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MicroRNA Biomarkers and Platelet Reactivity – the "Clot" Thickens

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Short Title: Non-coding RNAs in platelets

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Abstract

Over the last few years, several groups have evaluated the potential of microRNAs (miRNAs) as biomarkers for cardiometabolic disease. In this review, we discuss the emerging literature on the role of miRNAs and other small noncoding RNAs in platelets and in the circulation, and the potential use of miRNAs as biomarkers for platelet activation. Platelets are a major source of miRNAs, YRNAs, and circular RNAs. By harnessing multiomics approaches, we may gain valuable insights into their potential function. Because not all miRNAs are detectable in the circulation, we also created a gene ontology annotation for circulating miRNAs using the gene ontology term extracellular space as part of blood plasma. Finally, we share key insights for measuring circulating miRNAs. We propose ways to standardize miRNA measurements, in particular by using platelet-poor plasma to avoid confounding caused by residual platelets in plasma or by adding RNase inhibitors to serum to reduce degradation. This should enhance comparability of miRNA measurements across different cohorts. We provide recommendations for future miRNA biomarker studies, emphasizing the need for accurate interpretation within a biological and methodological context.

Non-Standard Abbreviations and Acronyms

circRNAs	-	circular RNAs
Ct	-	cycle threshold
GO	-	gene ontology
lncRNAs	-	long non-coding RNAs
miRNA	-	microRNA
MP	-	microparticle
mRNA	-	messenger RNA
NGS	-	next-generation sequencing
PCPT	-	phosphatidylcholine transfer protein
PPP	-	platelet-poor plasma
PRP	-	platelet-rich plasma
qPCR	-	quantitative polymerase chain reaction
rRNA	-	ribosomal RNA

Introduction

Platelets play a key role in hemostasis, initiating and propagating thrombosis.[1] Dysregulation and inappropriate activation of platelets underpin a wide range of important diseases, including stroke and myocardial infarction, whereas impairment of platelet function results in bleeding disorders. Platelets are anucleate and were previously considered to be simple cytoplasmic fragments of megakaryocytes. We now know that platelets house an array of molecules, including proteins, RNA, and the subcellular machinery of de novo protein synthesis. Platelets can synthesize new proteins, such as the integrin alpha3 (glycoprotein-IIIa) protein and the fibrinogen receptor.[2,3] They contain messenger RNA (mRNA), which codes for around a third of human genes.[4,5] More recently, the discoveries that platelets are an abundant source of microRNAs (miRNA),[6] and that miRNA expression profiles within platelets correlate with platelet reactivity,[7] raised the exciting possibility of novel therapeutic targets and disease biomarkers.

Initial studies by Mitchell et al[8] revealed that not all miRNAs in the circulation are intracellular. This pool of extracellular circulating miRNA consists of miRNA within microvesicles, exosomes, or bound to proteins. The finding that miRNAs can also be measured in cell-free serum and plasma was surprising, given their high RNase activity. Compared with mRNAs, miRNAs remain relatively stable in the circulation through several protective mechanisms: association with RNA-binding proteins and lipoproteins, or housing in shed microvesicles, such as exosomes or microparticles (MPs).[9–12] Platelets release MPs, particularly during activation. These vesicles contain a host of proteins, inflammatory mediators, and noncoding RNAs.[13–15] Microvesicles might be taken up by other cells, introducing another layer of complexity in terms of intercellular signaling by potentially allowing direct regulation of the recipient cell's mRNA profile and gene expression.[16,17] Given this emerging role for circulating miRNAs, plasma and serum miRNA profiles may find use in clinical practice as novel markers of disease, organ function, and prognosis.[18]

In this review, we bring together nearly a decade's experience with circulating noncoding RNAs, with particular focus on platelet-derived and associated miRNAs, YRNAs, long noncoding RNAs (lncRNAs), and circular RNAs (circRNAs). We conducted a literature search in PubMed as queried on November 23, 2016, using the search terms "platelet* AND (miRNA [MeSH Terms] OR micro RNA [MeSH Terms] OR micro-RNA OR microRNA)". Four hundred and thirty-four results were retrieved, of which 75 were relevant primary research papers and 39 were relevant literature reviews. From the literature to date, we collate the known platelet repertoire of noncoding RNAs and contemplate their physiological significance. We also examine their disease associations and potential clinical applications. Finally, we discuss the practical challenges in measuring and comparing miRNA levels, highlighting obstacles that will need to be overcome for this relatively new field to come of age.

Noncoding RNAs in Platelets and the Circulation

Different noncoding RNAs have been described (**Figure 1**).

miRNAs

The human genome codes for small (≈22 nucleotide) regulatory RNA molecules, termed miRNAs or miRs. Since miRNAs were first discovered in 1993, over 2500 have been defined.[19,20] They are highly evolutionarily conserved and important in the posttranscriptional regulation of protein expression—about one third to half of the human genes are estimated to be regulated by miRNAs.[21–23] MiRNAs act by imperfect Watson–Crick base pairing to the 3′untranslated region (UTR) of target mRNA to affect translation or induce degradation.[24,25] MiRNAs can be expressed as stand-alone scripts or alongside the parent gene mRNA. In either case, the primary miRNA transcript is processed by the nuclear microprocessor complex consisting of the endoribonuclease Drosha and the double-stranded RNA-binding protein Pasha/DGCR8.[26,27] The resultant hairpin pre-miRNA is then transferred to the cytoplasm by the nuclear membrane protein exportin-5 and further processed by the endoribonuclease Dicer.[28] The resulting mature miRNA duplex associates with an Argonaute (Ago) protein, which unwinds the double strand and incorporates one of the 2 complementary miRNA strands, forming the RNA-induced silencing complex.[29] The RNA-induced silencing complex stabilizes the miRNA strand and guides it to its target mRNA. Because platelets do not have a nucleus and thus cannot transcribe miRNAs, it is currently thought that they inherit their miRNA content during the shedding process from megakaryocytes.[30] Thus, the platelet miRNAome in the circulation may provide insights into megakaryocyte function in the bone marrow.

There are many more miRNAs encoded in the human genome than there are detectable in the circulation.[31] For example, we detected >200 miRNAs using next-generation sequencing (NGS) in plasma samples.[32] Detectable miRNAs greatly vary in abundance. Not all miRNAs that are present in the circulation can be consistently quantified with cycle threshold (Ct) values of <32 by quantitative polymerase chain reaction (qPCR), even after preamplification. Another striking observation is the high correlation among the abundant circulating miRNAs.[33] This may be because of their common platelet origin: in response to antiplatelet drugs, circulating levels of many miRNAs are reduced in platelet-poor plasma (PPP).[34] Research into miRNAs is hampered by the current lack of functional miRNA data in bioinformatics resources. Access to cataloged experimental data is crucial to enable simple questions to be asked, such as, Which miRNAs are present in the circulation?, as well as the ability to perform sophisticated pathway and network analyses.[35] This lack of freely accessible data is being addressed by the addition of gene ontology (GO) annotations for cardiovascular-related miRNAs.[36,37] To answer the question of circulatory miRNAs, we have created GO annotations for those miRNAs identified as being present in human plasma from our previous study.[32] In the parlance of GO, the annotations were created using the GO term extracellular space (GO:0005615) with the addition of contextual information describing the extracellular space as part of blood plasma (using the Uber Ontology term Uberon:0001969). A large number of miRNAs were identified in PPP and platelet-rich plasma (PRP).[32] For those with marked enrichment in PRP, as well as significantly increased levels in a platelet spike-in experiment, we were additionally able to include in the annotation that the miRNAs present in the extracellular space are likely to have originated from platelets (using the cell ontology term CL:0000233). In total, we have identified 230 human miRNAs as present in the circulation, and the annotations providing this information are available in the GO database.[38] Inclusion of functional annotations such as these into public databases will enable researchers to find and analyze miRNA data more easily, allowing for faster progression of their research.

