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Association of MicroRNAs and YRNAs with Platelet Function

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Abstract

Rationale—Platelets shed microRNAs (miRNAs). Plasma miRNAs change upon platelet inhibition. It is unclear if plasma miRNA levels correlate with platelet function.

Objective—To link small RNAs to platelet reactivity.

Methods and Results—Next-generation sequencing of small RNAs in plasma revealed two peaks at 22-23 and 32-33 nucleotides corresponding to miRNAs and YRNAs, respectively. Among YRNAs, predominantly fragments of RNY4 and RNY5 were detected. Plasma miRNAs and YRNAs were measured in 125 patients with a history of ACS who had undergone detailed assessment of platelet function 30 days after the acute event. Using quantitative real-time polymerase chain reactions, 92 miRNAs were assessed in ACS patients on different anti-platelet therapies. Key platelet-related miRNAs and YRNAs were correlated with platelet function tests. MiR-223 ($r_p=0.28$, $n=121$, $P=0.002$), miR-126 ($r_p=0.22$, $n=121$, $P=0.016$), other abundant platelet miRNAs and YRNAs showed significant positive correlations with the vasodilator-stimulated phosphoprotein phosphorylation assay. YRNAs, miR-126 and miR-223 were also among the small

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Disclosure

King's College London filed and licensed patent applications related to circulating miRNAs as biomarkers. R.F.S. reports receiving research grants, honoraria and/or consultancy fees from AstraZeneca, Aspen, ThermoFisher Scientific, Eli Lilly/Daiichi Sankyo, Merck, Accumetrics, Novartis, Corveio, PlaqueTec, Sanofi-Aventis, Medscape, Regeneron, Roche and The Medicines Company.

RNAs showing the greatest dependency on platelets, and strongly correlated with plasma levels of P-selectin, platelet factor 4 and platelet basic protein in the population-based Bruneck study (n=669). A single nucleotide polymorphism that facilitates processing of pri-miR-126 to mature miR-126 accounted for a rise in circulating platelet activation markers. Inhibition of miR-126 in mice reduced platelet aggregation. MiR-126 directly and indirectly affects ADAM9 and P2Y₁₂ receptor expression.

Conclusions—Levels of platelet-related plasma miRNAs and YRNAs correlate with platelet function tests in ACS patients and platelet activation markers in the general population. Alterations in miR-126 affect platelet reactivity.

Keywords

Acute coronary syndrome; antiplatelet therapy; biomarker; microRNA; platelet

Subject Terms

Functional Genomics; Secondary Prevention; Platelets; Treatment

Introduction

MiRNAs are small non-coding RNAs with cell-type specific expression patterns that are released by cells into the circulation as part of membranous particles or protein complexes.¹ Thus, miRNAs can be readily quantified by real-time polymerase chain reactions (qPCR) in plasma and serum and have generated increasing interest as potential new biomarkers.² Our group has previously identified platelet-related miRNA signatures that are predictive of cardiovascular events.³ Additionally, we measured miRNAs in healthy volunteers and in patients with symptomatic atherosclerosis before and after initiation of dual anti-platelet therapy and demonstrated reduced plasma levels of platelet-related miRNAs upon platelet inhibition.⁴

Dual oral antiplatelet therapy (aspirin (ASA) + a P2Y₁₂ inhibitor) is commonly used for the management of non-ST-elevation acute coronary syndromes (ACS) and ST-elevation myocardial infarction (STEMI).⁵ ASA irreversibly inhibits cyclooxygenase 1 in platelets, thereby repressing thromboxane A₂ (TxA₂) synthesis and, consequently, platelet activation. Clopidogrel, prasugrel and ticagrelor target the P2Y₁₂ receptor for adenosine diphosphate (ADP). However, interindividual variability in the platelet response to clopidogrel has been reported. Prasugrel and ticagrelor exhibit a more consistent anti-platelet effect and have shown benefits over clopidogrel in ACS patients but also increase the risk of bleeding.^{6,7} It is currently unclear whether plasma levels of platelet-related miRNAs correlate with the residual platelet activity in ACS patients and how different anti-platelet agents alter miRNAs.

In this study, we used RNA sequencing to characterize small RNAs in plasma. Then, we compared the effect of different anti-platelet agents and explored the association of small RNAs (miRNAs and YRNAs) with platelet function tests in ACS patients. Moreover, we correlated their plasma levels to platelet activation markers in the prospective, population-

based Bruneck study³ and investigated if a single nucleotide polymorphism (SNP) that facilitates miR-126 processing⁸ alters circulating miR-126 levels and platelet reactivity. These epidemiological observations were complemented by preclinical studies, assessing platelet function in mice upon treatment with antagomiRs directed against miR-126, and by mechanistic studies measuring miR-126 targets.

Methods

An expanded Methods section is available in the Online Data Supplement.

Next-generation Sequencing (NGS)

Small RNA libraries were generated from non-normalized RNA (ranging from 375 pg to 1 ng) extracted from equal volumes of platelet-poor (PPP) and platelet-rich plasma (PRP) from healthy human volunteers. Prior to library preparation, RNA was spiked with equal amounts of *C. elegans* miR-39 star (*cel-miR-39**) to assist in normalization. Libraries were prepared using the small RNA library preparation kit v2.0 (Illumina Cambridge Ltd) according to manufacturer's protocol with limited modifications. In brief, adapters were ligated to the 3' end and 5' end of RNA followed by cDNA preparation through RT. The cDNA was amplified with 12 cycles of PCR. The resulting library was purified using Agencourt Ampure XP beads (Beckman Coulter Inc). Libraries were quantified using Qubit (Life Technologies) and Bioanalyzer (Agilent). Since the total amount of libraries generated was limited, the libraries were mixed together in non-equimolar concentrations and sequenced on HiSeq2000 (Illumina Cambridge Ltd). Traces of the adapter (TGGAATTCTCGGGTGCCAAGG) at the end of the 22 nucleotides for miRNAs were removed using the "Trimalore" software, allowing for 10% mismatch and discarding reads that, after adapter removal, became shorter than 15 nucleotides. More than 99.5% of the reads were reduced in size after adapter removal.

RNA Isolation, Reverse Transcription (RT) and Pre-amplification

RNA isolation, RT reaction and pre-amplification as well as individual qPCRs for miRNAs were performed as described previously.^{3,4}

Custom-designed qPCR Plates

The expression profile of 92 miRNAs was assessed using custom-made Exiqon LNA qPCR plates (Exiqon Life Sciences) as described previously.⁴

ACS Study Population and Sample Collection

Plasma samples were obtained from a patient-based cohort of 125 patients with a history of ACS 30 days previously (STEMI, NSTEMI or unstable angina) who had undergone detailed assessment of platelet function.⁵ The Northern General Hospital, Sheffield, UK, is the only center providing PCI and cardiac surgery services to the surrounding population of approximately 1.8 million people and protocols for antiplatelet therapy are standardized and implemented synchronously across this region. This was a prospective observational study intended to phenotype ACS patients, including assessment of the effects of changing patterns of P2Y₁₂ inhibitor usage. Treatment algorithms for oral antiplatelet and other

secondary prevention therapies in ACS patients are described in Joshi *et al.*⁵ All the patients recruited for the study provided informed consent and the study was approved by the local research ethics committee. The exclusion criteria were less than 18 years of age, serious comorbidities, end-stage renal failure and pregnancy or suspected pregnancy. Patients enrolled in the study attended the Clinical Research Facility, Northern General Hospital, Sheffield, UK, at 30 days after the onset of ACS. Venepuncture with needle and syringe was used to obtain venous blood samples that were anticoagulated with 3.13% trisodium citrate dihydrate (“citrate”).

Human Platelet Function Testing

(1) *Light transmittance aggregometry (LTA)*: Citrate-anticoagulated blood was first centrifuged at 200 RCF for 10 min from which PRP was extracted. The remaining blood was then centrifuged again at 1500 RCF from which PPP was extracted. A platelet count was performed on the PRP and no dilution with PPP was performed unless the platelet count was greater than $400 \times 10^9/L$ in which case PPP was used to dilute the PRP to a platelet count of $400 \times 10^9/L$. Platelet aggregation response to ADP was determined using LTA (BioData PAP-8E optical aggregometer). The maximum platelet aggregation response to ADP 20 μM or arachidonic acid 2 mM was determined after 6 minutes. (2) *VerifyNow P2Y₁₂ assay*: Citrate-anticoagulated blood was analyzed using VerifyNow P2Y₁₂ cartridges and VerifyNow analyzer (Accumetrics, USA) according to the manufacturer’s instructions. Platelet reaction units (PRU) were recorded. (3) *VASP phosphorylation assay*: Citrate-anticoagulated blood was analyzed using VASP phosphorylation assay kits (BioCytex, France) and platelet reactivity index (PRI) was determined, according to the manufacturer’s instructions. Fluorescence of samples was measured using an LSRII flow cytometer (Becton Dickinson).

