MicroRNA Biomarkers Released by Platelet Activation

Jie Wang

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Committee:

Colin C. Pritchard

Jonathan F. Tait

Muneesh Tewari

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Contents

Abstract

MicroRNAs are a group of small noncoding RNA molecules that have been implicated in a variety of human diseases. Because of their remarkable stability and abundant quantity in blood, circulating microRNAs are promising as noninvasive biomarkers for diagnosis or prognosis. Platelets are likely to be a substantial contributor to circulating miRNAs. Upon activation, platelets shed membrane-bound microparticles, microvesicles and protein complexes into circulation. Recent studies suggest that circulating miRNAs are protected by encapsulation in vesicles or binding with Argonaute2 (Ago2) protein complex from blood RNase activity. However, the mechanisms of how platelets release miRNAs into circulation, and the role of platelet activation in miRNA release remains unclear. Therefore, I characterized four highly expressed platelet-released microRNAs: miR-16; miR-142-3p; miR-223 and let-7a, using size-exclusion chromatography. My results show that miRNAs are released by platelet activation. Further, my data suggests that platelet-released miRNAs are primarily associated with vesicle-free protein complexes rather than microparticles and microvesicles.

Key Words: biomarkers, miRNAs, platelet, platelet activation, microvesicles, protein-associated miRNA

List of Figures:

Lists of abbreviations

miRNA……..micro Ribonucleic Acid

RT……..……Reverse Transcription

qPCR……..….Quantitative Polymerase Chain Reaction

PPP……...Platelet-Poor Plasma

PRP…….……..Platelet-Rich Plasma

BSA…….Bovine Serum Albumin

Argonaute2……....Ago2

HDL……...…….. High-Density Lipoprotein

Acknowledgements

First, I would like to thank my advisor Dr. Pritchard for always being supportive. His enthusiasm for science and his dedication to helping others were truly impressive.

I would also like to thank my parents for their endless giving and not much taking back. Finally, I want to thank my husband Zhonghua and daughter Grace who encouraged, supported and cheered along the way.

Introduction:

MiRNAs are small non-coding RNA sequences, approximately 22 nucleotides long, that regulate gene expression via suppression of specific target mRNAs in animals (1). Expression of miRNA is dramatically changed in cancer and other disease states. Recent studies demonstrate that miRNAs are abundant in peripheral blood and surprisingly stable in plasma and serum (2-6). Circulating miRNAs have great potential to serve as noninvasive blood-based biomarkers. Human platelets have been shown to express more than 492 miRNAs (7, 8). I focused on studying four selected miRNAs: miR-16, miR-142-3p, miR-223 and let-7a, which are highly expressed in platelets (7). I found that the levels of these four miRNAs in platelet-rich plasma are significantly higher than in platelet-poor plasma. My results suggest that a substantial portion of circulating miRNAs is originated from platelets and platelet activation may alter the profile of circulating miRNAs.

Recent pioneering studies suggest that circulating miRNAs are protected from plasma RNase by vesicles or AGO2 ribonucleoprotein complexes (9). Although my research shows that platelet-released miRNAs levels are associated with the quantity of platelet microparticles which contain small vesicles and proteins, relatively little is known about the mechanism of how platelets release miRNAs into circulation. **My hypothesis** is that platelets release miRNA into plasma and serum upon activation, likely via vesicles that are shed during activation (10), and possibly via other mechanisms. The goal of my research is to test this hypothesis, and define the contribution of platelets to circulating miRNA. It is also the first step towards understanding the biological significance of platelet-released miRNA.

Methods:

Platelet Preparation - Human blood was drawn from healthy donors through 11-gauge needles into 10 ml syringes containing one-ninth volume of 109 mM sodium citrate with 1 µM Prostaglandin E1. Blood was immediately transferred to 10 ml tubes, and the platelet-rich plasma was obtained by centrifugation at 300 x g for 15 minutes at room temperature. Platelet-rich plasma was then passed through a leukocyte reduction filter to prevent leukocyte contamination. The platelets were pelleted by centrifugation at 1200 x g for 10 minutes at room temperature. The supernatant is platelet-poor plasma. Prostaglandin E1 was added to platelet washing buffer (113 mM NaCl, 4.3 mM K_2HPO_4, 4.2 mM Na_2HPO_4, 24.4 mM NaH_2PO_4, and 5.5 mM glucose, pH 6.5) to a final concentration of 1µM. The platelet pellet was resuspended in platelet washing buffer and washed twice. For platelet activation studies the platelets were suspended in platelet activation buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, 1 mg/ml bovine serum albumin).

