Review

Circulating microRNAs as biomarkers and mediators of platelet activation

Clemens Gutmann[1], Manuel Mayr[1]

[1] King's College London British Heart Foundation Centre, School of Cardiovascular Medicine and Sciences, London, UK.

Keywords

Platelets, microRNA, noncoding RNA, biomarker, cardiovascular disease, thrombosis

Abstract

Platelets are essential mediators of physiological haemostasis and pathological thrombosis. Currently available tests and markers of platelet activation did not prove successful in guiding treatment decisions for patients with cardiovascular disease, justifying further research into novel markers of platelet reactivity. Platelets contain a variety of microRNAs (miRNAs) and are a major contributor to the extracellular circulating miRNA pool. Levels of platelet-derived miRNAs in the circulation have been associated with different measures of platelet activation as well as antiplatelet therapy and have therefore been implied as potential new markers of platelet reactivity. In contrast to the *ex vivo* assessment of platelet reactivity by current platelet function tests, miRNA measurements may enable assessment of platelet reactivity *in vivo*. It remains to be seen however, whether miRNAs may aid clinical diagnostics. Major limitations in the platelet miRNA research field remain the susceptibility to preanalytical variation, non-standardised sample preparation and data normalization that hampers inter-study comparisons. In this review we provide an overview of the literature on circulating miRNAs as biomarkers of platelet activation, highlighting the underlying biology, the application in patients with cardiovascular disease and antiplatelet therapy and elaborating on technical limitations regarding their quantification in the circulation.

Introduction

Over a century ago, platelets were identified as the main mediators of physiologic haemostasis and pathological thrombosis [1]. Platelets are generated through membrane budding from megakaryocytes in the bone marrow and lungs [2,3] and hence enter the blood stream without a nucleus. Despite the absence of relevant amounts of genomic DNA, platelets inherit a vast amount of RNAs from their megakaryocyte precursors, including coding messenger RNAs (mRNAs) as well as noncoding microRNAs (miRNAs), long noncoding RNAs (lncRNAs), circular RNAs (circRNAs) and YRNAs. Among noncoding RNAs, most studies have investigated miRNAs in platelets. Given that platelets are enriched with miRNAs compared to mRNAs and that platelets are the second most abundant blood cell type, we and others demonstrated that platelets are the major source of miRNAs in plasma and serum [4–8]. Interestingly, both intraplatelet levels of certain miRNAs [9] as well as levels of platelet-derived miRNAs in the cell-free circulation [6] were found to correlate with platelet reactivity. Thus, miRNAs have been investigated as novel biomarkers of platelet reactivity, *i.e.* for monitoring the efficacy of antiplatelet therapy monitoring and assessing the risk of atherothrombosis, as highlighted in our previous reviews [5,10]. This review will provide an overview on miRNAs as biomarkers of platelet reactivity and highlight novel mechanisms that may have implications for the function of circulating miRNAs.

MicroRNA Biology

MiRNAs are evolutionary highly conserved small RNAs (~ 22 nucleotides), whose main function is to repress the synthesis of specific target proteins. This effect is typically mediated in complex with an Argonaute (Ago) protein, which guides one of the two complementary miRNA

strands to the target mRNA. Through sequence complementarity of the miRNA seed region (~ 8 nucleotides) to the 3'-untranslated region of the target mRNA, protein synthesis is repressed. Until recently, this canonical RNA interference (RNAi; Figure 1) has been assumed to be the only regulatory mechanism of miRNAs. First evidence of regulatory functions that extend beyond RNAi came from studies implying ligand-like roles of miRNAs [11–15] (Figure 1): i) extracellular miRNA let-7 was shown to activate Toll-like receptors (TLRs) on microglia, macrophages and neurons [12,13]; ii) miR-21 and miR-129a secreted by tumor cells were shown to activate TLRs of immune cells [14]; and iii) extracellular miR-711 was shown to activate the transient receptor potential cation channel subfamily A member 1 (TRPA1) on neurons [15]. The fact that intracellular miRNA levels are around 13 times higher than Ago protein levels [16] and that only a fraction of miRNAs are bound (intra- or extracellularly) to Ago [16,17] provide support to this concept of a noncanonical miRNA pathway. However, concerns about artefacts remain due to the high miRNA concentrations used in these early studies. The physiological relevance of noncanonical miRNA roles has gained traction through a recent study that used sub-pmol/L miRNA concentrations (close to endogenous intracellular miRNA levels) showing that cardiomyocyte miR-1 physically binds to plasma membrane Kir2.1 ion channels and modulates cardiac action potential [18]. Given that this noncanonical miR-1-Kir2.1 mechanism appeared to be evolutionary conserved between (mouse, guinea pig, canine and human) species [18], it is possible that this ligand-like interaction is part of a wider regulatory mechanism that has been overlooked; because neither screening methods, nor standard procedures used to study regulatory mechanisms would routinely identify biophysical activation of receptors by small RNAs. If confirmed, the implications could be substantial, especially for the field of circulating miRNAs given the range of different cell-free miRNAs in blood. Most studies on horizontal miRNA transfer