Bruneck Cohort

The Bruneck Study is a population-based, prospective survey of the epidemiology and pathogenesis of atherosclerosis and cardiovascular disease.^{3,9} The study protocol was reviewed and approved by the Ethics Committees of Verona and Bolzano, and all participants provided their written informed consent before entering the study. At the 1990 baseline evaluation, the study population comprised an age- and sex-stratified random sample of all inhabitants of Bruneck (125 men and 125 women from each of the fifth through eighth decades of age, all white). Samples from the year 2000 follow-up were used for the present study (n=669). As part of the 2000 follow-up, citrate plasma and serum samples were drawn after an overnight fast and 12 hours of abstinence from smoking. Samples were divided into aliquots and immediately stored at $-80^\circ C$.

Platelet Spike-in

Human platelets and plasma were isolated from 4 healthy volunteers. 16 mL of whole blood was drawn onto 4 mL of Acid-Citrate-Dextrose (ACD; stock solution: 2.5 g sodium citrate, 2.0 g glucose, 1.5 g citric acid, in 100 mL H₂O) using a 21-gauge needle and 20 mL syringe and centrifuged at $190 \times g$ for 30 min. All centrifugations were performed at room temperature without brake. The supernatant was transferred into a new tube. To prevent platelet activation, 1 nM prostaglandin E1 (Sigma-Aldrich) and 20 μM indomethacin

(Sigma-Aldrich) were added. To deplete leukocytes the supernatant was further centrifuged at $280 \times g$ for 10 min. 100 μ L of the leukocyte-depleted PRP was transferred to a 1.5 mL tube for RNA extraction. The remaining volume was transferred to a new tube and centrifuged at $1180 \times g$ for 10 min to pellet platelets. The resulting supernatant was PPP. The platelet pellet was washed twice with modified Tyrode's buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na_2HPO_4 , 12 mM NaHCO_3 , 20 mM HEPES, 1 mM MgCl_2 , pH 7.4; Glucose (45 mg/50 mL) was added just before use, and the solution was warmed to 37 °C in a water bath). Prostaglandin E1 and indomethacin were added during each wash at the concentrations mentioned above. The solution was centrifuged between washes at $1180 \times g$ for 10 min. The final platelet pellet was resuspended in 1/20 of the volume of original PRP to obtain a 20x stock platelet solution. The 20x platelet solution was then spiked back into the PPP from the same donor to achieve 200%, 100%, 50% and 5% spike-ins. Immediately after spiking PPP, QIAzol lysis reagent was added and RNA was extracted as described previously.^{3,4}

Systemic Inhibition of miR-126-3p

Male C57BL/6J mice (Harlan), aged 8 weeks, were treated with cholesterol-conjugated antagomiR constructs (Fidelity Systems, Gaithersburg, MD) or sterile PBS. Sequences were designed to target miR-126-3p (5'-C*G*CAUUAUUACUCACGGU* A*C*G*A*-Chol*T-3') or to serve as non-targeting control (55'-A*A*GGC AAGCUGACCCUGAA*G*U*U*Chol*T-3'). Intraperitoneal injections were performed on day 0, 1 and 2, in a dose of 25 mg/kg for the platelet function assays in whole blood, and 40 mg/kg for the aggregometry experiments carried out in PRP. On day 7, mice were anesthetized using pentobarbital before collection of blood from the inferior vena cava using syringes containing lepirudin (Refludan, 25 μ g/ml; Celgene, Windsor, UK). PRP was isolated as previously described¹⁰. Briefly, whole blood was diluted 1:1 with HEPES-Tyrode's buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 1 g/l BSA, 1 mM MgCl_2 , 2.7 mM KCl, 3.3 mM NaH_2PO_4) before centrifugation ($100 \times g$, 8 min, RT).

Platelet Function in Mice

Platelet function tests were carried out as described previously.¹¹ Half-area 96-well microtiter plates (Greiner Bio-One, Stonehouse, UK) were pre-coated with hydrogenated gelatin (0.75% w/v; Sigma, UK) in PBS to block non-specific activation of blood. 4 μ l of vehicle or agonist solution was then added to each well: arachidonic acid (AA; 0.03–0.6 mM; Sigma, Poole, UK), Horm collagen (0.1–3 μ g/ml; Nycomed, Linz, Austria), the PAR-4 activating peptide AYPGKF amide (PAR4-AP, 50–100 μ M; Bachem, Bubendorf, Switzerland), and the stable TxA_2 mimetic U46619 (0.1–10 μ M; Cayman Chemical Company, Ann Arbor, USA). To each well, 35 μ l of PRP or whole blood was added and the plate was then placed onto a heated plate shaker (Bioshake IQ, Q Instruments, Jena, Germany) at 37 °C for 5 min mixing at 1200 rpm. Where appropriate, light transmission of each well was determined using a 96-well plate reader (SunriseTM, Tecan, Männedorf, Switzerland) at 595 nm. Alternatively, samples were diluted 1:5 with an acid citrate dextrose solution (5 mM glucose, 6.8 mM trisodium citrate, 3.8 mM citric acid) before individual platelet counts of each were determined by flow cytometry. Platelets were labeled with APC-conjugated anti-CD41 (clone eBioMWR30) for 30 min before further dilution 1:50

in phosphate buffered saline containing 0.1% formalin (Sigma, UK), 0.1% dextrose and 0.2% bovine serum albumin (BSA) and addition of 104 CountBright™ absolute counting beads (Life Technologies). Labeled, diluted blood was then analyzed using a FACSCalibur flow cytometer (BD Biosciences, Oxford, UK).

Results

NGS of plasma miRNAs

In order to characterize circulating small RNAs, libraries of small RNAs were generated from platelet-rich (PRP) and platelet-poor (PPP) plasma for NGS. The presence of platelets and the absence of leukocyte contamination in PRP samples were verified by qPCR for ITGA2B and CD45, respectively (Online Figure IA). The quality of base-calls is represented by consecutive boxplots for each position on the 100 nucleotide read (Online Figure IB). The total number of digital reads correlated with the amount of RNA obtained from PPP and PRP ($r_p=0.94$). Two peaks were observed across all four samples, one at 22-23 nucleotides corresponding to miRNAs, and another centered around the 32-33 nucleotide range (Fig. 1A), which aligned predominantly to RNY4, a non-coding RNA, on chromosome 7. The 25 most abundant small RNAs in each sample are shown in Fig. 1B. At a threshold of 0.4 reads per million and without distinguishing between isomiRs, 224 miRNAs were consistently identified in all samples (Online Table I). There was a good correlation for the number of reads per miRNA (Online Figure IC). Coverage of RNY4 was predominantly observed on the 5' end, with some additional fragments from the 3' end. Sequences derived from other RNY genes were also detected; albeit at much lower read counts: For RNY1 and RNY5, like RNY4, mostly 5' fragments were found; for RNY3 the 3' fragments were more abundant (Online Figure II). 203 out of 224 miRNAs and both RNY4 fragments were detected at higher levels in PRP than PPP, suggesting that most circulating small RNAs are present in platelets (Online Figure III).

Effect of anti-platelet therapy

We have previously reported that anti-platelet therapy reduces plasma levels of platelet-related miRNAs, including miR-126 and miR-223.4 92 miRNAs were measured using custom-made Exiqon LNA qPCR plates. The plate layout has been published previously⁴ and is shown in Online Table II. In a PCA, the interindividual variability in plasma miRNA profiles decreased with prolonged platelet inhibition⁴ (Fig. 2A), reinforcing the concept that platelet activity is an important determinant of the plasma miRNA pool. To explore how different anti-platelet agents affect plasma miRNAs, the same 92 miRNAs were screened in a closely matched cohort of ACS patients who were either on ASA only (n=8), ASA+clopidogrel (n=8), ASA+prasugrel (n=8) or ASA+ticagrelor (n=8) for 30 days after the acute event. In the PCA analysis, no clear separation was obtained between the different anti-platelet agents but the least inter-patient variability was observed in the ASA+prasugrel group (Fig. 2B).

Correlation to platelet function in ACS patients

Next, platelet-related miRNAs (Online Figure IV) and abundant plasma YRNA fragments were measured by individual qPCR assays in the ACS patient cohort who had undergone

detailed assessment of platelet function (n=125, Online Table III). The correlations of small RNAs to measurements of platelet function are shown in Fig. 3. No correlation was found between miRNA and YRNA levels and optical aggregometry in response to arachidonic acid (AA) or ADP. In contrast, significant associations were obtained for the VASP phosphorylation assays, e.g. miR-223 ($r_p=0.28$, $P=0.002$), miR-24 ($r_p=0.25$, $P=0.006$), miR-191 ($r_p=0.24$, $P=0.009$), RNY4 3' ($r_p=0.23$, $P=0.012$), miR-126 ($r_p=0.22$, $P=0.016$) and RNY4 5' ($r_p=0.21$, $P=0.025$). For these miRNAs and YRNA fragments, the correlations with the VerifyNow P2Y₁₂ assay were of similar strength ($r_p=0.18-0.35$), but the statistical power was weaker in view of low numbers and nominal significance was only noted for miR-126 ($r_p=0.35$, $p=0.033$). No associations were obtained for miR-93, miR-106a, miR-146b and miR-150.

