to the control non-EV isolated milk (Figures 2.1 and 2.2). This higher proportion of protein in the ExoQuick EV isolate may increase the possibility of non-EV associated small RNAs in complex with other proteins. Because the ExoQuick methodology is based on a "net like" polymer pulling the EVs out of the milk, it is likely that other particles are dragged along with the EVs, explaining the large amounts of contaminating proteins in the ExoQuick EV isolate. The UC and SEC methods had considerably less protein in their EV enriched isolates, which is expected, as these methods rely on separating particles based on density and size, respectively. In the UC method, most proteins are less dense than EVs, and therefore remain in the supernatant and are removed from the final EV pellet. In the SEC

methodology, smaller proteins are trapped in the pores of the gel matrix and eluted from the column in later fractions, apart from the earlier more EV-enriched fractions.

The tetraspanin proteins CD81 and CD63 are known biomarkers for EVs. Thus, the western blot data (Figures 3.1, 3.2, and 3.3) confirms the presence of EVs across the SEC, UC, and ExoQuick methodologies as well as the control milk samples. In the SEC blot, the early fractions 7-11 for CD81 and CD63 are the most intense and likely contain the most EVs. However, fractions 12-32 also show bands for CD81 with progressively decreasing intensity. This indicates less EVs in later fractions, possibly EVs that are smaller in diameter and hindered in the pores in the gel matrix of the SEC column. Because the SEC samples show bands across multiple fractions which would eventually be pooled for total RNA sequencing, it is difficult to compare the total amounts of EVs across the CD81 enriched fractions to the other methodologies which only have a singular band for the total sample. The blots in Figure 3.3 show much stronger signal in CD81 bands in the UC samples than the ExoQuick, indicating that the UC samples contain more EVs. Therefore, SEC and UC likely contain more EVs than ExoQuick and may be better methodologies for the isolation of EVs in milk. However, these findings will eventually be supplemented with RNA sequencing data indicating which methods include more types and amount of small RNAs, particularly miRNA.

Representative TEM images in Figure 4 also visually confirm the presence of EVs in the UC and ExoQuick methods. Images taken of ExoQuick samples tended to show more sparse distribution of EVs, while UC images tended to show more EVs, some of which were single vesicles and some in aggregates as seen in the image above. The presence of more EVs in UC images is supported by the bands with stronger intensity for UC than ExoQuick in western blots for EV associated protein CD81 (Figure 3.3).

Nanoparticle tracking analysis is a standard method of assessing in EV research, however it is non-specific for EVs and therefore the particle sizes and amounts could reflect other types of particles in the EV-enriched isolate measured. The graph for the ExoQuick sample (Figure 4A) shows a single main peak of particles at 138 nm which is within the range of sizes for EVs. In the Nanosight plot for the UC sample (Figure 4B), there is a bimodal distribution of particles with the slightly higher peak of smaller particles at 58 nm along with a second peak of slightly less concentration at 148 nm. This distribution explains the lower mode particle diameter for UC but comparable average particle size compared to ExoQuick (Table 1). While the UC peaks at 148 nm and 243 nm may represent individual EVs, they may also be representing aggregates of vesicles such as the one seen in the representative UC TEM image. The UC sample also showed about 3 times higher concentration of particles than the ExoQuick solution, also supporting the CD81 western blots that indicated more EVs in the UC samples than the ExoQuick samples.

The representative Bioanalyzer traces in Figure 5, as well as several other Bioanalyzer traces not pictured here, indicate that small RNA was successfully extracted across all four EV isolation methodologies. The broader size ranges across the ExoQuick and exoRNeasy samples are likely due to the presence of small RNAs other than miRNA such as tRNA fragments or piRNA. The RNA sequencing results will elucidate the presence of different classes of small RNAs across the methodologies. Again, because the RNA for the SEC data is dispersed amongst several fractions as compared to a single RNA extraction in UC, ExoQuick and exoRNeasy samples, the relative amounts must be considered as a pool of multiple fractions. In Bioanalyzer traces across all fractions, not depicted in Figure 5, the SEC fractions showed a decrease in total RNA past fractions 12+13. This decrease across fractions is expected based on results from the western blots, which showed the largest bands of EV marker proteins in earlier fractions (7-11)

as well. Although the ExoQuick samples showed more total RNA in the Bioanalyzer results than UC, they also showed the highest amount of protein contamination in protein gels (Figure 2.2) and the least intense CD81 bands in western blot analysis (Figure 3.3), indicating that the ExoQuick samples may have more contaminating non-EV RNA. Thus, although ExoQuick is the simplest and fastest protocol, it may not be the most ideal for preparation of sequencing of EV associated miRNAs.

