Effect of Storage Conditions on Small Noncoding RNA Concentrations in Human Milk using the Size Exclusion Chromatography Method for Extracellular Vesicle Isolation.

Samantha Wu, Amy L. Non

Received August 21, 2023
Accepted September 21, 2023
Electronic access October 31, 2023

Small noncoding RNAs (ncRNAs) derived from extracellular vesicles (EV) in human milk are evidenced to epigenetically modulate gene expression in the developing infant. The types and quantities of certain ncRNAs, such as microRNAs, have been suggested to vary based on maternal conditions, such as disease status, diet, stress, or maternal age. Despite growing interest in this subject, methodological inconsistencies and challenges in the storage and handling of human milk samples remain, potentially compromising the quality and quantity of extracted EV-derived ncRNAs. The present study explores the effects of up to four freeze-thaw cycles at -80°C and overnight storage at 4°C, as well as potential confounding factors such as milk volume, on the quantity of small ncRNAs in human milk samples. Importantly, we employ Size Exclusion Chromatography (SEC), a method that separates molecules based on size. While rarely used on milk, SEC is evidenced to have greater efficacy in extracting extracellular vesicles from human milk than other methods because it is more specific at isolating EVs than polymer precipitation methods, and because it relies on gravity which is gentler than ultracentrifugation. We observed inconsistent trends across donors in RNA concentrations across freeze thaw-cycles and no effect on concentration for storing milk at 4C (840.1 pg/uL) overnight relative to processing same day (1092.8 pg/uL). Graphical analyses also revealed that the initial volume of human milk pumped was inversely related to the RNA concentration, as the woman with a high volume pumped had a lower RNA concentration than the woman with a lower volume pumped (mean=207.96 pg/uL and 1241.9pg/uL, respectively), suggesting that greater volume may lead to milk samples that are diluted in RNAs. While our findings are inconsistent across donors regarding freeze thaw cycles, generally it seems that overnight storage is not detrimental to RNA concentration, and that volume of milk was the most influential confounding factor in affecting RNA concentrations. However, given the small sample size, further analysis is necessary to determine whether these findings are replicable and generalizable to a larger population.

Introduction

Human milk (HM) is a biofluid known to aid the survival of the infant through conferring protection against chronic and infectious diseases [1], contributing to metabolic, microbiota [2,3], and neurological development [4]. Much of HM's potentness lies in its diverse nutritional and bioactive components, such as antibodies, microbes, lipids, whey and casein complexes [5], antimicrobial peptides [5,6], cortiso [7], and non-coding RNAs (ncRNAs) [8]. HM's composition is highly variable between different mothers [9], and has been hypothesized to not only vary with the characteristics of the developing infant [10], but also with maternal conditions and the environment [5,11]. Importantly, studies of HMEV-ncRNAs do not have standardized methods for sample storage or handling, which are factors that can influence RNA concentrations in the milk.

One bioactive component of interest is human milk-derived extracellular vesicles (EVs), which are 30–150nm structures that house a variety of non-coding RNAs [8] (ncRNAs). A primary component of HMEVs are miRNAs, a type of ncRNA, which have been analyzed in relation to maternal conditions, including stress [12], chronic [13] and infectious diseases [14], diet [15], and environmental pollution [11], and are associated with immune development, endocrine signaling, adipocyte differentiation, neurodevelopment, and cell community [16,17,18]. Moreover, extracellular vesicles in HM are evidenced to protect their RNA cargo from conditions of the infant's gastrointestinal tract. Not only do HMEV-miRNAs remain intact in simulated digestive conditions, but the absorption of extracellular vesicles and their bioactive cargo into intestinal epithelial cells is aided by acidic conditions [19,20]. This evidence supports the possibility that HMEVs deliver functional ncRNAs that modulate gene expression in their recipient cells. There is great diversity in methods used to isolate HMEV-ncRNAs. While Size Exclusion Chromatography (SEC) may be favored over other methods, as outlined below, the effects of storage conditions on HMEV-ncRNAs have yet to be explored using this method.

© The National High School Journal of Science 2023

Common HMEV-Isolation Methods

Most commonly used methods for isolating HMEVs vary greatly in consistency and yield. In a review, Li et al evaluated the common methods for isolating HMEVs for purity and yield recovery[21]. The method known as the gold standard for HMEV isolation, ultracentrifugation (UC), involves centrifuging samples at around 100,00 g for 90-120 minutes to sediment exosomes and other components of HM. Although UC is easy to operate and affordable, it has been evidenced to have the lowest and most inconsistent recovery rates of between 5-25% as a result of impurities and exosomes shears due to the large g-forces[22]. Another widely used method is the polymer precipitation method's ExoQuick precipitation kit, which uses a proprietary polymer to combine water molecules and precipitate exosomes. Despite its relative ease of use and less labor intensive approach, this method's pitfalls include poor recovery, low purity, and spoilage of exosomes due to the method's chemical additives. Of the methods evaluated, the polymer precipitation method has the second lowest recovery rate of 50%, making it difficult to draw valuable associations concerning the composition and yields of MEVs[21]. Another method is the Immunocapture method, which relies on antibodies for CD81, CD63, and CD9 to identify and isolate exosomes[23]. The immunocapture method has high specificity and purity, but is evidenced to lose exosomes due to the washing processes[21].

Size Exclusion Chromatography

A more rarely-used but promising method for HMEV isolation is the Size Exclusion Chromatography method (SEC),which has been described as the ideal method for the isolation of exosomes[24]. SEC relies on low-pressure gravitational flow of liquids through a column to separate components of HM into different fractions based on particle sizes. HMEVs isolated via the SEC method have high purity and sample reproducibility[21], making SEC a promising and cost-effective method for isolating HMEVs. In ongoing experiments in Dr. Non's lab, they discovered that even with a relatively small starting sample volume (typically 500uL), successful RNA isolation has been demonstrated. In a rare study extracting HMEVs with SEC, SEC followed UC as a secondary purification and enrichment method, rather than a primary extraction method[25]. However, higher expression of exosome marker proteins were observed with HM samples isolated exclusively with SEC rather than coupled with UC[26]. Moreover, SEC is effective in removing whey and casein proteins from EV membranes, and is scalable, gentle, and effective in isolating HMEVs with minimal loss for both large and small sample volumes[26]. In an ongoing experimental comparison of these EV-extraction methods by Dr. Non's lab, SEC yielded the highest miRNA proportions relative to other EV isolation methods. Therefore, the present study uses a UC-free SEC method to extract EVs. Fractions 6-11 were selected based on our preliminary experiments and findings from prior studies[24] (preprint, Dr. Non personal communication) that showed Western Blots indicating the highest presence of EV antibodies within these fractions.