Evidence for platelet origin

Although all candidate miRNAs were present in platelets (Online Figure IV), some reflected platelet activation better than others. To determine to what extent platelets may contribute to circulating miRNA and YRNA levels, we reconstituted PPP with washed platelets. Platelets were isolated from PRP and spiked back into PPP corresponding to 5, 50, 100 or 200% of the initial volume (Fig. 4A). For miR-126 and miR-223, a significant linear increase was observed with increasing platelet content (Fig. 4B). As expected, the liver-specific miRNA, miR-122, was not affected by addition of platelets. Notably, miR-126 showed the greatest dependency on platelets in comparison to 21 other miRNAs (Fig. 4C). This spike-in experiment provides further evidence for platelets being a major source of circulating miRNAs, including miR-126, which was previously implicated to be of endothelial origin. 12–14 Levels of RNY4 fragments were also strongly affected by addition of platelets (Fig. 4C). Unlike miRNAs, however, RNY4 was not associated with argonaute-2 complexes in MEG-01 cells, a human megakaryoblastic cell line (Online Figure V).

Mir-126 and platelet function in the general population

MiRNAs and RNY4 fragments were measured in the Bruneck study (n=669) and correlated to the platelet activation markers platelet factor 4 (PF4), pro-platelet basic protein (PPBP) and P-selectin (SELP) as quantified by ELISA (Fig. 5A). Substantial positive correlations of miRNAs and RNY4 fragments with all three platelet-activation markers were observed in plasma. These were more pronounced for the platelet-specific proteins PF4 and PPBP than for SELP, which is shed from platelets as well as endothelial cells.^{16,17} The liver-specific miRNA, miR-122, and other miRNAs, such as miR-150, showed no or considerably weaker correlations with platelet activation markers (Fig. 5A). Accordingly, there was a striking correlation between the platelet dependency of miRNAs in the spike experiment and the correlations of miRNAs with platelet activation markers in the general population ($r=0.92$ to 0.94 , Fig. 5B, Online Fig. VI). RNY4 fragments were strongly correlated with platelet-derived plasma miRNAs (Fig. 5C), suggesting a common platelet origin.

While circulating miRNAs have been shown to be affected by disease state, cardiovascular risk factors and drug treatment, the influence of genetic variability, especially single nucleotide polymorphisms (SNPs), on miRNA expression and function is poorly understood. Thus far, only one functional SNP has been described for miR-126 (Fig. 6A): The primary

sequence of human miR-126 contains a SNP (dbSNP: rs4636297) downstream of the pre-miR sequence.⁸ The genotype of this SNP has been shown to affect the processing of pri-miR-126: pri-miR-126 encoded by the major “G” allele is processed to a lesser extent than pri-miR-126 encoded by the minor “A” allele. In the Bruneck cohort (Online Table IV), there was a trend towards higher miR-126 levels in individuals homozygous for the minor allele (AA genotype), that facilitates processing of miR-126, compared to the GA + GG genotype in serum (+4.4%, $P=0.050$) and in plasma (+6.9%, $p=0.099$) (Online Fig. VII). Importantly, the AA genotype was associated with higher plasma levels of platelet activation markers: PF4 ($p=0.002$), PPBP ($P<0.001$) and SELP ($p=0.099$) (Fig. 6B). To compare the effect on platelet proteins to other plasma proteins, we measured 219 proteins, utilizing mass spectrometry for detection of high-abundant proteins ($n=84$) and proximity extension assays for detection of low-abundant proteins ($n=132$) as well as ELISA ($n=3$). Out of 62 proteins whose levels are significantly associated with the AA genotype (Online Fig. VIII), PPBP and PF4 showed the 2nd and 3rd highest fold change (mean ratios AA vs GA + GG of 1.49 and 1.47, respectively, Online Table V). The proteins associated with the AA genotype also showed an enrichment of the GO term annotation “platelet activation” ($p = 0.019$, Online Fig. VIII).

MiR-126 and platelet function in mice

The genetic associations of a SNP within miR-126 with parameters of platelet function prompted us to further investigate the role of miR-126 in platelet activation. It has recently been reported that oligonucleotides with a phosphorothioate backbone modification (PS, minimum length of 18 nt) can activate platelets.¹⁸ Thus, we incubated human PRP with fully PS-modified LNAs as well as antagomiRs with 2 and 4 PS-modified nucleotides at the 5' and 3' end, respectively. None of the tested oligonucleotides induced platelet aggregation (Online Fig. IX). Next, C57BL/6J mice were injected with PBS, a control antagomiR or antagomiR-126-3p at 25 mg/kg i.p. for three consecutive days and sacrificed at day 7 (Fig. 7A). The knockdown of miR-126 was confirmed in blood samples by qPCR (Online Fig. X). Whole blood was treated with different concentrations of agonists: arachidonic acid (AA), collagen, PAR4-AP and the TxA_2 analog U46619 (Fig. 7B). The aggregation response to arachidonic acid and U46619 was significantly reduced in platelets from mice treated with antagomiR-126-3p. At antagomiR doses of 25 mg/kg, however, there was no effect on platelet aggregation in response to collagen or PAR4-AP. Thus, we repeated the experiment with a higher antagomiR concentration (40 mg/kg i.p.). Again, the inhibition of miR-126 was confirmed by qPCR (Online Fig. X). To further address the effect of miR-126 in platelets and to minimize a potential influence of other cell types, PRP was used instead of whole blood for platelet aggregation. AntagomiRs against miR-126-3p blocked platelet aggregation induced by 0.6 mM AA (Fig. 7C), and led to a significant reduction of aggregation in response to 50 μ M PAR4-AP. The attenuated response to 0.3 μ g/ml collagen failed to reach statistical significance. No differences in platelet aggregation were observed at higher agonist concentrations of PAR4-AP (100 μ M) and collagen (3 μ g/ml) (data not shown).

Targets of miR-126 in platelets

To explore potential mechanisms, we measured the expression levels of known miR-126 targets with a role in platelet function alongside platelet-related genes (Online Table VI). We observed reduced expression of the P2Y₁₂ receptor in whole blood of antagomiR-126-3p treated mice (Fig. 8A). In a human megakaryoblastic cell line (MEG-01), mimics or LNA inhibitors of miR-126-3p (Fig. 8B-C, Online Fig. XI) regulated ADAM9, a confirmed target of miR-126 that has been shown to impact on collagen-induced platelet aggregation (Fig. 8D).¹⁹ Thus, miR-126 affects gene expression in megakaryocytes.

Discussion

In this study, miRNA measurements were performed in 669 subjects of a population-based study as well as in 125 patients with ACS. The study correlates miRNAs with platelet activation markers in the general population and with the residual platelet activity in ACS patients on anti-platelet therapy. Most but not all abundant platelet miRNAs were positively correlated with the VerifyNow P2Y₁₂ and VASP assays, which are standardized assays for assessing the effects of P2Y₁₂ inhibitors. Additionally, we show an association of plasma YRNAs with platelets and that inhibition of miR-126 attenuates platelet aggregation in response to low but not high agonist concentrations.

Platelet dependency of small RNAs in plasma

To demonstrate the platelet dependency of our candidate miRNAs, washed platelets were isolated from PRP and spiked back into PPP. This spike-in experiment rules out a cellular contamination, which can hamper direct comparisons between PRP and PPP, and provide further evidence that the selected miRNAs, including miR-126, are genuine platelet miRNAs.⁴ Furthermore, we provide evidence that RNY4 fragments in plasma also originate from platelets. YRNA fragments are enriched in exosomes.^{20,21} Our NGS data confirm that RNY4 fragments are abundant in plasma.²² Unlike miRNAs, however, RNY4 fragments were not present in the Ago2 complexes of MEG-01 cells.^{23,24} Recently, YRNA fragments were implicated as biomarkers for coronary artery disease.²⁵ Changes in circulating levels were attributed to apoptotic macrophages, which generate and secrete YRNA fragments.²⁵ In contrast, our data suggest that platelets are a major source of circulating YRNA fragments, and cellular origin has to be taken into account if YRNA fragments are considered as potential biomarkers for cardiovascular disease. The function of YRNA fragments remains unknown, but it has been suggested that YRNA fragments, including RNY4 5', act as small guide RNAs for tRNase Z^L, forming a tRNA-like duplex with a target RNA, thereby enabling its hydrolysis.²⁶ This mechanism has been shown for synthetic target RNAs in vitro, and it remains to be seen whether YRNA fragments can act as guide RNAs for tRNase Z^L in vivo.