YRNAs

In our most recent study, we have also identified YRNAs as another species of circulating noncoding RNAs that is platelet-derived.[32] YRNAs are small noncoding RNAs, ≈100 nucleotides in length, that originate from 4 human genes (*RNY1*, *RNY3*, *RNY4*, and *RNY5*and ≈900 pseudogenes).[39,40] Using NGS in PPP and PRP, we observed a dominant peak at sequences of 32 to 33 nucleotides long, which was mapped to YRNA genes (**Figure 2A**).[32]The full-length YRNAs have cytoplasmic or nuclear localization and are involved in the initiation of DNA replication, as well as in ribosomal RNA quality control.[41,42] They are components of Ro ribonucleoproteins, which constitute important autoantigens in autoimmune diseases, such as systemic lupus erythematosus and Sjögren's syndrome.[43]YRNA fragments are present in the circulation, with 5′ fragments being much more abundant than 3′ fragments for the majority of RNY genes.[32,44] YRNA fragments are generated during cell stress or apoptosis. Given that YRNA fragments are abundant in platelets, and strongly correlate to platelet-derived proteins in the circulation (**Figure 2B**),[32]we speculate that the YRNA fragments are formed during the budding of platelets from megakaryocytes. In contrast to the processing of mature miRNAs from precursor miRNAs, YRNA fragments are generated by a Dicer-independent mechanism.[45] Unlike miRNAs, we also failed to detect YRNAs in Ago2 complexes of MEG-01 cells, a human megakaryoblastic cell line.32 The predominant platelet origin of YRNA fragments in human plasma is further supported by the results from platelet spike-in experiments.[32]

Ribosomal RNA

Ribosomal RNA is a key regulator of intracellular protein synthesis and is also detected in the circulation. Tissue damage and unregulated cell death result in significant quantities of ribosomal RNA shifting into the extracellular compartment.[46,47] As reviewed recently,[48]extracellular ribosomal RNA may have several important pathophysiological roles in atherosclerotic plaques, including promotion of vascular hyperpermeability, initiation of coagulation and thrombus formation, recruitment of leukocytes, promotion of a proinflammatory phenotype of monocytes/macrophages, and promotion of cardiomyocyte death.

Long Noncoding RNAs

Recently, attention has shifted from small noncoding to lncRNAs. Unlike miRNAs and YRNA fragments, which are mainly found in the cytosol, lncRNAs are predominantly retained in the nucleus.[49] This nuclear localization has important implications for their release mechanism. Although miRNAs and YRNA fragments are secreted as part of any budding process of microvesicles with cytoplasmic components, even in the absence of injury, the secretion of lncRNAs may be dependent on cellular damage that is sufficient to release nuclear material. On release, the lncRNAs require protection from RNase degradation. In general, lncRNAs may be more susceptible to RNase activity than small noncoding RNAs because the longer sequence is more vulnerable to cleavage and not as easily complexed and protected by RNA-binding proteins. Although lncRNAs are readily detected in whole blood, levels in plasma or serum tend to be low, at least in healthy individuals without tissue injury. The release of mitochondria-associated lncRNAs has been described in patients with myocardial infarction.[50] It is possible that mitochondrial lncRNAs are protected from RNase

degradation. Further, because cardiomyocytes are particularly rich in mitochondria, these lncRNAs may indeed be cardiac-derived. However, unlike troponins, lncRNAs are not cardiac muscle-specific, so contributions from other cell types cannot be excluded.[51] Because platelets are anuclear and have a low mitochondrial content, most lncRNAs may not be detectable in platelets, with the notable exception of circRNAs.

Circular RNAs

Recent evidence suggests that platelets are relatively abundant in circRNAs,[52] an emerging class of noncoding RNAs that had been described as early as 1976.[53] Development of advanced NGS techniques led to their recent rediscovery,[54] showing ubiquitous expression in both humans and mice.[55] Several pathways exist to generate circRNAs.[56] Most commonly, they are derived from exons of protein-coding genes through back-splicing, whereas some arise from intronic or intergenic regions via alternative splicing. circRNAs are generally localized in the cytoplasm, but how they are exported from the nucleus remains unknown. RNA-binding proteins like Quaking and Muscleblind are thought to affect the abundance of circRNAs in human cells through binding of the specific RNA-binding proteins to flanking intronic sequence motifs. Interaction between the RNA-binding proteins on each end approximates both ends of the exon and facilitates the formation of a circle. Quaking regulates human circRNA biogenesis, and knockdown of Quaking decreases the expression of abundant circRNAs.[57] CircRNAs were found to be enriched in platelets, where they are generated by exon back-splicing.[52] Using RNase R to selectively remove linear transcripts, several distinct circRNAs were identified in platelets. Although the majority of circRNAs are not cell type-specific, human platelets and erythrocytes were found to be highly enriched for circRNAs compared with nucleated cell types. Several circRNAs were expressed at much higher levels than the linear form of the RNA in platelets and erythrocytes. Interestingly, the relative proportion of circRNAs in cultured megakaryocytes is much lower compared with mature platelets. Combined with the finding that circRNA decays at a significantly slower rate than linear RNA, and platelets lose >90% of their progenitor mRNA, the enrichment of circRNA in platelets might be the result of linear RNA degradation.[52] This process has a profound effect on the composition of RNA because anucleate platelets cannot transcribe new RNA. Recent reports demonstrated that circRNAs can serve as miRNA sponges, which are believed to negatively regulate miRNAs by sequestering miRNA molecules.[58] This raises questions of how circRNAs interact with miRNAs and mRNAs in platelets and how circRNA transcripts are formed in platelets. With their enrichment being a signature of mRNA decay, platelet circRNAs may reflect platelet function or ageing.

miRNA Repertoire of Platelets

Landry et al[6] first reported a large number of platelet miRNAs by using locked nucleic acid–based microarray profiling. NGS has enabled the discovery of novel miRNA species. Plé et al[59] identified nearly 500 species, with the let-7 family of miRNAs comprising nearly 50% of the total. Since then, Bray et al[4] have identified many more, bringing the total of recognized platelet miRNAs to ≈750. The variation in reported platelet miRNA expression is not solely because of interindividual differences, such as sex and age.[60] Sample preparation, storage, normalization procedures, and measurement platforms play an important role. Studies aimed at defining the platelet miRNA profile use a variety of techniques, including microarrays, qPCR-based methods, and NGS. For example, Wang et al[61] demonstrated platform-associated amplification differences in miR-107, which showed much higher amplification with locked nucleic acid–based assays compared with TaqMan assays. In clinical laboratory conditions, Yu et al[62] showed upregulation of several apoptosis-related platelet miRNAs after prolonged blood bank storage of platelets. As platelet preparation from whole blood can lead to contamination with leukocytes and erythrocytes, themselves abundant sources of miRNAs, controlling cellular contamination of platelet preparations is essential.[61] Variation in preparation and storage conditions can drastically affect platelet miRNA expression, which may explain why interstudy miRNA expression ranks are highly variable. Based on the current literature, a combined interstudy miRNA expression rank list is presented in **Figure 3**.

Association of Platelet miRNAs With Disease

Antiplatelet drugs reduce the risk of cardiovascular events, but patients on antiplatelet therapy still suffer thrombotic events. Ever more potent antiplatelet strategies carry the risk of bleeding complications. Ideally, a so-called "sweet spot" could be defined for each patient, which offers maximum platelet inhibition with minimal bleeding risk.[63] For optimal platelet inhibition, platelet turnover is important: with a life span of 7 to 9 days, 10% to 15% of the platelet pool must be replenished every day, equivalent to 10^{10} to 10^{11} platelets per day.[64] The platelet turnover rate may be important for the efficiency of drugs like aspirin with a short half-life. Aspirin irreversibly binds cyclooxygenase, so is effective for the full life span of the platelet. However, increased platelet turnover can lead to budding of new platelets without inactive cyclooxygenase between doses. Furthermore, newly formed platelets are larger, more reticulated, more reactive, and more prone to participate in thrombus formation compared with older platelets. They also contain a higher amount of RNA and are more able to produce proteins, in particular, alpha granule proteins. Counting the subset of reticulated platelets may be a parameter for platelet turnover. However, the intrinsic properties of platelets could be even more important for platelet reactivity than their turnover rate.[65] In light of the enrichment of miRNAs and circRNAs, and the described effects of mRNA degradation during the lifetime of platelets, the noncoding RNA content of platelets may better reflect their intrinsic properties.