Platelet Activation - Before activating platelets, $CaCl_2$ was added to the platelets to a final concentration of 2.5 mM. Three different agonists were selected to activate platelets: 1) 5 µM calcium ionophore, 2) 20 µg/ml collagen, or 3) 20 µg/ml collagen plus 1 unit/ml thrombin at room temperature for 15 minutes. The activated platelets were centrifuged at 12,000 x g for 8 minutes, and the supernatant was transferred to a new 1.5ml microfuge tube.

Measurement of microparticles released by platelets–The Multisizer^{TM} 4 coulter counter (Beckman) was used to measure particles from 0.40 µm to 12 µm in diameter. Ten µl of supernatant from platelet activation was added to 10 ml of isotone in a cuvette and run on the Multisizer^{TM}4 coulter counter. The Multisizer^{TM}4 coulter counter automatically counts numbers

and measure size distribution of particles by Multisizer 4 control software. The number of platelet-released microparticles can be measured by selecting the size range from 0.4 um to 1.0 um.

Size-Exclusion Chromatography– 0.5 ml of platelet-poor plasma or supernatant of activated platelets was injected into a column packed with Sephacryl S-500 resin (GE Healthcare) and equilibrated with 1X PBS, pH 7.4. The samples were eluted with 1X PBS at a flow rate of 30ml/hour. Fractions were collected and immediately added to 1 ml of Qiazol, then stored at -80°C before use.

RNA Isolation – 5µl of non-human exogenous (*C. elegans*) spike-in miRNA was added to 200 µl samples. Samples were incubated for 5 minutes at room temperature followed by addition of 0.2 volumes of chloroform. The mixture was centrifuged for 15 minutes at 12,000 x g at 4°C and the upper aqueous phase was transferred to a new tube and applied to a Qiagen miRNeasy kit, and was processed according to the modified manufacturer's protocol. RNA was eluted with 52 µl RNase-free water and stored at -80°C.

Reverse Transcription – 5 µl of the 52 µl extracted RNA was used as input into a multiplex reverse transcriptase reaction (Applied Biosystems, Inc.) with miRNA-specific stem-loop primers. The thermal cycles used to amplify the samples were 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes.

MicroRNA Quantitation Using Taqman qPCR–the diluted cDNA produced in the reverse transcriptase reaction was amplified in 384-well optical plate in duplicate 5µl reactions using a ViiA7 real-time PCR instrument (Applied Biosystems, Inc.). The thermal cycles were 95°C for

10 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 1 minute, and hold at 4°C. Raw data were analyzed using ViiA7 software version 1.1 which automatically sets the baseline and cycle threshold (Ct) for Ct determination. MiRNA absolute copies were measured by standard curves of synthetic oligos. Exogenous non-human miRNA (cel-miR-39) was used as a spike-in normalization control (11,12).

Results

I. miRNA profiles in platelet rich plasma and platelet poor plasma

Recent work from our lab and others indicate that blood cells, including red blood cells, platelets and white blood cells, are a major contributor to circulating miRNAs (12-14). Although we have observed miRNAs in platelets, the biology of miRNA in platelets and mechanisms of miRNA release are largely unexplored. To investigate to which level platelets contribute to the circulating miRNAs, I used individual Taqman qRT-PCR examine expression of 4 well-characterized miRNAs: let-7a, miR-16, miR-223 and miR-142-3p in platelet-rich plasma and platelet-poor plasma. I chose these 4 miRNAs because they are 1) abundant in platelets, 2) they represent a spectrum of vesicle-associated (let-7a, miR-142-3p), protein complex associated (miR-16) and mixed vesicle/protein complex-associated (miR-223) circulating miRNAs in plasma based on our earlier work, and 3) they have been proposed as circulating miRNA biomarkers in the literature. Platelet-rich plasma was obtained from freshly drawn blood containing sodium citrate/prostaglandin E1 anticoagulant with standard plasma protocols. The effect of centrifugation on platelet levels was studied by spinning (1200g, 10 min) platelet-rich plasma to obtain platelet-poor plasma. An average platelet count of 1 X 10^3/µl was observed in

the platelet-poor plasma from healthy donors (n = 3), compared to 281 X 10^3/µl in platelet-rich plasma, as measured by automated complete blood cell counts. In platelet-poor plasma, approximately 99.6% of residual platelets were removed from platelet-rich plasma. This result was also confirmed by coulter counter (Fig. 1). I determined absolute normalized quantification of miRNA copies using synthetic spiked-in *Caenorhabdities elegans* miRNAs as controls, and observed that 29% of miR-16, 6% of miR-142-3p, 6% of miR-223 and 5% of let-7a from platelet-rich plasma were recovered in platelet-poor plasma. In the other words, 71% of miR-16, 94% miR-142-3p, 94% of miR-223 and 95% of let-7a in platelet-rich plasma are likely to come from platelets (Fig. 2). Therefore, I concluded that there is a substantial platelet contribution to the circulating miRNAs.

