CHAPTER 6

Aspirin treatment hampers the use of plasma microRNA-126 as biomarker for the progression of vascular disease

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Abstract

MiR-126 facilitates angiogenesis and regulates endothelial cell function. Recent data suggest that miR-126 can serve as a biomarker for vascular disease. Although endothelial cells are enriched for miR-126, platelets also contain miR-126. In this paper, we investigated the contribution of platelets to the pool of miR-126 in plasma from patients with type 2 diabetes and how this is affected by aspirin.

In vitro platelet activation resulted in the transfer of miR-126 from the platelet- to the plasma-compartment, which was prevented by aspirin. *In vivo* platelet activation, monitored in patients with type 2 diabetes by measuring soluble P-selectin, correlated directly with circulating levels of miR-126. Administration of aspirin resulted both in platelet inhibition and concomitantly reduced circulating levels of platelet derived microRNAs including miR-126.

Platelets are a major source of circulating miR-126. Consequently, in patho-physiological conditions associated with platelet activation, such as diabetes type 2, administration of aspirin may lead to reduced levels of circulating miR-126. Thus, the use of platelet inhibitors should be taken into account when using plasma-levels of miR-126 as a biomarker for the progression of vascular disease.

Introduction

MicroRNAs (miRNAs) are short non-coding RNAs that function as post-transcriptional, negative regulators of gene expression. While native RNA molecules are rapidly degraded in plasma, miRNAs display exceptional stability in the circulation due to their association with argonaute protein [1], high density lipoprotein [2] or their inclusion into exosomes or microparticles [3]. Since many miRNAs are tissue-specific and differentially expressed in patho-physiology, miRNA-profiles in the circulation may serve as biomarkers for disease progression. Indeed, altered levels of selected miRNAs have been reported in various cardiovascular diseases, such as acute myocardial infarction [4], myocarditis [5], acute and chronic heart failure [6]. MiR-126, which has been shown to be enriched in endothelial cells [7], has received particular interest. Alterations in circulating miR-126 have been proposed as a marker for endothelial dysfunction in type 2 diabetes (DM2) [8] and coronary artery disease (CAD) [9]. However, next to its endothelial origin, miR-126 also constitutes one of the most abundantly expressed miRNAs in platelets [10, 11]. Here, we investigated whether platelets are a possible source of circulating miR-126 and how aspirin treatment affects its plasma level in patients with DM2.

Material and methods

In vitro platelet activation

To establish a relation between the activation of platelets and miR-126, sodium citrate-anticoagulated (3.8% Na-citrate containing vacuum tubes, Becton Dickinson) whole blood samples were obtained from four healthy, male volunteers. From these samples, platelet-rich-plasma (PRP) was obtained by centrifugation (15 min at 150 g at room temperature, no brake) and divided into three samples (60 µL per sample). One sample served as non-stimulated control. The other two samples were incubated with arachidonic acid (AA, 1.5 mmol/L, Hart Biologicals, UK) in the absence or presence of aspirin (asp, 330 µmol/L, Sigma). This concentration of aspirin is equivalent to the amount that patients receive when they are treated with 300 g/day of aspirin. We chose for AA, since this platelet activator is inhibited optimally by aspirin whereas two other routinely used platelet-activators collagen or adenosine diphosphate are only inhibited by aspirin for approximately 50% [12]. After AA-incubation (10 min at 37°C), 50 µL of the PRP was transferred to 450 µL of paraformaldehyde (1% w/v, PFA) to fixate the platelets. From the remaining sample, platelet-free plasma (PFP) and a platelet-pellet was obtained by centrifugation (5 min at 5000 rpm). To check whether the PFP preparations were cell-free, all samples were analyzed with an automated cell-counter (Sysmex®) and by FACS. To illustrate that the PRP did not contain any leukocytes, the forward (FSC)/ side scatter (SSC) FACS-plots (logarithmic scales) are shown from one donor (Supplemental Figure S1): non-incubated (tube A), incubated with arachidonic acid and aspirin (+AA/+asp; tube B) or AA alone (+AA, tube C), as indicated. Each tube contained a small portion of small particles (debris in grey), while the platelets (anthracite) are clearly distinguishable from the debris, not only on the FSC/SSC-plot, but especially when the expression of P-selectin is measured (second row of dot plots) after activation with AA in the absence of aspirin (tube C). The number of events in the debris- and platelet-gate accounted for 99.4 % (tube A), 99.3 % (tube B) and 99.4% (tube C) of the total number of events, of which 99% could be identified as P-selectin positive when incubated with arachidonic acid (histogram, tube C). This indicates that leukocytes were not detectable in these PRP samples, not by cell-counter (not shown), nor by FACS, and also not in the PFP samples obtained from these PRP-samples at a later stage. Samples were stored at -80°C until further analysis. All *in vitro* experiments were performed in triplicate. Representative results are shown.