Functions of Non-Coding RNAs in Human Milk

HMEV small ncRNAs include microRNAs (miRNAs), piRNAs, and circRNAs[8], each functioning in a variety of pathways. The most commonly studied HMEV-derived ncRNAs are miRNAs, which are single stranded, noncoding, regulatory RNAs 18-25 nucleotides in length[27]. MiRNAs function as post-transcriptional modulators by preventing the translation of specific mRNAs into proteins. The most abundant miRNA in milk, miR-148a-3p16, is known to decrease the expression of DNA methyltransferase 1 (DNMT-1), an enzyme central to the epigenetic pathway of gene methylation and implicated in the silencing of tumor suppressor genes[28]. Four of the ten most abundant miRNAs in human milk are involved in immune modulation[16], and pathway analysis across studies suggest HMEV-miRNA involvement in adipocyte differentiation, immune system development, and neurodevelopment[17]. Furthermore, HMEV-miRNAs may play a vital role in the prevention of leukemia and Hodgkin's lymphoma, as well as infectious diseases[14]. Other ncRNAs derived from HMEVs may be biologically active. For example, circRNAs regulate gene expression primarily through miRNA sponging, which involves inhibiting the activity of one or more miRNAs through attachment to a binding site. Pathway analysis of exosomal circRNAs in human colostrum found circRNAs most significantly involved in the VEGF signaling pathway, which may contribute to angiogenesis and vasculogenesis, as well as bone development[29]. Other examples of ncRNAs found in HM are piwiRNAs, which help modulate the methylation of histone 3 lysine 932; or vaultRNAs, which function as immunomodulators and putative tumor suppressors[30]. Therefore, the types and quantities of HMEV-ncRNAs may have potential implications for the development of the infant. While HMEV-derived ncRNAs present many pathways for epigenetic modulation of an infant's development, little research has been conducted on the optimal methods for the storage, handling, and extraction of HMEVs. In part, this may be a result of the relative novelty of research on ncRNAs[31], and low attention given to human milk as an important topic of medical research[32]. Optimization and purity of yield is foundational for identifying accurate and reliable associations between HMEV-ncRNA compositions and maternal conditions to make possible valuable real-life applications, such as improvement of infant formula, or understanding conditions under which it is safe to breast-

© The National High School Journal of Science 2023

feed. These questions are particularly important for the many studies that collect milk in the field, where there is inconsistency in storage conditions prior to lab analyses.

Handling, Storage, and Freeze-Thaws of HM Samples

Storage conditions and the process of freezing and thawing samples has been suggested affect the purity and viability of HMEV samples. Following the freezing of HM at both $-80°$C and 4°C, cell death and disintegration was observed, leading to storage-induced vesicles that could contaminate analysis of naturally present EV vesicles[33]. Another study examining the effect of storage at 4°C and $-80°$C on HM exosomes found that while exosome counts followed a decreasing trend over 4 weeks in storage, the decrease over time was only statistically significant for samples stored at 4°C without preservatives[34].

The number of freeze-thaw cycles of human milk has generally been thought to decrease HM EV-ncRNA purity and yield. Kosaka et al subjected two HM samples to three consecutive freeze thaw cycles, and found no significant decrease in EV-derived hsa-miR-21 and hsa-miR-181a, using a magnetic bead isolation method with the anti-CD63 antibody[20]. However, human EVs also contain CD9 and CD81 tetraspanin proteins embedded in their phospholipid bilayers that were not considered in this analysis, potentially leading to an incomplete isolation[23]. Zhou et al[16] subjected four HM samples to a variety of harsh treatments, including six freeze-thaw cycles. Eventually, they concluded that although the expression level of the top ten exosomal miRNAs displays a decreasing trend of miRNA expression levels over freeze-thaw cycles, they remained stable through the freeze-thaws. However, this study used the ExoQuick Precipitation Method for isolation of exosomes, which is known for poor recovery and inconsistency[21].

To our knowledge, the use of SEC to isolate HMEVs and measure the effects of storage conditions and freeze thaws on HMEV-ncRNAs has not been attempted. Therefore, the present investigation aims to 1) study the influence of storage conditions and multiple freeze thaws on the quantity of HMEV-ncRNAs extracted using the SEC method; and 2) to explore the potential confounding experimental factors, such as the number of times the column was used, and the volume of milk pumped, that impact SEC method for the purposes of HMEV extraction.

Results

In order to investigate the stability of EV-small RNAs in HM, we subjected samples to different conditions, including up to 4 freeze thaws, and storage overnight at 4°C. Estimated EV-small RNA concentrations varied across samples and conditions from 59-3202 ng/mL.

RNA Size Distribution

From the bioanalyzer data, we determined that the majority of RNAs were below 500 nucleotides long. This verifies the success of our RNA extraction, and the existence of small ncRNAs rather than the long mRNAs in our sample. Figure 1 displays a graphical distribution of the fluorescence units and microfluidic capillary gel electrophoresis migration times, which are used as parameters for estimates of concentration and RNA length. From here on, we describe small noncoding RNAs in our study as RNAs.

Replicate Analysis

Upon visual inspection of Figure 2, replicates seem to be approximately similar. We determined that the sample replicates were highly correlated using Pearson's Correlation Test (r=0.961), a value that is similar to that obtained by Zhou et al (r=0.94)[16], supporting the experimental reliability of our methods.

Effect of Multiple Freeze Thaws on RNA Concentrations

In our analyses of the effect of multiple freeze-thaw cycles on ncRNA concentrations, we noted sufficient RNA was obtained for most downstream sequencing analyses from all samples. However, after taking the mean of each technical replicate, we graphically observed an increasing trend from freeze thaw cycle 0-3, and a decreasing trend from freeze thaw 3-4, primarily in milk from only one of the two donors (Mom 4) (Figure 3.0). There was minimal variation in Mom 3 across Freeze thaw cycles, potentially due to very low volumes of RNA in that sample overall.