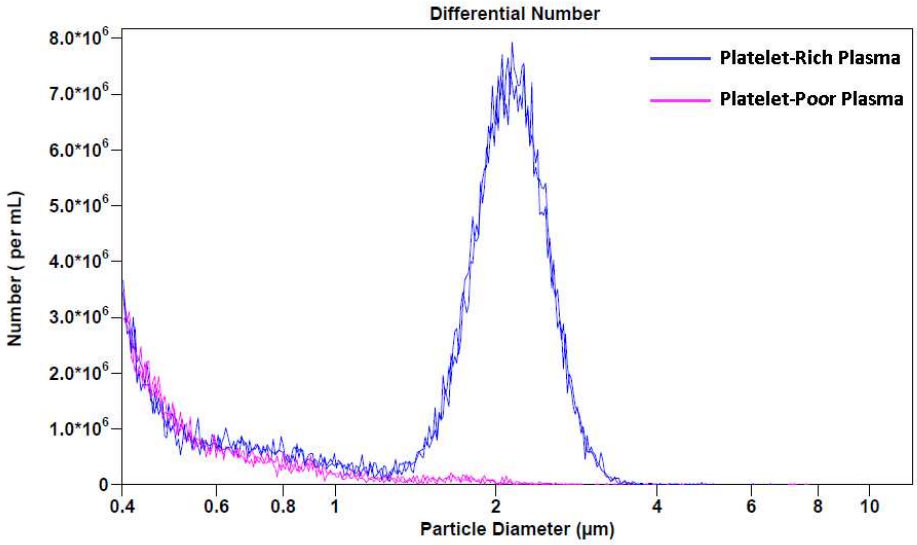

Figure 1. Profiles of platelet-rich plasma (blue line) and platelet-poor plasma (pink line) determined by Coulter counter.

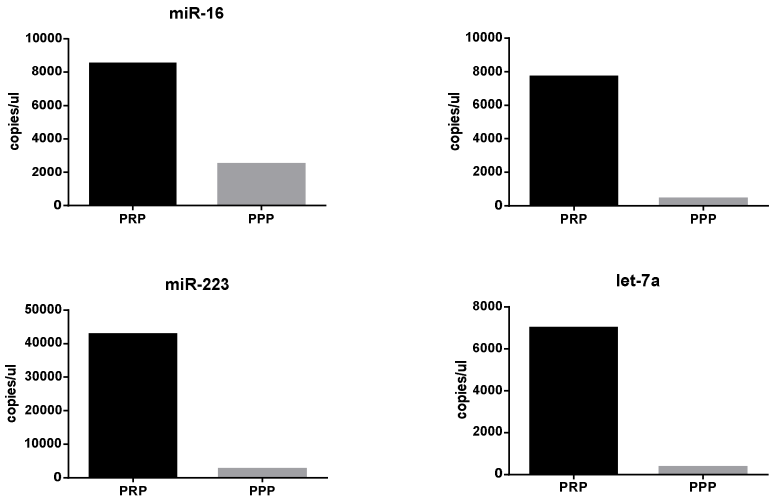

Figure 2. Platelets contribute substantially to circulating plasma miRNAs. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) from a healthy donor were assayed for miR-16, miR-142-3p, miR-223 and let-7a by using Taqman qRT-PCR. Bars represent absolute copies of miRNA recovered from 1μL of PRP (black) and PPP (grey).