Platelet miRNA expression profiles in healthy volunteers remain stable over the life-span of a platelet.[66] This expression profile is significantly altered in various disease states, including myocardial infarction,[67,68] diabetes mellitus,[69] and cancer.[70] There is, therefore, the opportunity to find new biomarkers or therapeutic targets and increase our understanding of the pathophysiological mechanisms of these diseases. Anucleate platelets lack transcriptional control, so differences in miRNA signatures during platelet-activating disease states have been ascribed to several mechanisms: shedding of MPs,[71] synthesis of mature miRNA from precursor miRNAs,[69] and posttranscriptional modifications.[59] We undertook the first systematic analysis of circulating miRNAs in a large population-based study (the Bruneck study) and revealed a loss of miRNAs in type 2 diabetes mellitus.[72] Subsequent studies indicated that this loss may be attributed to activation of calpain, which can affect platelet levels of Dicer.[73] Because this endoribonuclease is key in the processing of precursor miRNAs to mature miRNAs, its loss resulted in decreased levels of several platelet-enriched miRNAs.[69,74] These findings are in line with studies showing that circulating miRNA changes in cardiovascular risk can be attributed to platelets.[32,34] Additionally, we demonstrated that a combination of miRNAs may predict future cardiovascular events.[75] This has been confirmed by other investigators.[76] In continuation of our work on circulating miRNAs in diabetes mellitus,[72] we have identified 2 angiogenic miRNAs as biomarkers for diabetic retinopathy.[77]

Findings by other investigators and our group suggest that platelet miRNAs might not only reflect but also affect platelet function.

miR-96 and Vesicle-Associated Membrane Protein 8

In 2010, Kondkar et al[78] reported a role for miR-96 in platelet reactivity. They showed that miR-96 may act through regulation of vesicle-associated membrane protein 8

(VAMP8), an important SNARE protein involved in platelet degranulation. Healthy volunteers' platelets were categorized as either hyper- or hyporeactive. mRNA profiling revealed differential expression of a range of scripts, including VAMP8 mRNA (4.8-fold decrease in the hyporeactive versus hyper-reactive platelets). This translated to a 2.5-fold lower VAMP8 protein expression in the hyporeactive platelets. miR-96 levels were upregulated in the hyporeactive platelets (2.6-fold higher). Transfection of VAMP8-expressing human colorectal cancer cells with miR-96 was associated with dose-dependent reductions in VAMP8 mRNA and protein levels; a similar finding was reported in cells of a megakaryocyte lineage. miR-96 was predicted to bind the 3′UTR of VAMP8. In contrast with this study, Shi et al[79] reported no association between platelet response to clopidogrel and miR-96 expression, and miR-96 was not among the most highly expressed miRNAs found in human platelets. Although this does not discredit a role for miR-96 in regulating platelet reactivity, these findings need to be validated and explained in a larger study.

MiR-376c, miR-599, and Platelet Phosphatidylcholine Transfer Protein

There is a racial disparity in survival in coronary heart disease between patients with Caucasian and Afro-Caribbean background, even after adjusting for all known factors. Edelstein et al[80] investigated whether racial differences in platelet function underpin an increased thrombotic risk. One hundred and fifty-four healthy black and white volunteers were recruited, and platelet reactivity to thrombin was measured. Higher aggregation and platelet calcium responses to PAR4-AP (thrombin receptor 4 activating peptide) in Afro-Caribbean individuals were found. PCTP (phosphatidylcholine transfer protein)–encoding mRNA was found to be upregulated in Afro-Caribbeans. After profiling platelet miRNA signatures, 178 miRNAs, including miR-376c, were predicted to target PCTP mRNA. miR-376c levels inversely correlated with PCTP mRNA levels, PCTP protein levels, and PAR4 reactivity. miR-376c is part of a cluster of miRNAs differentially expressed by ethnic origin, which may underpin differences in platelet function. Interestingly, a statistical cluster of 24 miRNAs all mapped to chromosome 14q32.2. Among other differentially expressed miRNAs were some of the most abundant platelet miRNAs: miR-223, miR-21, miR-23b, miR-107, and let-7c. Plé et al[81] also found evidence of miRNA regulation of PCTP. Their analysis predicted miR-599 targeting of the 3′UTR of PCTP mRNA and showed downregulation of a reporter gene containing the PCTP 3′UTR in HEK293 (human embryonic kidney) cells transfected with pre-miR-599.

miR-223 and P2Y$_{12}$ Receptor Expression

miR-223 is highly expressed in platelets and megakaryocytes, where it is thought to regulate thrombopoiesis. One of the most interesting features of miR-223 is its ability to bind to the 3′UTR of human P2Y$_{12}$ receptor mRNA.[6] The P2Y$_{12}$ receptor mediates important platelet functions, including aggregation and granule secretion, and is activated by ADP, the physiological agonist of this Gi$_2$ protein-coupled receptor. Landry et al[6] showed that platelet P2Y$_{12}$ receptor mRNA coprecipitates with Ago2, suggesting that miR-223 could regulate receptor levels and, thereby, platelet function. Platelet response to ADP antagonists is clinically relevant given the widespread use of drugs such as clopidogrel, prasugrel, and ticagrelor in the treatment of myocardial infarction and ischemic stroke.

Because of the proposed regulation of $P2Y_{12}$ receptor function, miR-223 has been the focus of many clinically orientated studies. In particular, there is great interest in relation to platelet responsiveness to clopidogrel, a prodrug that requires hepatic cytochrome P450 enzymes to become active. Shi et al[79] reported decreased levels of platelet miR-223 in association with high platelet reactivity, despite treatment with clopidogrel. They looked at patients presenting with non–ST-elevation acute coronary syndrome, in terms of their platelet reactivity index, who were treated with aspirin and clopidogrel. In patients who had a reduced response to clopidogrel, platelet miR-223 was significantly downregulated, whereas miR-96 was unchanged. Platelet miR-223 also inversely correlated with the platelet reactivity index. Of note, neither miR-223 nor miR-96 were significantly different between normal and low responders when ADP-induced platelet aggregometry was used. These results suggest that platelet miR-223 may be a predictor of normal and low responders to clopidogrel. This is supported by the fact that $P2Y_{12}$ mRNA can be found in Ago2 immunoprecipitates.[6]

In a preliminary report including 21 males presenting with non–ST-elevation acute coronary syndrome, Chyrchel et al[82] studied miR-223 plasma levels. Patients were treated with aspirin plus either clopidogrel, prasugrel, or ticagrelor. Although there were no differences in plasma miR-223 levels between drug types, when all the data were pooled, miR-223 positively correlated with the level of platelet inhibition.[82] These results seem in line with those of Shi et al[79] who found that high platelet miR-223 levels predicted efficacious platelet inhibition with clopidogrel. Similarly, Zhang et al[83] report decreased plasma miR-223 to be associated with clopidogrel low responders. In this study of 62 patients with unstable angina, miR-223 was found to be the only independent predictor of low responders to clopidogrel and was higher with greater degrees of platelet inhibition.

miR-223 is also expressed in mouse platelets. In response to high agonist concentrations, miR-223 knockout mice do not show any detectable alteration in aggregation, speed of clot retraction, platelet adhesion, or bleeding time. $P2Y_{12}$ mRNA expression levels are also unchanged.[84] This is probably because of mouse $P2Y_{12}$ mRNA lacking the specific binding site for miR-223, rendering mouse models difficult to interpret in this context. Nonetheless, Elgheznawy et al[69] subsequently demonstrated that miR-223 deletion in mice did result in modestly higher platelet activation at lower agonist concentrations. Proteomic analysis revealed differential expression of kindlin-3 and coagulation factor XIII-A in platelets from miR-223-deficient mice.