Pearson correlations (Figure 3.1) revealed a moderate positive correlation between number of freeze-thaw cycles and concentrations of RNAs in participant #4 (Pearson Correlation Coefficient$= 0.62$), and a null correlation for participant #3 (Pearson Correlation Coefficient$= -0.04$). The combined Pearson's correlation coefficient for the two samples was a moderate positive correlation of 0.40.

Comparison Between Milk Stored at 4°C overnight and Fresh Milk

Similarly, we conducted a two-way ANOVA test to determine whether there were significant differences in the RNA concen-

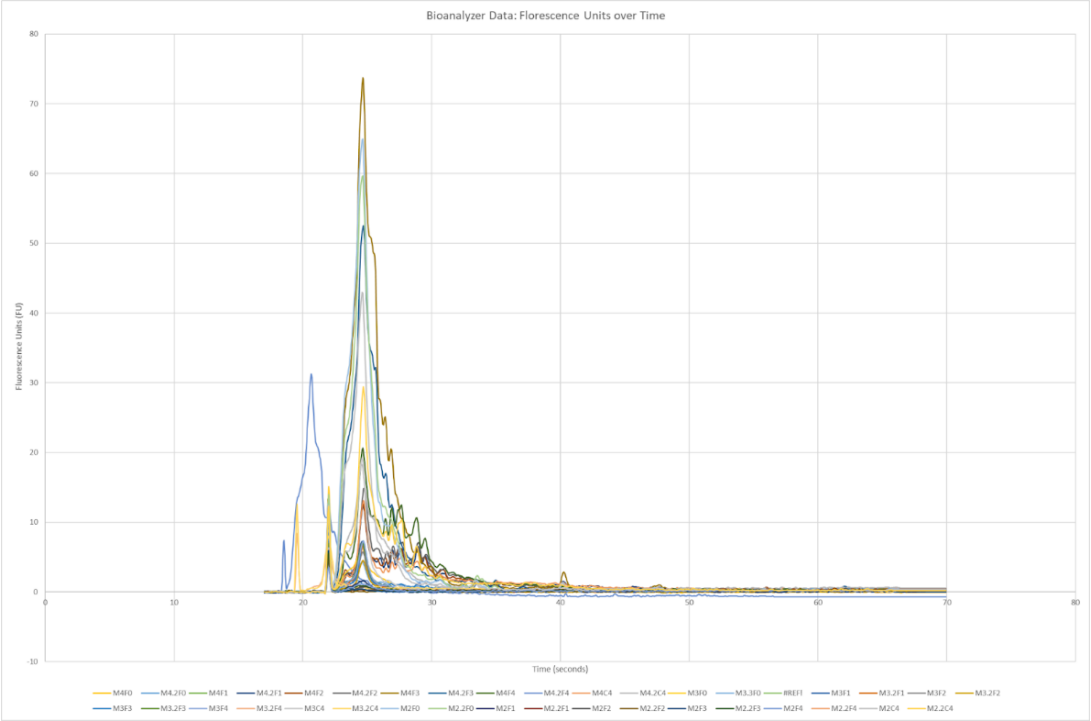

Fig. 1: Live Plot from the Agilent 2100 Bioanalyzer: Migration Time against Fluorescence Units. Higher intensity values (FU) indicate higher RNA concentrations, and greater migration times (s) indicate longer length of RNAs. Samples are RNA solutions extracted from human milk under each storage condition. The peak representing the standard ladder was not included in the plot as it differed for each curve.

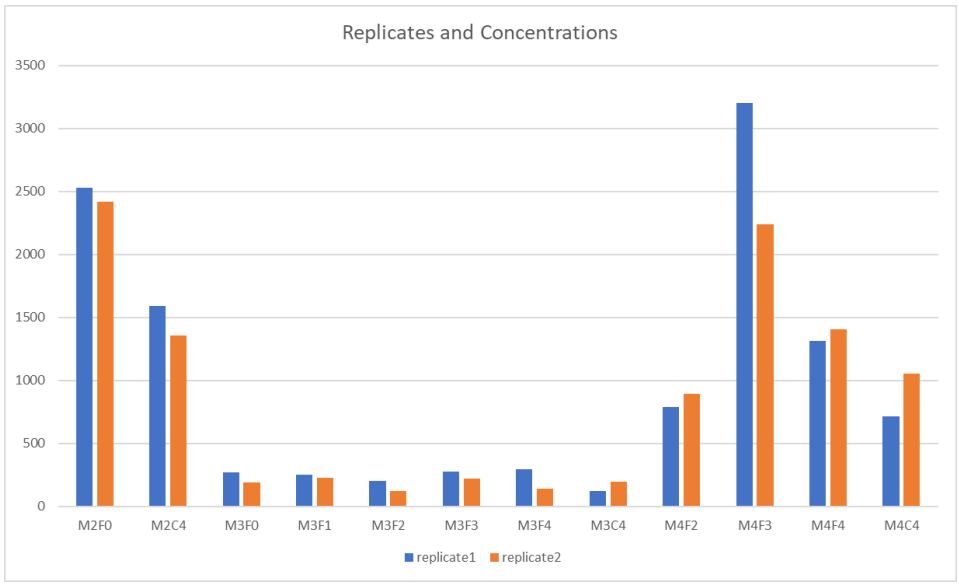

Fig. 2: Visual Distribution of Technical Replicates for each experimental condition (24 data points). Bar graph for comparing milk sample replicates based on estimated RNA concentrations (pg/uL).