Surface-expression of P-selectin by platelets

To assess the expression of P-selectin by platelets as a measure of AA-activation and to assess the inhibitory effect of aspirin treatment, PFA-fixed platelets were washed with FACS-buffer (PBS supplemented with 1% bovine serum albumin and 0.05% Na-azide), incubated with mouse IgG directed against human P-selec-

tin or an isotype-matched control IgG (both 5 µg/mL, BD Biosciences), washed with FACS buffer and incubated with goat-anti-mouse IgG conjugated with Alexa-488 (Molecular Probes). P-selectin expression was measured using the LSRII (Becton Dickinson). Platelets incubated with isotype-matched control IgG, were gated (gate P2), which represents background staining. Gate P3 was set to detect fluorescent signals above background and represents P-selectin-positive platelets. Mean fluorescent intensity (MFI) was measured from the platelets in P3.

Measurement of soluble P-selectin and von Willebrand factor

Soluble P-selectin (sP-sel)was measured in PFP using a commercially available ELISA kit, according to the instructions of the manufacturers (R&D Systems). Measurements were performed in duplicate and average values were used. Von Willebrand factor (vWF) was measured routinely at the Clinical Chemistry Laboratory of the Leiden University Medical Center according to standard procedures.

MiRNA-expression levels

Total RNA from EDTA-samples was isolated using Trizol reagent (Invitrogen, Breda, The Netherlands). To serve as a technical control 5 fmol synthetic, exogenous C.Elegans miR-238 (Biolegio, Nijmegen, The Netherlands) was spiked into 200 µL plasma or platelet isolates. Subsequently, RNA was isolated and expression levels of miR-126, miR-16, miR-223, miR-423 and miR-238 were validated in triplicate by quantitative RT-PCR (qPCR). Reverse transcription was performed using a 5 minute incubation at 65°C of 2.0 uL (plasma samples) or 250 ng total RNA (platelet isolates) with specific Taqman® microRNA probes (miR-126, Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). cDNA was synthesized using a M-MLV First-Strand Synthesis system (Invitrogen). Results were normalized using Gene Expression Analysis for iCycler IQ® RT-PCR Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands). To compare miRNA-levels, the concentration (fmol/mL) of miRNAs was calculated by correlating the mean miR-238 CT-values with the spiked concentration of miR-238 added to the samples or miRNA-expression was shown as "fold change", calculated with standard $\Delta\Delta$CT method.

Plasma samples of type 2 diabetes mellitus patients

Platelet-free plasma samples were acquired from patients diagnosed with DM type 2 (DM2), who had entered a prospective, randomized study with a placebo-controlled, double-blind, crossover design. A detailed description of the inclusion criteria of these patients and the study has been published previously [12, 13]. All subjects gave written informed consent and the study was approved by the institutional review committee and performed in accordance with the Declaration of Helsinki [12, 14]. In short, all subjects (n=40) received one period placebo

and the other period aspirin (100 or 300 mg/day). The first treatment period with aspirin or placebo for 6 weeks was followed by a washout period of 4 weeks. Thereafter, those assigned to placebo in the first period received aspirin for 6 weeks and those assigned to aspirin received placebo for additional 6 weeks. At each visit, EDTA-anticoagulated peripheral blood samples were drawn from antecubital veins. All plasma-samples were stored at -80°C until further analysis. Based on our definition of aspirin-responders and non-responders (see main text), 19 patients were considered responders, 19 patients were non-responders and for 2 patients the aspirin-responsiveness could not be determined, since a sP-selectin value was missing. These patients were excluded. For 2 patients insufficient amounts of plasma were present to determine levels of miR-126, yielding 18 miR-126 values for both the "responder" and the "non-responder" group.

Statistical analysis

All calculations were performed with Graphpad Prism software. For linear regression analysis P-, β- and r^2-values are reported. To calculate significant differences ($P<0.05$), paired T-tests were used.

