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

Hetty C. de Boer 1*, Coen van Solingen 1, Jurriën Prins 1, Jacques M.G.J. Duijs 1, Menno V. Huisman 2, Ton J. Rabelink 1, and Anton Jan van Zonneveld 1

1Department of Nephrology and the Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands; and 2Department of Thrombosis-Haemostasis and the Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands

Received 6 August 2012; revised 23 November 2012; accepted 3 January 2013; online publish-ahead-of-print 4 February 2013

See page 3400 for the editorial comment on this article (doi:10.1093/eurheartj/eht032)

Aims

MicroRNA-126 (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 of patients with type 2 diabetes (DM2) and how this is affected by aspirin.

Methods and results

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 DM2 by measuring soluble P-selectin, correlated directly with circulating levels of miR-126. The administration of aspirin resulted both in platelet inhibition and concomitantly reduced circulating levels of platelet-derived microRNAs including miR-126.

Conclusion

Platelets are a major source of circulating miR-126. Consequently, in patho-physiological conditions associated with platelet activation, such as diabetes type 2, the 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.

Keywords

miR-126 • platelets • aspirin • diabetes mellitus type 2

Introduction

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

Methods

In vitro platelet activation

To establish a relationship 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...
whether the PFP preparations were cell free, all samples were analysed to check that the PRP did not contain any leukocytes, the forward (FSC)/side (SSC) FACS-plots (logarithmic scales) are shown from one donor (Supplementary material online, 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 (anthractase) 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 99.9% 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 (phosphate-buffered saline supplemented with 1% bovine serum albumin and 0.05% Na-azide), incubated with mouse IgG directed against human P-selectin 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 the fluorescent signals above background and represents P-selectin-positive platelets. The 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.

MiR-expression levels
Total RNA 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 the 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-min incubation at 65°C of 2.0 μL (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 an M-MLV First-Strand Synthesis system (Invitrogen). The results were normalized using Gene Expression Analysis for iCycler IQ® RT-PCR Detection System (Bio-Rad Laboratories, Venendaal, The Netherlands). To compare the miR-levels, the concentration (fmol/mL) of miRs was calculated by correlating the mean miR-238 CT values with the spiked concentration of miR-238 added to the samples or miR-expression was shown as ‘fold change’, calculated with a standard ΔΔCT method.

Plasma samples of type 2 diabetes mellitus patients
Platelet-free plasma samples were obtained from patients diagnosed with DM2, who previously entered a prospective, randomized study with a placebo-controlled, double-blind, crossover design of which details have been published previously. All patients gave written informed consent and the study was approved by the institutional medical ethics committee. The baseline characteristics of this patient cohort are shown in Supplementary material online, Table S1. Subjects were excluded if they had any history of cardiovascular disease (defined as myocardial infarction, acute coronary syndrome, percutaneous coronary intervention, coronary artery bypass grafting, heart failure, severe cardiac arrhythmia, cerebrovascular accident, transient ischaemic attack, or peripheral vascular disease) or known contraindications to use of aspirin (defined as history of asthma, any bleeding disorder, gastrointestinal tract bleeding or known allergy to acetylsalicylic acid). Other exclusion criteria were the presence of uncontrolled hypertension, severe renal or hepatic dysfunction, pregnancy, concurrent participation in other research projects or blood donation and use of non-steroidal anti-inflammatory drugs, anticoagulant medication, corticosteroids, or statins.

In summary, 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-sel value was missing. These patients were excluded. For two patients insufficient amounts of plasma were present to determine the levels of miR-126, yielding 18 miR-126 values for both the ‘responder’ and the ‘non-responder’ groups.

Statistical analysis
All calculations were performed with Graphpad Prism software. Approximate normal distributions were determined by D’Agostino and
Pearson normality test. To calculate significant differences \((P < 0.05)\) for more than two variables (Figures 1 and 2) a repeated measures one-way ANOVA test was performed with Bonferroni multiple comparison test correction; for two variables a two-tailed Mann–Whitney test was performed since the values were not distributed normally (Supplementary material online, Table S1, Figure 4A, B and C). For correlation analysis, Spearman \(r\) correlation coefficients and P-values were determined, since these values were also not distributed normally (Figures 3, 5 and Supplementary material online, Figure S2).