© The National High School Journal of Science 2023

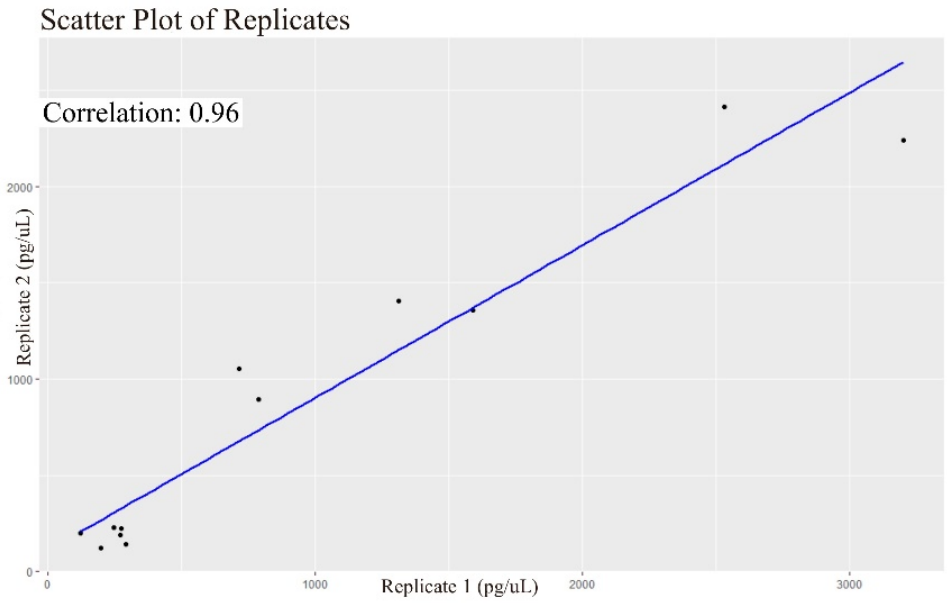

Fig. 2.1: Scatter plot and Line of Best Fit Demonstrating Correlation between Technical Replicates of Milk Samples. Pearson's Correlation Coefficient was calculated (r=0.961) based on comparison of RNA concentrations (pg/uL).

trations of milk stored for 24 hours at 4°C and fresh milk. We found no significant difference (p= 0.536), with a high likelihood that the variation observed was due to random chance (F= 0.415). Figure 4.0 visually displays the lack of significant differences between these two conditions.

Relationship between Column Use and RNA Concentration

After observing minor differences ($p < 0.05$) in pooled fraction volumes (ranging from 2992-3314 uL) from the Size Exclusion Chromatography step, we analyzed whether the number of times a column had been used affected the concentration of RNAs recovered. Although negative correlations between column use and RNA concentrations were observed, R2-values from linear regression analyses indicated little correlation (Figure 5.0). Furthermore, a two-way ANOVA analysis considering participant and column use as independent variables suggest no significant differences ($p = 0.875, F = 0.026$).

Differences in RNA Concentration and Volume

Using the two-factor ANOVA test and treating donor and volume as independent variables, we determined a significant difference in RNA concentration across different initial milk volumes ($F = 28.39, p = 1.82e-05$). This significant difference was also observed with a one-factor ANOVA test with only volume as an independent variable ($F = 24.52, p = 4.7e-05$).Upon visual inspection of Figure 6.0, there seems to be a clear correlation between volume of milk pumped and the RNA concentration.

Discussion

To our knowledge, only two other studies have employed the SEC method for isolating EVs from human milk[26,35]. While SEC has rarely been used to date for HM, it has been described as the ideal method for the isolation of exosomes across different body fluids[24].

Notably, all sample conditions yielded sufficient RNAs for downstream sequencing analyses. Moreover, the concentration of RNAs extracted were between 59ng/mL to 3202 ng/mL, which is a higher range than what had been previously reported (9.7ng/mL to 228.2 ng/mL)[20]. This may suggest that the SEC method captured a greater proportion of existing EVs in human milk samples than the study using magnetic beads, anti-CD63 antibody[20]. However, differences in RNA concentration between studies may be influenced by variation across individual donors. EV-extraction methods can be more accurately compared using the same donor samples.

Our results indicated a surprising positive trend between the concentration of RNAs between freeze-thaws 1-3, and a decline from 3-4, though only based on two samples. The positive association between RNA concentration and number of

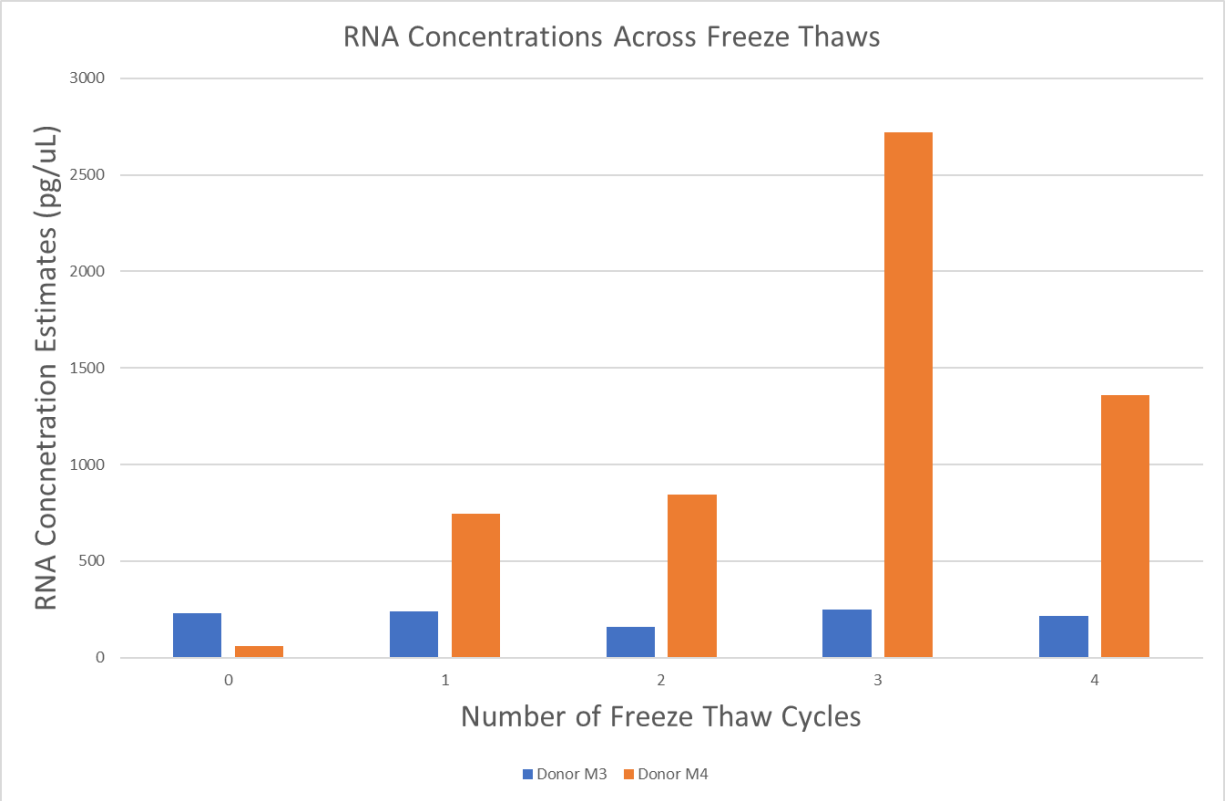

Fig. 3: RNA concentrations (pg/uL) across number of Freeze-Thaw cycles (0-4). Bar graph comparing means of replicates for each freeze-thaw condition of milk samples based on estimated RNA concentrations. Donor M2 was excluded due to the loss of sample during processing, as detailed in the methods.

freeze-thaws may support the hypothesis of storage-induced vesicles. Freeze-thaw cycles cause cells to lyse, potentially inducing the disintegration of dead cells into vesicles. This suggests that our observed trend may not be indicative of an increase in naturally occurring EVs, but a contamination of EVs introduced upon sample storage[33]. However, given the small sample size, it is premature to draw firm conclusions about positive effects of freeze thaws. Future studies should investigate this question with larger sample sizes, and could also investigate whether additional filtration methods, such as 0.45um PVDF filtration[16], should be applied on milk supernatants to remove excess cellular debris before freezing.