II. miRNAs released by platelet activation

Since a significant portion of circulating miRNA copies come from platelets, platelet activation may change the profile of circulating miRNAs. To test whether miRNAs are released by platelet activation, I performed platelet activation under three conditions by using different platelet agonists: 1) calcium ionophore; 2) collagen; 3) collagen plus thrombin. Purified platelets without any agonist were used as control. Purified platelets were prepared by centrifugation of platelet-rich plasma, discarding platelet-poor plasma, washing twice using platelet washing buffer, then suspending in platelet activation buffer. The quality and purity of platelets were assessed by coulter counter (Fig. 3). The supernatant of activated purified platelets contains platelet-released microparticles. The number and size distribution of these microparticles of supernatant were also detected by coulter counter (Fig. 4). Among these three agonists, calcium ionophore stimulates the largest platelet activation and results in the largest quantity of microparticles shed by

platelets, followed by collagen plus thrombin. Collagen has the weakest effect on platelet activation. It is unlikely that microparticles were released in the supernatant as a result of platelet rupture under the force of centrifugation because only trace quantities of microparticles were observed in the supernatant of no-agonist controls (Fig. 4). Supernatants of activated platelets and control were then assayed for miR-16, miR-142-3p, miR-223 and let-7a by Taqman qRT-PCR. I observed that the supernatant of platelets activated by calcium ionophore had the largest amount of miRNAs, and, conversely, the supernatant of platelets activated by collagen contains the least of miRNAs. MiRNA copies in the supernatant of platelets stimulated by thrombin plus collagen were between the other two stimulating conditions for all four miRNAs (Fig. 5). I subsequently analyzed the relationship between miRNAs and microparticles released by platelets. I found that although the platelet-released miRNAs level was positively associated with the number of microparticles shed by platelets on activation, the relationship between them was not linear (Fig. 6). It suggested that platelets may release miRNAs through smaller particles such as exosomes or small protein complexes. These results demonstrated that 1) Platelets release miRNAs upon activation; 2) The copies of miRNAs released from platelets depend on the nature of the agonist(s) 3) Microparticles are probably not the only carriers of miRNAs released by platelets.

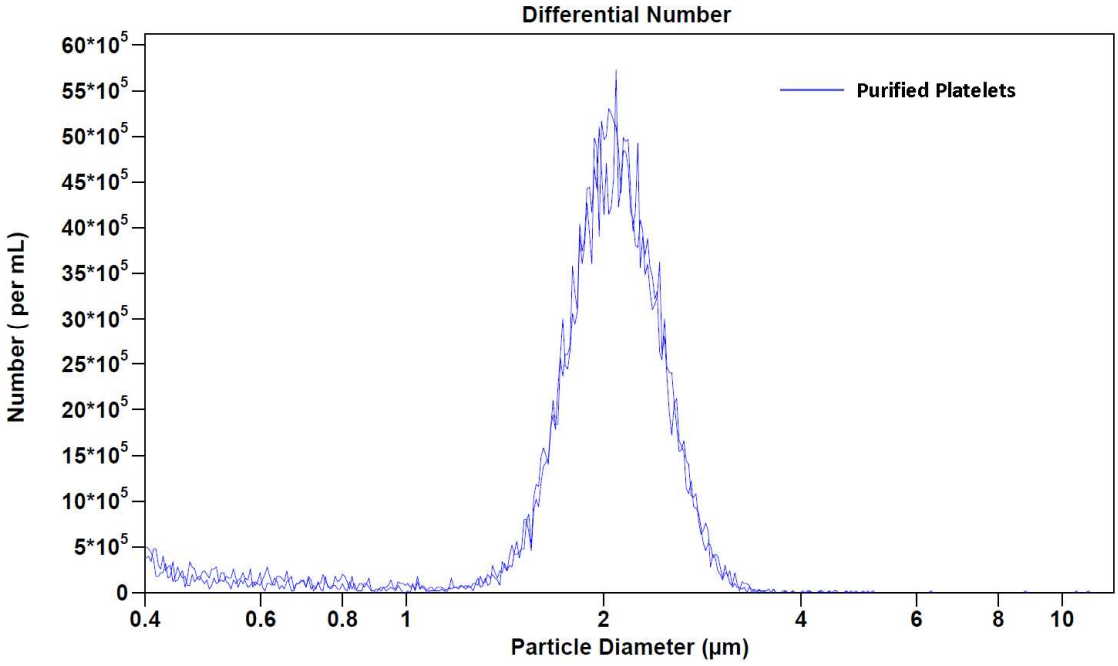

Figure 3. Purified platelets assessed by Coulter counter. The particles of purified platelets (with very limited activation) are 1.5 - 3 µm in diameter.

