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Circulating MicroRNAs as Novel Biomarkers for Platelet Activation

Peter Willeit,* Anna Zampetaki,* Katarzyna Dudek, Dorothee Kaudewitz, Alice King, Nicholas S. Kirkby, Roxanne Crosby-Nwaobi, Marianna Prokopi, Ignat Drozdov, Sarah R. Langley, Sobha Sivaprasad, Hugh S. Markus, Jane A. Mitchell, Timothy D. Warner, Stefan Kiechl, Manuel Mayr

Rationale: MicroRNA (miRNA) biomarkers are attracting considerable interest. Effects of medication, however, have not been investigated thus far.

Objective: To analyze changes in plasma miRNAs in response to antiplatelet therapy.

Methods and Results: Profiling for 377 miRNAs was performed in platelets, platelet microparticles, platelet-rich plasma, platelet-poor plasma, and serum. Platelet-rich plasma showed markedly higher levels of miRNAs than serum and platelet-poor plasma. Few abundant platelet miRNAs, such as miR-24, miR-197, miR-191, and miR-223, were also increased in serum compared with platelet-poor plasma. In contrast, antiplatelet therapy significantly reduced miRNA levels. Using custom-made quantitative real-time polymerase chain reaction plates, 92 miRNAs were assessed in a dose-escalation study in healthy volunteers at 4 different time points: at baseline without therapy, at 1 week with 10 mg prasugrel, at 2 weeks with 10 mg prasugrel plus 75 mg aspirin, and at 3 weeks with 10 mg prasugrel plus 300 mg aspirin. Findings in healthy volunteers were confirmed by individual TaqMan quantitative real-time polymerase chain reaction assays (n=9). Validation was performed in an independent cohort of patients with symptomatic atherosclerosis (n=33), who received low-dose aspirin at baseline. Plasma levels of platelet miRNAs, such as miR-223, miR-191, and others, that is, miR-126 and miR-150, decreased on further platelet inhibition.

Conclusions: Our study demonstrated a substantial platelet contribution to the circulating miRNA pool and identified miRNAs responsive to antiplatelet therapy. It also highlights that antiplatelet therapy and preparation of blood samples could be confounding factors in case-control studies relating plasma miRNAs to cardiovascular disease. (_Circ Res_. 2013;112:595-600.)

Key Words: antiplatelet therapy ■ biomarkers ■ microRNAs ■ platelets ■ platelet inhibitors

Pioneering studies have revealed the presence of endogenous microRNAs (miRNAs) in the circulation that are not cell-associated.[1] The cellular origin and the biological function of circulating miRNAs, however, are less clear.[2]

Editorial, see p 576

We have previously quantified circulating miRNAs in a large population-based cohort, the Bruneck study.[3,4] Using concepts of network topology,[5] we identified altered miRNA signatures in patients with type 2 diabetes mellitus[3] and with future myocardial infarction.[4] In addition, we subjected healthy volunteers to limb ischemia-reperfusion generated by thigh cuff inflation.[4] Computational analysis identified 6 distinct miRNA clusters.[4] One cluster included all miRNAs associated with risk of myocardial infarction and consisted of miRNAs predominantly expressed in platelets. Microarray screening revealed that miR-126, miR-197, miR-223, miR-24, and miR-21 are among the most highly expressed miRNAs in platelets and platelet microparticles (PMPs), and their circulating levels correlated with PMPs as quantified by flow cytometry.

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From the Department of Neurology, Medical University Innsbruck, Austria (P.W., S.K.); Department of Public Health and Primary Care, University of Cambridge, United Kingdom (P.W.); King's British Heart Foundation Centre, King's College London, United Kingdom (A.Z., K.D., D.K., M.P., I.D., S.R.L., M.M.); Stroke and Dementia Research Centre, St George's University of London, London, United Kingdom (A.K., H.S.M.); National Heart and Lung Institute, Imperial College, London, United Kingdom (N.S.K., J.A.M.); William Harvey Research Institute, Queen Mary, University of London, London, United Kingdom (N.S.K., T.D.W.); Florence Nightingale School of Nursing and Midwifery, King's College London, United Kingdom (R.C.-N.); and Laser and Retinal Unit, King's College Hospital, London, United Kingdom (S.S.).

*These authors contributed equally to this study.

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Nonstandard Abbreviations and Acronyms	
miRNA	microRNA
PMP	platelet microparticles
PPP	platelet-poor plasma
qPCR	quantitative real-time polymerase chain reaction

On the basis of our recent finding that circulating miRNAs may reflect platelet activation, we further investigate the platelet contribution to circulating miRNAs. We provide proof-of-concept for the potential of platelet miRNAs as a surrogate marker of efficacy of antiplatelet therapy.

Methods

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

Study Subjects

The following samples were obtained: (1) platelets, PMPs, serum, platelet-rich plasma, and platelet-poor (PPP) plasma were isolated from healthy volunteers (n=3)[6]; (2) serum and PPP were collected from patients with type II diabetes mellitus (n=19, Online Table I); (3) PPP was collected from healthy young men (<40 years, n=9) participating in a dose-escalation study of dual antiplatelet therapy at 4 timepoints[7]; (4) validation was performed in PPP of patients with symptomatic carotid atherosclerosis[8] (n=33, Online Table II).

Real-Time Polymerase Chain Reaction

MiRNAs were measured as described previously.[3,4] The layout of custom-made quantitative polymerase chain reaction (qPCR) plates is shown in Online Table III. For details and statistics or bioinformatics analysis, see the Online Data Supplement.

Results

Platelet Contribution to Circulating miRNAs

TaqMan miRNA fluidic cards (Human Pool Cards A v2.1) were used to assess a total of 377 miRNAs in platelets, PMPs, serum, platelet-rich plasma, and PPP of healthy individuals. Consistent with the expression data from platelets and PMPs (Online Figure I), miR-223 was the most differentially expressed miRNA in platelet-rich plasma (Figure 1). Similarly, when a panel of 28 miRNAs was compared in PPP and serum of diabetic patients (n=19), miR-223 showed the highest degree of classification potential (Figure 2A). On examination of the minimum miRNA signature that could discriminate serum from PPP, circulating levels of miR-223 provided the best efficiency of prediction (Figure 2B). Other miRNAs present in platelets, such as miR-24, miR-191, and miR-197, had less efficient prediction accuracy.

Pharmacological Intervention in Healthy Volunteers

Given the lack of a gold standard for assessing platelet function, we explored the potential of platelet miRNAs as a

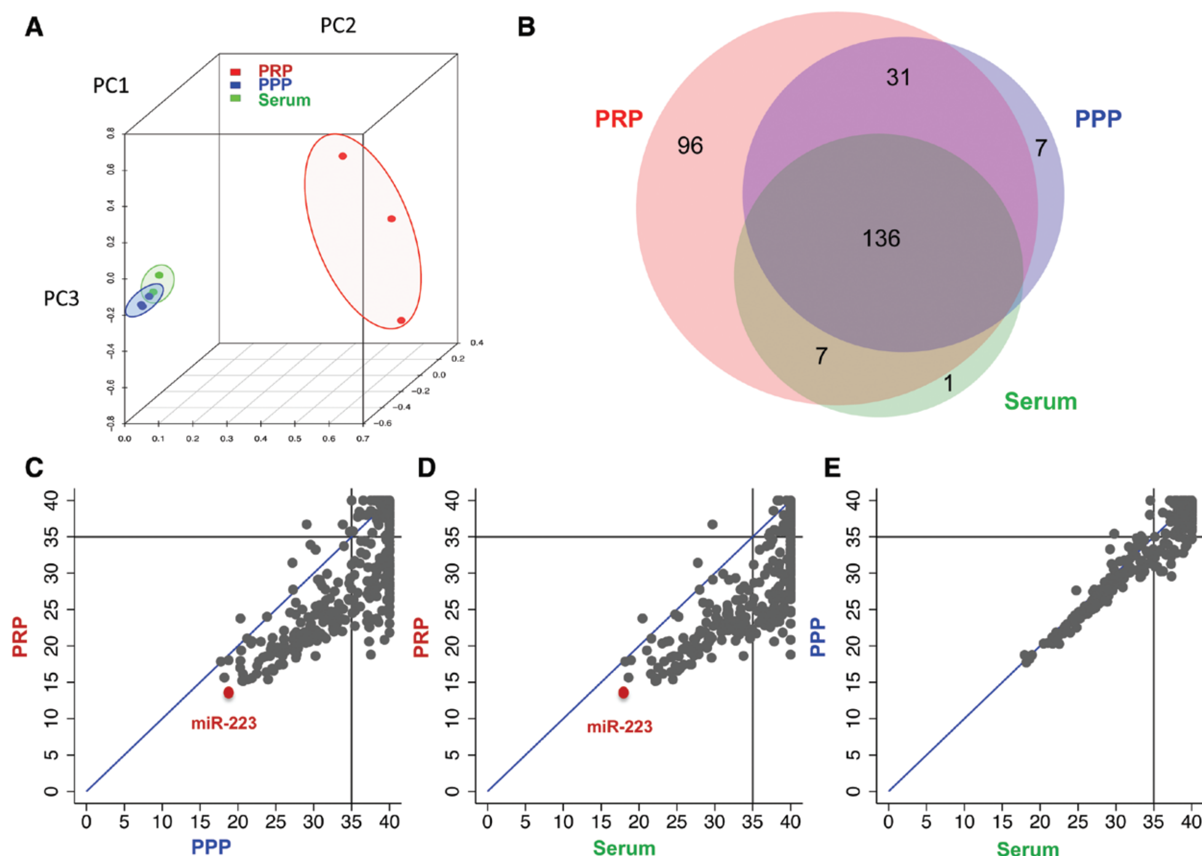

Figure 1. Platelet contribution to circulating miRNAs. Three hundred seventy-seven miRNAs were assessed in platelet-rich plasma (PRP), platelet-poor plasma (PPP), and serum of the same healthy individuals (n=3). A principal component analysis revealed clear discrimination of the samples (**A**) with most miRNAs being detected in PRP (cycle threshold [Ct] <35) (**B**). Levels of miRNAs were consistently higher in PRP compared with PPP (**C**) and serum (**D**). Differences between PPP and serum were much less pronounced (**E**). Blue lines are lines of equal levels. Added black lines on *x* and *y* axis indicate the detection threshold, assumed to be at a Ct value of 35.