However, another study on plasma using ultracentrifugation for extraction and flow cytometry for quantification revealed a loss of 10-15% in microvesicle count after one freeze thaw39, indicating that naturally-present extracellular vesicles may degrade over freeze-thaw cycles. Effects may vary by sample type and method used to isolate EV-miRNAs. The only two studies that have investigated the effects of freeze-thaw cycles on HM found no significant change in relative RNA expression levels in freeze thaws using either magnetic beads or ExoQuick to isolate EVs, followed by qRT-PCR analysis[16,20]. However, these studies used potentially less reliable methods for isolating EVs. Further studies with larger sample sizes using SEC are required to determine whether this positive trend is generalizable to other methods. We also tested the effects of storage overnight at 4°C on the concentrations of RNAs, finding these concentrations were not significantly from those of fresh milk samples. However, it is important to note that another study using ultracentrifugation as an EV isolation method showed a significant decrease to 49% ± 13% the original vesicle count when stored for 4 weeks at 4°C. The same study also found that storage at -80oC for 4 weeks did not lead to a significant loss of vesicles, with 71% ± 45% the original count remaining.[34] These findings combined with ours suggests that when collecting milk samples, temporary storage for $<$ 24 hours in a mother's refrigerator may not compromise the structural integrity of RNAs in human milk, but -80°C conditions are more ideal for extended periods of storage. As it is common practice for freshly expressed human milk to be stored in the refrigerator for up to 4 days40, future studies should use the SEC method to determine whether the

© The National High School Journal of Science 2023

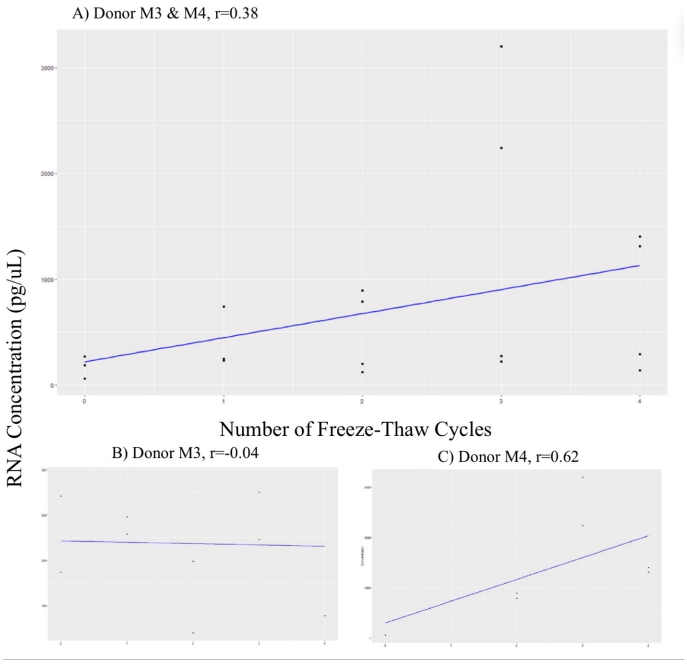

Fig. 3.2: Correlations between number of freeze-thaws and RNA concentrations, for each condition and replicate. ANOVA scatterplot and linear regression comparing samples and their replicates, across freeze-thaw cycles, based on RNA concentrations (pg/uL) from A) donor M3 and M4, B) donor M3, C) donor M4. Pearson Correlation Coefficients were calculated.

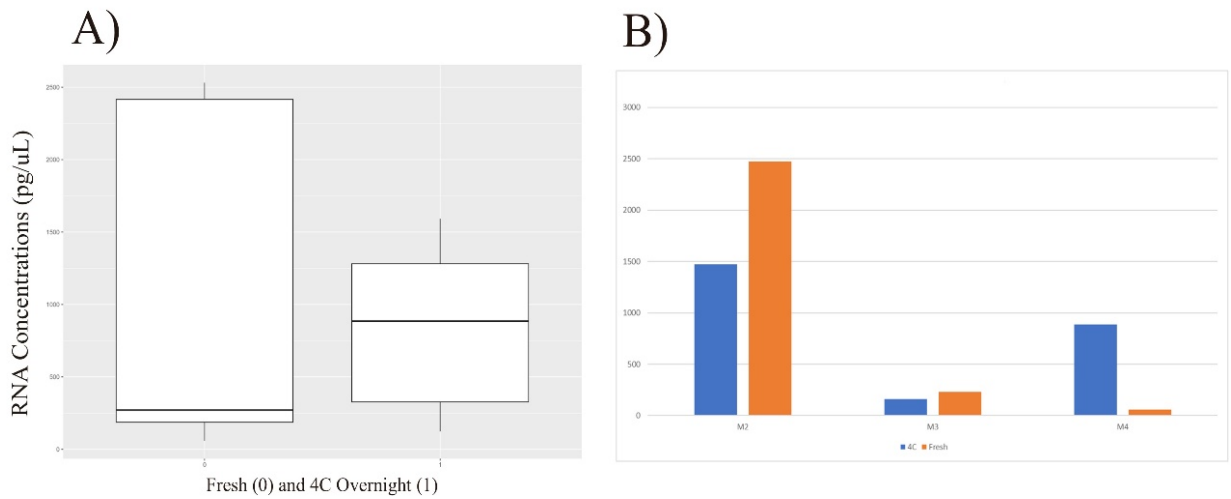

Fig. 4: RNA concentrations from fresh milk and milk stored at 4°C overnight. A) Standard box plot, comparing individual samples and replicates for each condition (Fresh milk 0, 4°C 1) based on RNA concentration (pg/uL). B) Bar graph comparing differences in RNA concentrations in 4°C and fresh milk across donor M2, M3, and M4 (averaged replicates).