**Results**

**In vitro platelet activation**

To investigate the correlation between platelet activation and miR-126 concentration in plasma, peripheral blood was withdrawn from healthy volunteers. After obtaining PRP, platelet activation was induced with arachidonic acid (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 (Supplementary material online, 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, \(P < 0.05\)). AA stimulation resulted in a significant increase in sP-sel in the plasma compartment (PFP) obtained from PRP (Figure 1A, right axes, \(P < 0.01\)). The addition of aspirin alone did not alter the 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 \((P < 0.01)\). Apparently, aspirin is able to prevent the shedding of sP-sel from the platelets, while membrane expression of P-selectin is not inhibited completely.

MiR-126 levels were measured in PFP or the platelet pellet, isolated from the same PRP sample and the relative distribution ratio (PFP/plts) was calculated. Non-activated PRP showed a distribution ratio of \(\sim 1:9\) (PFP/plts, \(0.109 \pm 0.036\), Figure 2) and AA activation resulted in a significant transfer of miR-126 \((P < 0.01)\) from the platelet to the plasma compartment, yielding a relative ratio of 1:4 \((+\text{AA, } 0.259 \pm 0.05)\). AA activation in the presence of aspirin prevented the transfer of miR-126 towards the plasma compartment \((P < 0.01)\), which showed a similar ratio of 1:8 \((0.121 \pm 0.018)\) as non-activated PRP. As three other platelet-enriched miRs (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 the shedding of P-selectin from the platelet membrane and the release of miRs from intracellular platelet stores.

**In vivo platelet activation**

To investigate the relationship between plasma levels of miR-126 and in vivo platelet activation, we studied patients with DM2, who exhibit a disease-mediated platelet activation.\(^{14}\) A description of the baseline characteristics of the patient cohort is available in the Supplementary material online, Table S1. These patients had participated in a randomized, placebo-controlled cross-over trial, in which 40 patients were 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,\(^ {15}\) which is consistent with increased platelet aggregability in chronic vascular diseases, such as DM2.\(^ {14}\)

Figure 3A shows that in the total cohort of patients, a significant correlation was observed after placebo \((P = 0.006, r = 0.446)\) between the extent of in vivo platelet activation, as measured by the absolute level of sP-sel (ng/mL) and the level of circulating miR-126 (fold change). Also after treatment with aspirin, regardless of the dose, a significant correlation was found (Figure 3B, asp-100 + 300, \(P = 0.003, r = 0.495\)). When discriminating for aspirin dose, only the higher dose of 300 mg/day showed a significant correlation \((P = 0.015, r = 0.578)\), while the lower dose was not significant \((P = 0.128, r = 0.373)\), which is most likely due to...
**Figure 2** The relative distribution ratio of miR-126, miR-16, miR-223 (left axis), and miR-423 (right axis) in platelet-free plasma vs. platelets (PFP/plts) is shown in treated vs. non-treated platelet-rich plasma obtained from healthy volunteers. As negative control miR-263, not present in the platelets, was measured. *P* < 0.05, **P** < 0.01.

**Figure 3** *In vivo* platelet activation in type 2 diabetes patients shows a correlation with miR-126 levels. Spearman correlation analysis was performed on (A) miR-126 (fold change) vs. soluble P-selectin (ng/mL) in the plasma of type 2 diabetes patients after the placebo period (n = 18) and (B) after aspirin treatment in all patients (asp-100 + 300) or subdivided in patients with low dose (asp-100) or high dose (asp-300) of aspirin. (C) The change in miR-126 (ratio asp/plac) vs. delta soluble P-selectin values (asp-plac) is shown or (D) the change in miR-126 vs. delta vWF values (asp-plac) in response to aspirin treatment. Note the logarithmic scale for miR-126 in (C) and (D).
the large variation between the values, since the three fitted correlative lines practically overlapped.

Of note, aspirin inhibits the enzyme cyclo-oxygenase (COX), which is expressed in two isoforms, COX-1 and COX-2. Platelets predominantly express COX-1 which is more sensitive to inhibition by aspirin than COX-2, while endothelial cells mainly express COX-2. In vivo, the pool of circulating miR-126 may be derived from both the platelets and the endothelium, but aspirin treatment will have a dominant inhibitory effect on the release of the platelet-derived miR-126. To have insight into the platelet-mediated effects, changes in circulating sP-sel (delta sP-sel: sP-sel values at the time of aspirin treatment minus placebo values; asp-plac) and in circulating miR-126 (ratio miR-126: miR-126 values at the time of aspirin treatment divided by the placebo values; asp/plac) were calculated. Correlation analysis showed that delta sP-sel positively correlated with the ratio of miR-126 (Figure 3C, \( P < 0.0001, r = 0.675 \)). When discriminating for aspirin dose, correlation analysis showed almost identical statistically significant values (Supplementary material online, Figure S2: aspirin 100 mg/day: \( P = 0.001 \), Spearman \( r = 0.682 \); aspirin 300 mg/day: \( P = 0.008 \), Spearman \( r = 0.640 \)).