Results

In vitro platelet activation

To investigate the correlation between platelet activation and miR-126 in plasma, peripheral blood was withdrawn from healthy volunteers. After obtaining PRP, platelet activation was induced with AA in the absence or presence of aspirin. Of note, PRP samples did not contain contaminating leukocytes as was determined with an automated cell-counter (data not shown) and by FACS (Supplemental Figure S1).

Platelet activation was monitored by surface-expression of P-selectin or by shedding of the soluble form (sP-sel) into the plasma. Non-activated platelets did not express P-selectin (Figure 1B). AA induced P-selectin expression on all platelets (99%, Figure 1D) and in all donors (Figure 1A, left axis). Aspirin (+asp) inhibited the AA-induced expression of P-selectin significantly ($P=0.001$), although this expression was not inhibited on all platelets (Figure 1C) and was not inhibited to non-stimulated levels (Figure 1A, $P<0.001$). AA-stimulation resulted in a significant increase of sP-sel in the plasma-compartment (PFP) obtained from PRP (Figure 1A, right axes, $P=0.025$). Addition of aspirin alone did not alter expression of P-selectin on the platelets or sP-sel levels in the PFP (data not shown). When aspirin was added during AA-stimulation, shedding of sP-sel was completely inhibited to basal levels in each donor. Apparently, aspirin is able to prevent the shedding of sP-sel from the platelets, while membrane expression of P-selectin is not inhibited completely.

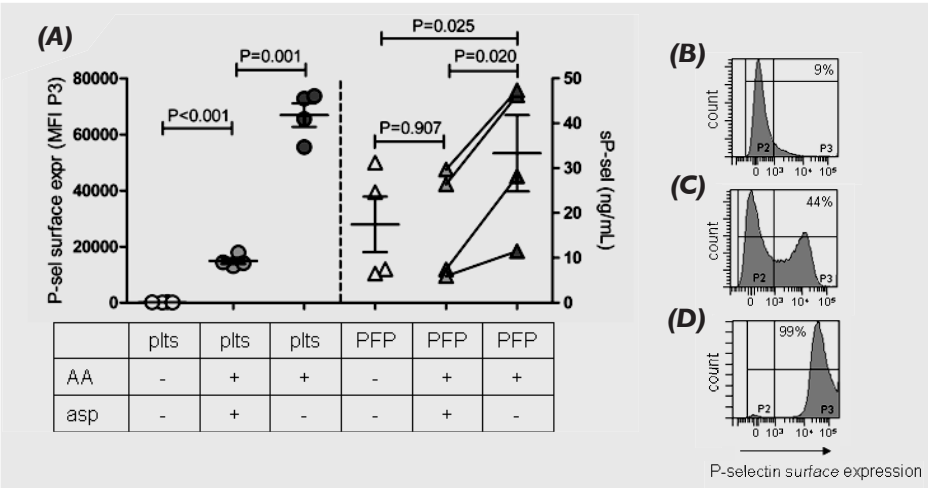


Figure 1. In vitro platelet activation leads to transfer of miR-126 from the platelet- to the plasma-compartment

(A) PRP obtained from healthy volunteers was incubated with arachidonic acid (+AA) in the absence (-asp) or presence of aspirin (+asp). Surface expression of P-selectin, expressed in mean fluorescent intensity (MFI of P3-gated platelets) was measured (left axis), on plts/-asp/-AA ***(B)***, plts/+asp/+AA ***(C)*** or plts/-asp/+AA ***(D)***. Soluble P-selectin (sP-sel; right axis) was measured in platelet free plasma (PFP) after AA-induced platelet activation plus or minus aspirin.