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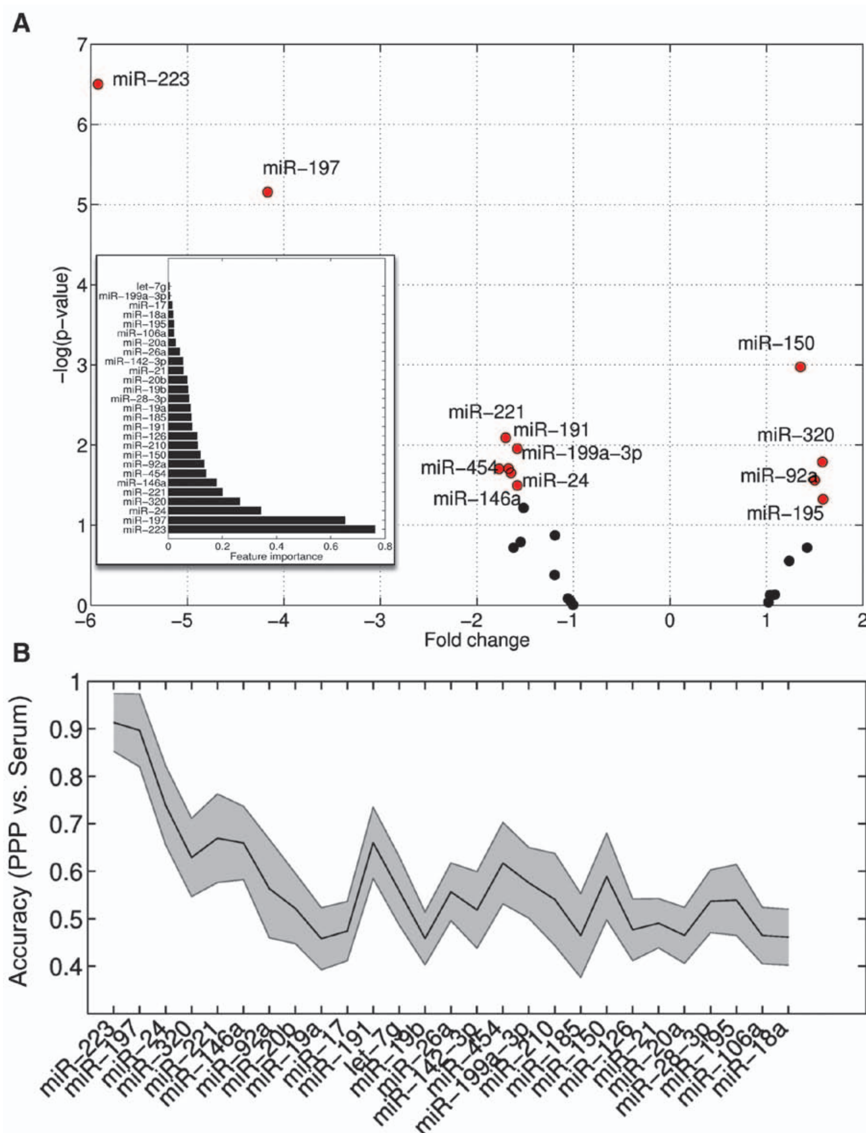

Figure 2. MiRNAs in patients with type 2 diabetes mellitus. A panel of 28 miRNAs was assessed in platelet-poor plasma (PPP) and serum of the same diabetic patients (n=19) by individual TaqMan quantitative real-time polymerase chain reaction assays. Levels of miR-24, miR-191, miR-197, and miR-223 were significantly higher in serum compared with PPP (**A**). miR-223, miR-197, and miR-24 showed the highest degree of classification potential (**inset**), with miR-223 levels giving the best efficiency of prediction (**B**).

surrogate marker of efficacy of antiplatelet therapy. Healthy young men (<40 years, n=9) were given 10 mg prasugrel (week 1), followed by a combination therapy with low-dose aspirin (75 mg, week 2) and higher-dose aspirin (300 mg, week 3). This dose escalation of aspirin in combination with prasugrel resulted in increasing platelet inhibition. As reported previously,[7] platelet function was assessed by 96-well plate aggregometry and the formation of thromboxane A_2 (measured as thromboxane B_2) by clotting blood, with response to treatment being additionally assessed by VerifyNow, and urine samples being retained for quantification of prostanoid metabolites on days 0, 7, 14, and 21 (Online Figure II). Using custom-designed qPCR plates preplated by the TaqMan custom plating service, 92 miRNAs were tested in PPP of a subset of 6 individuals. An exogenous spike-in control (*Caenorhabditis elegans* miRNA *cel-miR-39*) was used for normalization. Significant differences were identified for 15 miRNAs over the 4 time points (Online Figure III). To confirm the effect of dual antiplatelet therapy, selected miRNAs were quantified in all participants by individual TaqMan qPCR assays (Figure 3). Correlation coefficients with custom-made qPCR plates were >0.9. Notably, U6, a noncoding RNA frequently used for normalization, was affected by antiplatelet medication at week 1 and week 2 (P<0.05 in paired t test).

Pharmacological Intervention in Patients

Findings in healthy volunteers were corroborated by miRNA measurements in patients with recently symptomatic carotid atherosclerosis (n=33, Online Table II) participating in a randomized trial to determine whether treatment with dipyridamole or clopidogrel, in addition to aspirin, was more effective at reducing embolization.[8] Both treatment regimens had similar efficacy in reducing embolization, as evaluated using transcranial Doppler detection of cerebral embolic signals.[8] Twelve patients were randomly selected for miRNA analysis. All patients were on 75 mg of aspirin at baseline. Eight were randomized and received dipyridamole and 4 clopidogrel in

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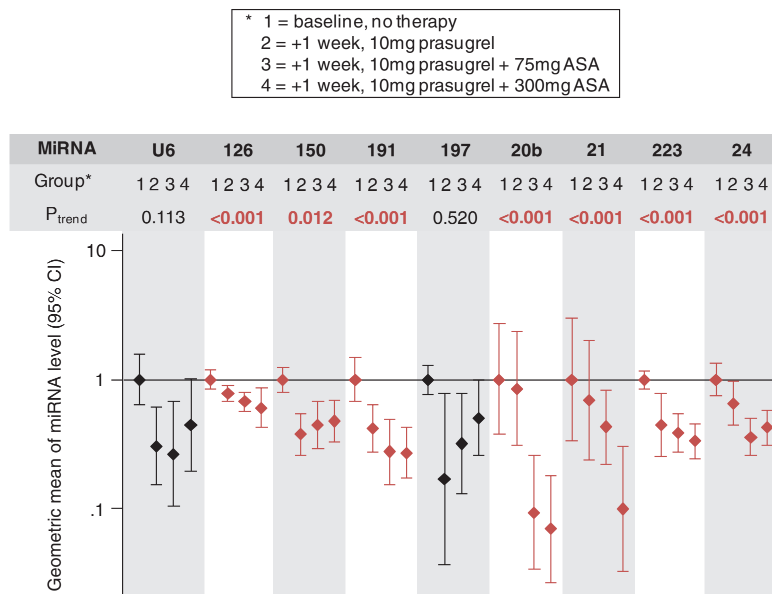

Figure 3. Response to antiplatelet therapy in healthy individuals. After screening of 92 miRNAs using custom-made quantitative real-time polymerase chain reaction (qPCR) plates (Online Figure III), a panel of 8 miRNAs plus U6 was assessed by individual Taqman qPCR assays in platelet-poor plasma from all healthy volunteers (n=9) participating in a dose-escalation study for antiplatelet therapy. Probability values are from linear mixed models with random intercepts comparing miRNA levels over the 4 time points. ASA indicates acetylsalicylic acid.

addition to aspirin. After 48 hours, effects on miRNAs were assessed in PPP using custom-designed miRNA qPCR plates based on Exiqon's locked nucleic acid technology to further ensure robustness of data independent of the technological platform (Online Figure IV). Selected miRNAs were quantified in all patients (n=33) by individual TaqMan qPCR assays (Figure 4). As in healthy volunteers, more potent platelet inhibition resulted in a reduction of miR-126 (P<0.001), miR-150 (P=0.003), miR-191 (P=0.004), and miR-223 (P=0.016), providing independent confirmation of our findings in a patient cohort, who were not naive for antiplatelet agents. In comparison, changes in ex vivo measurements of peak aggregation to ADP and collagen were less pronounced (Online Table II).

Discussion

The present study identified circulating platelet miRNAs that are responsive to antiplatelet therapy. Particular strengths of our study are as follows: (1) the repeated measurements in healthy individuals; (2) the additional investigation in patients on dual antiplatelet therapy; (3) the large number of miRNAs that were assessed by using custom-designed miRNA qPCR plates based on 2 different technologies (locked nucleic acid and TaqMan).[9]

Circulating Platelet miRNAs

Platelets represent the second most abundant cell type in blood. Although their miRNA content is low compared with other cells, platelets contribute substantially to the circulating miRNA pool. Any inconsistencies in plasma preparation will have profound effects on the miRNA content. Also, platelets shed microparticles on activation. Reduced microparticle shedding on platelet inhibition is likely to be responsible for the observed decrease in plasma miRNAs. Antiplatelet therapy was probably a confounding factor in previous case–control studies

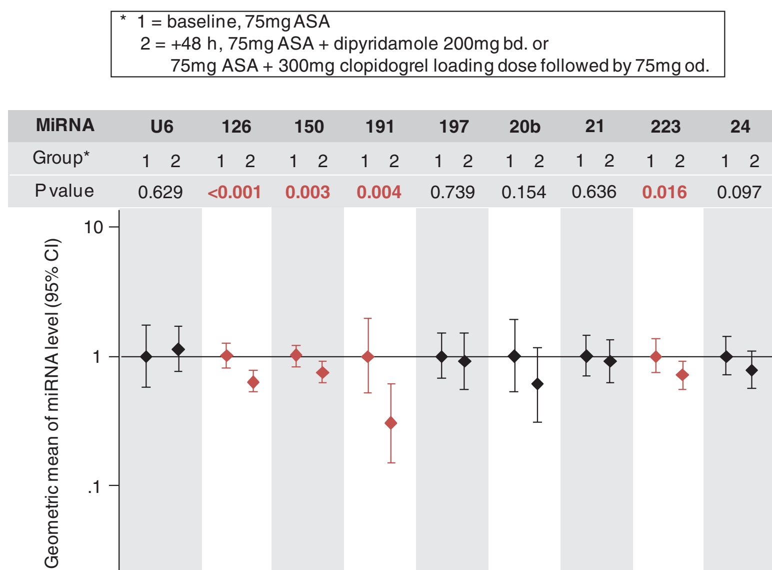

Figure 4. Response to antiplatelet therapy in patients. After screening of 92 miRNAs using custom-made quantitative real-time polymerase chain reaction plates (Online Figure IV), a panel of 8 miRNAs plus U6 was assessed in patients with symptomatic carotid atherosclerosis (n=33) who were on 75 mg aspirin (ASA) at baseline. Samples were taken 48 h after initiation of dual antiplatelet therapy with either dipyridamole or clopidogrel. *Groups depicted in red differ significantly in their average ΔCt value from the baseline group (tested by paired t tests, critical probability value of 0.05).

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reporting a loss of miRNAs in patients with coronary artery disease.[10] Similarly, the transcoronary concentration gradients of noncardiac miRNAs may be related to platelet adhesion in the coronary circulation.[11] Given our limited knowledge about circulating miRNAs,[12] well-controlled intervention studies are needed to fill the significant gaps in our current knowledge about the effects of medication on circulating miRNAs.