Zampetaki et al[75] found that serum miR-223 levels are inversely associated with risk of future myocardial infarction and showed a stronger association with fatal than nonfatal events. Badrnya et al[85] showed reduced platelet microvesicle miR-223 in smokers. These findings are potentially complementary to those relating low platelet miR-223 level to increased $P2Y_{12}$ activity in humans and to those showing higher platelet reactivity in response to miR-223 deletion in mice. Subsequent clinical studies have investigated platelet miR-223, including in a small cohort of patients who suffered an ischemic stroke. Duan et al[86] report that platelet miR-223 and miR-146a were lower in stroke patients with diabetes mellitus versus controls. Platelet miR-223 and miR-146a levels significantly correlated with platelet reactivity as measured by P-selectin expression and also correlated to plasma levels, suggesting that platelets are a major source of circulating miR-223.

In summary, miR-223 targets the 3′UTR of the human $P2Y_{12}$ platelet receptor mRNA. The $P2Y_{12}$ receptor is important in platelet activation and is the target of many drugs used to inhibit platelet function. Lower levels of platelet miR-223, therefore, may result in more $P2Y_{12}$ receptor activity and, thus, higher platelet reactivity. In this way, lower platelet miR-223 levels may increase the risk of thrombotic disease, such as myocardial infarction. This also may underpin the observation that lower platelet miR-223 levels are associated with reduced efficacy of $P2Y_{12}$ receptor antagonists, such as clopidogrel. Mice lack the specific miR-223-binding site on the $P2Y_{12}$ mRNA, and so comparisons to human data is difficult. Nonetheless, miR-223-deficient mice do seem to have altered platelet function, perhaps through regulation of other proteins.

miR-126 and ADAM9 (A Disintegrin and Metalloproteinase Domain-Containing Protein 9)

Early studies, including one from our group,[72] stated that miR-126 is endothelial specific. Although miR-126 is enriched in endothelial cells and supports endothelial integrity, its circulating levels cannot be attributed solely to endothelial cells. In fact, megakaryocytes and endothelial cells both express miR-126, and circulating miR-126 levels seem to be predominantly platelet derived.[34,77]

de Boer et al[87] investigated the effects of aspirin on the platelet contribution of circulating miR-126 in patients with type 2 diabetes mellitus. PRP from subjects was treated with either arachidonic acid (a platelet activator) or arachidonic acid plus aspirin. Once the PRP had been treated, it was centrifuged to separate the platelets from the now PPP. Platelet activation resulted in a considerable transfer of miR-126 to the PPP fraction, and this could be rescued with the addition of aspirin. miR-223, miR-16, and miR-423 showed a similar pattern, suggesting that they are all released from platelets on activation and that aspirin attenuates this release. miR-126 levels also correlated with platelet activation as measured by soluble P-selectin. As part of another clinical trial involving patients with type 2 diabetes mellitus, the platelet activation state (measured via P-selectin expression) was assessed in 40 patients treated with increasing doses of aspirin. Platelet activation positively correlated with plasma miR-126 and aspirin reduced circulating levels of miRNA-126. Arguing against an endothelial origin of miR-126, the authors point out that aspirin primarily inhibits platelet-enriched COX-1, whereas the endothelium predominantly expresses COX-2. They also demonstrated no change in circulating von Willebrand factor.[87] Taken together, these findings suggest that aspirin has the potential to significantly alter the miRNA signature of plasma, and careful consideration should be given when designing clinical studies in pursuit of novel biomarkers.

In our experiments, we demonstrated that plasma miR-126 levels (1) correlate to platelet MPs, (2) are reduced on platelet inhibition, and (3) are associated with markers of platelet activation in the general population.[32] These associations were further substantiated by genetic evidence. The single nucleotide polymorphism rs4636297 is located in the primary transcript-encoding region of miR-126 and affects its processing to mature miR-126. The minor allele is associated with increased processing of the primary miRNA to mature miR-126. In a population-based study, carriers of the minor allele had higher levels of miR-126 in plasma and in serum.[32] The increase in circulating miR-126 was associated with a rise in platelet activation markers, such as platelet factor 4 and proplatelet basic protein. The genetic data on miR-126 and

platelet function were in line with findings in the Bruneck cohort, in which miR-126 serum levels were positively associated with risk of myocardial infarction after adjustment for miR-223 and miR-197, again with platelets being identified as the most probable source.[75] A study by Yu et al[88] showed that higher plasma miR-126 predicted major adverse cardiac events in 491 patients after a percutaneous coronary intervention, while on dual antiplatelet therapy.

In mice, inhibition of miR-126 by antagomiRs (synthetic inhibitors of miRNAs) decreased thromboxane A2-dependent platelet aggregation. This effect is likely because of megakaryocyte miR-126, but because antagomiRs are administered systemically, the contribution of other cell types, in particular endothelial cells, cannot be excluded. However, inhibition of miR-126 expression in endothelial cells would be expected to result in endothelial dysfunction and increased platelet activation. Similarly, if the effects of the single nucleotide polymorphism rs4636297 were limited to endothelial cells, one might expect that higher miR-126 levels are protective for endothelial cells and, thus, reduce platelet activity. The opposite was observed in mice and men. In MEG-01 cells, ADAM9 was confirmed as a direct miR-126 target that attenuates the adhesion of platelets to collagen. Inhibition of miR-126 by antagomiRs also had indirect effects on P2Y$_{12}$ expression levels, at least in mice. Thus, in addition to endothelial cells, miR-126 affects megakaryocytes. It remains to be seen whether platelet-specific deletion of miR-126 can recapitulate the accelerated atherosclerosis phenotype seen in miR-126-deficient mice.[89]

In summary, higher plasma or serum levels of miR-126 are associated with an increased risk of myocardial infarction and other major adverse cardiac events. This may, at least in part, occur via increased platelet activation. Inhibition of miR-126 with antagomiR-126 reduces platelet activation in mice, which may be explained by blocking of ADAM9's negative effect on platelet:collagen adhesion or via indirectly affecting platelet P2Y$_{12}$ expression levels.

Cellular Crosstalk Via miRNAs?

Several studies suggest that secreted miRNAs may act as paracrine mediators. The concept is appealing, but most evidence is currently limited to in vitro studies. By overexpressing a miRNA in one cell type, harvesting microvesicles from the conditioned media, and adding these microvesicles to recipient cells, miRNA transfer and downregulation of miRNA targets is detectable, suggesting that the miRNA uptake has biological function. There are several caveats to these in vitro studies:

1. MiRNAs are not abundant in the circulation, and preamplification is usually required for all but the most abundant miRNAs for reliable detection by qPCR. It is difficult to envisage how the few copies of miRNAs would have a potent effect on target gene expression. The amount of circulating miRNAs is several orders of magnitude below the cellular miRNA content, and exogenous miRNAs would have to compete with the endogenous miRNAs in the recipient cells for loading into the RNA-induced silencing complex. Unless there is a receptor that recognizes miRNAs and initiates a downstream signaling cascade, it is difficult to envisage how uptake of exogenous miRNAs could mediate biological effects.
2. MPs and exosomes are rich in proteins. The protein content of microvesicles may be functionally more important than their miRNA load. RNase treatment does not unambiguously prove that the observed biological effect is because of miRNAs. Protein degradation may also occur during the RNase digestion step.
3. In situ hybridization studies for a particular miRNA tend to reveal a characteristic staining pattern. For example, staining for miR-126 is confined to endothelial cells, whereas staining for miR-143 and miR-145 is observed in vascular smooth muscle cells. If miRNA transfer was a common phenomenon, such specificity would not be detected, and a more homogenous staining pattern would be expected.
4. Importantly, the tissue architecture, including basement membranes and extracellular matrix, should act as a physical barrier for the exchange of microvesicles.
5. Routine cell culture uses fetal calf serum, which is a source of bovine microvesicles.

Thus, we currently lack convincing evidence that the exchange of miRNAs is more than an in vitro phenomenon. In uninjured tissues, cellular miRNA exchange might be low. It is conceivable that miRNA transfer could occur under pathological conditions, such as platelet activation and clearance by inflammatory cells. Several experiments shed light on the role of platelet miRNA as a putative paracrine regulator of cellular function via regulation of other cells' mRNA and protein expression. For abundant platelet miRNAs, such as miR-223, target genes have been described, many of which are associated with inflammation. This miRNA is also expressed in leukocytes, further indicating a potential role. Given that platelets are already heavily implicated in several disease processes with an inflammatory basis, an interplay between endothelial cells, leukocytes, monocytes, and other cell types by endogenous and transferred miRNA seems plausible and an important area for further investigation.