Platelet miRNAs in patients post ACS

Plasma was taken 30 days after the acute event when the inflammation associated with the acute injury has receded and anti-platelet drugs had been administered for a month. Importantly, none of the ACS patients had received heparin at the time of sampling.^{27,28} Levels of miR-126 and other platelet-related miRNAs showed a positive correlation with the

VerifyNow P2Y₁₂ and VASP phosphorylation assays. VerifyNow P2Y₁₂ is a commercial assay that uses whole blood to monitor P2Y₁₂ inhibition.²⁹ The VASP assay is considered to be among the most specific assays to monitor P2Y₁₂ inhibition since it does not rely on co-activation of the P2Y₁ receptor by ADP.³⁰ In contrast, optical aggregometry is performed on isolated platelets. Theoretically, aggregometry responds to ADP and the VASP and Verify Now P2Y₁₂ assays assess the same parameter: P2Y₁₂ receptor activation. However, the variability of the aggregometry results in isolated platelets being higher compared to tests performed in whole blood may explain the loss of associations with plasma miRNAs. Previous studies have suggested that VerifyNow P2Y₁₂ and VASP assays are more discriminating of clopidogrel response and its genetic influences compared to light transmittance aggregometry, and there are only moderate correlations between the different platelet function assays.^{30,31} Moreover, aspirin is such a potent inhibitor of the platelet response to AA that any variation between patients is expected to be low. Aspirin inhibits the production of TxA₂. Clopidogrel and prasugrel act by blocking the platelet P2Y₁₂ receptor.^{5,29} Thus, their mechanisms are complementary. Clopidogrel and prasugrel are both thienopyridine prodrugs that are converted via hepatic CYP isoenzymes to their active metabolite but prasugrel is more efficiently converted to its active form and so achieves more reliable P2Y₁₂ inhibition. Ticagrelor is a novel P2Y₁₂ receptor antagonist but its clinical profile, both in terms of efficacy and adverse events, differs from that of the thienopyridine prodrugs.³² Ticagrelor is also supposed to have a dual mode of action as its P2Y₁₂ antagonism is complemented by inhibition of adenosine cell uptake via inhibition of the equilibrative nucleoside transporter 1 thereby increasing extracellular adenosine level and mediating adenosine-receptor activation.³²

Platelet miRNAs and platelet activation in the general population

It is currently unclear to what extent platelet miRNAs are mechanistically involved in platelet activation. A SNP (rs4636297) in the miR-126 locus has previously been demonstrated to affect the expression of mature miR-126 in cells overexpressing the different variants of pri-miR-126. An effect on endogenous miR-126 levels has not been investigated thus far.⁸ In plasma and serum from the Bruneck cohort, we observed a trend towards higher levels of circulating miR-126 in individuals carrying the minor allele (AA genotype), which facilitates the processing of pri-miR-126. Importantly, the SNP genotype affected the plasma concentrations of three platelet activation markers: PF4 (p=0.002), PPBP (P<0.001) and SELP (P=0.099) were all positively correlated with the AA genotype. Moreover, for proteins showing an association with the AA genotype in a panel of 219 plasma proteins measured in the Bruneck cohort, there was a significant enrichment of proteins linked to platelet activation (p=0.019, Online Fig. VIII). These genetic associations, however, await confirmation in independent cohorts. MiR-126 is abundant in endothelial cells and platelets.³ While miR-126 is known to be an important regulator of endothelial cell function^{12,33}, its role in platelets is unknown. It is therefore conceivable that higher miR-126 levels in individuals with the AA genotype influence platelet activity either directly by changing platelet function or indirectly by affecting endothelial cells. Recently, the A allele of SNP rs4636297 has been shown to be associated with sight-threatening diabetic retinopathy in patients with type II diabetes mellitus³⁴, corroborating the relevance of genetic variability of miRNAs for susceptibility to disease.

MiR-126 and platelet aggregation in mice

Despite numerous reports on miRNAs, surprisingly little is known about their function in platelets. MiR-223 targets the P2Y₁₂ receptor.³⁵ MiR-223 deficient mice form larger thrombi and have a delayed clot retraction compared to wild type mice.³⁶ Platelets of miR-223 deficient mice display increased aggregation in response to thrombin and collagen but not to fibronectin and the TxA₂ analog U44619. MiR-96 regulates VAMP8/endobrevin, a protein involved in platelet granule secretion that is upregulated in hyperreactive platelets.³⁷ Our experiments demonstrate that inhibition of miR-126 in mice attenuates platelet aggregation in response to AA, U44619, and PAR4-AP. Collagen-induced platelet aggregation may also be affected, but we did not obtain statistical significance, probably due to low numbers. Platelet aggregation was reduced in whole blood and in PRP. Thus, the involvement of other cell types is less likely. Moreover, MEG-01 cells express miR-126 in abundance and mimics of miR-126 reduced the expression of ADAM9 while inhibition of miR-126 had the opposite effect. ADAM9 is a predicted and experimentally confirmed target of miR-126 that attenuates the adhesion of platelets to collagen.¹⁹ Given its function as a protease of the ADAM family, it may alter the platelet response by cleaving membrane proteins. In whole blood from antagomiR-treated mice, ADAM9 was not differentially expressed. This is expected because ADAM9 is present in many other blood cells, which lack miR-126 and therefore are not affected. Notably, the expression of the P2Y₁₂ receptor was reduced in blood of antagomiR-126-3p treated mice. P2Y₁₂ levels are much higher in platelets than in leukocytes³⁸ and erythrocytes.³⁹ Thus, the latter finding may, at least in part, explain why platelets from antagomiR-126-3p treated mice display an attenuated aggregation response: The aggregation responses to strong platelet agonists, such as collagen, thrombin receptor-activating peptides and particularly either TxA₂ (generated from AA) or its mimetic U46619, are amplified by ADP, which is released from the platelet dense granules in response to the agonists and activates the P2Y₁₂ receptor. On the other hand, higher concentrations of these agonists or less effective P2Y₁₂ inhibition or deficiency allows a more robust platelet aggregation response. P2Y₁₂ activation may also contribute to the generation of TxA₂ from AA.⁴⁰

Study limitations

Causality cannot be inferred from associations of miRNAs with platelet function tests in ACS patients. Although recent studies implicated miR-126 in atherogenesis^{41,42} and miR-223 has been used for categorization of patients as 'responder' and 'non-responder' to the P2Y₁₂ inhibitor clopidogrel⁴³, larger cohorts with prolonged follow-up are required to determine if miRNA levels are associated with clinical outcomes in ACS patients. Since the P2Y₁₂ receptor plays such an important role in platelet reactivity, relationships between the extent of P2Y₁₂ receptor inhibition and miRNA levels have to be further explored in future studies. Similarly, the reported association of a SNP for miR-126 with platelet activation markers requires replication in independent cohorts. MiR-126 is also abundant in endothelial cells, and indirect effects on platelets cannot be excluded. On the other hand, gene expression in MEG-01 cells may be subject to different regulation mechanisms than in primary megakaryocytes and platelets, i.e. in undifferentiated MEG-01 cells P2Y₁₂ expression is not detectable.

Conclusion

Numerous studies have demonstrated the importance of platelet reactivity in the risk of clinical events such as stent thrombosis following PCI or recurrent arterial thrombotic events following ACS. Exciting opportunities exist to further pursue platelet miRNAs as potential biomarkers for treatment response in ACS patients. 44 miRNAs can be measured in frozen samples, which could offer a potential advantage compared to other platelet function tests currently available. Besides their biomarker potential, some miRNAs, such as miR-126 and miR-223,36 may also regulate platelet reactivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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Nonstandard Abbreviations and Acronyms

ACS	acute coronary syndrome
ASA	acetylsalicylic acid
Ct	cycle threshold
ELISA	enzyme-linked immunosorbent assay
miRNA	microRNA
PAR4-AP	PAR-4 activating peptide AYPGKF amide
PC	principal component
PF4	platelet factor 4
PPBP	pro-platelet basic protein
PPP	platelet-poor plasma
PRP	platelet-rich plasma
qPCR	real-time polymerase chain reaction

SELP	P-selectin
SNP	single nucleotide polymorphism
STEMI	ST-elevation myocardial infarction
TxA₂	thromboxane A ₂

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Novelty and Significance

What Is Known?

- Plasma microRNAs are highly correlated.
- Platelets contain and release microRNAs.
- Platelet inhibition reduces microRNA levels in platelet-poor plasma.

What New Information Does This Article Contribute?

- Besides microRNAs, YRNA fragments in plasma are also platelet-derived.
- Platelet microRNAs and YRNA fragments correlate with indices of platelet function in patients on dual anti-platelet therapy.
- MicroRNA-126 alters platelet activity.

This study provides evidence for platelets being a source of microRNAs and YRNA fragments in plasma. A strong platelet dependency of microRNAs and YRNA fragments was observed in a spike-in experiment. There was also a striking correlation of microRNAs and YRNA fragments with platelet activation markers in the general population. Plasma microRNA and YRNA levels are associated with residual platelet activity in patients on dual anti-platelet therapy. MicroRNA-126, previously considered to be endothelial specific, is present in platelets and in a human megakaryoblastic cell line. A single nucleotide polymorphism that facilitates processing of microRNA-126 increases plasma levels of platelet activation markers. Treatment with antagomiRs to microRNA-126 reduces platelet activation in mice. MicroRNAs may not just be markers of platelet activity but also alter their function, most probably by influencing gene expression in megakaryocytes.