from the circulation to the cells could not convincingly explain the mechanism how circulating miRNAs enter specific target cells, evade degradation upon cellular entry, associate with Ago and compete for biological effects with the more abundant intracellular miRNA pool of the target cell [10]. The concept that circulating miRNAs regulate cell surface receptors via physical interactions is appealing because signals induced by miRNA-receptor ligation could be amplified by downstream signalling, allowing small amounts of circulating miRNAs to mediate biological effects. One argument against this concept of miRNA-cell surface receptor ligation, however, is that miRNAs in circulation are contained in vesicles or protein complexes that protect miRNAs from degradation by RNases, which are abundant in the circulation. MiRNAs would therefore need to either be released from vesicles or disassociate from their protein carriers for binding to cell surface receptors. Alternatively, the miRNA-protein complex may be able to bind receptors. The latter hypothesis could then involve a more active role for the miRNA carrier, potentially guiding the miRNA to its receptor target, similar to the role of Ago in RNAi. Experimental evidence for a noncanonical regulatory mechanism by miRNA ligands has yet to be shown but recent ground-breaking developments in RNA biology; such as the finding that small RNAs can be glycosylated and presented on the cell surface, where they interact with receptors of immune cells [19]; are expected to prompt further studies in this evolving area of research.

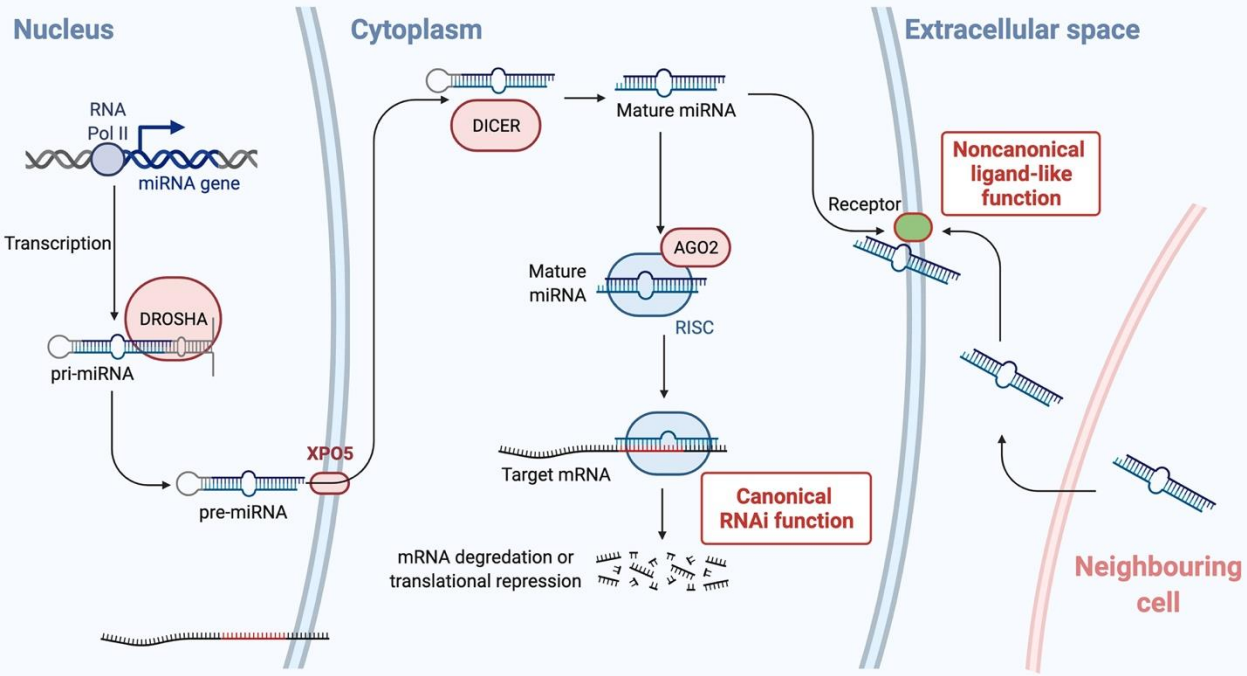

Figure 1. ***Canonical and noncanonical miRNA functions.*** *In the nucleus, primary miRNA transcripts (pri-miRNA) are processed into precursor miRNAs (pre-miRNA) by a complex containing the endoribonuclease Drosha. Pre-miRNAs are then transferred to the cytoplasm through exportin-5 (XPO5), where the endoribonuclease Dicer removes the hairpin structure of pre-miRNAs and generates mature miRNA duplexes. For canonical RNA interference (RNAi), miRNA duplexes associate with Argonaute-2 (AGO2) proteins, which incorporate one of the two strands and form the RNA-induced silencing complex (RISC), guiding the miRNA seed sequence to the target mRNA. Noncanonical ligand-like roles of miRNAs have only been documented in few studies but involve a ligand-like receptor interaction of miRNAs. Noncanonical ligand-like roles may potentially be mediated by both intra- and extracellular miRNAs. Extracellular miRNAs may be shuttled within vesicles or proteins.*

Intraplatelet MicroRNAs and Platelet Function

The first comprehensive assessment of miRNAs in platelets was performed in 2009, identifying 219 miRNAs using microarray profiling [20]. This number later increased to approximately 750 miRNAs with next generation sequencing (NGS), equivalent to approximately 30% of all known miRNA species [21]. The three most extensively studied platelet miRNAs are miR-223, miR-21 and miR-126, which we will describe in the following section.