Jansen et al[90] sought to define the miRNA content of circulating microvesicles and vesicle-free plasma in 176 patients with stable coronary artery disease undergoing coronary angiography. Over 6 years follow-up, microvesicle miR-126 and miR-199a levels above the median were associated with significantly fewer major adverse

cardiac events and percutaneous coronary interventions. There was no association between miRNAs from PPP and cardiovascular events. Microvesicle miR-126 was significantly reduced, and miR-199a trended toward reduction, in coronary artery disease patients versus age-matched controls. Again, no such difference was found in the PPP. Through microvesicle typing of surface markers, they established that miR-126 expression is highest in CD31$^+$/CD42b$^-$microvesicles (ie, endothelial), consistent with known expression patterns in endothelial cells, and miR-199a is highest in CD31$^+$/CD42b$^+$ microvesicles (ie, megakaryocyte-derived). These results are in contrast to results for circulating miR-126 levels. However, this study by Jansen et al[90] looked specifically at the microvesicular compartment, making cross-study comparisons difficult.

Laffont et al[17] showed that activated platelets can deliver regulatory Ago2–miRNA complexes to endothelial cells and that this occurs via MPs. First, they confirmed that activated platelets release substantially more MPs than quiescent platelets. Profiling the miRNA content of the MPs showed that the miR-223 content was greatly increased after platelet activation, suggesting selective packaging of miRNAs into platelet MPs. They then showed that the MPs contain functional Ago2:miR-223 complexes, which could cleave a complementary miR-223 RNA sensor, suggesting that these complexes are functionally relevant. Next, they used fluorescently labeled MPs to demonstrate internalization by human umbilical vein endothelial cells. Endothelial miR-223 levels increased 22-fold after incubation with MPs for 1 hour, an effect that persisted for 48 hours. Again addressing functional relevance, endothelial cells demonstrated a 44% reduction in a transfected reporter gene under miR-223 control when incubated with MPs. Significant downregulation of endogenous RNA levels occurred within 6 hours of exposure to platelet MPs, an effect that was rescued with the addition of an miR-223-neutralizing sponge.[17]

Pan et al[91] also showed increased MP release and MP miRNA enrichment after platelet activation. In the apolipoprotein E-null mouse model of atherosclerosis, MP miR-223 content was higher than in controls. This finding was also tested in a small selection of humans, in whom miR-223 expression was found to be higher in platelets and MPs of patients with inflammatory diseases compared with healthy controls. The authors demonstrated that endothelial cells take up MPs in an ATP-dependent manner, and endothelial miR-223 levels increase without an increase in pre-miR-223, suggesting that the increase was not because of de novo synthesis in recipient cells. Both an miR-223 mimic and incubation with MPs were shown to reduce insulin-like growth factor 1 receptor protein levels. This effect was blocked by anti-miR-223. miR-223 delivery increased advanced glycation end products–induced apoptosis, potentially via reduced levels of the insulin-like growth factor 1 receptor.[91]

Finally, Gidlöf et al[67] showed that patients with ST-segment–elevation myocardial infarction had reduced levels of miR-22, miR-185, miR-320b, and miR-423-5p in platelets compared with controls and an even greater reduction in aspirated coronary artery thrombus. The authors concluded that this is because of platelet activation and miRNA release. In vitro, they demonstrated miR-22, miR-185, miR-320b, and miR-423-5p release into the supernatant after thrombin-induced platelet activation. Using platelets transfected with a scrambled, fluorescently labeled miRNA, they showed effective transfer to endothelial cells when incubated with activated platelets. Furthermore, this was shown to be MP-dependent because the addition of brefeldin A

(a vesicle formation inhibitor) prevented miRNA transfer. Overexpression of miR-22 and miR-320b in endothelial cells suggested that these miRNAs were involved in the regulation of intercellular adhesion molecule 1. When endothelial cells were incubated with platelet releasate, intercellular adhesion molecule 1 expression was downregulated by 30%. Knockdown of endogenous endothelial miR-22 and miR-320b caused an increase in intercellular adhesion molecule 1 expression, confirming that the effect was because of MP miR-22 or miR-320b. Interestingly, this could be rescued by incubating the cells with platelet releasate.

In summary, there is now good evidence that (1) platelets transfer miRNAs and (2) that this is mediated by MPs.[92,93] However, it is less certain whether this transfer is functionally relevant at physiological concentrations in vivo and to what extent the observed effects are mediated by the miRNA content rather than the protein content of the platelet MP material. Although most studies have focused on platelet transfer of miRNA to endothelial cells, Risitano et al[71] showed that RNA transfer can occur between circulating platelets and monocytes.

Guidance on How to Measure Circulating miRNAs

Research on circulating miRNAs is attracting increasing attention because noncoding RNAs may constitute an entirely new entity of biomarkers. A prerequisite for advances in this area is the generation of comparative data by independent groups. To help this, we have suggested a set of minimum-reporting standards and recommended methods for circulating miRNA data (**Table**).

Plasma or Serum?

A fundamental question is the choice of plasma or serum for measuring miRNAs (**Figure 4**). The origin of miRNAs in plasma and serum may not be the same because platelets are activated during clotting and may contribute a larger proportion of miRNAs to serum compared with plasma. In plasma, the cells are removed by centrifugation in the presence of an anticoagulant. Citrate and EDTA are the preferred anticoagulants. Heparin plasma, for the reasons outlined below, is less suitable for miRNA measurements. Platelet activation during plasma centrifugation is another concern. Serum, on the other hand, is obtained by clotting blood at room temperature for 30 minutes to 1 hour. The clotting process involves the activation of thrombin, which cleaves fibrinogen to fibrin. Thrombin is a protease and its substrate specificity is not restricted to fibrinogen. The coagulation cascade involves a series of proteases, which may degrade carrier proteins of miRNAs. This might result in a loss of miRNAs, which can be mitigated by adding RNase inhibitors.[94] If platelets release miRNAs on activation, then findings in plasma and serum might give opposite results: plasma miRNAs may reflect the platelet release prior to blood sampling, whereas serum miRNAs may reflect the extracellular and platelet miRNA content combined—the more the release, the less the content. Thus, for our most recent studies, we used PPP to link miRNAs to in vivo platelet activation.32,34

Platelets, Microvesicles, and Exosomes

An understanding of which specific fraction of the circulation is being measured is of the utmost importance for reproducibility and adequate interstudy comparison. Ideally, one would be able to easily separate different circulating compartments: the cellular compartments (erythrocytes, leukocytes, and platelets), the microvesicle compartment, the exosome compartment, and the particle-free compartment devoid of membrane-bound vesicles or cells. Although isolating erythrocytes and leukocytes is relatively easy based on their size and density, isolating the smaller and less dense compartments poses a greater challenge. Isolating platelets from PRP is done routinely but requires care to avoid activating the platelets during the early centrifugation steps. As platelets release microvesicles on activation, this could affect the measured miRNAs in platelets, as well as plasma.

Residual Platelets

Platelets contain a substantial amount of miRNAs and are likely to be major contributors to the circulating miRNA pool, as discussed earlier.[34] This further complicates sample preparation for miRNA analysis: differences in plasma preparation, centrifugation speed, duration of coagulation, and the residual platelet content will have an impact on multiple miRNAs.[95,96] In RNA extracts from plasma

samples, the detection of the platelet- and megakaryocyte-specific transcript integrin α-IIb can be used to assess platelet content. Notably, the expression of integrin α-IIb correlates with the expression of abundant platelet-derived plasma miRNAs (miR-24, miR-126, miR-191, and miR-223). If plasma samples have a high residual platelet content, their miRNA profile may be more similar to that of serum and reflect predominantly platelet miRNA content rather than platelet miRNA release. Plasma platelet content must be uniform to allow comparisons between samples because residual platelets may introduce confounding. When plasma samples have already been collected, an additional centrifugation prior to RNA extraction may reduce the platelet content.[95] However, centrifugation of plasma after freezing is still subject to the risk of contamination from platelets lysed during the freeze/thaw cycle. Ideally, a method for the preparation of PPP prior to storage should be used to minimize the effect of residual platelets on miRNA measurements.