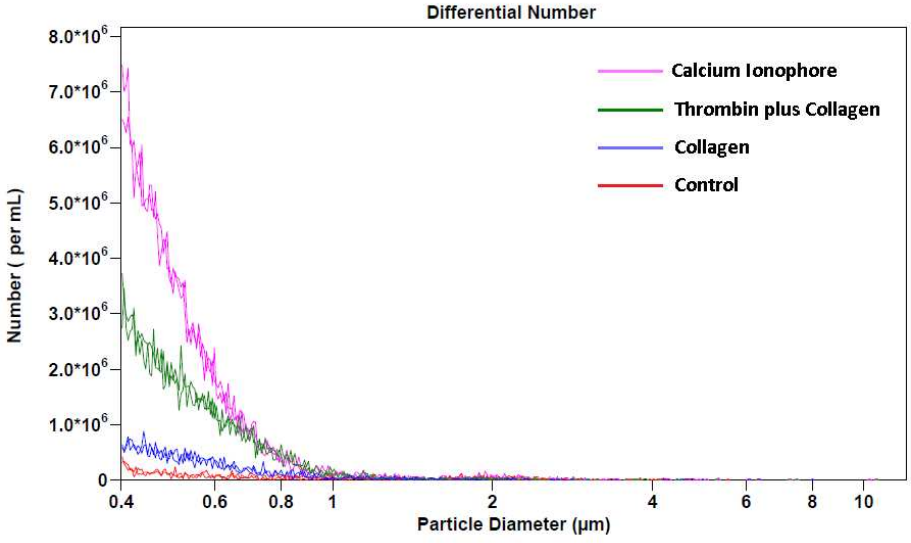

Figure 4. Microparticles released by platelets measured by coulter counter under different activation conditions: Calcium ionophore (pink line); thrombin plus collagen (green line); collagen (blue line); control (red line).

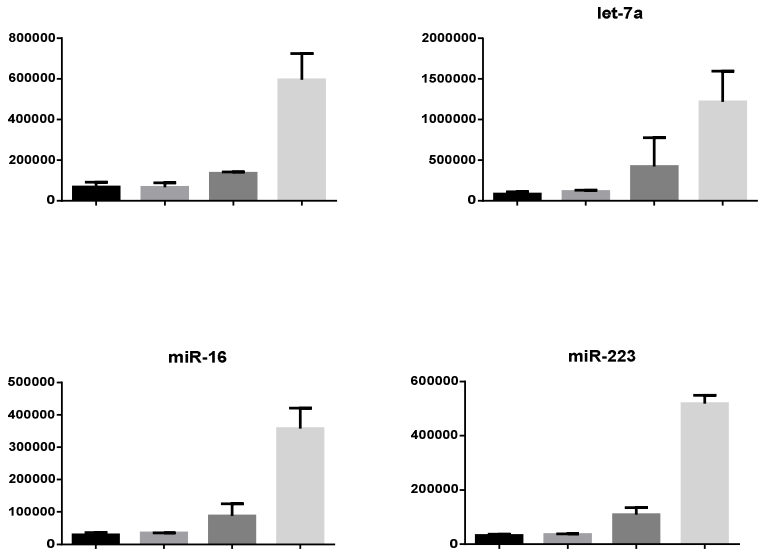

Figure 5. miRNAs are released by platelet activation. The supernatants from different activation conditions were assayed for miR-16 (A), miR-142-3p (B), miR-223 (C) and let-7a (D) by Taqman qRT-PCR. Bars represent absolute copies of miRNA in 1µl of supernatant.

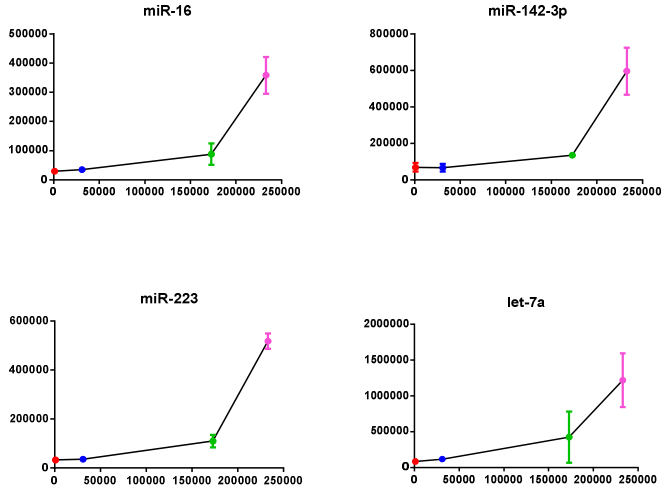

Figure 6. The relationship between microparticles and miRNAs released by platelets. Points represent absolute copies of miRNA under different activation conditions: Calcium ionophore (pink dot); thrombin plus collagen (green dot); collagen (blue dot); control (red dot).