As shown previously, 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 vWF in the plasma. When calculating delta vWF values (asp-plac), no difference was observed between the responder and non-responder groups (Figure 4C, \( P = 0.459 \)) and correlation analysis for delta-vWF values and the ratio of miR-126 showed no significance (Figure 3D, \( P = 0.317 \), \( r = -0.177 \) and Supplementary material online, Figure S2B), indicating that sP-sel levels, and thus miR-126 levels, were not changed due to activation/damage of the endothelium. These data illustrate that platelets are an important cellular source of circulating miR-126 and that anti-platelet medication may be a confounder when studying patients with manifest vascular disease, such as DM2 (this paper), CAD, or myocardial infarction.

**Discussion**

Previous reports show that miRs in plasma display exceptional stability in the circulation due to their association with argonaute protein, high-density lipoprotein, or their inclusion into exosomes or microparticles. In fact, it has recently been shown that the main fraction of miRs in human plasma is localized in microparticles of which 41–45% originate from platelets. These miR-containing microparticles may actually play a role in cardiovascular diseases by transferring their miR 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 and miR-126-containing microvesicles protected the kidney against ischaemia/reperfusion injury in mice. These functional properties and the notion that circulating miRs may be derived from cells in the vascular system has stirred current interest in the use of circulating miR profiles as diagnostic- and maybe even prognostic biomarkers for the progression of cardiovascular disease.

The patho-physiological mechanisms involved in vascular diseases, such as DM2 and CAD, include EC dysfunction and ongoing vascular injury. Since miR-126 is enriched in endothelial cells, these vascular diseases would predict a release of endothelial-derived miR-126 into the circulation. However, in a
recent study, a counter-intuitive decrease in miR-126 was observed in CAD patients in comparison with 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. Our in vivo observations described in this paper show that aspirin administration to DM2 patients resulted in platelet inhibition and concomitant reduction of circulating levels of miR-126. As a consequence, aspirin use could explain the reported decrease in circulating miR-126 in the CAD patient cohort.9 Furthermore, an overall systemic inhibition of platelet activation would predict a decrease in other platelet-derived miRs as well. Indeed, our in vitro platelet activation assay showed that the transfer from the platelet to the plasma compartment of three other miRs 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 miRs 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 miRs10,11 of which miR-126 was ranked number 5. This is further illustrated in Figure 5, in which the platelet-miR profiles from two independent papers are combined10,11 displaying a very similar rank order with a highly significant regression for quantified signals of the miR arrays ($P < 0.0001$, Spearman $r = 0.752$). In contrast, of the 20 miRs reported to be upregulated to the highest extent in the aspirin-treated CAD patients,9 only 4 miRs are present in the list of platelet-associated miRs and these 4 miRs 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 study17 and the CAD study,9 the release of platelet-derived miRs 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

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

### Limitations of the study

In the current study, statin use was one of the criteria to exclude patients from entering the trial.13 From a clinical point of view, this may be a drawback since statins are a baseline treatment, thus rendering our patient cohort not fully representative for DM2 patients on standard treatment. Nevertheless, next to a negative effect of aspirin on the level of circulating miR-126 in the CAD patients, Fichtlscherer et al.9 also noticed a negative effect of statin use. Furthermore, Luzak et al.25 showed in vitro, that the inhibiting effect of aspirin on ADP-induced platelet activation and aggregation was potentiated in the presence of statins. These data would predict that for the prevention of cardiovascular events in patients with diabetes mellitus, treatment with a combination of aspirin and statin, as recommended by Udell et al.,26 may show an additive

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**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), shows a significant linear regression ($P < 0.0001$, Spearman $r = 0.752$). The top 23 miRs that were shown to be significantly downregulated in the plasma of aspirin-treated coronary artery disease patients9 are shown in grey circles. The location of miR-126, miR-223, miR-16, and miR-423 are shown separately. The optimal regression curve (correlation coefficient = 1.0) is depicted as underbroken line.
inhibitory effect on platelet activity and thus on the level of plasma miR-126.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

**Funding**

This work was supported in part by grants from the Netherlands Heart Foundation (2006B145), the Dutch Kidney Foundation (C07.2227) and the European Union (project CIRCULATING CELLS, grant 01C-102).

**Conflict of interest:** none declared.

**References**