miR-223. Based on data from multiple studies, miR-223 appears to be the most abundant platelet miRNA [5]. Interestingly, intraplatelet levels of specific miRNAs have been associated with hyporeactive and hyperreactive platelet responses to agonist stimulation [9] and conversely, several intraplatelet miRNAs are differentially expressed upon platelet activation [22], an effect that seems independent of the activation stimulus [23]. Several miRNAs were also shown to have functional roles in platelet activation (Figure 2). For instance, Landry et al. [20] have used a reporter gene assay to show that miR-223 pairs to the 3'UTR of the platelet $P2Y_{12}$ receptor and found $P2Y_{12}$ mRNA in Ago immunoprecipitates. The findings by Landry et al. [20] suggest that miR-223 regulates the platelet $P2Y_{12}$ receptor, but whether this mechanism significantly regulates $P2Y_{12}$ mRNA and protein levels *in vivo* is unclear. MiR-223 deficiency in mice leads to increased thrombus size and prolonged clot retraction [24], providing support for a potential effect of miR-223 on platelet activation. Similarly, in patients with type II diabetes, intraplatelet levels of miR-223 are reduced, whilst $P2Y_{12}$ levels and platelet reactivity are increased [25]. The $P2Y_{12}$ receptor, being mediator of the adenosine diphosphate platelet activation pathway, is a target of antiplatelet drugs like clopidogrel, prasugrel and ticagrelor. Accordingly, non-ST elevation myocardial infarction (NSTEMI) patients with a low response to clopidogrel were found to have lower intraplatelet levels of miR-223 [26].

miR-21. In another study in patients with acute coronary syndrome (ACS), levels of miR-223, miR-221 and miR-21 were upregulated in those patients with a high response to clopidogrel [27]. Notably, miR-21 is also known for its role in tissue fibrosis and progressed to clinical trials for Alport syndrome. Our group demonstrated that miR-21 manipulation in fibroblasts induced only marginal changes in extracellular matrix protein secretion. Instead, we reported a previously unrecognized effect of miR-21 inhibition on platelets. In plasma samples from the community-based Bruneck Study, we found a marked correlation for miR-21 levels with several platelet-derived pro-fibrotic factors, including transforming growth factor beta-1 (TGF-β1). Platelet α-granule secretion of TGF-β1 is a trigger for fibrotic responses. Pharmacological miR-21 inhibition with an antagomiR reduced the platelet release of TGF-β1 in mice. Mechanistically, Wiskott-Aldrich Syndrome protein is regulated by miR-21, which is a negative regulator of TGF-β1 secretion in platelets [28].