Hemolysis

Erythrocytes are the most abundant cell type in blood. The rupture of erythrocytes can occur because of high shear forces in the needle during blood collection or improper handling during sample processing. Because hemolytic samples contain a high amount of red blood cell–derived miRNAs, the levels of multiple miRNAs are altered in hemolytic samples.[97] Hemolysis can be assessed in plasma and serum by measuring hemoglobin spectrophotometrically or in the respective RNA extracts by analyzing miRNAs known to be affected by hemolysis, such as miR-451.[98] It is advisable to exclude hemolytic samples from further analyses. Alternatively, such compromised samples can only be used for miRNAs of interest that are unaffected by hemolysis.[99]

Leukocyte Contamination

Cells shed miRNAs via MPs and exosomes into the extracellular space. The miRNA content of the MPs and exosomes differs depending on the stimulus of the parent cells. However, the intracellular concentration of miRNAs is much higher than that in plasma or serum. Considering the low concentration of extracellular, circulating miRNAs, any contamination of plasma or serum samples with cellular material, for example, leukocytes, will greatly affect the miRNA profile. Thus, studies reporting a surprisingly large number of circulating miRNAs compared with other publications may have analyzed samples that were contaminated with cellular material.

Batch Effects

Batch effects can occur when analyzing the expression of miRNAs on multiple qPCR plates. To minimize their effect, several precautions should be taken to obtain reliable data: (1) to correct for variability between plates, a calibrator sample should be included across all plates. (2) The same batch of plasticware and reagents, such as PCR Mastermix, primers, and probes should be used for the entire study cohort. We have previously observed lot-to-lot-variability for miRNA reagents, even from leading manufacturers. (3) Reagents should be aliquoted because freeze-thaw cycles affect their quality and performance. (4) Samples from different groups should be distributed equally across all batches. These precautions minimize the effect of batch effects and help to obtain more reliable data.

Endogenous Normalization Controls

Normalization procedures should eliminate technical and experimental variation across samples to more reliably identify biological differences. In qPCR analysis, normalization controls are used to correct for variability in RNA extraction, reverse transcription, and PCR. Ideally, a normalization control is stably expressed and not affected by experimental conditions or clinical parameters. For the analysis of miRNAs in cells or tissue, small nucleolar RNAs are routinely used to normalize data. In general, they accurately reflect the small RNA content of a sample, although there are limitations and drawbacks. For example, small nucleolar RNAs can correlate with cancer pathology and prognosis, which can introduce bias when they are used as normalization controls for cancer samples.[100] For circulating miRNAs, however, there is no generally accepted endogenous control. Small nucleolar RNAs are intracellular RNA molecules, and their levels in the circulation are very low. U6 RNA has been used as a normalization control in plasma and serum samples but circulating levels of U6 display interindividual variability and are affected by inflammatory processes, reducing its reliability.[101] Single miRNAs or panels of miRNAs have been used or suggested as endogenous controls: miRNAs that have been used as normalizers include miR-16, miR-21, miR-93, miR-103, miR-191, miR-192, miR-423, and miR-425. These miRNAs, however, may well be affected by other uninvestigated diseases or medications. Antiplatelet therapy, for example, has been demonstrated to lower the circulating levels of plasma-derived miRNAs, including miR-21 and miR-191.[34] Alternatively, data can be normalized to the average Ct of multiple or all analyzed miRNAs.[102] Generally, combinations of miRNAs may be a more robust approach, based on the assumption that overall miRNA expression is stable and not affected by disease to the same degree as individual miRNAs. The more miRNAs that are combined, the more robust the control should be. However, this normalization method can be problematic when several of the miRNAs used to normalize are dysregulated in a similar direction, potentially causing false-positive or false-negative findings. Nonetheless, accepting that no better alternatives exist, a combination of miRNAs represents the most stringent endogenous normalization control.

Exogenous Normalization Controls

The most commonly used exogenous normalization method for circulating miRNA analysis is the use of a spike-in control. For this purpose, synthetic oligonucleotides of miRNA sequences from other species are used, typically from the nematode *Caenorhabditis elegans* (eg, cel-miR-39). Such spike-in controls are added in equal amounts to each sample during RNA extraction or reverse transcription. A spike-in control added at the RNA extraction step can normalize for differences in extraction efficiency, reverse transcription, or qPCR efficiency, which may be caused by the presence of inhibitors in the sample or pipetting errors. Spike-in controls generally reflect miRNA quantities in different samples, but we have observed that RNA samples display a lower quantity of cel-miR-39 when they were stored at −80°C for a longer period of time, impairing analysis in older samples. Moreover, heparin medication can affect cel-miR-39, as well as the measurement of miRNAs, depending on their origin.[103,104] The cel-miR-39 measurements across samples must be stable. Variability exceeding 0.5 to 1 Ct values should be cause for concern.

MiRNA Measurements

The gold standard for miRNA measurements is qPCR. It provides independent and robust amplification and quantitative detection of minute RNA amounts across the wide dynamic range of miRNA expression levels. Among the drawbacks of the technology is the fact that its high-throughput capabilities are limited. We have previously compared commercial qPCR assays and obtained good agreement between assays from 2 suppliers, for individual Taqman miRNA assays and customized qPCR plates from Exiqon. For customized qPCR plates, however, there is the possibility that variations in the manufacturing process remain undetected. This is less likely to occur in standard miRNA assays because multiple users will rapidly detect manufacturing problems. Thus, it is important to know the expected measurement range for miRNAs of interest when using customized qPCR plates and to compare the results from each batch of customized qPCR plates with individual qPCR assays.

We and others have noted difficulties comparing miRNA measurements from different technologies. In particular, there is poor agreement between microarrays and qPCRs when assessing quantitative changes. Microarray-based technologies use hybridization and are suitable for detecting the presence of miRNA species, but have limited quantitative accuracy and dynamic range. Similarly, NGS has several drawbacks, in particular, requiring much larger sample volumes than qPCR analysis and the introduction of amplification bias for certain miRNAs during library preparation. For example, in our study, we obtained most reads for miR-486, a miRNA that is present in the circulation but has a relatively high Ct value by qPCR, suggesting that it is much less abundant than other circulating miRNAs. Thus, miRNAs with more sequencing reads are not necessarily more abundant (**Figure 5**). Quantitative comparison must only be made by comparing sequencing reads for the same miRNAs.

Freedman et al used high-throughput microfluidic quantitative PCR to analyze the miRNA signature in blood (whole blood, platelets, mononuclear cells, plasma, and serum) collected from 5 healthy individuals. They screened for 852 miRNAs in 83 patients undergoing diagnostic coronary angiography and categorized their patients into 2 groups: with coronary artery stenosis ≥70% (n=34) and without (n=49). Increased plasma levels of miR-494, miR-769-3p, and miR-490-3p were associated with ≥70% coronary stenosis, and logistic regression analysis found that antihypertensive therapy, smoking, and miR-769-3p were significantly associated with coronary status.[105] In this study, few of the commonly reported highly expressed miRNAs were amongst the top 10 ranking miRNAs in platelets (**Figure 3**). It is likely that there are similar discrepancies with plasma and serum miRNA measurements. One cause for this difference may be the high-throughput BioMark Real-Time Fluidigm PCR system used in this study to increase the number of miRNA measurements that can be performed simultaneously; no direct comparison with conventional qPCR was provided.

Potential for Clinical Translation

The miRNA biomarker field is currently nascent. Although there is much to link the expression of miRNAs to various diseases, the clinical utility or validity of miRNA detection has not been demonstrated. Currently, miRNAs are manually extracted, presenting the largest obstacle to increasing throughput. If new technological platforms provide the opportunity for faster miRNA extraction or direct analysis of miRNAs without the need for miRNA extraction, they will significantly improve the ease-of-use in clinical settings. Increasing throughput, however, must not compromise the quantitative accuracy of the analysis for both low- and high-abundance miRNAs. Ideally, miRNA measurements need to be able to outperform current diagnostic methods to reach the clinical mainstream.