concentration of RNAs change when human milk is stored at 4°C for more than 24 hours. An unexpected finding in our study was the strong negative association between the volume of the full breast pumping and the quantity of RNAs present in the solution. We obtained a large volume of human milk from a single pumping from donor M3, and the RNA concentrations from this donor were an order of magnitude smaller than those of donor M4. Donor M3 exclusively pumps her milk rather than traditional breastfeeding, which may have caused a larger volume to be expressed in a single pumping. More-

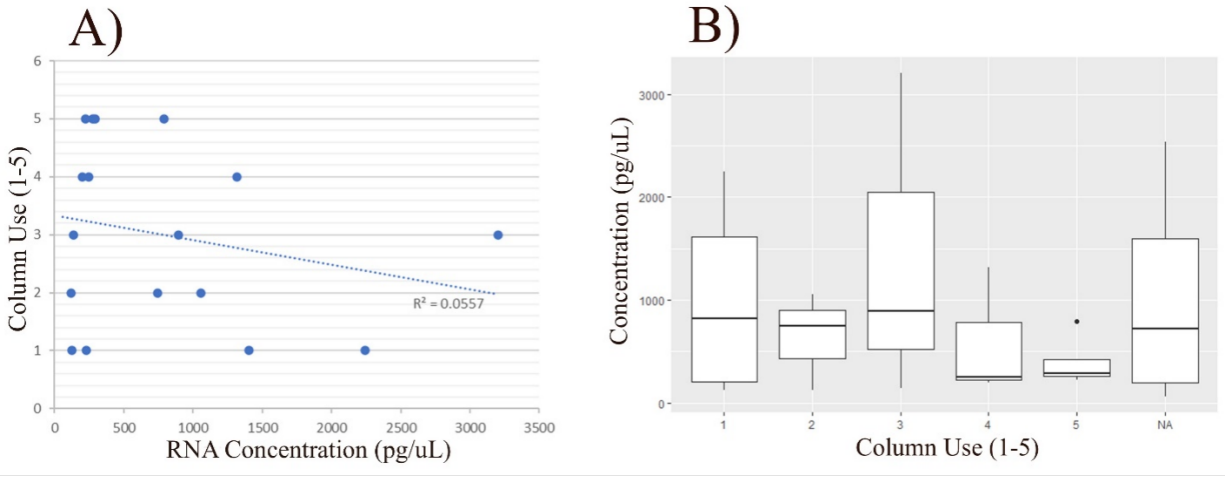

Fig. 5: Fraction volume and column use in relation to RNA concentrations. A) displays the number of times a column was used when the sample was run, over the RNA concentration. A Pearson correlation coefficient was calculated. B) shows a box plot for each column use and its respective concentration distribution, in pg/uL.

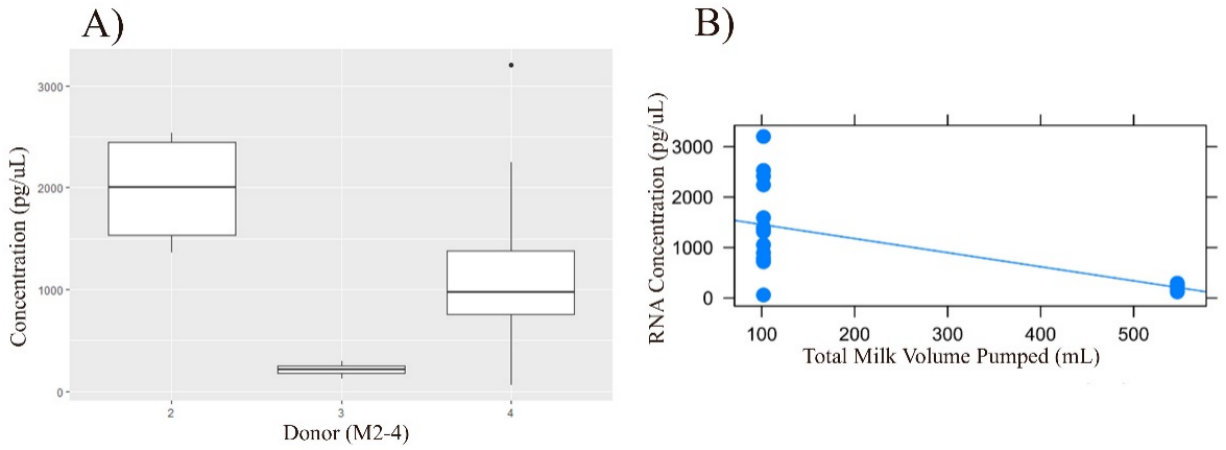

Fig. 6: Donor and Volume in relation to RNA concentrations. A) Box plot of RNA concentrations grouped by donor, B) Scatterplot of milk volume in relation to RNA concentration.

over, milk samples from donor M3 were visually lower in fat content. The differences in fat content and RNAs with the high volume of milk may suggest that exclusively pumping and/or overproduction can lead to lower micronutrient and bioactive molecule content. However, these differences may also be explained by the differences in lactation stages, since donor M3 had been expressing milk for 4 months, and donor M2 had been expressing milk for two months. Future studies should determine whether exclusively pumping affects the RNA content in human milk, and whether this may affect the health of the infant.

Strengths and Limitations

Our study is strengthened by careful experimental design that included a wide range of conditions tested, such as same day processing of fresh milk, the use of multiple biological and technical replicates, use of the optimal SEC method for isolating EVs and the specific Norgen kit optimal for isolating exosomal RNAs. Our study was also strengthened by careful use of RNAse free tubes and reagents, and successful high yield of RNAs. Our study may represent the first to explore storage and handling conditions of milk using these methods.