III. Physical state of miRNAs released by platelets

A comprehensive study that investigated the populations of circulating miRNAs was published by Arroyo et al. in 2011 (9). The study reported that although miRNAs are remarkable stable in the RNase-rich environment of blood, circulating miRNAs are not intrinsically resistant to endogenous RNase activity. They are protected by mechanisms which likely include encapsulation in membrane-bound vesicles, and stabilization by Argonaute2 complexes. However, no studies have yet addressed the mechanisms of platelet-released miRNAs. To determine whether miRNAs released by platelets are associated with microvesicles or vesicle-free protein complexes, I used size-exclusion chromatography to characterize platelet-released miRNAs.

I chose to activate platelets using thrombin plus collagen because it had relatively strong activation according to my platelets activation experiment, and is more physiologically relevant than calcium ionophore as a platelet agonist. Residual platelets were pelleted as described in the methods and the contents released by platelets remained in supernatant after centrifugation. Before I fractionated the supernatant, I set up microvesicle and protein standards by analyzing the elution of synthetic lipid vesicles with normal average diameter of 100 nm and BSA, respectively. Based on vesicle and protein standards, I determined that the particles 120 nm and larger are eluted near fraction 8; and particles 1 nm or smaller are eluted near fraction 20 (Fig. 7). I then injected never-frozen supernatant containing platelet-released miRNAs under gentle conditions into Sephacryl S-500 column and collected fractions. The copies of miR-16, miR-142-3p, miR-223 and let-7a were quantified in each fraction by individual Taqman qRT-PCR assays and normalized to spiked-in synthetic *C. elegans* cel-miR-39 to avoid fraction-to-fraction technical differences during miRNA extraction. Absorbance at 280 nm was used to determine protein abundance of each fraction.

The results indicated that two populations of platelets released miRNAs separated based on the size of their associated particles. For all four miRNAs: miR-16, miR-142-3p, miR-223 and let-7a, a minority of platelet-released miRNAs eluted earlier in fractions 8 to 10, which are expected to contain vesicles,

whereas the majority of miRNAs co-fractionated with proteins in fractions 15 to 21. Negative controls of the supernatant of platelets without activation showed even distribution close to the baseline (Fig. 9).

In addition, I analyzed platelet-poor plasma from the same donors and observed that the majority of miR-142-3p and let-7a eluted with vesicles (fractions 8 to 10). In contrast, the majority of miR-16 eluted with proteins (fractions 15 to 21), only low levels of miR-16 were detected in the early fractions. MiR-223 in platelet-poor plasma eluted in both peaks (Fig. 8). Therefore, miRNAs in the platelet-poor plasma can be divided to three classes: 1) early eluting: miR-142-3p and let-7a, 2) late eluting: miR-16, 3) distributed in both peaks: miR-223. My findings were similar to what was reported before in the Arroyo et al. 2011 study (9), and served as proof-of-principal that my size exclusion chromatography methods were comparable to that study.

Comparing the distributions of miRNAs in platelet-poor plasma with the distributions of platelet-released miRNAs in the supernatant, only miR-16 had a similar distribution. All four of these miRNAs released by platelets predominately eluted in late fractions 15 to 21 (Fig. 9). Thus, by using size-exclusion chromatography with appropriate controls, my results suggest that miRNAs released by platelets upon activation are more likely to be associated with proteins rather than microvesicles. A correlate to this finding is that the vesicle-associated let-7a and miR-142-3p miRNA I observed in platelet-poor plasma is likely not derived from platelets, but from another vesicle source.

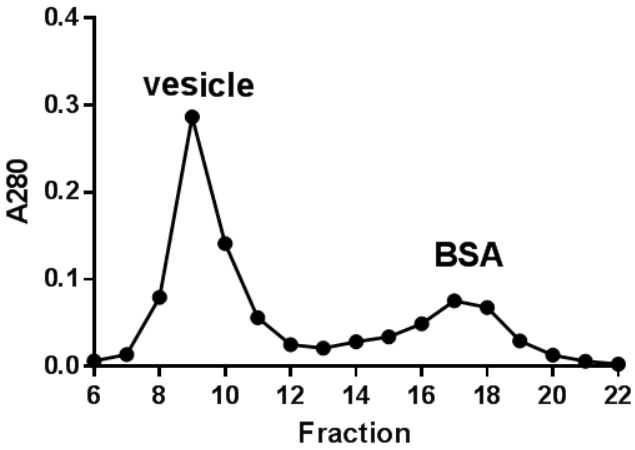

Figure 7. Elution profiles of vesicles with average 100 nm diameter and BSA standards were determined by absorbance at 280 nm (A280).