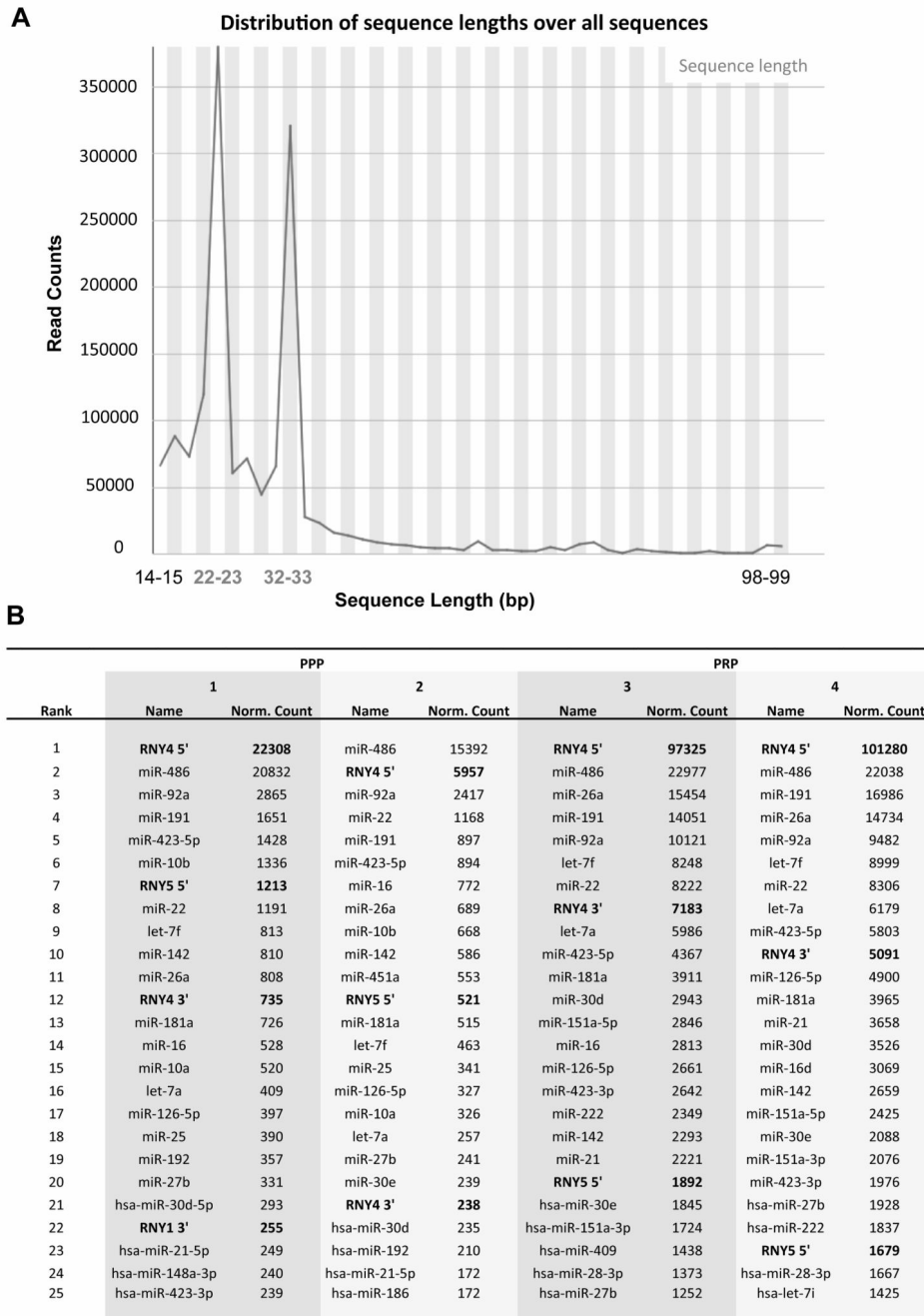


Figure 1. NGS of small RNAs in plasma. (A) Representative histogram of sequence lengths for PPP after processing for adapter removal and lower bound on length. (B) Summary of the top 25 small RNAs (reads per million total reads) in each sample. YRNA fragments are highlighted in bold.

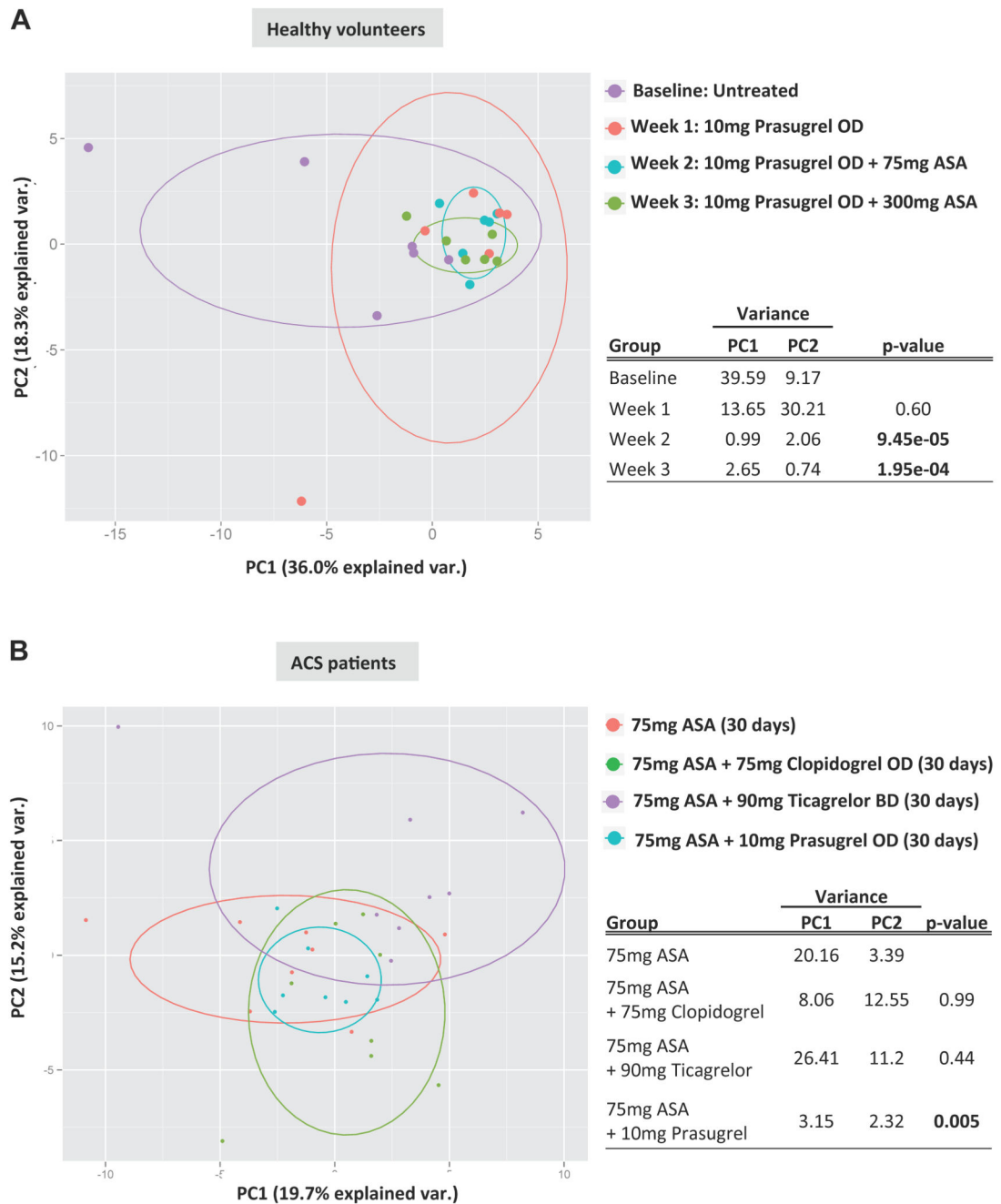


Figure 2. MiRNAs and anti-platelet therapy.

PC analyses based on screening of 92 plasma miRNAs using custom-made qPCR plates. **(A)** Decreasing variability of plasma miRNA profiles in healthy volunteers after 2 and 3 weeks of platelet inhibition (n=6 at four time points). **(B)** Effects of different anti-platelet agents in patients with ACS (n=8 per group). The tables show the variances of the two principal components PC1 and PC2. An F-test was used to calculate the differences in variance; P-values reflect difference of variance compared to baseline **(A)** or 75mg ASA **(B)**. “OD” denotes once daily, “BD” twice daily.