Effects of Antiplatelet Therapy

Antiplatelet therapy plays a prime role in treatment and prevention of myocardial infarction and strokes. Yet, there is still no widely agreed and ideal measure of platelet activation to assess antiplatelet efficacy.[13] Importantly, the combination therapy of aspirin plus $P2Y_{12}$ inhibitors is associated with a significant bleeding risk, which can be life-threatening in a small but significant number of patients.[14] Combination therapy is commonly used for the management of non–ST-elevation acute coronary syndromes and ST-elevation myocardial infarction. Aspirin inhibits the production of thromboxanes. $P2Y_{12}$ inhibitors, such as clopidogrel, prasugrel, and ticagrelor, act by inhibiting ADP receptors. Thus, their mechanisms are complementary but variability in response to clopidogrel has been associated with gastrointestinal absorption, drug interactions, and P450 isoenzyme activity.[15] There is currently no one measure or standard definition in tests for antiplatelet efficacy.[13] Our study provides evidence that platelet miRNAs should be explored as a point-of-care test for tailoring antiplatelet therapies. These findings could become even more important as new potent antiplatelet agents are currently developed and tested in clinical trials.

Clinical Implications

miRNAs are stable and readily determined by qPCR, a technique widely used for viral diagnostics in clinical laboratories. Furthermore, quantification of miRNAs may well provide information about platelet reactivity within the circulation, which is a result of a balance between pro- and antiaggregatory stimuli. Tests of platelet reactivity to date have concentrated on assessing platelet responses ex vivo. Such approaches have notably failed to provide any useful guidance to antiplatelet medication. Thus, a clinical introduction of platelet miRNA assays could address the need to provide tailored effective antiplatelet therapy. Future studies will need to explore whether platelet miRNA-guided dosing of therapy can improve therapeutic outcomes.

Conclusions

Repeated measurements in healthy volunteers and patients on antiplatelet therapy provided proof-of-concept that miRNAs could serve as novel biomarkers of platelet activation and codiagnostic for efficacy of antiplatelet therapy. This awaits confirmation in larger studies. Our findings also highlight that antiplatelet therapy is a potential confounding factor for miRNA measurements in case–control studies of cardiovascular disease.

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Disclosures

The authors filed patent applications related to circulating miRNAs as cardiovascular biomarkers.

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Novelty and Significance

What Is Known?

- Platelets and platelet microparticles contain microRNAs.
- Platelets shed microparticles on activation.
- Platelet microparticles correlate to plasma microRNAs.

What New Information Does This Article Contribute?

- Extent of platelet contribution to circulating microRNAs.
- Identification of plasma microRNAs responsive to platelet inhibition

Tests of platelet reactivity to date have concentrated on assessing platelet responses ex vivo. Such approaches have limited use in guiding antiplatelet medication. Platelets shed microparticles on activation. Microparticles contain platelet microRNAs, which can be readily determined by real-time polymerase chain reactions. Thus, a clinical introduction of platelet microRNA assays could be used to tailor antiplatelet therapy and reduce the risk of life-threatening bleeding complications. Future studies are required to determine whether tailoring antiplatelet therapies according to platelet microRNA levels might improve therapeutic outcomes in patients on dual antiplatelet therapy.

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SUPPLEMENTAL MATERIAL

BRIEF ULTRARAPID COMMUNICATION

Circulating MicroRNAs as Novel Biomarkers for Platelet Activation

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Online Figures: 4, **Online Tables**: 3

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MATERIALS AND METHODS

Study subjects. The appropriate Ethics Committees approved the studies, and all study subjects gave their written informed consent before entering the studies. The following samples were obtained: *(a)* Plasma and serum samples were collected from the same diabetic patients (n=19). Baseline clinical and laboratory characteristics of the 19 subjects are shown in Online Table I. *(b)* Healthy young males (<40 yrs, n=9) underwent a dual anti-platelet therapy and plasma samples were collected at baseline (timepoint 1), following 1 week of 10 mg prasugrel treatment (timepoint 2), one week of 10 mg prasugrel and 75 mg of aspirin treatment (timepoint 3) and one week of 10 mg of prasugrel and 300 mg of aspirin (timepoint 4)[1]. *(c)* Findings in healthy volunteers were corroborated by miRNA measurements in 33 patients (Online Table II) participating in a randomized trial to determine whether treatment with dipyridamole or clopidogrel, in addition to aspirin, was more effective at reducing embolization in patients with recent symptomatic carotid stenosis[2]. Treatment efficiency was evaluated using transcranial Doppler detection of embolic signals and platelet aggregometry[2].

MiRNA screening in platelets and PMPs. Platelets were isolated from three healthy volunteers[3]. In brief, blood was drawn using acid citrate dextrose as anticoagulant (ACD: 120 mmol/L sodium citrate, 110 mmol/L glucose, 80 mmol/L citric acid, 1:7 vol/vol) and centrifuged for 17 minutes at 200g and 30°C in the presence of indomethacin (10 µmol/L; Sigma-Aldrich). The platelet-rich plasma (PRP) was then centrifuged for another 10 minutes at 1000g in the presence of prostacyclin (0.1 µg/mL; Sigma-Aldrich). The supernatant was kept as platelet-poor plasma (PPP). The pelleted platelets were resuspended in modified Tyrode-HEPES buffer (145 mmol/L NaCl, 2.9 mmol/L KCl, 10 mmol/L HEPES, 1 mmol/L $MgCl_2$, 5 mmol/L glucose, pH 7.3) at a concentration of 4 x 10^8/mL. PMPs were isolated following platelet activation with thrombin (0.1 U/mL; Sigma-Aldrich). Platelet aggregation was monitored with a turbidometric method (Chronolog 490; Chronolog). PMPs were harvested by ultracentrifugation at 100000g for 90 minutes at 4°C. The pellet was lysed in Qiazol reagent and RNA was extracted as described above. Total RNA was eluted in 25µl of nuclease free H_2O. RNA was quantified using the NanoDrop spectrophotometer and 20ng of total RNA were used for reverse transcription. The expression profile of platelets and PMPs was assessed using the Human TaqMan miRNA Array Card A (Applied Biosystems) as described previously[4].

RNA isolation, reverse transcription and pre-amplification. MiRNAs were extracted using the miRNeasy kit (Qiagen) as described previously[4,5]. A fixed volume of 3µl of the 25µl RNA eluate was used as input in each reverse transcription (RT) reaction. An RT reaction and pre-amplification step were performed as described previously[4,5]. In brief, miRNAs were reversely transcribed using Megaplex Primer Pools (Human Pools A v2.1, Applied Biosystems). RT reaction products were further amplified using the Megaplex PreAmp Primers (Primers A v2.1) as recommended by the manufacturer. Both RT and PreAmp products were stored at -20°C.

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TaqMan qPCR assay. TaqMan miRNA assays were used to assess the expression of individual miRNAs. 0.5 µl diluted pre-amplification product were combined with 0.25 µl TaqMan miRNA Assay (20x) (Applied Biosystems) and 2.5 µl TaqMan Universal PCR Master Mix No AmpErase UNG (2x) to a final volume of 5 µl. QPCR was performed on an Applied Biosystems 7900HT thermocycler at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. All samples were run in duplicates. Relative quantification was performed using the software SDS2.2 (Applied Biosystems). Exogenous miRNA (*cel-miR-39*) was used as a spike-in normalization control as described previously[4].

TaqMan miRNA custom-designed qPCR plates. The expression profile of miRNAs in platelet-poor plasma samples (PPP) of healthy volunteers participating in the dose escalation study was determined using custom-made Human TaqMan miRNA qPCR assay plates. A total of 92 miRNAs previously identified as consistently present in the circulation and a set of 4 targets that served as normalization controls or negative controls were quantified. The layout of the custom-made qPCR array is shown in Online Table III.

Exiqon miRNA custom-designed qPCR plates. The expression profile of miRNAs in PPP of patients with recently symptomatic carotid atherosclerosis was determined using custom-made human Exiqon LNA qPCR plates. A total of 90 miRNAs were measured using the miCURY LNA Universal RT microRNA PCR protocol which is a two-part protocol consisting of first-strand cDNA synthesis followed real-time PCR amplification. For cDNA synthesis, 4µl of 5x Reaction buffer were combined with 2 µl of 10xEnzyme mix and 4µl of RNA to a final volume of 20µl. Reverse transcription was performed by incubating the samples at 42°C for 1h and subsequent heat inactivation of the enzyme at 95°C for 5 min. RT products were stored at -20°C. For PCR amplification, 2x SYBR® green master mix was used for cDNA with the reaction conditions being identical as described for TaqMan qPCR plates.

Bioinformatics and statistics. Predictive significance of all miRNAs was calculated using bootstrap aggregation for an ensemble of 10,000 decision trees. Each miRNA profile was assigned an importance value based on how well it can differentiate PPP and serum samples of the same diabetic patient (value range is from 0 to 1 with one being the greatest discriminatory power). Accuracy to discriminate plasma and serum samples for all miRNAs was quantified using the Support Vector Machines (SVM) algorithm, a supervised learning method, and a 10-fold cross validation approach. SVM were trained on half of the data selected at random and validated on the remaining samples. This procedure was repeated 10 times and the final correct classification rate (accuracy) was presented as an average of all SVM iterations. The changes in miRNA levels in response to anti-platelet therapy were evaluated using paired t-tests for comparison between two time points, with the baseline levels as reference group. For more than two time points, P-values were calculated with linear mixed models with a random intercept. Unlike ANOVA, this test takes the auto-correlation of measurements within individuals into account. Because raw miRNA expression levels were

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markedly skewed, the arithmetic mean and its confidence interval were calculated on a logarithmic scale for statistical analyses, which correspond to the geometric mean and its confidence interval after back-transformation to the normal scale. Stata version 12.0 MP was used for statistical analysis, with two-sided tests and $P<0.05$.

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Online Figures

Online Figure I. MiRNA profile of platelets and PMPs. MiRNAs were determined by microarray screening. The average Ct value was used as a normalization control. Expression levels (2^{-DCt}) were log transformed.

Online Figure II. Platelet function measurements upon platelet inhibition. TxB2, denotes thromboxane B2; Tx-M, urinary metabolites of thromboxane; PGI-M, urinary prostanoid metabolites; TRAP-6, thrombin receptor activator for peptide 6.

Online Figure III. MiRNA response to anti-platelet therapy in healthy individuals. Levels of 92 miRNAs were measured at four time points in PPP from healthy volunteers (n=6) using custom-made TaqMan qPCR plates. *Groups depicted **in red** differ significantly according to the p values derived from linear mixed models with random intercepts comparing miRNA levels over the four time points.

Online Figure IV. MiRNA response to anti-platelet therapy in patients. Patients with symptomatic carotid atherosclerosis (n=12) were on 75mg aspirin (ASA) at baseline and samples were taken 48h after initiation of dual anti-platelet therapy with either dipyridamole or clopidogrel. MiRNAs were assessed in PPP using custom designed miRNA qPCR plates based on Exiqon's miRCURY LNA™ Universal RT miRNA PCR system. *Groups depicted **in red** differ significantly in their average ΔCt value from the baseline group (tested by paired t-tests, critical P value 0.05).