miR-126. A functional role in platelets has also been shown for miR-126: miR-126 inhibition with antagomirs led to reduced aggregation in mice [4], whilst transfection of miR-126 into $CD34^+$-derived megakaryocytes increases reactivity of its platelet-like structures [29]. In line with these findings, a single-nucleotide polymorphism favouring miR-126 processing to a mature miRNA was found to be associated with higher levels of protein markers of platelet activation [4]. The inhibition of disintegrin and metalloproteinase domain-containing protein 9 (ADAM9; an inhibitor of platelet-collagen adhesion) and upregulation of the $P2Y_{12}$ receptor [4,30,31] might be responsible for these effects. Moreover, miR-126 appears to have a key role in platelet-supported thrombin generation, based on *in vivo* miR-126 overexpression in zebrafish, *ex vivo* miR-126 transfection of human megakaryocytes and association data from patients with cardiovascular disease (n = 185) [32].

Other miRNAs. A role for platelet activation despite aspirin treatment has recently been attributed to platelet miR-26b. MiR-26b targets the multidrug resistance protein-4 involved in aspirin resistance, and miR-26b was downregulated upon aspirin treatment [33]. Platelet miR-204 was reported to regulate platelet reactivity through cell division control protein 42 (CDC42) downregulation and fibrinogen receptor expression [34]. In another recent study, healthy volunteers were grouped according to very high and very low platelet reactivity as assessed by thromboelastography, identifying increased miR-150 levels in the high reactivity group [35]. These findings were then replicated in ACS patients, where patients with high on-treatment platelet reactivity had elevated miR-150 levels in platelets [35]. Another example of how platelet function may be regulated by miRNAs is platelet miR-96, which targets vesicle-associated membrane protein 8 (VAMP8), involved in platelet α-granule secretion [36].