Because our datasets only include a sample size of two to three participants, we avoid presenting any statistical analyses,

© The National High School Journal of Science 2023

other than correlations between technical replicates. To avoid overstating findings with a small dataset, our conclusions rely on general trends in the data, and not on statistical significance tests. Our sample size was limited by some technical issues that resulted in the loss of ten samples, including nearly all of the second donor freeze-thaw comparisons. Given the large variation in volume and age of infants between donors, which appear to have a large effect on RNA concentrations, we recommend further investigation with larger sample sizes of varying volumes and ages to confirm our findings. We also note that our study sample had a narrow age range of 28-32 years, with infants less than 1 year, and only healthy mothers with ethnicities of White, Middle Eastern, and East Asian. Future studies with more diverse samples will be important to see if trends are generalizable. Other confounders may be important to explore in future studies as well, including maternal diet, hydration, and stress levels. It is also important to note that the Bioanalyzer approach, particularly for the 'Total RNA Analysis pg Sensitivity Eukaryote Chip,' is not optimal for accurately quantifying small RNA concentrations. However, the shape of the Bioanalyzer live plot provides a useful qualitative indicator of presence of non-degraded small RNAs, and generally correlated with the quantitative estimates of concentrations. The shape of all Bioanalyzer live plots in our study indicated good quality RNA, but this can only be confirmed after amplifying specific miRNAs with qPCR or whole genome sequencing, which should be done in future studies. Finally, we note that study methods used to isolate EVs and quantify miRNAs can affect yield, and our findings are only relevant to studies using SEC and Norgen RNA isolation kits, and quantifying with the Bioanalyzer.

Conclusion

In conclusion, we found minimal effects of multiple freeze-thaws on HM EV-derived small ncRNA preservation. We found a surprising positive correlation between freeze-thaw cycles and RNA quantity, contrary to our expectations. However, there was no significant effect of storing at 4°C overnight relative to freshly processed milk. Graphical trends suggests there may also be differences in RNA concentration due to differences in donor volume and/or lactation month between the milk donors. These findings may imply that milk EV RNAs are generally robust to storage conditions, but this requires further confirmation with larger sample sizes. However, given large variation across the very few donors tested, and the effect of sample volume on RNA concentrations, care should be taken when comparing RNA concentrations across studies.Once these methodological questions are settled, future studies of milk can provide more consistent results, which could lead to improvement of infant formulas as well as more knowledge on how various maternal conditions affect milk miRNAs and the health of the infant. As miRNAs are essential for regulating gene expression, and the most abundant milk miRNA, 148a-3p, is important for regulating expression of the DNA methyltransferase gene which regulates methylation of all genes in the genome, this is an important frontier for epigenetics research. Milk represents an interface between mother and infant, so understanding the interactions between mother's environment, milk content, and infant health may be essential for ensuring epigenetic health across generations.

Methods

Participants

Our initial sample size consisted of three healthy women from San Diego, California, who volunteered to participate in this methodology study. Detailed demographics are listed in Table 1.0. Inclusion criteria were that the women were not on any medications, had no major health conditions, and gave birth to either primiparous or multiparous healthy infants of singleton births (demographics and health were self-reported). This study was deemed exempt by the Institutional Review Board at the University of San Diego, and written informed consent was obtained from participants. All study protocols were in line with Helsinki Principles for conduct of ethical research.

Milk Expression and Collection

Participants donated a full pumping of milk before their infant's first feeding of the day. Bags of freshly pumped milk were stored in donors' 4°C fridge until retrieval, and then stored on ice during transportation to the laboratory for analysis on the same morning. Milk from each mother was aliquoted into 50mL conical tubes, where they were centrifuged twice; first at 200 x g for 10 min to remove the lipid layer, and then at 12,000 x g for 30 min to remove cellular debris from the supernatant. The resulting supernatant (skim milk with no debris) was aliquoted into 1.5mL conical tubes for each (number of conditions) test condition.

Study Design and Tests

We extracted HM EV-derived RNAs from a total of 36 HM samples. For each condition we analyzed 6 samples (three distinct donors as biological replicates, and two technical replicates per donor). The 6 different storage conditions were as follows: 1) fresh milk kept on ice and processed within a few hours of collection, 2) stored overnight at $-4°C$, and 3) 1 freeze-thaw at $-80°C$, 4) 2 freeze-thaws at 80°C, 5) 3 freeze-thaws at 80°C, and 6) 4 freeze thaw cycles. All samples frozen at 80oC were slowly thawed on Armor Metallic Beads that had

	Sample M2	Sample M3	Sample M4
Maternal Ethnicity	White/Caucasian	East Asian	Middle Eastern
Maternal Age	29 years	32 years	28 years
Infant Gender	Female	Male	Female
Parity	1	2	1
Infant Age (months)	2 months	4 months	10 weeks/2 months
Time of Day	6:20AM	8:15AM	8:30AM
Volume of Milk	102mL	18-19oz(547mL)	102mL

Table 1: Maternal demographic and milk donation characteristics

been stored at $-20°$C, allowing for a more gradual thaw process taking up to 2 hours. All handling of milk and exosomes was conducted at 4°C on Armor Metallic Beads.

Exosome Isolation

Exosomes from the fresh milk condition were isolated on the same day using the SEC method. The procedure for SEC followed manufacturer's recommendations for use of Izon qEV size-exclusion columns (qEVoriginal/70nm Legacy columns, Izon Science, UK) and a BioRad Model 2110 Fraction Collector. A volume of 500uL of HM supernatant was gently loaded onto the column, followed by 1 column volume (10ml) of PBS. Each sample was separated into 14 fractions of 0.5 mL volume. Fractions 6-11, which western blots indicate to contain HM EVs, were pooled and stored at $-80°$C. Columns were stored and cleaned per the manufacturer's recommendations.

RNA Isolation

EV-RNA was isolated from the pooled exosome fractions, thawed on ice, for each sample using the NORGEN Plasma/Serum Circulating and Exosomal RNA Purification 96-Well Kit, Slurry Format, following standard protocol, except for half the sample volume was used. A total of 500uL of sample was added to 900uL of BME/Lysis A solution, and incubated at 60°C for 10 minutes. 1.5mL of ethanol was added and the mixture was spun for 2 minutes at 1000rpm. All centrifugations in the spin plate were performed at 3900 rpm. Additional minutes of spin were added after each wash to ensure sample was fully dry.To elute the sample, a total of 28uL of RNAse free water at 50°C was added and spun at 3900rpm for three minutes. Resulting eluted solutions were stored at -80oC in DNA LoBind tubes. We successfully extracted RNAs from 26 of the initial 36 samples. We lost two samples, one fresh milk and one freeze-thawed once, both from donor M4 due to pipetting error. Due to technological failure with our centrifuge during the RNA extraction step, we lost eight samples from the freeze-thaw cycle conditions of donor M2.