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Online Table I: Clinical Characteristics of the Diabetic Cohort (n=19)

Male, n(%)	9 (47%)
Ethnicity	
Asian, n(%)	4 (21%)
Black, n(%)	6 (32%)
White, n(%)	9 (47%)
Age, years	68.5±2.5
Age at diagnosis, years	46±2.4
BMI, kg/m2	31.4±1.7
Systolic BP, mmHg	136.0±3.2
Diastolic BP, mmHg	75.5±2.8
HbA1c, % total hemoglobin	7.9±0.4%
HDL, mmol/L	1.2±0.1
LDL, mmol/L	2.0±0.2
Triglycerides, mmol/L	1.2±0.1
Total cholesterol/HDL ratio	3.2±0.3
eGFR, mLs/min/1.73m2	69.7±3.3
Hb, g/dL	12.3±0.4

Values are given as percentage or means±SE

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Online Table II: Clinical Characteristics of the Patient Cohort (n=33)

Characteristic	Value
Male, n(%)	27 (81.8%)
Age, years	72.1±1.9
Dual Antiplatelet	
ASA + Dipyridamole, n(%)	18 (54.5%)
ASA +Clopidogrel, n(%)	15 (45.5%)
Embolic Signal (ES) at baseline, n(%)	23 (69.7%)
ES Change from Baseline, n	4.3±2.1
ES Change from Baseline, %	43.5%±24.4%
Time from Recent Symptoms, days	7.9±1.0
Type of Event	
TIA/TMB, n(%)	10 (30.3%)
Stroke, n(%)	23 (69.7%)
No. Events in the Last Month	1.4±0.2
Grade of Stenosis, % stenosis	73.7±2.5%
Smoker	
Never, n(%)	6 (18.2%)
Ex-Smoker, n(%)	18 (54.5%)
Current Smoker, n(%)	9 (27.3%)
Diabetes, n(%)	6 (18.2%)
Cholesterol	
None n(%)	11 (33.3%)
Untreated, n(%)	6 (18.2%)
Treated, n(%)	16 (48.5%)
Hypertension	
None n(%)	4 (12.1%)
Untreated, n(%)	4 (12.1%)
Treated, n(%)	25 (75.8%)
Previous MI, n(%)	3 (9.1%)
Previous Vascular Disease	4 (12.1%)
Previous Stroke	6 (18.2%)
Family History of Stroke	8 (24.2%)
Anti-platelet at Event	
None n(%)	20 (60.6%)
Asprin, n(%)	10 (30.3%)
Asprin + Dipyridamole, n(%)	3 (9.1%)
Proton Pump Inhibitor	
None n(%)	29 (87.9%)
Omeprazole, n(%)	1 (3.0%)
Lansoprazole, n(%)	3 (9.1%)
Event During 30 Day Follow-Up	1 (8.3%)
None n(%)	30 (87.9%)
TMB, n(%)	1 (3.0%)
TIA, n(%)	2 (6.1%)
ADP Difference in Peak Aggregation, %	9.4±3.1%
Collagen Difference in Peak Aggregation, %	2.2±3.4%

Values are given as percentage or means±SE

TMB, transient monocular blindness; TIA, transient ischemic attack.

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Online Table III: Lay-out of custom-designed qPCR plates

Well	Detector	Task
A1	hsa-let-7e-002406	Target
A2	hsa-miR-16-000391	Target
A3	hsa-miR-17-002308	Target
A4	hsa-miR-19b-000396	Target
A5	hsa-miR-20a-000580	Target
A6	hsa-miR-20b-001014	Target
A7	hsa-miR-24-000402	Target
A8	hsa-miR-28-3p-002446	Target
A9	hsa-miR-30b-000602	Target
A10	hsa-miR-30c-000419	Target
A11	hsa-miR-92a-000431	Target
A12	hsa-miR-93-5p-001090	Target
A13	hsa-miR-106a-002169	Target
A14	hsa-miR-125a-5p-002198	Target
A15	hsa-miR-126-002228	Target
A16	hsa-miR-139-5p-002289	Target
A17	hsa-miR-146a-000468	Target
A18	hsa-miR-146b-001097	Target
A19	hsa-miR-150-000473	Target
A20	hsa-miR-186-002285	Target
A21	hsa-miR-191-002299	Target
A22	hsa-miR-197-000497	Target
A23	hsa-miR-222-002276	Target
A24	hsa-miR-223-002295	Target
B1	hsa-miR-320-002277	Target
B2	hsa-miR-331-000545	Target
B3	hsa-miR-342-3p-002260	Target
B4	hsa-let-7b-002619	Target
B5	hsa-miR-451a-001141	Target
B6	hsa-miR-454-002323	Target
B7	hsa-miR-483-5p-002338	Target
B8	hsa-miR-484-001821	Target
B9	hsa-miR-486-001278	Target
B10	U6 snRNA-001973	Control
B11	ath-miR159a-000338	Control
B12	H2O	Control
B13	cel-miR-39-000200	Control
B14	hsa-let-7g-002282	Target
B15	hsa-miR-19a-000395	Target
B16	hsa-miR-21-000397	Target
B17	hsa-miR-25-000403	Target
B18	hsa-miR-26a-000405	Target
B19	hsa-miR-26b-000407	Target
B20	hsa-miR-27a-000408	Target
B21	hsa-miR-27b-000409	Target
B22	hsa-miR-28-000411	Target
B23	hsa-miR-29a-002112	Target
B24	hsa-miR-29b-000413	Target
C1	hsa-miR-103-000439	Target
C2	hsa-miR-107-000443	Target
C3	hsa-miR-122-002245	Target
C4	hsa-miR-125b-000449	Target
C5	hsa-miR-127-000452	Target
C6	hsa-miR-130a-000454	Target
C7	hsa-miR-139-3p-002313	Target
C8	hsa-miR-140-5p-001187	Target
C9	hsa-miR-142-3p-000464	Target
C10	hsa-miR-145-002278	Target
C11	hsa-miR-152-000475	Target
C12	hsa-miR-185-002271	Target
C13	hsa-miR-192-000491	Target
C14	hsa-miR-193b-002367	Target
C15	hsa-miR-194-000493	Target
C16	hsa-miR-195-000494	Target
C17	hsa-miR-199a-3p-002304	Target
C18	hsa-miR-200b-002251	Target
C19	hsa-miR-221-000524	Target
C20	hsa-miR-18a-002422	Target
C21	hsa-miR-503-001048	Target
C22	hsa-miR-328-000543	Target
C23	hsa-miR-335-000546	Target
C24	hsa-miR-339-3p-002184	Target
D1	hsa-miR-155-002623	Target
D2	hsa-miR-340-002258	Target
D3	hsa-miR-363-001271	Target
D4	hsa-miR-374-000563	Target
D5	hsa-miR-381-000571	Target
D6	hsa-miR-423-5p-002340	Target
D7	hsa-miR-518b-001156	Target
D8	hsa-miR-518f-002388	Target
D9	hsa-miR-532-3p-002355	Target
D10	hsa-miR-574-3p-002349	Target
D11	hsa-miR-590-5p-001984	Target
D12	hsa-miR-618-001593	Target
D13	hsa-miR-628-5p-002433	Target
D14	hsa-miR-660-001515	Target
D15	hsa-miR-744-002324	Target
D16	hsa-miR-758-001990	Target
D17	hsa-miR-210-000512	Target
D18	hsa-miR-214-002306	Target
D19	hsa-miR-885-5p-002296	Target
D20	hsa-miR-10a-000387	Target
D21	hsa-miR-15a-000389	Target
D22	hsa-miR-130b-000456	Target
D23	hsa-miR-375-000564	Target
D24	hsa-miR-202-002363	Target

Substitutes for miRNAs without LNA primers (red font):

hsa-miR-126*
hsa-miR-376c
hsa-miR-195*

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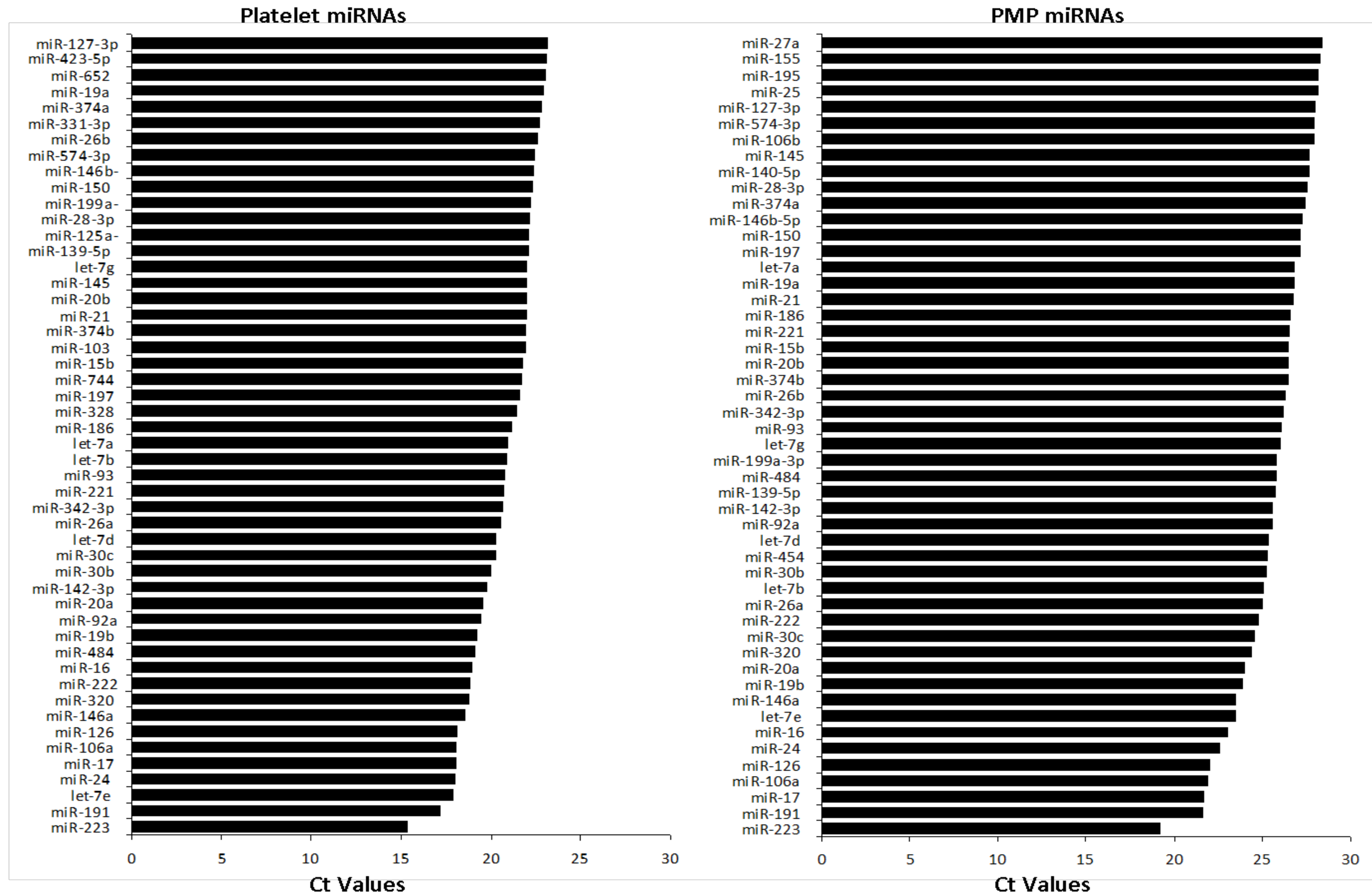
Online Figure I