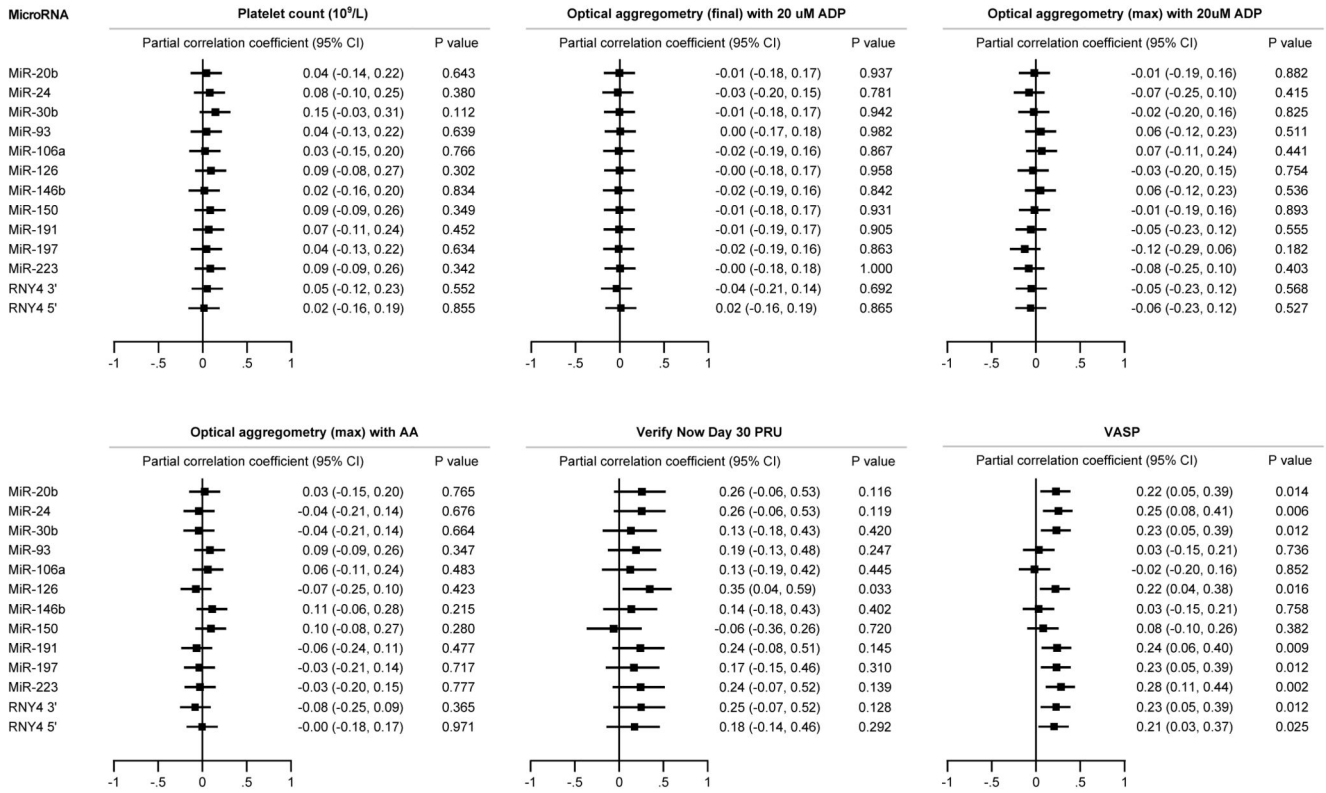


Figure 3. Pearson correlation forest plot depicting associations of 11 miRNAs and two YRNA fragments to platelet counts and platelet function tests in ACS patients (n=125). Measurements of RNAs by individual Taqman assays were normalized to exogenous *Cel-miR-39*. Note that fewer samples were measured with the Verify Now (n=40) compared to the VASP aggregation assay (n=121). ADP, denotes adenosine diphosphate; AA, arachidonic acid; PRU, P2Y₁₂ Reaction Units; VASP, vasodilator-stimulated phosphoprotein.

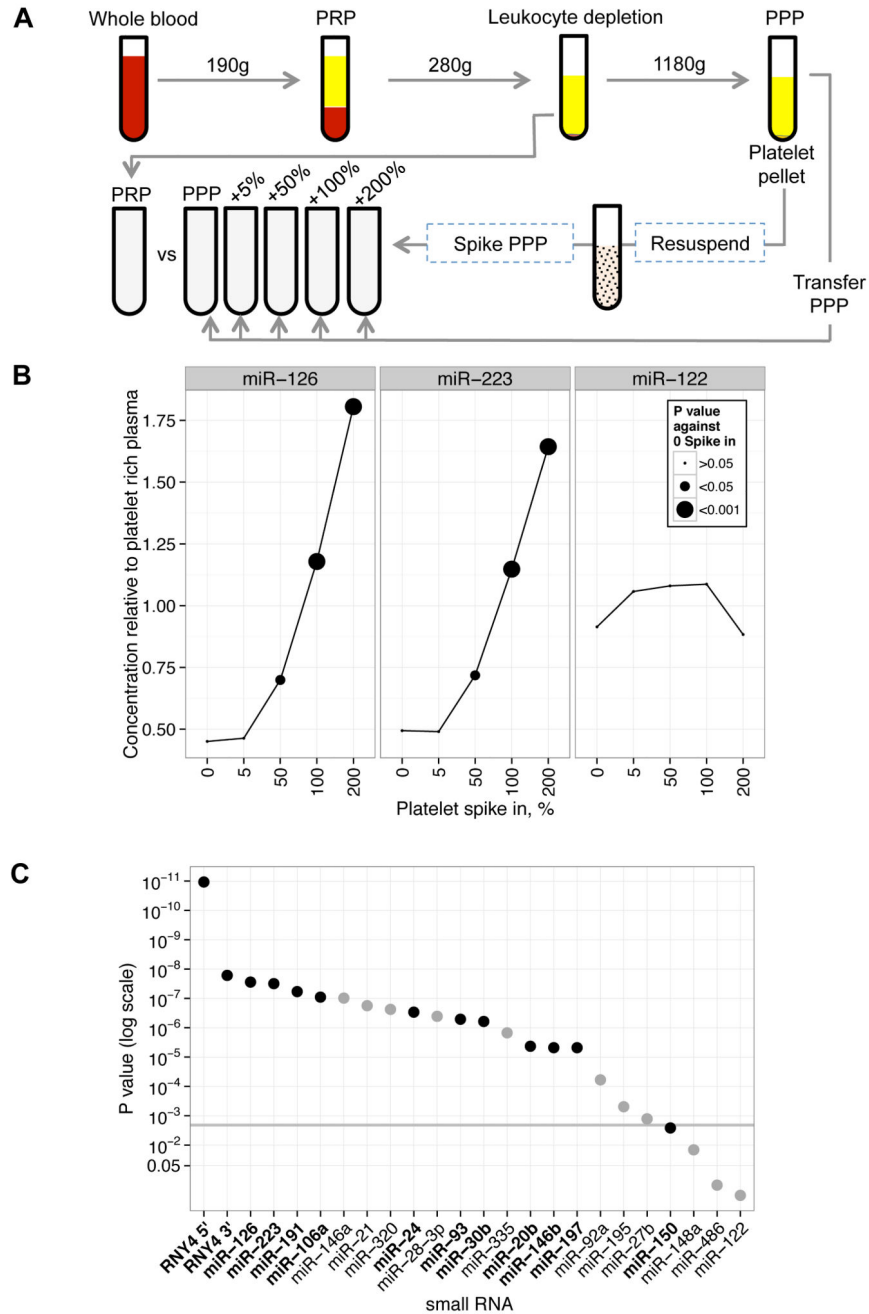


Figure 4. Platelet spike-in experiment.

(A) Schematic summary of workflow. PPP and PRP denote platelet-poor and platelet-rich plasma. (B) Dependence of miR-126, miR-223 and miR-122 levels on platelet spike-in. (C) P values for the dependence of levels of 22 microRNAs and two RNY4 fragments on platelet spike-in. RNAs marked in bold were tested in the ACS cohort. The grey line represents the significance threshold for a P value of 0.05 after Bonferroni correction. (B, C) Results shown are derived from linear mixed models featuring fixed effects for platelet spike-in in

categorical form (**B**) or in continuous form (**C**), random intercepts for subjects, and a general (unconstrained) covariance structure.