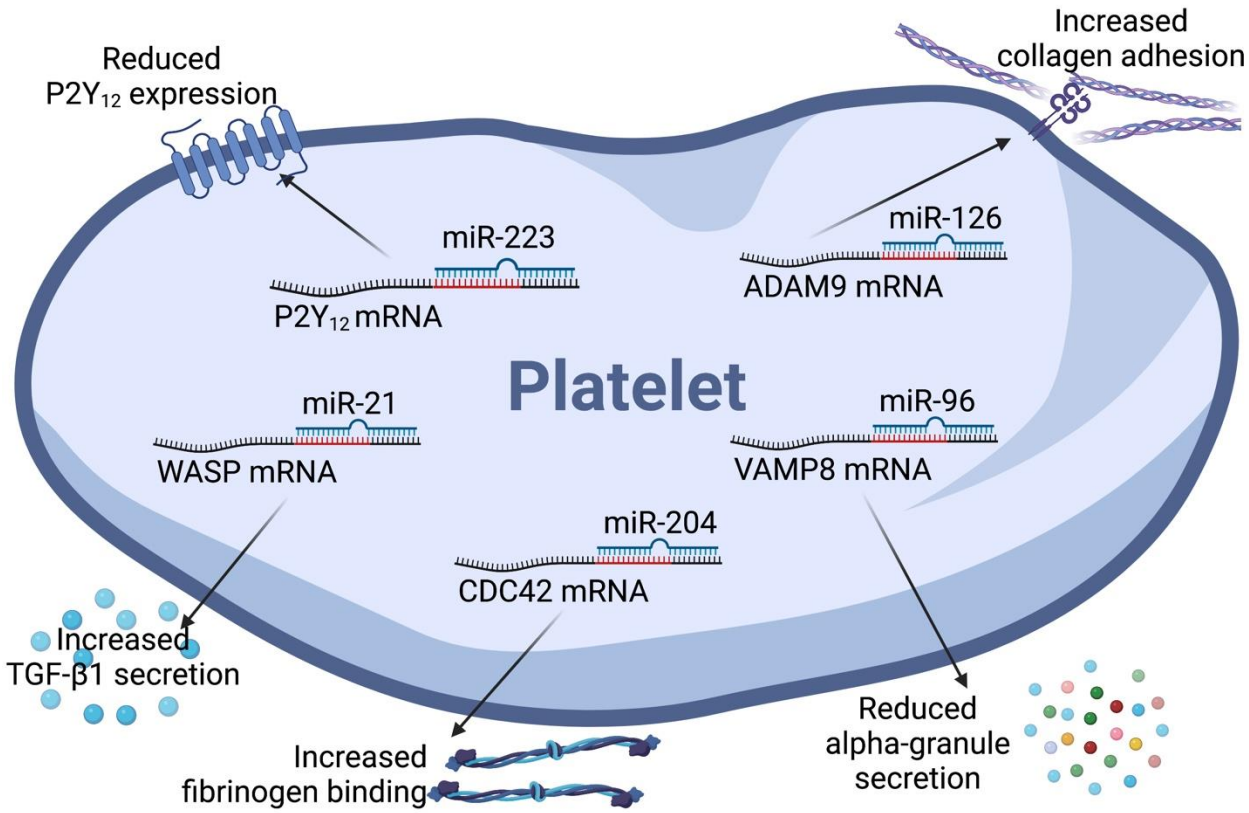

Figure 2. ***Intraplatelet miRNAs with known functions in platelet reactivity.*** *MiR-126 targets ADAM9, which is an inhibitor of platelet-collagen adhesion. MiR-96 targets VAMP8, which is involved in platelet alpha-granule secretion. MiR-204 targets CDC42, leading to increased fibrinogen binding. MiR-21 targets WASP, a negative regulator of platelet TGF-β1 secretion. MiR-223 downregulates $P2Y_{12}$, a key receptor for adenosine diphosphate platelet activation pathways.*

Circulating Platelet-Derived MicroRNAs and Platelet Function

The discovery of miRNAs in the cell-free circulation in 2008 [37] was surprising, given that RNases are abundant in the circulation and quickly degrade free RNA [38]. RNase-resistance of circulating miRNAs was later attributed to protection by proteins or vesicles, which act as miRNA carriers in the circulation [10]. A large proportion of circulating miRNAs is derived from platelets, predominantly secreted within vesicles upon platelet activation [4–8]. Differences in the miRNAome between platelet-derived vesicles and platelets have been suggested to be the result of a specific packaging mechanism rather than an unspecific bulk release [39], although experimental data for a specific release mechanism are lacking. Given that the platelet transcriptome is derived from megakaryocytes, the measurement of platelet-derived transcripts in the circulation also offers insight into the megakaryocyte transcriptome through a liquid biopsy.

In line with the concept of miRNAs being released upon platelet activation (Table I), Willeit et al. [6] analysed changes in plasma miRNAs in response to antiplatelet therapy, showing that antiplatelet therapy was associated with significantly reduced miRNA levels [6]. Similarly, another study showed that the transfer of miR-126 from platelets to the plasma compartment upon platelet stimulation is prevented by aspirin [40]. Kaudewitz et al. [4] looked at the association of platelet-derived miRNAs with platelet reactivity in the community-based Bruneck study as well as in