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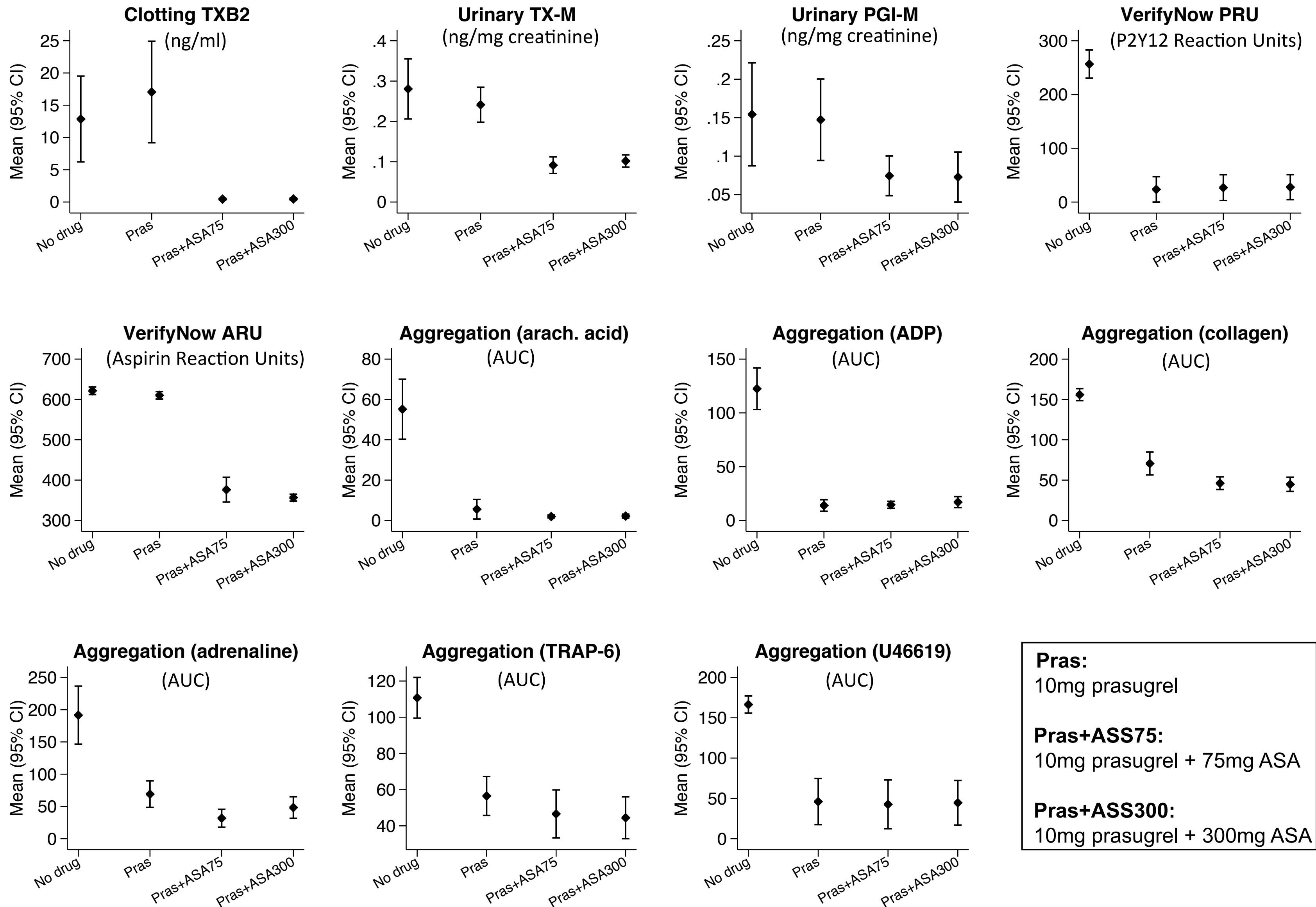

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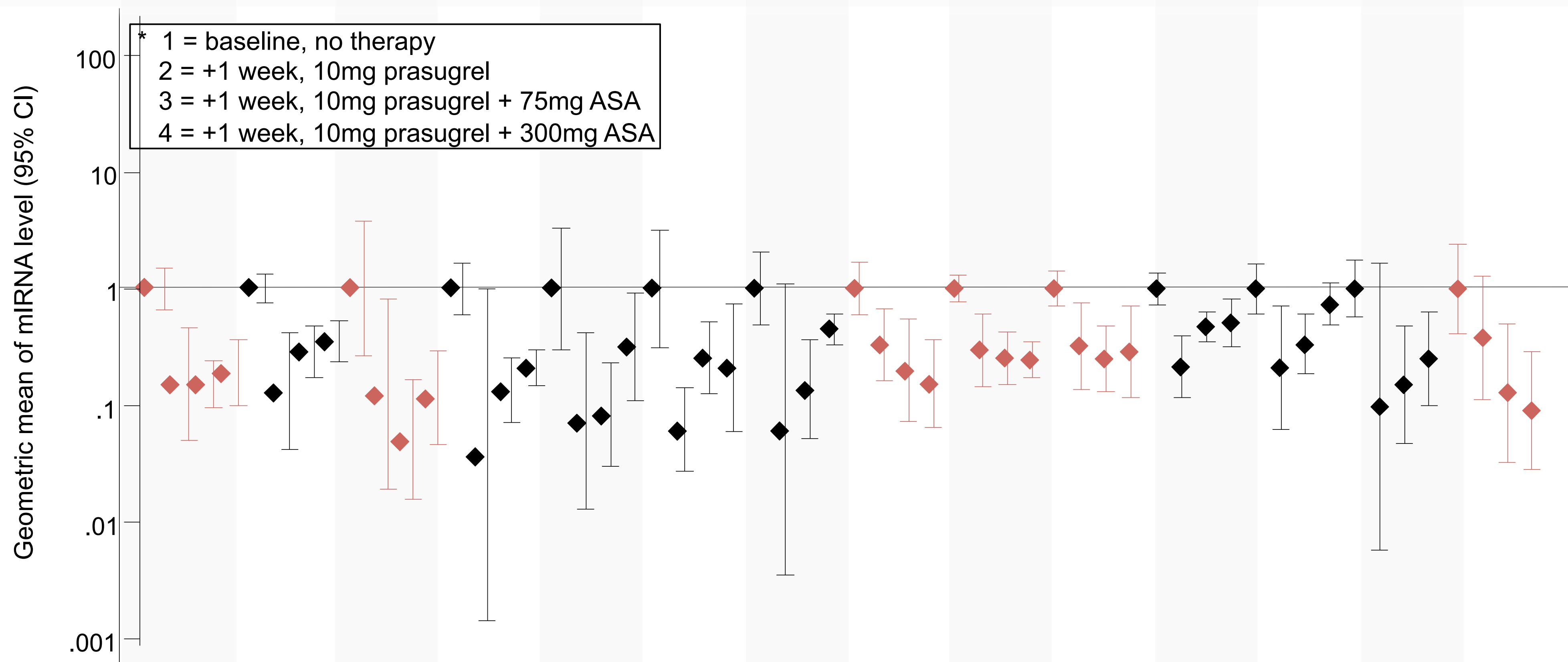
Online Figure III

MiRNA	191	30b	185	484	186	16	92a	19a	223	24	126	150	328	106a
Group*	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4
P_{trend}	**0.006**	0.211	**0.011**	0.403	0.311	0.229	0.685	**<0.001**	**<0.001**	**0.013**	0.347	0.816	0.309	**0.001**



P values are from linear mixed models with random intercepts comparing miRNA levels over the four time points.

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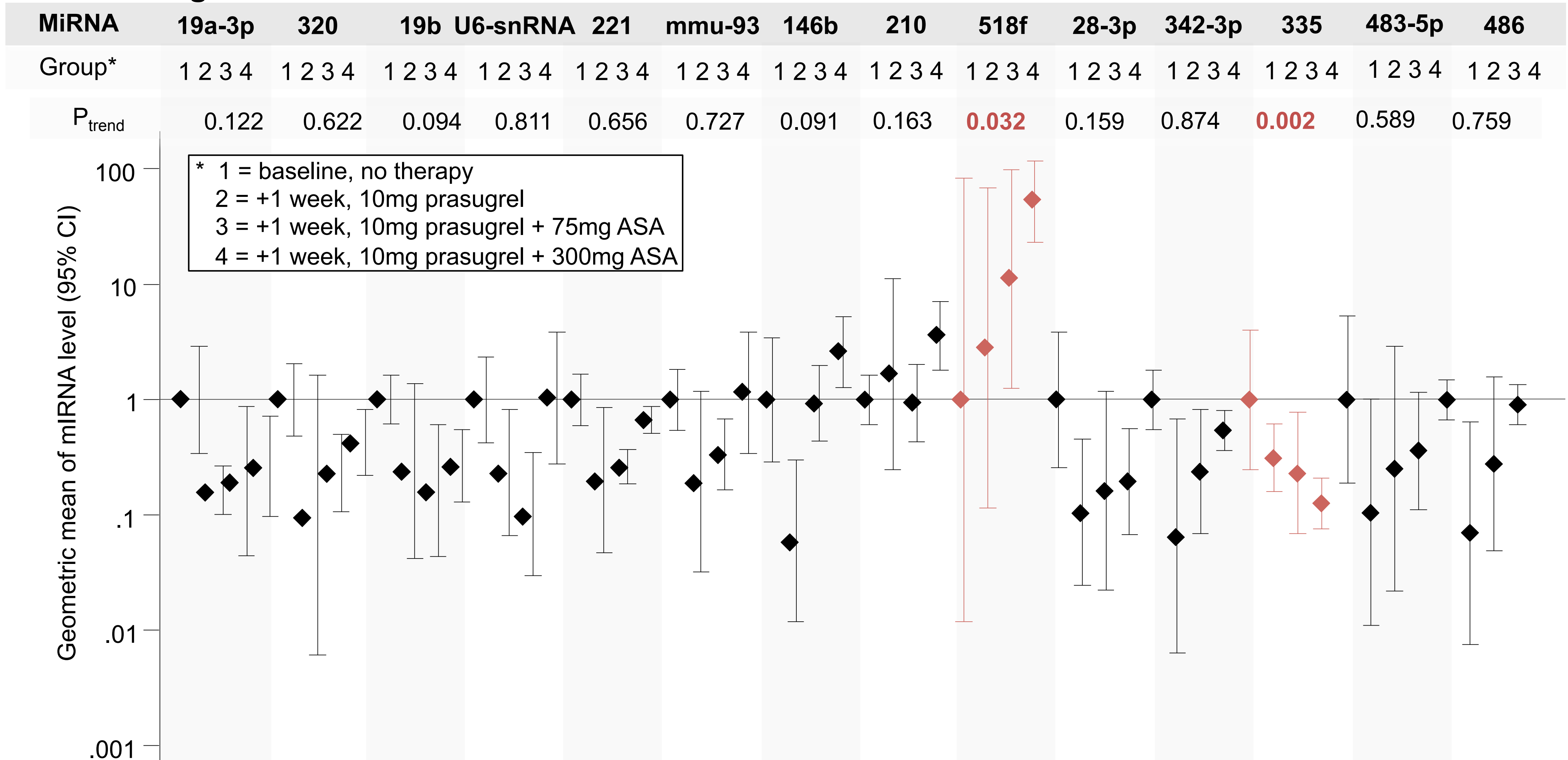
Online Figure III



P values are from linear mixed models with random intercepts comparing miRNA levels over the four time points.