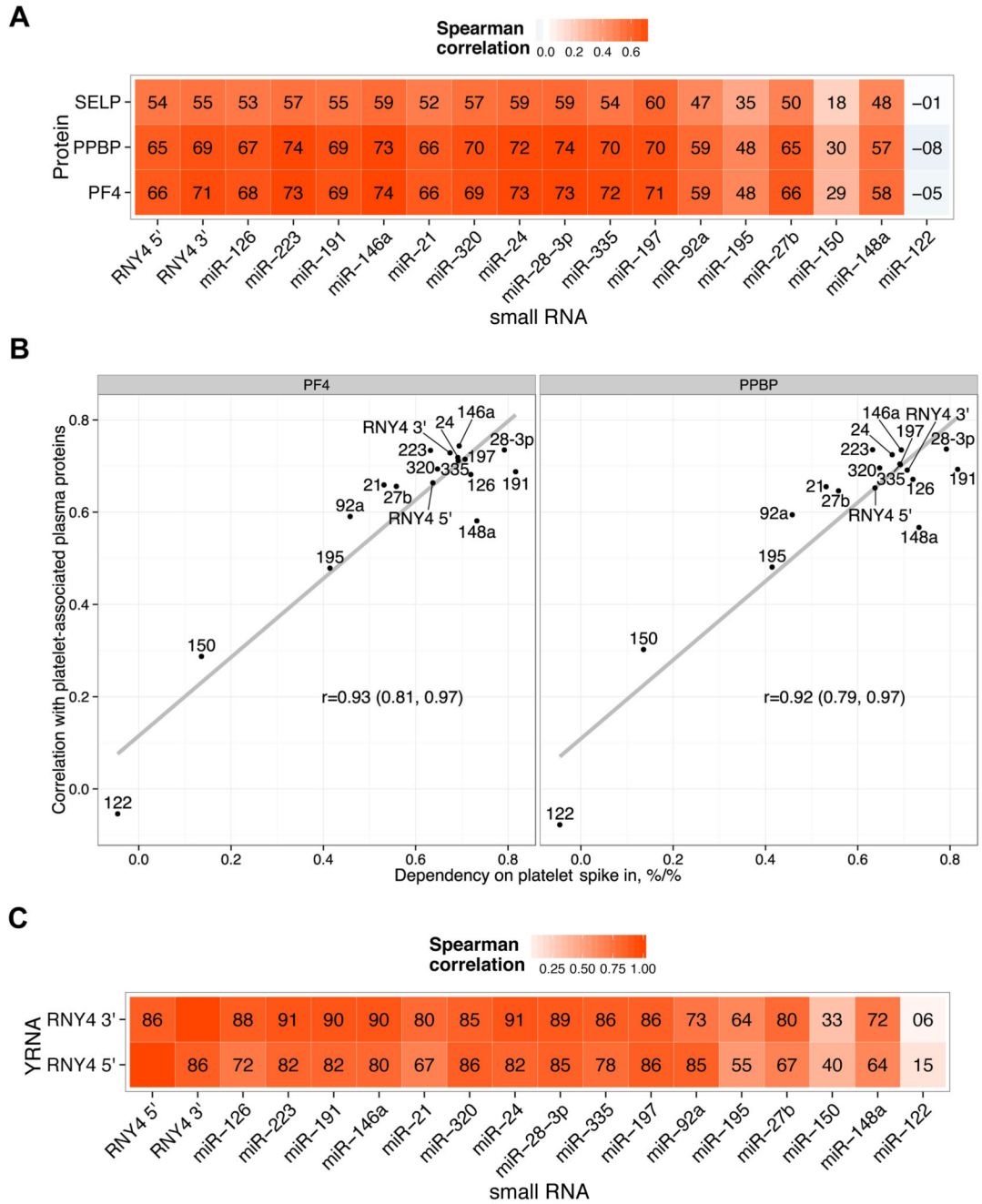


Figure 5. Small RNAs and platelet proteins in the Bruneck cohort.

(A) Association of plasma miRNAs and YRNA fragments with three platelet activation markers (PF4, PPBP, SELP) in the population-based Bruneck study (n=669). (B) Relationship between the dependency of small RNA levels on platelets in the spike-in experiment (x-axis, see Fig. 4) and the correlation of small RNA levels with platelet-derived protein concentrations (PF4, PPBP) in the general population (y-axis, see A) for 16 miRNAs and two RNY4 fragments. Lines are Deming regression lines and r denotes Pearson correlation with 95% confidence interval. (C) Association of plasma YRNA fragments with

plasma miRNAs measured in the Bruneck cohort (n=669). (**A, C**) Tile color codes for direction and magnitude of correlation, while tile text gives its sign and first two decimal digits. PF4, platelet factor 4; PPBP, pro-platelet basic protein; SELP, P-selectin.

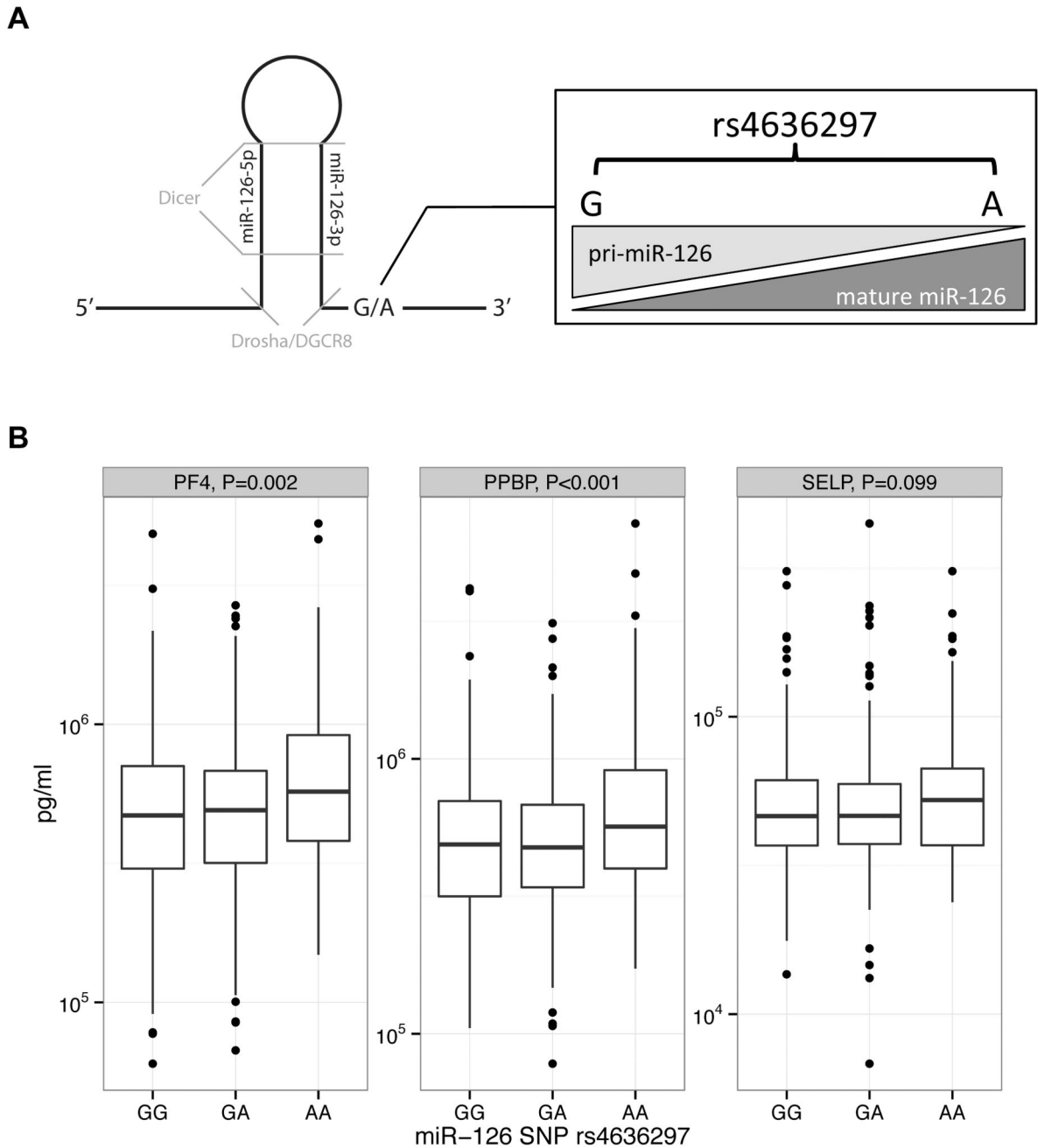


Figure 6. Association of rs4636297 with plasma markers of platelet activation.

(A) Schematic representation of pri-miR-126. Cleavage sites for Drosha/DGCR8 and Dicer are indicated in gray. The SNP rs4636297 is located downstream of the stem-loop (pre-miR-126). pri-miR-126 carrying the major “G” genotype, is less efficiently processed than the minor “A” genotype. (B) Plasma levels of platelet activation markers are associated with the rs4636297 genotype in the Bruneck cohort (n=628), P values are shown for one-way ANOVA. PF4, platelet factor 4; PPBP, pro-platelet basic protein; SELP, P-selectin.