Bioanalyzer

Estimate concentrations and tracer plots of RNA size distributions from each sample were obtained using the Agilent 2100 Bioanalyzer System with the Total RNA Analysis pg sensitivity (Eukaryote) chip.

Testing for Protein Contamination in SEC Columns

Izon Columns have been used to extract EVs from human plasma and serum with high efficiency, yet have rarely been used on human milk. Therefore, we assessed whether the manufacturer's recommendation of flushing between samples with 10mL of NaOH is sufficient for decontamination, since HM has higher concentrations of proteins and other macromolecules. Protein concentrations of PBS solutions run through columns that had been used and cleaned 5x were analyzed for presence of Protein A-280 on the ND-1000 Spectrophotometer (NanoDrop). Protein concentrations (mg/mL) were undetectable, providing evidence that Izon's recommended decontamination protocol was sufficient for HM.

Data Analysis

Small RNA concentration estimates obtained from the Bioanalyzer were used as our primary outcome for all analyses. We compared mean estimated levels of small RNAs across samples under each condition using ANOVA and paired t-tests. We also used hierarchical linear regression models, with fixed effect for donor, to analyze the effects of confounding factors, including the number of times an Izon qEV size-exclusion column was used and the initial volume of the full pumping on small RNA concentrations, in order to elucidate the influence of details in methodology on small RNA yields. All data analyses took place in Microsoft Excel and R version 4.2.2.

Acknowledgements

First and foremost, the first author would like to thank Dr. Amy L. Non for the opportunity to contribute to her lab's

 © The National High School Journal of Science 2023

ongoing work through partnering with her on this research project, and for her kindness, mentorship, and dedication. The authors would also like to thank members of Dr. Non's Epigenetic Anthropology Lab at UC San Diego for their support, both in the lab and during discussions; as well as Dr. Louise Laurent's lab, for allowing the authors to utilize their Bioanalyzer. Finally, the authors would like to thank the mothers who volunteered to donate freshly pumped breast milk samples for this study.

References

1 G. A. Lokossou, L. Kouakanou, A. Schumacher and A. C. Zenclussen, *Frontiers in immunology*, 2022, **13**, 849012.
2 V. Notarbartolo, M. Giuffrè, C. Montante, G. Corsello and M. Carta, *Pediatr. Gastroenterol. Hepatol. Nutr.*, 2022, **25**, 194–210.
3 K. E. Lyons, C. A. Ryan, E. M. Dempsey, R. P. Ross and C. Stanton, *Nutrients*, 2020, **12**, 1039.
4 M. B. Belfort, *Breastfeed. Med.*, 2017, **12**, 459–461.
5 O. Ballard and A. L. Morrow, *Pediatr. Clin. North Am.*, 2013, **60**, 49–74.
6 M. Tomita, M. Takase, H. Wakabayashi and W. Bellamy, *Adv. Exp. Med. Biol.*, 1994, **357**, 209–218.
7 M. Aparicio and et al., *PLOS ONE*, 2020, **15**, e0233554.
8 L. Tingö and et al., *Front. Immunol.*, 2021, **12**, 725323.
9 G. E. Leghi and et al., *Sci. Rep.*, 2021, **11**, 10224.
10 F. Hassiotou and et al., *Clin. Transl. Immunol.*, 2013, **2**, e3.
11 M. De Angelis and K.-W. Schramm, *Mol. Aspects Med.*, 2022, **87**, 100988.
12 A. Ziomkiewicz and et al., *Sci. Rep.*, 2021, **11**, 11576.
13 K. B. Shah and et al., *Nutrients*, 2021, **13**, 1091.
14 B. M. Henrick, X.-D. Yao, L. Nasser, A. Roozrogousheh and K. L. Rosenthal, *Front. Immunol.*, 2017, **8**, year.
15 F. Bravi and et al., *Am. J. Clin. Nutr.*, 2016, **104**, 646–662.
16 Q. Zhou and et al., *Int. J. Biol. Sci.*, 2011, **8**, 118–123.
17 A. Kupsco and et al., *Sci. Rep.*, 2021, **11**, 5840.
18 E. M. Munch and et al., *PLoS ONE*, 2013, **8**, e50564.
19 Y. Liao, X. Du, J. Li and B. Lönnerdal, *Mol. Nutr. Food Res.*, 2017, **61**, 1700082.
20 N. Kosaka, H. Izumi, K. Sekine and T. Ochiya, *Silence*, 2010, **1**, 7.
21 X. Li and et al., *Front. Nutr.*, 2022, **9**, 871346.
22 D. K. Jeppesen and et al., *J. Extracell. Vesicles*, 2014, **3**, 25011.
23 A. Bobrie, M. Colombo, G. Raposo and C. Théry, *Traffic*, 2011, **12**, 1659–1668.
24 K. Sidhom, P. O. Obi and A. Saleem, *Int. J. Mol. Sci.*, 2020, **21**, 6466.
25 K. Vaswani and et al., *J. Nutr. Metab.*, 2019, **2019**, 5764740.
26 K. Blans and et al., *J. Extracell. Vesicles*, 2017, **6**, 1294340.
27 S.-Y. Ying, D. C. Chang and S.-L. Lin, *Mol. Biotechnol.*, 2008, **38**, 257–268.
28 Y. Wang, Y. Hu, J. Guo and L. Wang, *Genet. Test. Mol. Biomark.*, 2019, **23**, 98–104.
29 M. Rubio and et al., *PloS One*, 2018, **13**, e0193527.
30 P. K. Berger, M. L. Ong, L. Bode and M. B. Belfort, *Nutrients*, 2023, **15**, 719.
31 M. I. Zonneveld and et al., *J. Extracell. Vesicles*, 2014, **3**, year.
32 A. Leiferman, J. Shu, B. Upadhyaya, J. Cui and J. Zempleni, *J. Pediatr. Gastroenterol. Nutr.*, 2019, **69**, 235–238.
33 S. Chutipongtanate and et al., *BioRxiv Prepr. Serv. Biol.*, 2023, 2023.06.01.543234.
34 M. Jayachandran, V. M. Miller, J. A. Heit and W. G. Owen, *J. Immunol. Methods*, 2012, **375**, 207–214.
35 Centers for Disease Control and Prevention, *Proper Storage and Preparation of Breast Milk*, 2023, `https://www.cdc.gov/breastfeeding/recommendations/handling_breastmilk.htm`.