This exploratory methodological study is strengthened by the inclusion of three biological replicates (three distinct mothers' milk) and three technical replicates from each of those three mothers' milk. This will ultimately allow for the comparison of replicability of the EV isolation and miRNA sequencing pipeline as well as the comparison of miRNA profiles between distinct individuals. Each methodology also received the same input volume of skim milk (500 μL) which allows for a consistent comparison of raw material being processed. Additionally, the EVs from different isolation methods were characterized using multiple standards for characterizing EVs, i.e., western blots probed with known EV markers, TEM images, and nanoparticle tracking analysis data. When the comparative EV isolation method samples have been sequenced and analyzed, the resulting study may be the first to systematically examine different EV isolation techniques and the resulting small RNA seq profiles from these techniques. This information is vital to understanding the most appropriate methods for further experiments in the field of human milk epigenetics.

There are also several limitations in this study. One important consideration is the potential presence of contaminating, non-EV small RNAs in the final EV-enriched isolate of each methodology. In the course of this study, western blots of SEC fractions were probed for potential contaminating RNPs such as Ago2 and ApoA1, but the results were inconclusive and

therefore not included. For the ultracentrifuge methodology data presented, only one protocol and spin time and speed were tested. However, in the EV isolation literature there are several different proposed methods for potentially increasing EV yield and intact morphology such as alternate speeds, washes, or gradient ultracentrifugation. The literature in the EV field also lacks consensus about the importance of pre-filtering milk before EV isolation. In this study, the milk samples for ultracentrifuge, ExoQuick, and exoRNeasy methodologies were filtered with 0.45 μm PVDF filters but not filtered for the SEC methodology. Another limitation that also provides an avenue for continued studies is the consistency of miRNA profiles in human milk through the post-natal period of breastfeeding. Some factors such as age of the infant and time of day of the milk collection may influence the composition of the milk. Because our mothers had infants of various ages (though all under 1 year old), and milk samples were potentially collected at different times of day, this may influence the EV composition and levels or types of miRNAs between the samples. Some studies use fresh, unfrozen milk as the initial input for EV isolation, but the milk in this study was stored at -80˚C and underwent at least one freeze-thaw cycle prior to EV isolation. Although this could potentially impact the original composition of the milk, it should also be noted that the majority of published studies also use frozen milk because access to fresh milk is rare, as most biobanks freeze their donated samples. Additionally, because the exoRNeasy methodology does not have an intermediate EV isolation step and continues through small RNA extraction, there is no data presented here on the characterization or visualization of EVs associated with those samples.

There are several future steps that will be performed to complete the ultimate goals of this study. Firstly, the addition of TEM images and Nanosight data for the SEC EV isolation method will provide a more complete profiling of the EVs across methodologies prior to RNA

extraction and sequencing. More western blots with different antibodies are also being run on these samples in order to better understand the protein content of the EV-enriched isolates across methods, especially in regard to potential RNPs. In the future, identification of the presence and relative of amounts of potential RNPs will aid in concluding the purity of EV enriched isolates for all four methods. Currently, the 144 RNA extractions from the four EV isolation methodologies have been converted into cDNA libraries, quality controlled, and are being prepared for size selection and pooling in anticipation of small RNA sequencing. These data should reveal the relative amounts and types of small RNA (e.g., miRNA, tRNA fragments, piRNA, Y RNA) found in each method. This will help in drawing comparisons for which method is ideal for isolating miRNA as compared to other small RNA types. Additionally, the amounts of miRNA found in the sequencing of SEC fractions and fraction pools will aid in selecting the most appropriate fractions upon which to focus in future studies.

The continued exploration of these methods will determine the design of an ongoing study investigating associations between maternal stress and mood and EV-miRNA profiles. Several studies point to an association between stress and miRNA levels in blood and brain tissue, suggesting that miRNA activity may be involved in gene expression changes in response to stressful circumstances. One study in male medical students before an important exam showed an elevation of certain miRNAs in their blood and a decrease in their mRNA targets. When the miRNA levels were measured again a month after the exam, the elevated group of miRNAs had decreased (Honda et al., 2013). Another study found global downregulation of miRNA activity to be associated with depression in the prefrontal cortex of suicide subjects (Smalheiser et al., 2012). These findings and others suggest that miRNAs have the potential to modulate responses to stress and depression. Maternal stress and mood have been linked with altered miRNA

expression in sperm, blood, and brain tissue of mice and humans (Meerson et al., 2010; Melnik and Schmitz, 2017; Rinaldi et al., 2010) but has yet to be thoroughly explored been in milk. Only one published study has tested maternal stress in relation to EV miRNA profiles and found several miRNAs associated with short- and long-term stress measures (Bozack et al., 2021). Their study utilized an exoEasy Maxi Kit to isolate EVs, which is spin-column based method that is similar to the exoRNeasy kit but without the RNA extraction process included. They also utilized a microarray with miRNA specific probes rather than full small RNA sequencing, and some measures of maternal stress but not maternal mood.

More broadly, this research will help improve our knowledge about the natural variation in EVs and miRNAs in human milk and their potential for transmission of intergenerational epigenetic signals. Also, research in this field can ultimately be utilized to improve maternal and infant health outcomes.

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