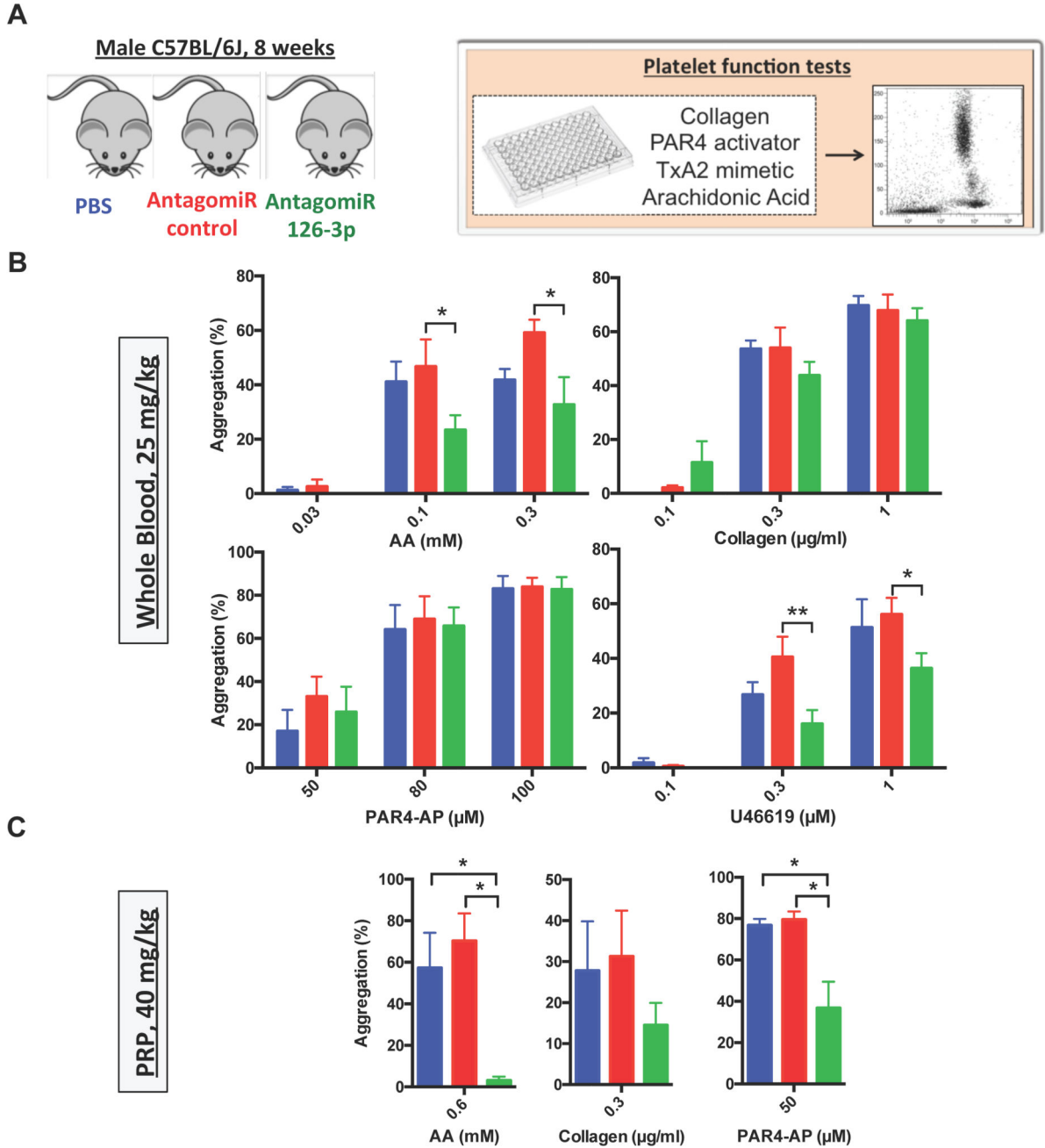
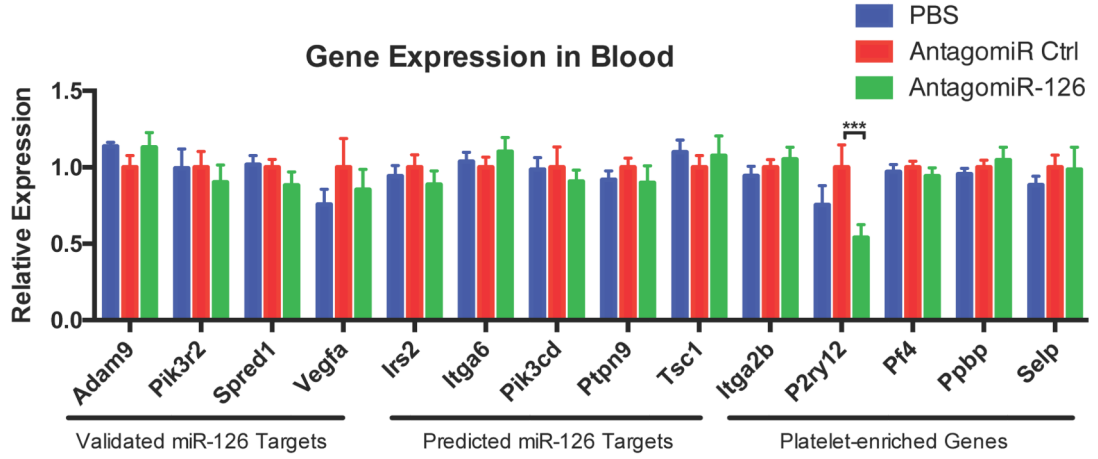


Figure 7. Platelet function in mice treated with antagomiR against miR-126-3p.

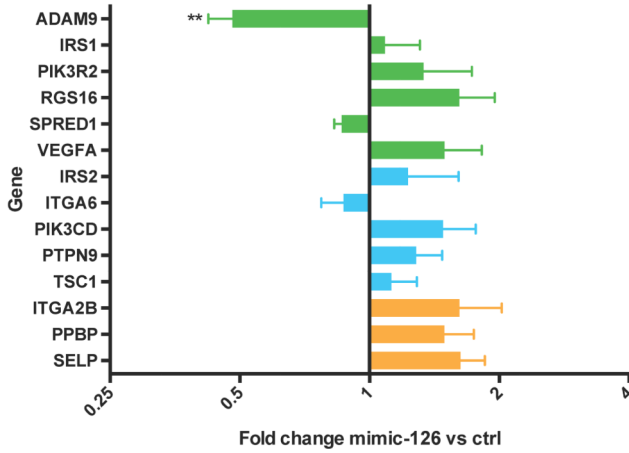
(A) 8 week old male C57BL/6J mice were injected with PBS, control antagomiR or antagomiR-126-3p for three consecutive days and sacrificed on day 7 for platelet function tests. (B) Platelet aggregation was measured in whole blood from mice injected i.p. with 25 mg antagomiR/kg in response to different concentrations of the indicated agonists. Asterisks denote significant difference in a two-way ANOVA with Bonferroni post-test (*, $P < 0.05$; **, $P < 0.01$). (C) Platelet aggregation was assessed in PRP in mice injected i.p. with 40 mg antagomiR/kg. Asterisks denote significant difference in a one-way ANOVA with

Bonferroni post-test (*, $P < 0.05$). (**B**, **C**) Data are shown as mean +SEM, $n=4$ per condition. AA, arachidonic acid.

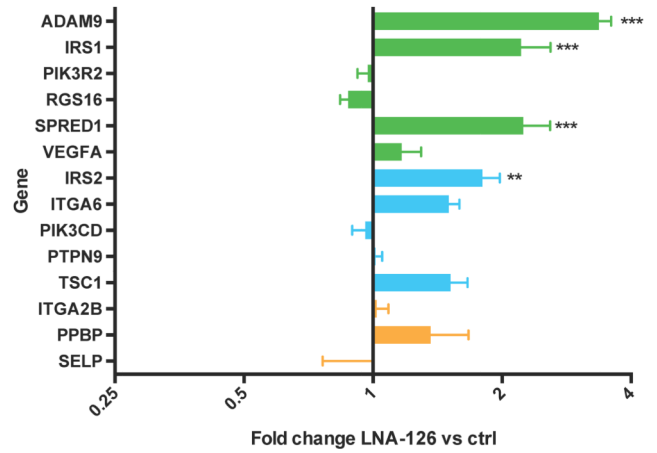
A



B



C



D

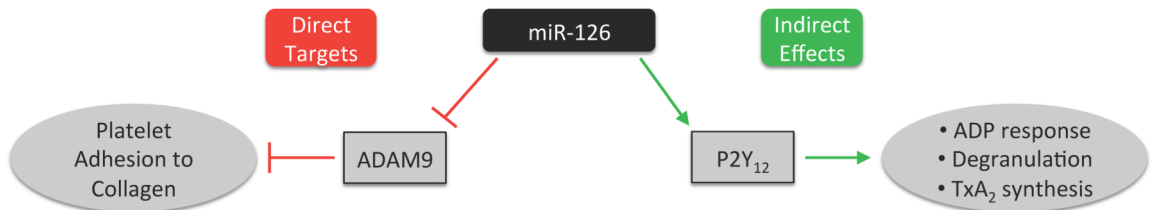


Figure 8. Effects of miR-126 in mice and MEG-01 cells.

(A) Gene expression was analysed in whole blood from mice treated with 25 or 40 mg/kg antagomiR, n=8 per group. Gene expression was normalised to Actb, Gapdh and Ppia. Gene expression was analysed in MEG-01 cells transfected with mimic-126 (B) or LNA-126 (C) and the respective controls. Gene expression was normalised to GAPDH and SP1. Validated targets of miR-126 are shown in green, predicted targets are shown in blue, platelet-enriched genes are shown in orange. (B, C): n=4 per condition. Graphs represent mean+SEM, asterisks denote statistical significance in a two-way ANOVA with Bonferroni post-test (*, **), *** p < 0.001.

p<0.05; **, p<0.01; ***, p<0.001). **(D)** Schematic illustration of potential miR-126-dependent effects on platelet function.