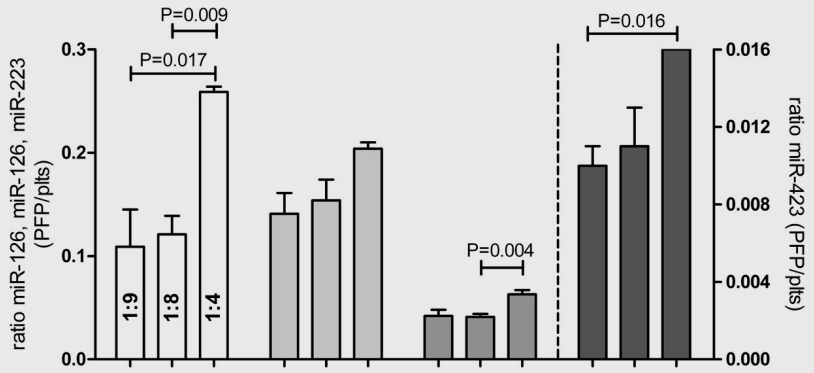
miR-126 levels were measured in PFP or the platelet-pellet, both isolated from the same PRP-sample and the relative distribution ratio (PFP/plts) was calculated. Non-activated PRP showed a distribution ratio of approximately 1:9 (PFP/plts, 0.109±0.036, Figure 2) and AA-activation resulted in a significant transfer of miR-126 (P=0.017) from the platelet- to the plasma-compartment, yielding a relative ratio of 1:4 (+AA, 0.259±0.05). AA-activation in the presence of aspirin prevented the transfer of miR-126 towards the plasma-compartment (P=0.009), which showed a similar ratio of 1:8 (0.121±0.018) as non-activated PRP. As three other platelet-enriched miRNAs (miR-16, miR-223 and miR-423) were released from activated platelets in a similar fashion (Figure 2) our data imply that platelet activation leads to shedding of P-selectin from the platelet-membrane and release of miRNAs from intracellular platelet-stores.

In vivo platelet activation

To investigate the relation between plasma levels of miR-126 and *in vivo* platelet activation, we studied patients with DM2, who exhibit a disease-mediated platelet activation [15]. These patients had participated in a placebo-controlled cross-over study, in which 40 patients were randomly assigned to a period of aspirin-treatment (100 or 300 mg/day) or placebo [12]. This resulted in a wide range of

DM2-induced *in vivo* platelet activation, monitored by sP-sel [16], which is consistent with increased platelet aggregability in chronic vascular diseases, such as DM2 [15].

Figure 3A shows that the absolute level of sP-sel yielded a positive linear regression with circulating levels of miR-126 (expressed as fold change) (P=0.006, β=1.123, r^2=0.197), irrespective of treatment and aspirin-dose. When treatment was taken into account, linear regression analysis showed that the change of sP-sel (delta sP-sel; asp-plac) positively correlated with the change in miR-126 levels (ratio asp/plac, Figure 3B, P=0.010, β=1.992, r^2=0.180).



miR	miR-126			miR-16			miR-223			miR-423		
AA	-	+	+	-	+	+	-	+	+	-	+	+
asp	-	+	-	-	+	-	-	+	-	-	+	-

Figure 2. The relative distribution ratio of miRs
miR-126, miR-16 and miR-223 (left axis) and miR-423 (right axis) in PFP versus platelets (PFP/plts) is shown in treated versus non-treated PRP obtained from healthy volunteers. As negative control miR-263, not present in platelets, was measured.

As shown previously [14], patients were considered aspirin-responders, when their sP-sel level decreased upon aspirin treatment, while patients were defined as non-responders when their sP-sel was either not affected or was even increased. When discriminating for responders (resp) and non-responders (non-resp), the expected maximal difference for delta sP-sel (Figure 4A) coincided with a highly significant difference in the ratio of miR-126 (Figure 4B; P<0.001). Since *in vivo*, endothelial cell activation may contribute to the plasma pool of sP-sel, we also measured the established endothelial cell marker von Willebrand factor (vWF) in the plasma [17]. When calculating delta vWF-values (asp-plac), no difference was observed between the responder and non-responder group (Figure 4C, P=0.350) and linear regression analysis for delta-vWF values and the ratio of miR-126 showed no significance (Figure 3C, P=0.450, β=-0.008, r^2=0.017), indicating that sP-sel levels, and thus miR-126 levels, were not changed due to activation/damage of the endothelium.