In addition to current practical limitations, miRNA biomarkers will also need to be presented in the context of related miRNAs to be adequately interpreted. There are relatively few tissue-specific miRNAs, with miR-122 being an exception given its exquisite liver specificity.[106] We have recently shown that high circulating miR-122 levels are associated with an adverse lipid profile and predict development of metabolic syndrome and diabetes mellitus type 2.[107] However, most circulating miRNAs are ubiquitously expressed, and numerous cell types can release miRNAs. This complicates the clinical application because levels of individual miRNAs can greatly vary between patients, without any pathological significance. Therefore, it is crucial to represent relative expression levels of a miRNA in relation to a network of several other miRNAs. The use of a so-called miRNA signature can greatly strengthen the predictive and diagnostic value.

Because the origin of many plasma miRNAs is unknown, De Rosa et al[108] investigated transcoronary gradients of various miRNAs in patients with acute coronary syndrome in a bid to define coronary-specific changes in the setting of myocardial damage. Samples were taken from the aorta and coronary sinus. They found an upregulation of the muscle-enriched miR-499, miR-133a, and miR-208a in acute coronary syndrome patients, of which miR-499 correlated with troponin levels, an established clinical marker of myocardial damage. Contrary to their predictions, miR-126 decreased across the coronary circulation, despite its high expression in endothelial cells. They also described a negative trend for miR-223 across the coronary circulation in the acute coronary syndrome group but not in the stable coronary artery disease group; however, this did not reach statistical significance.[108] An alternative explanation for the reduction in miR-126 and miR-223 across the coronary circulation is the retention of platelets and platelet MPs in the affected myocardium or differential activation of platelets in venous and arterial settings.

Pharmacological inhibitors can be used to identify the cellular origins of miRNAs. By comparing plasma miRNA levels in the same individuals before and after initiation of antiplatelet therapy, we identified a set of plasma miRNAs that is responsive to antiplatelet therapy.[34] The miRNA candidates were abundant in platelets, but they were not platelet-specific. Moreover, not every abundant platelet miRNA was equally affected by antiplatelet medication. Some miRNAs, despite their abundance in platelets, may be expressed at higher levels in other cell types. Consequently, their plasma levels may be less dependent on platelets. Given the platelet origin of many abundant circulating miRNAs, it is not surprising that the same miRNAs are

dysregulated in multiple diseases. The question, however, remains whether the circulating miRNA changes are cause or consequence. In cancer, for example, many of the circulating miRNA alterations may be because of platelet activation or inflammation, rather than the release of miRNAs from tumor tissue.

Early studies compared platelet samples from different individuals. Longitudinal sampling eliminates interindividual variability. For example, using serial measurements before and after antiplatelet medication, we investigated the effect of 10 mg prasugrel followed by the addition of an escalating aspirin dose regimen (75 mg daily in week 2 and 300 mg daily in week 3) on the PPP miRNA signature of healthy young volunteers. Using custom-made miRNA qPCR plates, 92 targets were measured at 4 different time points. Antiplatelet therapy was associated with a reduction in plasma miR-223, miR-126, miR-15, miR-191, miR-20b, miR-21, and miR-24. The findings were replicated in plasma of patients with recent symptomatic carotid atherosclerosis who had begun treatment with dipyridamole or clopidogrel, in addition to aspirin. These patients showed a reduction in miR-223, miR-126, miR-150, and miR-191.[34] The effect was less pronounced, presumably because the symptomatic patients were on aspirin medication at baseline and the follow-up measurement was only taken after 48 hours. The reduction in platelet miRNAs on platelet inhibition complements previous findings by Zampetaki et al[75] in an ischemia/reperfusion injury model by thigh cuff inflation, in which plasma miRNAs increased on platelet activation.

The clinical use of platelet function tests is limited at present, despite clear differences in response to pharmacological platelet inhibition. In the case of clopidogrel, a prodrug, a large percentage of this variation can be attributed to different cytochrome P450 isoforms involved in its bioactivation.[109] However, this in itself does not explain all of the variation in efficacy. Measurements of platelet activity by light transmission aggregometry are considered the gold standard, but this test is performed ex vivo and not well standardized between laboratories. Vasodilator-stimulated phosphoprotein (VASP) phosphorylation and VerifyNow $P2Y_{12}$ assays measure platelet activation in full blood but only in response to a single pathway, ADP stimulation. Platelet miRNAs may offer an opportunity to obtain an alternative read-out for platelet activation in vivo that integrates activation by multiple agonists rather than just ADP (**Figure 6**). Platelet miRNA measurements, however, will only be useful if they provide diagnostic advantages. They also must be easy to perform and capable of discriminating the degree of interindividual variability in platelet inhibition or platelet turnover.

Conclusions

Initially, circulating miRNAs were thought to originate from tissue injury. This is indeed the case for cardiac miRNAs that are released after myocardial infarction akin to troponins. For basal miRNA levels, however, the importance of circulating cells is now apparent, and the contribution from anucleate cells such as erythrocytes and platelets must not be underestimated. Several challenges face the accurate detection and use of plasma and serum miRNAs:

1. Variability in samples' cell composition affect miRNA profiles. This includes anuclear cells such as erythrocytes and residual platelets. Preanalytic variation in sample preparation or handling must be minimized to avoid alterations of results. Given the platelet origin of many abundant circulating miRNAs, the use of PPP would seem to be an appropriate substrate for miRNA measurements. In conventional plasma samples, the number of residual platelets can vary. Also, without inhibitors, platelet activation may occur during centrifugation. In serum, the stability of miRNAs may be affected, and miRNAs may get trapped in the clot. This could introduce bias that affects results, in particular, if the clotting time varies between sampling.

2. The concentration of extracellularly circulating miRNAs is low, and the miRNA yield of plasma and serum is insufficient for conventional quality control methods, such as optical densitometry or Bioanalyzer. Generally, equal volumes of plasma or serum are used for extraction. No correction is performed for RNA content. This approach of normalizing by volume is, however, analogous to protein measurements. In both cases, the total RNA or total protein amount of plasma or serum would not be a suitable normalization control for the miRNA or protein biomarker of interest.

3. Currently, there is no endogenous control for the normalization of circulating miRNAs. Instead, exogenous spike-in controls are used for normalizing the extraction procedure. The values of spike-in controls such as cel-miR-39 relate entirely to the consistency of the isolation procedure, not to the starting RNA quantity. By measuring a broader panel of unrelated miRNAs, the mean Ct value can be used for normalization.

4. Circulating miRNAs are highly correlated. Assessing individual miRNAs without integration into a wider miRNA network is of limited utility. Relative changes in miRNAs to one another (so-called miRNA signatures) can be more informative than the changes of individual miRNAs.

These points will need to be addressed in future publications on miRNA biomarkers to facilitate comparability between studies, something that has been difficult with variations between measurement platforms and lack of guidelines for standardization. Standardized miRNA measurement platforms are currently under development, which could reduce measurement variability, while facilitating cross-study comparisons. In addition to the need for consensus on sample preparation and analytic procedures, future research will need to move on from reporting on single miRNAs and focus more on miRNA signatures and integration with predicted target genes.[77,107,110] These issues are not insurmountable, and once addressed, future studies can further evaluate the potential of miRNA biomarkers for clinical use and whether miRNA assays will outperform existing clinical measurements.

Disclosures

M. Mayr is named inventor on patents that detail claims related to circulating miRNAs as biomarkers. M. Mayr has received a research grant from Astra Zeneca for investigating the effects of antiplatelets on circulating miRNAs. The other authors report no conflicts.

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Table 1. Minimum reporting standards and methodological recommendations for the analysis of circulating miRNAs.