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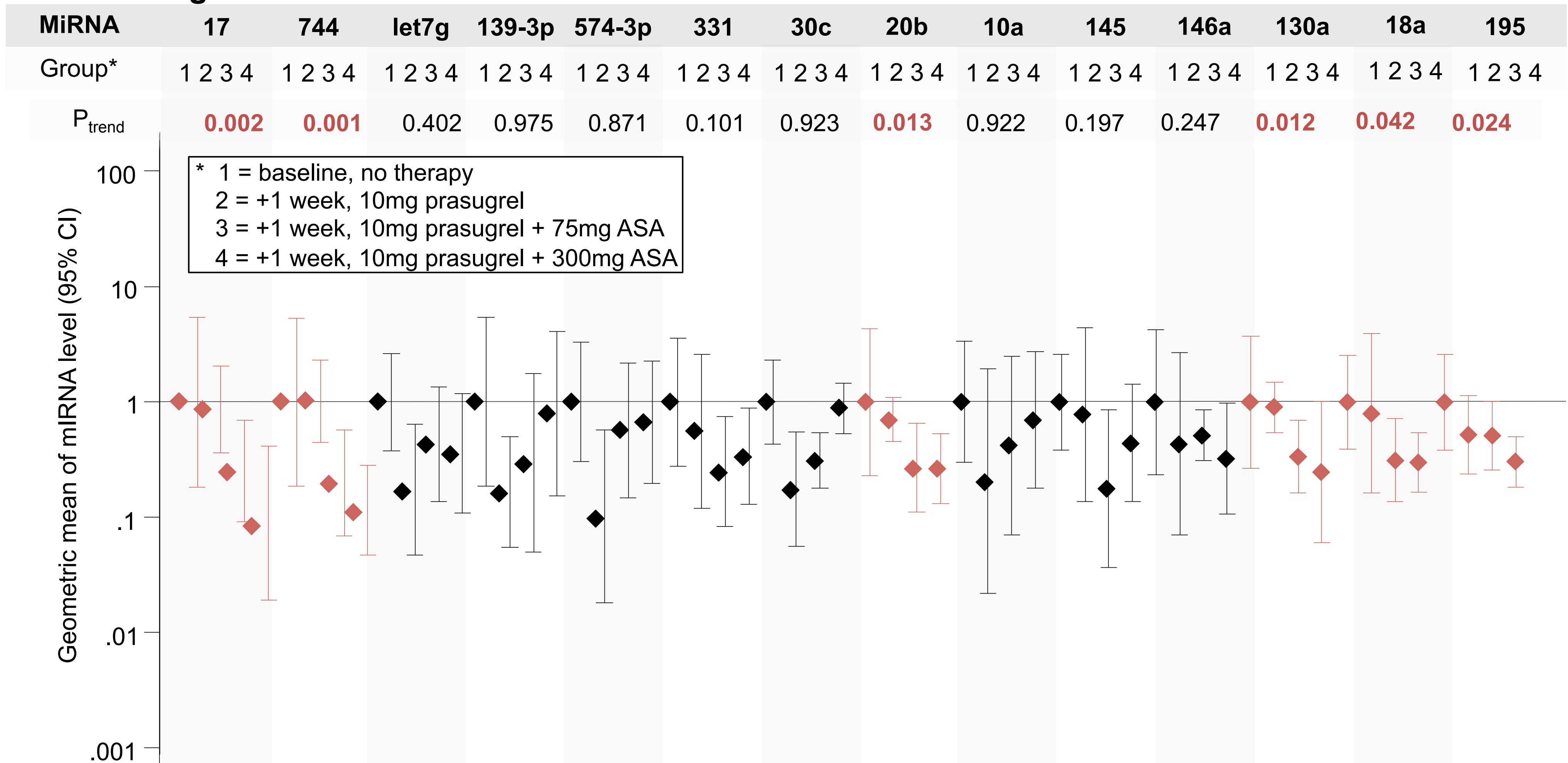
Online Figure III



P values are from linear mixed models with random intercepts comparing miRNA levels over the four time points.

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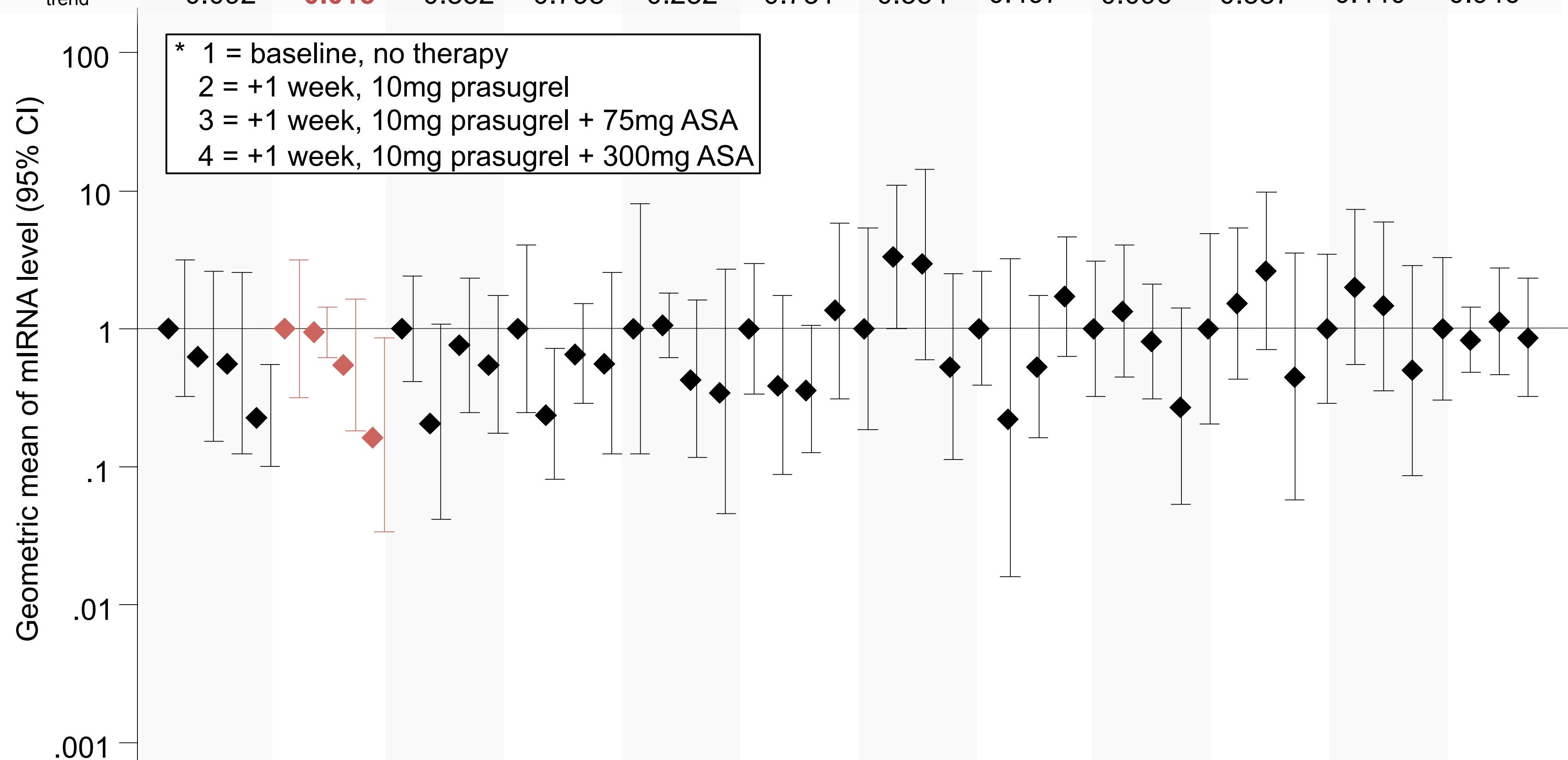
Online Figure III

MiRNA	660	21	193b	26a	142-3p	let7b	27a	197	20a	mmu451	192	29a
Group*	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4
P_{trend}	0.092	**0.015**	0.852	0.798	0.232	0.731	0.554	0.497	0.096	0.587	0.440	0.946



P values are from linear mixed models with random intercepts comparing miRNA levels over the four time points.

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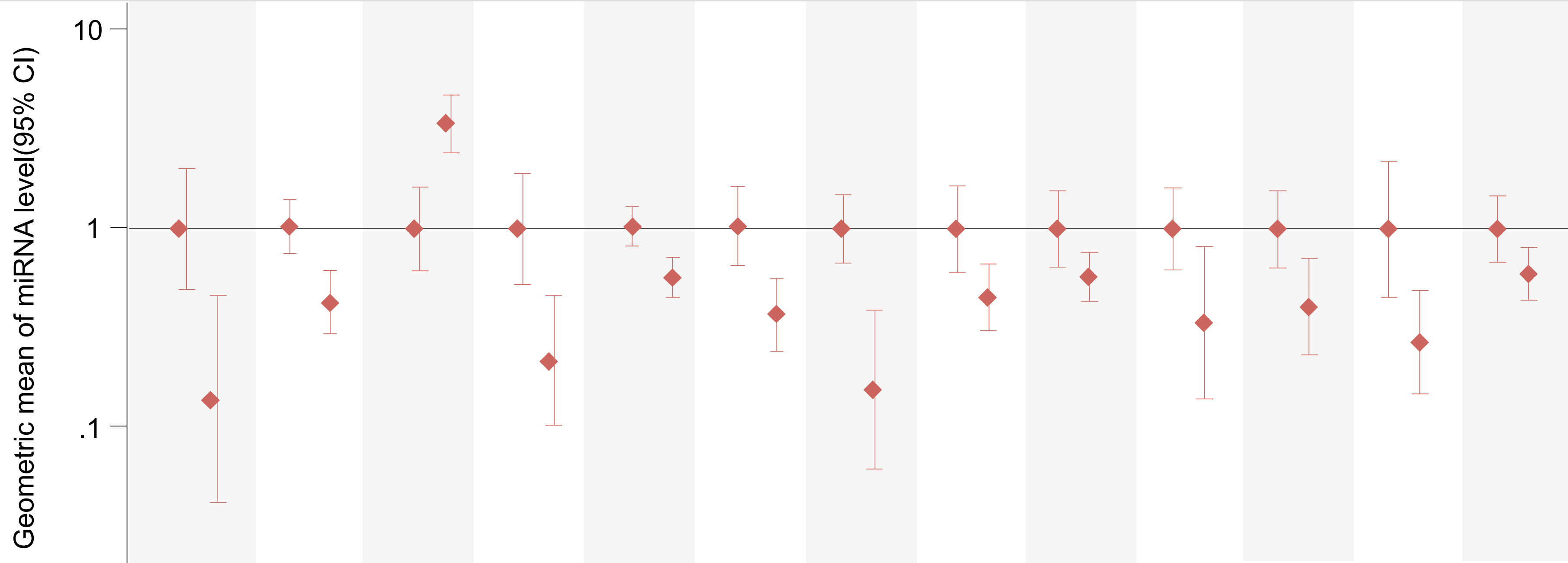
Online Figure IV

MiRNA	210		223		214		152		126		24		106a		340		142-3p		30c		146a		28-5p		26a	
Group*	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
P value	<0.001		<0.001		0.004		0.004		0.004		0.008		0.008		0.020		0.021		0.022		0.025		0.029		0.030	



* 1 = baseline, 75 mg ASA
2 = +48 h, 75 mg ASA + dipyridamole 200 mg bd. or
75 mg ASA + 300 mg clopidogrel loading dose followed by 75 mg od.