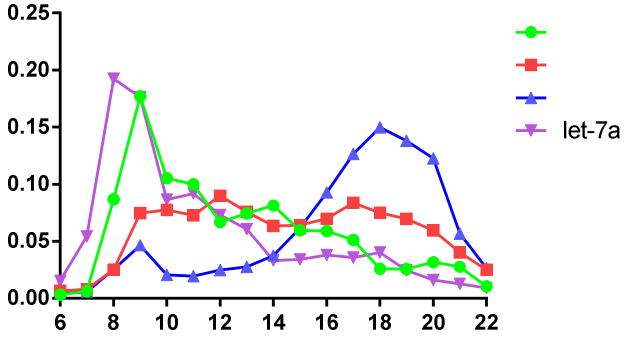

Figure 8. The distribution of miRNAs in platelet-poor plasma determined by size-exclusion chromatography. Platelet-poor plasma was fractionated on a Sephacryl S-500 column. Fractions were assayed for miR-16 (blue), miR-142-3p (green), miR-223 (red) and let-7a (purple).

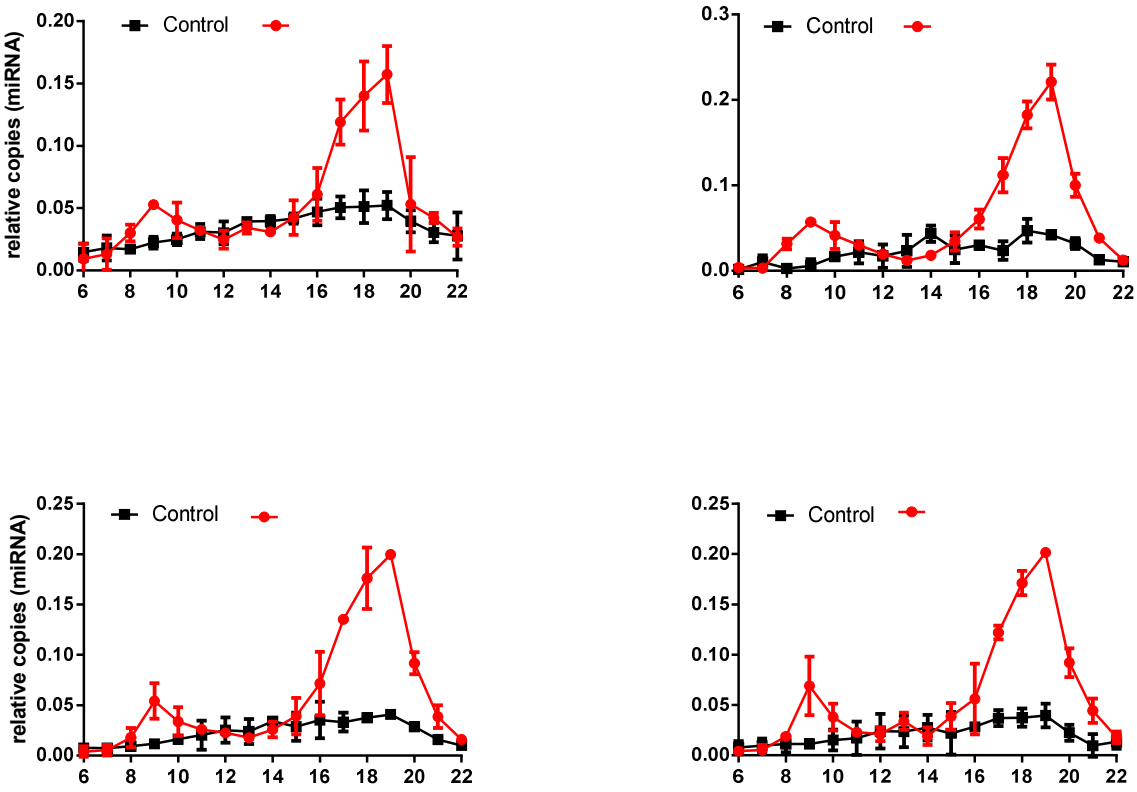

Figure 9. The distribution of miRNAs in supernatant of platelets activated by thrombin plus collagen determined by size-exclusion chromatography. The supernatants were fractionated on a Sephacryl S-500 column. Fractions were assayed for miR-16 (A), miR-142-3p (B), miR-223 (C) and let-7a (D).