patients with ACS [4], showing that platelet-derived miRNAs such as miR-223 and miR-126 associate with platelet-derived proteins such as platelet factor 4, platelet basic protein and P-selectin in the general population, and associate with platelet reactivity in ACS patients as assessed by the vasodilator-stimulated phosphoprotein phosphorylation (VASP) assay [4]. MiRNAs also appear to capture rather nuanced changes in platelet reactivity, given that the switch from the weaker $P2Y_{12}$ inhibitor clopidogrel to the stronger $P2Y_{12}$ inhibitor ticagrelor was associated with a greater reduction in platelet-derived miR-126, miR-150 and miR-223 [41]. In a low dose endotoxemia model, the rise of miR-223, miR-223* and miR-197 was suggestive of platelet activation despite $P2Y_{12}$ inhibitor treatment [42]. Similarly, it was recently shown that platelet-derived miR-21, miR-197 and miR-223 rise with COVID-19 severity [43], potentially reflecting the prothrombotic state in COVID-19. Interestingly, data from the prospective Bruneck study also showed that a combination of platelet-derived miR-126, miR-223 and miR-197 levels at baseline predicted incident cardiovascular events over a 10-year observation period [44]. Similarly, another study found that the risk for arterial thrombotic events in patients with coronary heart disease is associated with circulating vesicle-bound miR-199a and miR-126 [45]. Furthermore, plasma miR-150 levels measured within 72 hours of stroke symptom onset, were found to predict 90-day mortality in a cohort of ischemic stroke patients (n = 329) [46]. Associations between platelet reactivity and several platelet-derived miRNAs, such as miR-126, were also found in a large clinical trial of patients with NSTEMI (TRILOGY-ACS trial, n = 878) [47]. Similar to many protein biomarkers, levels of miRNA biomarkers may be confounded by medication, comorbidities as well as demographic factors such as age, sex, or body mass index [48]. One confounder appears to be type II diabetes, which associates with a reduction of several platelet-derived miRNAs [49], and other metabolic parameters such as obesity and lipid metabolism [50].

Data from diabetic patients on antiplatelet therapy, however, suggests that antiplatelet therapy may, at least partially, be responsible for the decrease of circulating platelet-derived miRNAs in diabetes: in a recent double-blind randomised controlled trial, treatment of diabetic patients with the strong $P2Y_{12}$ inhibitor prasugrel led to a decrease of circulating miR-24, miR-191, miR-197 and miR-223 compared to aspirin [51]. However, not all studies found significant associations between platelet-derived miRNAs and platelet reactivity: in a small randomized controlled trial investigating the effects of periodontal treatment on platelet function, as assessed by flow cytometry and miRNA measurements, platelet activation and reactivity indices did not correlate with the levels of platelet-derived miRNAs [52]. Other conflicting data from a small study showed that circulating platelet-related miRNAs are inversely associated with platelet reactivity as suggested by Chyrchel et al. [53]. In their study, higher miR-223 levels were associated with stronger platelet inhibition [53]. Moreover, a discontinuation of long-term $P2Y_{12}$ inhibitor treatment did not lead to an expected increase of circulating platelet-derived miRNAs such as miR-21, miR-126, miR-150 and miR-223 [54]. Such conflicting data in studies of circulating miRNAs cannot only be explained by biological confounders but may also arise from preanalytical issues related to the measurement of miRNAs.

Table I. Selected studies on platelet-derived miRNAs as markers of platelet reactivity.

Ref.	Year	miRNAs	Key findings
[44]	2012	miR-21, miR-24, miR-126, miR-197, miR-223	Patients with subsequent myocardial infarction showed co-expression patterns of circulating platelet-derived miRNAs.
[6]	2013	miR-20b, miR-21, miR-24, miR-126, miR-150, miR-191, miR-197, miR-223	Platelets were found to be a major source of circulating miRNAs. MiRNA levels were responsive to APT.
[40]	2013	miR-126	Platelets were found to be a major source of circulating miR-126. Aspirin use was associated with reduced miR-126 levels.
[53]	2014	miR-223	Circulating platelet miR-223 was found to be inversely associated with platelet reactivity.
[45]	2014	miR-126, miR-199a	MiRNAs in microvesicles were found to predict cardiovascular events in CAD patients.
[41]	2016	miR-96, miR-126, miR-150, miR-223	A switch from weaker to stronger $P2Y_{12}$ inhibitor associated with reduction in circulating miRNAs.
[4]	2016	miR-126, miR-223	Circulating miRNA levels correlated with platelet function tests.
[46]	2017	miR-150	In ischaemic stroke patients, miR-150 predicted 90-day mortality.
[54]	2019	miR-21, miR-126, miR-150, miR-223	Cessation $P2Y_{12}$ inhibitor treatment did not lead to an expected increase of circulating platelet-derived miRNAs.
[51]	2020	miR-21, miR-24, miR-191, miR-197, miR-223	In type II diabetes patients, addition of the $P2Y_{12}$ inhibitor prasugrel to aspirin lead to a further decrease in platelet-derived miRNAs.
[47]	2020	miR-15b, miR-93, miR-126	Circulating miRNAs in ACS patients were found to correlate with platelet function tests.
[42]	2020	miR-197, miR-223, miR-223*	Rise of platelet-derived miRNAs with endotoxemia reflected platelet activation despite $P2Y_{12}$ inhibition.
[52]	2021	miR-1, miR-21, miR-23a, miR-24, miR-27b, miR-28, miR-29b, miR-33a, miR-126, miR-150, miR-191, miR-197, miR-223, miR-320a, miR-451a	In periodontitis patients, platelet-derived miRNA levels were not associated with platelet activation.
[43]	2021	miR-21, miR-126, miR-197, miR-223	Circulating platelet-derived miRNAs were found to rise with COVID-19 severity.