P values are from paired t-tests comparing miRNA levels at baseline (75mg ASA) and 48 hours later.

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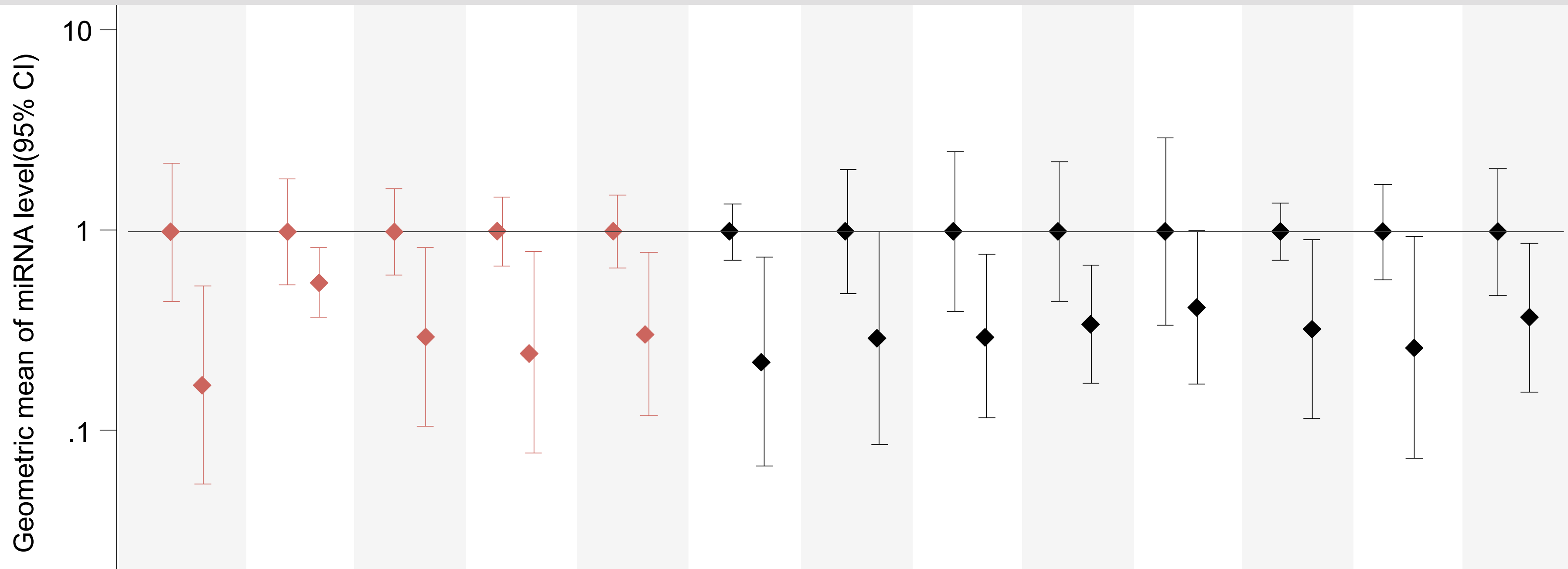
Online Figure IV

MiRNA	590-5p	574-3p	19b	103	199a-3p	93	885-5p	375	454	130b	107	335	17
Group*	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2
P value	0.032	0.038	0.042	0.045	0.049	0.051	0.053	0.064	0.072	0.079	0.086	0.097	0.109



* 1 = baseline, 75 mg ASA
2 = +48 h, 75 mg ASA + dipyridamole 200 mg bd. or
75 mg ASA + 300 mg clopidogrel loading dose followed by 75 mg od.

P values are from paired t-tests comparing miRNA levels at baseline (75mg ASA) and 48 hours later.

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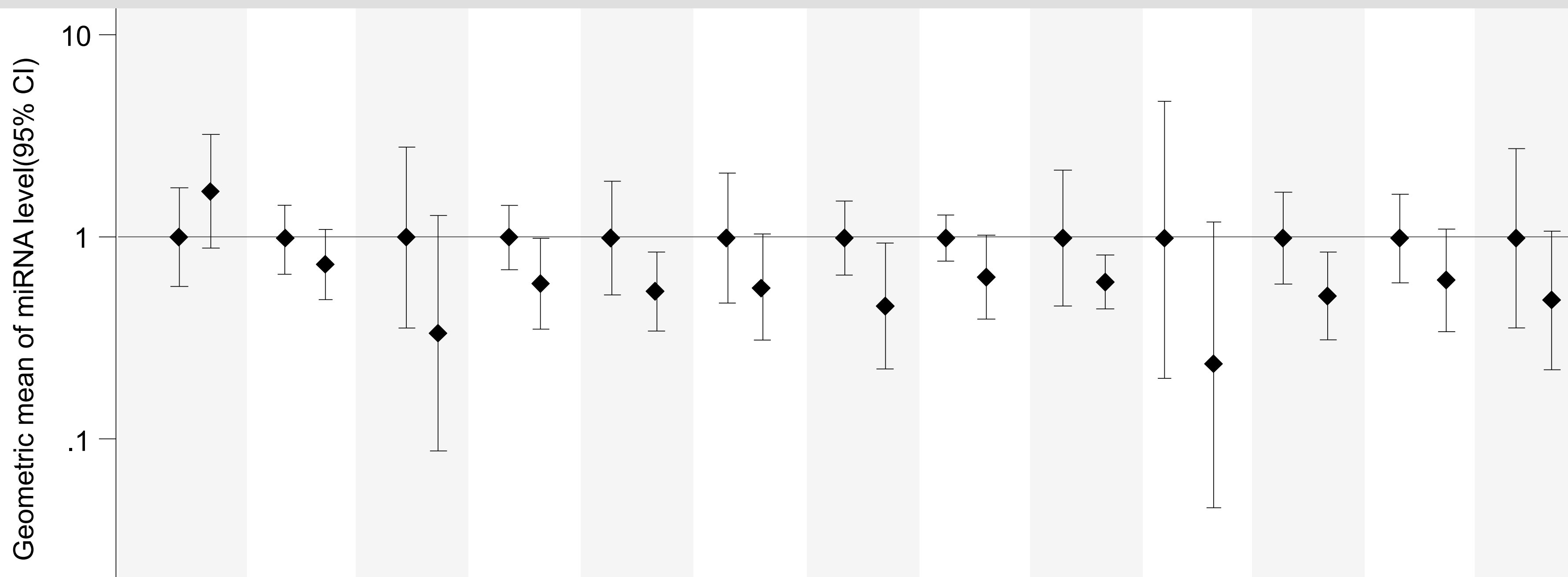
Online Figure IV

MiRNA	16	150	328	15a	27b	140-5p	28-3p	20a	200b	U6	27a	26b	376c
Group*	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2
P value	0.112	0.116	0.121	0.121	0.130	0.133	0.144	0.151	0.156	0.157	0.164	0.169	0.179



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75 mg ASA + 300 mg clopidogrel loading dose followed by 75 mg od.

P values are from paired t-tests comparing miRNA levels at baseline (75mg ASA) and 48 hours later.

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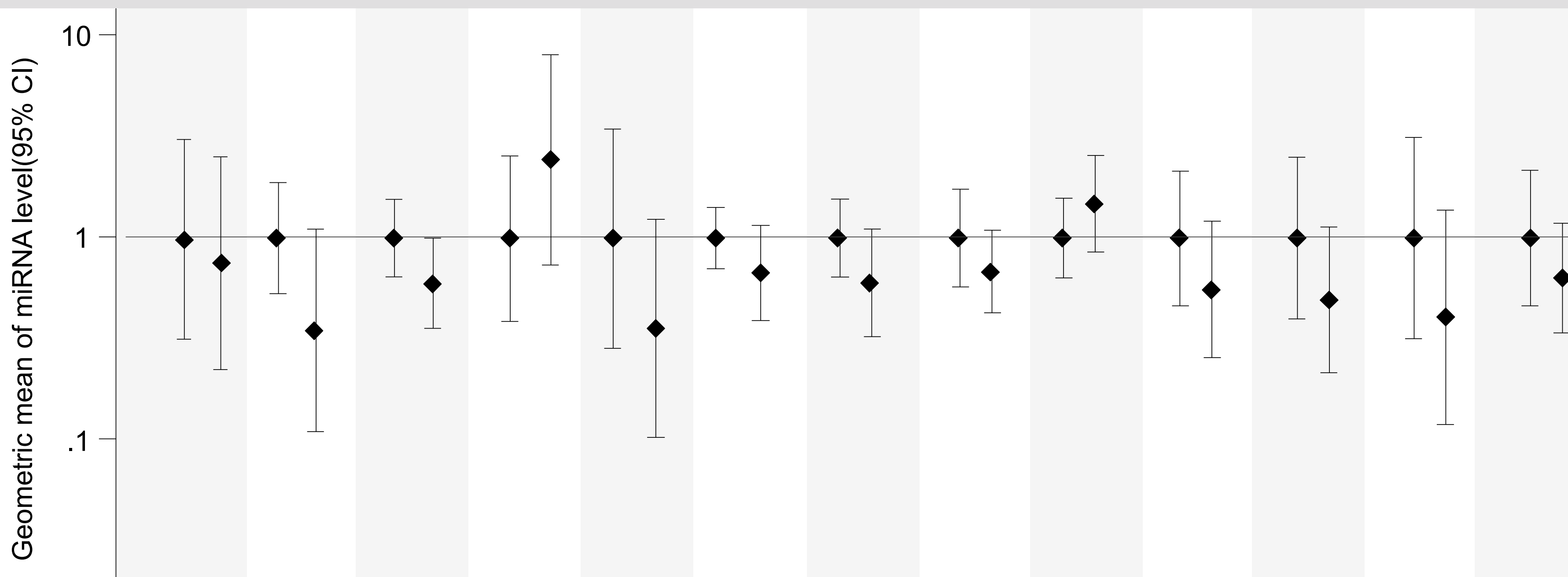
Online Figure IV

MiRNA	21		30b		320a		143		192		92a		423-5p		374a		628-5p		29b		18a		221		146b-5p	
Group*	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
P value	0.183		0.190		0.196		0.203		0.208		0.241		0.245		0.251		0.266		0.271		0.304		0.321		0.325	



* 1 = baseline, 75 mg ASA
2 = +48 h, 75 mg ASA + dipyridamole 200 mg bd. or
75 mg ASA + 300 mg clopidogrel loading dose followed by 75 mg od.