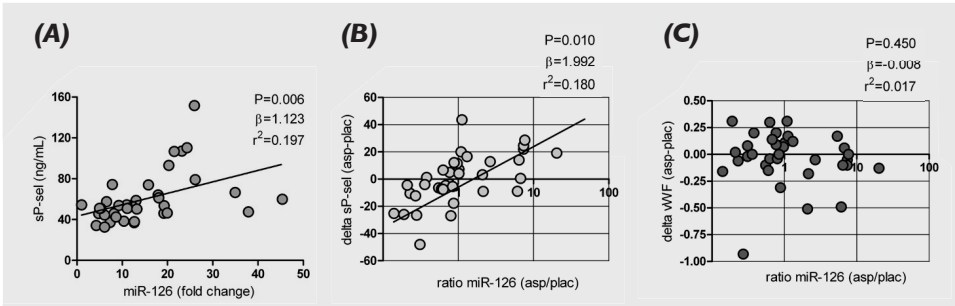


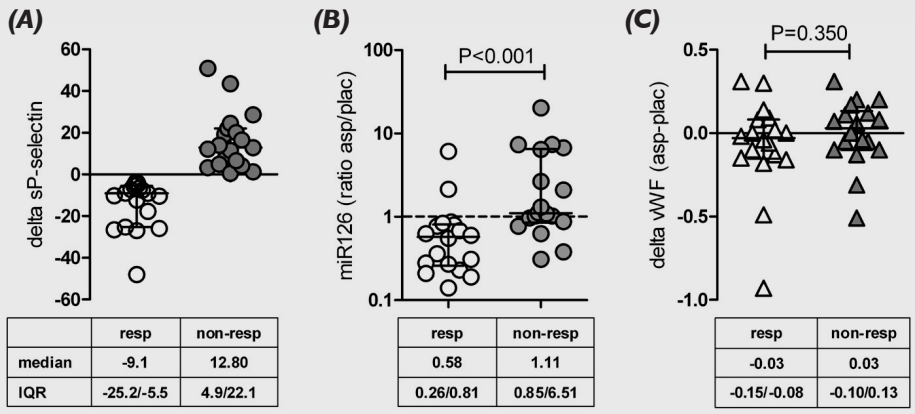
Figure 3. In vivo platelet activation in DM2-patients shows a correlation with miR-126 levels

Linear regression analysis was performed on ***(A)*** miR-126 (fold change) versus sP-sel (ng/mL) in plasma of DM2 patients (n=36) and ***(B)*** the change in miR-126 (ratio asp/plac) versus delta sPsel values (asp-plac) or ***(C)*** delta vWF values (asp-plac) in response to aspirin treatment. Note the logarithmic scale for miR-126 in panels (B) and (C).

Discussion

Previous reports show that miRNAs in plasma display exceptional stability in the circulation due to their association with argonaute protein [1], high density lipoprotein [2] or their inclusion into exosomes or microparticles [3]. In fact, it has recently been shown that the main fraction of miRNAs in human plasma is localized in microparticles of which 41-45% originates from platelets [18]. These miRNA-containing microparticles may actually play a role in cardiovascular diseases by transferring their miRNA-content to target cells. For example, Zernecke et al. have shown that miR-126-containing apoptotic bodies were able to mediate athero-protective effects in mouse models of atherosclerosis [19] and miR-126-containing microvesicles protected the kidney against ischemia/reperfusion injury in mice [20]. These functional properties and the notion that circulating miRNAs may be derived from cells in the vascular system has stirred current interest in the use of circulating miRNA profiles as diagnostic and maybe even prognostic biomarkers for the progression of cardiovascular disease [4-6].

The pathophysiological mechanisms involved in vascular diseases, such as DM2 and CAD, include EC-dysfunction [21] and ongoing vascular injury [22]. Since miR-126 is enriched in endothelial cells [7], these vascular diseases would predict a release of endothelial-derived miR-126 into the circulation. However, in a recent study, a counter-intuitive decrease of miR-126 was observed in CAD patients in comparison to normal age- and gender-matched controls [9]. Interestingly we noticed that all CAD-patients were treated with aspirin, while none of the healthy controls used aspirin. According to our observations, this



	resp	non-resp
median	-9.1	12.80
IQR	-25.2/-5.5	4.9/22.1

resp	non-resp
0.58	1.11
0.26/0.81	0.85/6.51

resp	non-resp
-0.03	0.03
-0.15/-0.08	-0.10/0.13

Figure 4. Classification in subgroups of DM2 patients in aspirin-responders and non-responders

(A) Based on their delta sP-sel levels. Patients subgroups show a significant difference in ***(B)*** the change in miR-126 (ratio asp/plac), but not in ***(C)*** delta vWF levels (asp-plac). Note the logarithmic scale for miR-126 in panel (B). Absolute values represent median and interquartile range (IQR).

could explain the decreased miR-126 levels. Furthermore, an overall systemic inhibition of platelet activation would predict a decrease of other platelet-derived miRNAs as well. Indeed, our *in vitro* platelet activation assay showed that the transfer from the platelet- to the plasma-compartment of 3 other miRNAs that are abundantly present in platelets [11], miR-16, miR-223 and miR-423, were likewise inhibited in the presence of aspirin.