Abbreviations: *ACS: acute coronary syndrome, APT: antiplatelet therapy, CAD: coronary artery disease, Ref: reference.*

Analytical and Preanalytical Issues Related to the Measurement of MicroRNAs

Measurements of circulating platelet-derived miRNAs are impacted by sample preparation and data normalization (Figure 3). One major problem is the release of miRNAs from platelets that are activated after blood draw, thus overestimating *in vivo* miRNA levels. This problem can be minimized by rigorous standardization of sample collection and sample processing. The choice of anticoagulant used in blood tubes and differences in centrifugation speed or temperature may be responsible for inter-study differences [5]. The biggest discrepancies, however, are observed when platelet-derived miRNA levels are compared between plasma and serum: the preparation of serum involves activation of the coagulation cascade, which in turn results in uncontrolled platelet activation as well as potential proteolytic cleavage of protein miRNA-carriers. Thus, plasma is considered a more suitable matrix, but routine preparation of plasma often leads to substantial platelet activation or contamination with residual platelets. We have proposed the use of platelet-poor plasma for circulating miRNA measurements, generated through two consecutive centrifugation steps in the presence of the platelet inhibitor prostacyclin [5]. Residual leukocytes in plasma are also a concern, since the total RNA content of a single leukocyte is around three orders of magnitudes higher than that of a single platelet [7].

Another important confounder is heparin, which is commonly administered to hospitalized patients or to patients undergoing coronary interventions. Heparin interferes with the polymerase chain reaction [55]. This effect can be overcome by treating RNA extracts with heparinase [56]. Most studies fail to report the administration of heparin or the treatment of RNA with heparinase. In addition to its effect on polymerase chain reactions, heparin may increase the release of platelet-derived extracellular vesicles, thus artefactually increasing platelet-derived miRNAs [57,58]. Moreover, neutrophil extracellular trap formation may be increased in heparinized samples [59].

Other important confounders are RNases, which can be introduced during or after RNA isolation and substantially affect the miRNA measurements even in trace amounts through RNA degradation. Similarly, RNA degradation may occur due to prolonged storage or upon repeated freeze-thaw cycles.

Finally, different data normalization strategies may yield different results [5]. Various single miRNAs or combinations of endogenous miRNAs, such as miR-16, miR-93, miR-103, miR-423 and miR-425 as well U6 RNA, have been proposed as normalizers based on stable expression of these RNAs [5]. Universal applicability of these normalizers, however, is questionable because the effect of many diseases or drugs on miRNA normalizers has not been sufficiently investigated yet. In the absence of a universal normalizer, the most robust approach may be to normalize to the average expression of a large panel of miRNAs [60], in addition to using an exogenous normalization control (e.g. Cel-miR-39-3p), that is spiked into the samples during RNA isolation [5].

Besides measurements of circulating miRNAs being impacted by analytical and preanalytical factors, miRNAs may also serve as markers of sample quality in platelet concentrates used for transfusion purposes. Platelet concentrates stored in blood banks may undergo storage lesions, which impair platelet structure and function [61]. Several studies have looked at the differential expression of miRNAs during prolonged storage and have revealed platelet miRNAs that are up- and downregulated with prolonged storage [61]. Accordingly, the ratios of certain miRNAs, such as a low miR-127/miR-320 ratio, have been implicated as a marker of platelet storage lesions [62].