P values are from paired t-tests comparing miRNA levels at baseline (75mg ASA) and 48 hours later.

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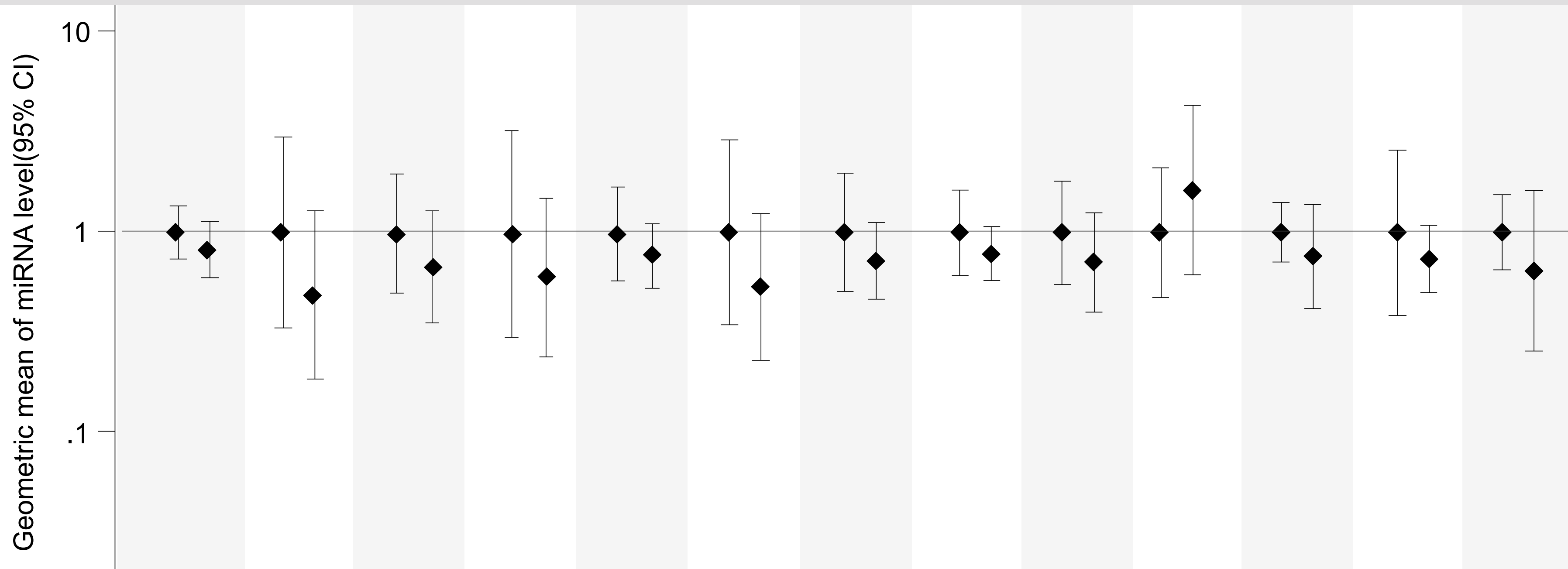
Online Figure IV



* 1 = baseline, 75 mg ASA
2 = +48 h, 75 mg ASA + dipyridamole 200 mg bd. or
75 mg ASA + 300 mg clopidogrel loading dose followed by 75 mg od.

P values are from paired t-tests comparing miRNA levels at baseline (75mg ASA) and 48 hours later.

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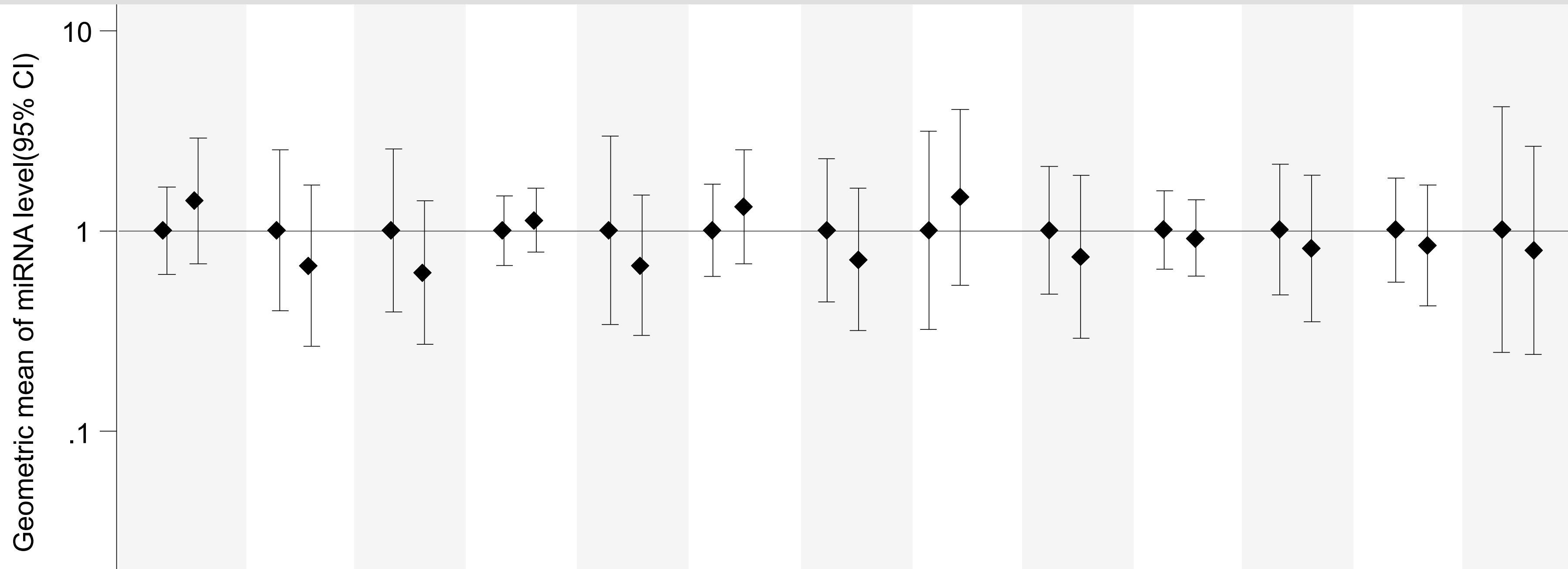
Online Figure IV

MiRNA	381	660	29a	127-3p	195	25	331-3p	342-3p	10a	744	194	486-5p	193b
Group*	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2
P value	0.479	0.498	0.513	0.535	0.577	0.578	0.587	0.641	0.644	0.654	0.673	0.702	0.740



* 1 = baseline, 75 mg ASA
2 = +48 h, 75 mg ASA + dipyridamole 200 mg bd. or
75 mg ASA + 300 mg clopidogrel loading dose followed by 75 mg od.

P values are from paired t-tests comparing miRNA levels at baseline (75mg ASA) and 48 hours later.

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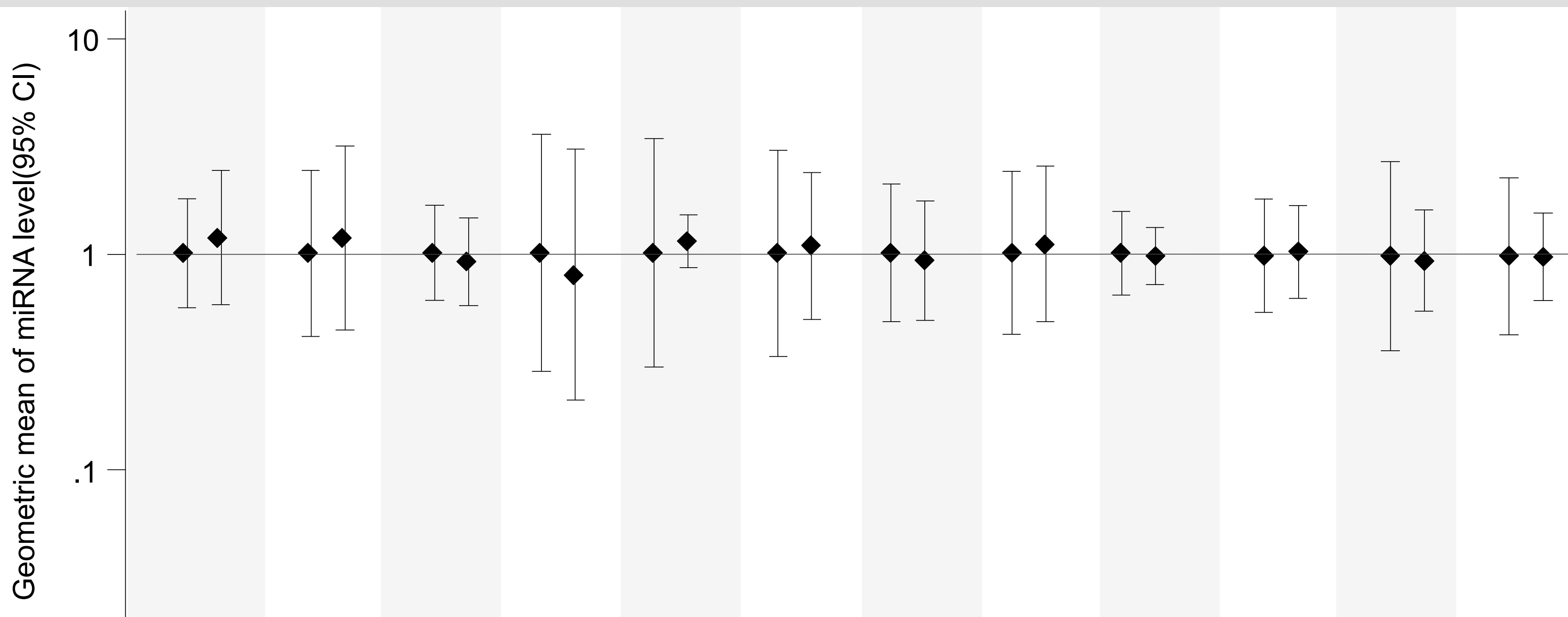
Online Figure IV

MiRNA	503		let7e		139-5p		532-3p		let7b		185		130a		125a-5p		202		484		122		18b	
Group*	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
P value	0.759		0.760		0.778		0.829		0.836		0.853		0.862		0.882		0.890		0.893		0.914		0.982	



* 1 = baseline, 75 mg ASA
2 = +48 h, 75 mg ASA + dipyridamole 200 mg bd. or
75 mg ASA + 300 mg clopidogrel loading dose followed by 75 mg od.

P values are from paired t-tests comparing miRNA levels at baseline (75mg ASA) and 48 hours later.

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