Minimum reporting standards	Recommendations
1. Phlebotomy protocol	Sample venous blood using butterfly or regular blood collection needle; standardize relevant pre-analytical variables (e.g. fasting, smoking, exercise)
2. Anticoagulant used	Use citrate or EDTA; avoid heparin due to interference with qPCR
3. Time until processing	Complete sample processing within 2 hours from sampling to minimize variability and degradation
4. Sample processing	Perform an initial soft spin to collect plasma, followed by a faster platelet depletion spin with added inhibitors of platelet activation.
5. Sample storage	Store samples at -80°C, avoiding freeze-thaw cycles prior to analysis.
6. RNA protocol	Use a validated miRNA or total RNA extraction kit; use a carrier (MS2 protein or glycogen); avoid potential batch effects by co-isolating RNA from across
7. miRNA measurement platform	Avoid batch effects of different technical lots for used laboratory material and equipment
8. Average Ct levels for each assay	Assays with an average Ct value >32 (when using a 40-cycle protocol) should be avoided. Report average values to facilitate adequate interpretation of presented
9. Variability of the normalization reference	Across-sample variability of quality control markers such as an exogenous spike-in miRNA should be no more than 1-2 PCR cycles
10. Normalization method(s)	Normalize to the average Ct value of multiple assays or to an exogenous spike-in control.

Figure legends

Figure 1. Overview of non-coding RNAs in platelets. Although platelets are anucleate, they contain genetic material in the form of non-coding RNAs. These include small non- coding RNAs, such as microRNAs (miRNAs) and YRNA fragments as well as long non- coding RNAs, such as circular RNAs (circRNAs). Secondary structures of RNA sequences were predicted using RTips.

Figure 2. Small non-coding RNAs in plasma. A) Two peaks corresponding to miRNAs and YRNAs were observed by small RNA sequencing in plasma. B) Correlation of YRNA fragments to plasma levels of platelet factor 4 (PF4), pro-platelet basic protein (PPBP), and P-selectin (SELP). Lipocalin 2 (LCN2) was used as control. N=667.

Figure 3. Platelet miRNA levels. Relative abundance of miRNAs in platelets were reported in 8 studies (indicated by first author). The 50 highest ranking on average are presented here, indicating marked variation between different studies for the miRNAs beyond the top- 10.

Figure 4. Choice of samples. A) Workflow for the preparation of PPP from PRP. Addition of prostacyclin inhibits platelet activation during centrifugation. B) Concerns with regards to miRNA measurements in the different samples range from a contamination with leukocytes in PRP, proteolytic activity in serum to residual platelets in plasma. PRP and serum should reflect platelet miRNA content; PPP platelet miRNA release; plasma samples may reflect either, platelet miRNA content or release, depending on the amount of residual platelets in the plasma samples or platelet activation during plasma preparation, in particular during centrifugation.

Figure 5. Discrepancy between miRNA quantitation by RT-qPCR and RNA sequencing. Average expression of 92 miRNAs was assessed in platelet-poor plasma from healthy subjects (n=12), using custom-made Exiqon LNA qPCR plates. Cycle-to-threshold (Ct) values are shown as 40-Ct (x-axis). Small RNA sequencing was performed with platelet- poor plasma from healthy subjects (n=2). miR-486-5p is highlighted, showing high sequencing reads (y-axis) despite inferred mid-range abundance by qPCR.

Figure 6. MiRNAs and platelet reactivity. Association of miR-126 and miR-223 levels in PPP to the Verify Now P2Y12 aggregation test (A) and the VASP phosphorylation assay (B) in patients on dual anti-platelet therapy for 30 days post acute coronary syndrome. Note that fewer samples were measured with the Verify Now (n=39) compared to the VASP assay (n=123). PRU, denotes P2Y12 Reaction Units (x-axis). Higher PRU values reflect higher P2Y12-mediated platelet reactivity.

Figure 1

Stem-Loop pre-miRNA

MicroRNAs

- ➤ 22-23 nucleotides in length
- ➤ >200 miRNAs in platelet-poor plasma
- ➤ Regulate mRNA translation

microRNA-126

5' strand

3' strand

Mature miR-126-5p strand

c a u u a u u a c u u u u g g u a c g c g

Mature miR-126-3p strand

u c g u a c c g u g a g u a a u a a u g c g

Y-RNAs

- ➤ 4 Y-RNA genes
- ➤ Essential for DNA transcription
- ➤ Linked to cell stress and apoptosis
- ➤ 32-33 nucleotides length fragments are detected in plasma

Human Y-RNA 4

5' fragment

3' fragment

DNA transcription

Ro60 binding

Circular RNAs

- ➤ Back-splicing of exons
- ➤ Originate from RNA decay
- ➤ May act as miRNA sponges

LPAR circular RNA

Figure 2

Figure 3

Platelet miRNA ranks

	Wang	Stratz	Osman	Nagalla	Landry	Plé	Pan	Freedman
miR-126-3p	4	43	2	5	9		5	12
miR-191-5p	25	7	6	15	15	12	2	11
miR-16-5p	3	14	3	11	35	32	9	2
miR-24-3p	12	3	4	9	30	30	7	37
miR-223-3p	1	73	1	1	3	13	1	48
miR-17	55	9	7	21			12	25
miR-106a	16	10	8	23	22		11	61
miR-103a-3p	20	1	53	22	10	4	70	5
miR-15b	22	6	32	18	23		44	20
miR-320a	17	49	29	41	11	21	27	21
miR-20a	13	22	15	33	24		57	30
miR-146a	50	60	10	35	17		4	49
miR-21	7	70	28	6	19	10	77	41
miR-199a-3p	57	28	20		36	3	39	53
miR-185-5p	23	2	72	34	5	11	75	50
miR-92a-3p	15	37	35	107		24	56	3
miR-151a-5p	36	4	66	46	29	50	67	26
miR-27a-3p	11	12	45	40	58	34	50	78
miR-93-5p	24	35	30	42		41	72	45
miR-106b	95	5	54	30	37		49	28
miR-26a-5p	10	13	12	3	14	22	28	240
miR-130a	63	16	80		13			44
miR-221-3p	27	75	36	57	44	16	66	34
let-7i-5p	76	125		36	7	19		15
miR-720	45	62	31	10			46	107
miR-222	61	131	14	45	27		15	63
let-7d-5p	97	23	69	39	43	9	99	32
let-7g-5p	40	103	43	26	84	6	68	51
miR-30b	35	27	11	17	16		24	243
miR-19b	6	8	5	64	97		13	195
let-7a-5p	65	71	94	19	4	2	171	24
miR-22	90	11	87	8	34		117	59
miR-342-3p	72	68	23	84	86		10	88
miR-486-5p	30	57	22	92	121		78	35
miR-19a	46	50	17		21		58	181
miR-29a-3p	129	56	56	58	33	23	40	116
miR-484	37	18	9	166	150		3	68
miR-151a-3p	39	55	19	71	39		22	209
miR-197	59	30	33	119	109		45	
miR-107	31	24	150	48	25	17	169	
miR-27b-3p	18	20	102	123	52	44	121	58
miR-25	43	51	88	91		7	102	101
miR-423-3p	44	36		56	169	46		69
miR-30d-5p	42	78	49	66	69	37	128	96
let-7e-5p	133	88	51	70	117	27	86	13
miR-30c	38	86	13	24	12		16	327
miR-28-5p	86	39	83	52	94		92	
let-7b-5p	41	157	111	55	8	8	201	18
miR-15a	32	32	60	31	68		193	113
miR-18b	49	112	92	78	48			

Color scale: 1, 5, 10, 15, 20, 25, 35, 50, 75, 100, 150, 250

Figure 4

A   3.2% NaCitrate
     1:9 to blood

190g
PRP

1000g
PPP

PPP

Transfer PRP
+ Prostacyclin

Transfer PPP

Erythrocyte pellet
+ 'buffy coat' (leukocytes)

Platelet pellet

B

Platelet miRNA content

PRP
Leukocyte
contamination

Serum
Platelet activation
during coagulation

Conventional Plasma
Residual platelets
Platelet activation during preparation

PPP

Figure 5

Figure 6

A

MiR-126
$r_p = 0.347$; P=0.033

MiR-223
$r_p = 0.245$; P=0.139

B

MiR-126
$r_p = 0.224$; P=0.013

MiR-223
$r_p = 0.264$; P=0.003