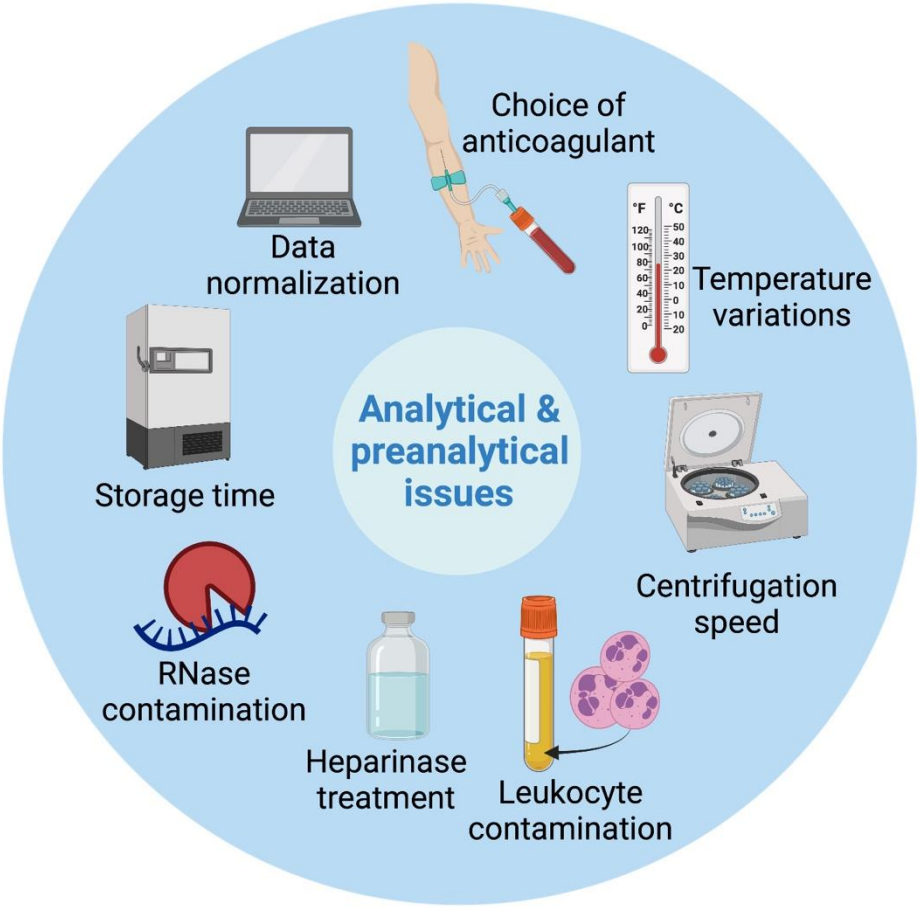

Figure 3. ***Analytical and preanalytical issues related to the measurement of miRNAs.***

Conclusions and Perspectives

Recent developments in RNA biology, such as the finding that miRNAs may have functional roles as receptor ligands independent of RNAi [18], or that small RNAs anchored on the cell surface may interact with immune cell receptors [19], have potential to advance our understanding of biological function of circulating cell-free miRNAs. A substantial proportion of circulating cell-free miRNAs is derived from platelets, and the levels of platelet-derived miRNAs are correlated with platelet activation. The clinical utility of miRNAs as biomarkers for platelet reactivity remains to be demonstrated in large clinical trials and preanalytical issues need to be

taken into consideration. The use of "biomarker signatures", *i.e.* the use of a combination of measurements rather than single markers, is certainly applicable to the miRNA field as well. The need for novel biomarkers of *in vivo* platelet reactivity is highlighted by the fact that existing *ex vivo* platelet function tests might not be as reliable, in particular in acute settings such as MI due to an *in vivo* pre-activation of platelets leading to a hypo-responsiveness *ex vivo* [63]. Accordingly, existing platelet function tests are not commonly used to inform clinical decisions with regards to choice and dosage of antiplatelet therapy [64]. It remains to be seen whether platelet reactivity signatures based on miRNAs or other molecular measurements can address this clinical need or improve risk management of thrombotic complications.

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Declaration of Interest

M.M. has filed and licensed patent applications on miRNAs as platelet biomarkers.

ORCID

Clemens Gutmann https://orcid.org/0000-0003-0675-8632

Manuel Mayr https://orcid.org/0000-0002-0597-829X

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