Discussion

The importance of circulating microRNA has become increasingly appreciated as a potential biomarker for cancer and other diseases (2-7), but the biology of platelet microRNA is largely unexplored. In my master's thesis study I have focused on how platelet activation affects the quantities of microRNAs and the biological characteristics of platelets-released miRNAs. I

assayed four platelet microRNAs levels using three different types of platelet agonists and found that platelet activation changes the levels of these miRNAs. The different platelet agonists stimulated platelet activation to different levels: among these three platelet agonists, calcium ionophore had the strongest effect on platelet activation, and collagen was the weakest agonist. Collagen plus thrombin was chosen as the platelet agonist in my further studies because it had the ability to trigger greater platelet activation than collagen or thrombin alone, and was a physiologically relevant agonist. Both collagen and thrombin are endogenous components of blood, simulating conditions *in vivo*. However, it is important to note that my *in vitro* platelet-activation protocols may not fully recapitulate mechanisms occurring in the body. Future studies of plasma from patients with *in vivo* platelet activation, such as patients with disseminated intravascular coagulation, essential thrombocythemia, or ongoing thrombosis, may help us better understand the characteristics and function of platelet-released miRNAs and how they affect the profile of circulating miRNAs.

Platelets shed microparticles (0.1 – 1 μm in diameter), microvesicles or exosomes (40 – 100 nm in diameter) and vesicle-free proteins into circulation upon activation (16, 17). Recent studies reported that activated platelets are likely to release miRNAs via microparticles (18, 19). However, I found that the relationship between the number of microparticles and miRNAs level is not linear (Fig. 6), implying that platelets may also release miRNAs through other mechanisms. By analyzing the data of size-exclusion chromatography, I demonstrated vesicle-free protein complexes are likely the predominant carriers for miRNAs released by platelets rather than microparticles and microvsicles (Fig. 9).

Future work is needed to further characterize the mechanism of miRNA release from platelets. Although my results support that platelets release the majority of miRNA in a protein-bound form, I did not test what proteins they are bound to. Circulating miRNAs may be delivered to target cells via Ago2 or HDL (9, 20). Arroyo et al. reported that circulating Ago2 complexes are a very important mechanism responsible for the stability of plasma miRNAs (9). Vickers et al suggested that HDL can transport a subset circulating miRNAs and deliver these miRNAs to recipient cells via specific receptors-mediated uptake with functional effects on target cells (20). Future work could use immunoprecipitation of platelet-released miRNAs to characterize the presumed protein complexes, using antibodies against candidate proteins such as Ago2 or HDL. Platelets contain three granule types: α-granules, dense granules, and lysosomes (21). It is likely that miRNAs are contained in one or more of these granule types, and that platelet activation stimulates release of miRNAs via one or more of these granules. One could use immunogold electron microscopy to characterize the relationship of platelet miRNAs to specific intracellular granule compartments.

I chose to focus on only four miRNAs from the approximately 492 miRNAs expressed in platelets: miR-16, miR-142-3p, miR-223 and let-7a. These miRNAs were selected because they are highly expressed in human platelets, which can improve the accuracy and sensitivity of miRNA detection. In addition, these 4 miRNAs represent three different classes of miRNAs in platelet-poor plasma defined by their distributions of elution by size exclusion chromatography (Fig. 7 and 8). This facilitated my analysis of the size distribution characteristics of miRNAs in platelet-poor plasma compared to purified platelet-released miRNAs. My results suggest that platelets are more likely to release miRNAs via a vesicle-free pathway and that proteins are the predominant carriers of miRNAs released by platelets.

In my study, the comparison of miRNAs profiles of platelet-poor plasma and platelet-rich plasma indicates that platelets release substantial quantities of miRNA upon activation. MiRNAs released by platelet activation are likely to contribute to a meaningful extent to the pool of circulating miRNAs, with implications for circulating biomarkers. For example, platelet activation *ex vivo* during sample handling, or *in vivo* (for example in a patient with ongoing thrombosis), is likely to impact levels of proposed miRNA biomarkers that are expressed highly in platelets. My study also demonstrates that platelet-released miRNAs are primarily associated with protein complexes rather than vesicles. My hope is that my thesis work will provide useful insights on key pre-analytical parameters of miRNA biomarkers in clinical serum and plasma specimens that are now being moved into clinical practice.

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