Consistent with our current findings [22], of the 25 miRNAs that were reported to be downregulated to the highest extent (0.50-0.76 fold) in the aspirin-treated CAD patients [9] were located in the top highest-expressed platelet-miRs [10, 11] of which miR-126 was ranked number 5. This is further illustrated in Figure 5, in which the platelet-miRNA-profiles from 2 independent papers are combined [10, 11] displaying a very similar rank-order with a highly significant regression for quantified signals of the miRNA-arrays ($P<0.0001$, $\beta=0.607$, $r^2=0.337$). In contrast, of the 20 miRNAs reported to be upregulated to the highest extent in the aspirin-treated CAD patients [9], only 4 miRNAs are present in the list of platelet-associated miRNAs and these 4 miRNAs are even located in the lowest region of rank-order [11].

These data suggest that when *in vivo* platelet activation is inhibited, as was the case in our DM2 study [14] and the CAD study [9], the release of platelet-derived miRNAs in general and miR-126 in particular, is inhibited accordingly. In fact, the CAD-study showed an actual negative influence of aspirin on circulating miR-126 ($P<0.001$, $R=-0.469$) [9].

Interestingly, in patients with troponin-positive acute coronary syndrome

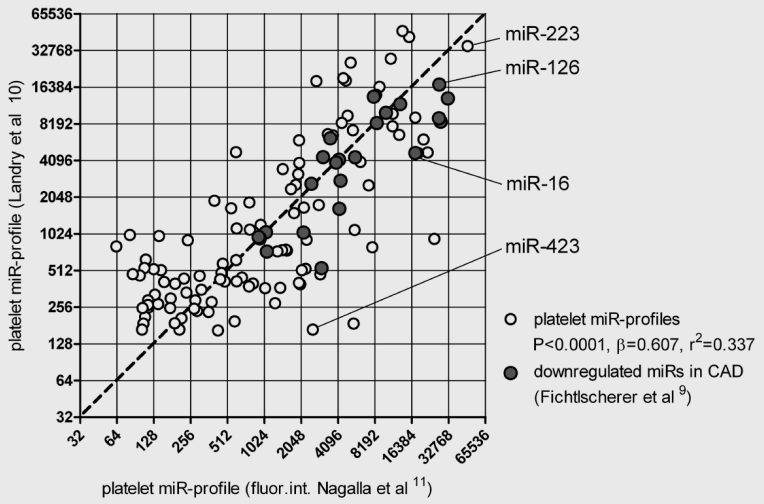


Figure 5. Rank correlation analysis of platelet-derived miRs
Expressed in fluorescence intensities (fluor. int; log 2 scales) as reported by Nagalla et al [11] (X-axis) and by Landry et al [10] (Y-axis), show a significant linear regression ($P<0.0001$). The top 23 miRs that were shown to be significantly down-regulated in the plasma of aspirin-treated CAD patients [9] are shown in grey circles. The location of miR-126, miR-223, miR-16 and miR-423 are shown separately. The optimal regression curve ($\beta=1.0$) is depicted as dotted line.

(ACS), which implies extensive myocardial injury, increased concentrations of systemic miR-126 were documented as compared to patients with CAD, displaying non-elevated troponin levels23. In addition, a significant increase was reported for sP-sel in troponin-positive ACS patients as compared to troponin-negative patients and a positive correlation was found between troponin levels and sP-sel [24].

Of note, in both studies, all patients (CAD and ACS) were treated with aspirin. Apparently, in the acute phase of the coronary syndrome platelet activation exceeds the inhibitory effect of aspirin, leading to increased levels of both sP-sel and miR-126, while in the chronic phase of the disease, as in CAD, effective platelet inhibition by aspirin may lead to corresponding reduced levels of both miR-126 and sP-sel.

In conclusion, aspirin-use should be taken into account when using circulating miR-126 and probably other platelet-associated miRNAs, as diagnostic biomarker for cardiovascular diseases or when studying a possible role of these miRNAs as mediators of cardiovascular disease and/or in athero-protective effects.

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