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(71) Applicant: **KING'S COLLEGE LONDON** [GB/GB]; An Institute Incorporated by Royal Charter of Strand, London WC2R 2LS (GB).

(72) Inventor: **MAYR, Manuel**; King's British Heart Foundation Centre, King's College London, 125 Coldharbour Lane, London SE5 9NU (GB).

(74) Agent: **TOMBLING, Adrian**; Withers & Rogers LLP, 4 More London Riverside, London SE1 2AU (GB).

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(54) Title: DETERMINATION OF MICRORNA EXPRESSION LEVELS FOR THE DIAGNOSIS OF A PLATELET-RELATED DISORDER

(57) Abstract: The present invention relates to a method of monitoring the efficacy of an anti-platelet therapy. The present invention also relates to a method of determining platelet activity, a method of predicting and/or diagnosing a platelet-related disorder and a method of determining the progression of a platelet-related disorder.

DETERMINATION OF MICRORNA EXPRESSION LEVELS FOR THE DIAGNOSIS OF A PLATELET-RELATED DISORDER

The present invention relates to a method of monitoring the efficacy of an anti-platelet therapy. The present invention also relates to a method of determining platelet activity, a method of predicting and/or diagnosing a platelet-related disorder and a method of determining the progression of a platelet-related disorder.

Platelets are one of the key elements of human blood. They play an important role in thrombogenesis, atherogenesis and the progression of atherosclerotic lesions.

Platelets are not only an important contributor to blood-related diseases, such as those characterised by prolonged bleeding, abnormal platelet activity has also been associated with the pathogenesis of a number of other diseases. For instance, the interaction of platelet with the vessel wall and its subsequent contribution to atheroma formation and thrombosis is of pivotal importance in the aetiology and pathogenesis of peripheral, coronary, cerebrovascular and other vascular diseases.

Accumulating evidence also suggests a role for platelet activation in cancer progression and an increase in platelet activity has been seen in patients with metastatic cancer.

Whilst there is increasing realization that inappropriate platelet activation plays a prime role in these diseases, there is still no generally accepted ideal measure of platelet activation that would indicate a state of 'high risk'. There is also a need to monitor the efficacy of anti-platelet therapy so that the level of treatment can be monitored and the effectiveness of anti-platelet therapies can be determined.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that function as translational repressors. They bind through canonical base pairing to a complementary site in the 3' untranslated region (UTR) of their target mRNAs and can direct the degradation or translational repression of these transcripts. Although the degree of target downregulation by miRNAs tends to be small, miRNAs exert a potent effect on cellular processes due to their ability to control multiple genes that function at different steps in the same biological

pathways. MiRNAs have been shown to play important roles in development, stress responses, angiogenesis and oncogenesis.

Recently, Mitchell *et al.* highlighted the presence of miRNAs in plasma. These plasma miRNAs are not cell-associated, but packaged in microvesicles that protect them from endogenous RNase activity. Interestingly, plasma miRNAs can display unique expression profiles: specific tumour miRNAs were identified in cancer patients, while tissue-derived miRNAs constitute a marker for injury. Altered levels of plasma miRNAs have been reported in patients with heart failure, coronary artery disease and diabetes.

According to a first aspect of the present invention, there is provided a method of monitoring the efficacy of an anti-platelet therapy, the method comprising the steps of determining the level of at least one microRNA selected from the group consisting of miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195 in a sample obtained from an individual before the therapy, determining the level of the at least one microRNA in a sample obtained from an individual during or after the therapy, and comparing the determined levels in the individual before and during or after the therapy.

It has been found that miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195 are expressed in platelets and that their levels change in response to anti-platelet therapy. Accordingly, making the determination set out above allows the efficacy of an anti-platelet therapy to be determined.

An increased level of one or more of miR-191, miR-185, miR-19a, miR-106a, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195, and/or a decreased level of miR-518f, during or after the therapy is indicative of an increased platelet miRNA content and, therefore, increased platelet activity.

Since levels of circulating microRNAs represent the net effect of shedding (microparticles) and clearance (D-dimer, which is a marker for thrombus resolution), an increased level of miR-191, miR-185, miR-19a, miR-106a, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195, and/or a decreased level of miR-518f, is also indicative of

an increased level of platelet miRNA shedding into the circulation, a decreased platelet microparticle clearance and/or enhanced thrombus resolution.

If the anti-platelet therapy reduces platelet microRNA shedding, then a decreased level of one or more of miR-191, miR-185, miR-19a, miR-106a, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195, and/or an increased level of miR-518f, during or after the therapy is a positive indication of the efficacy of the therapy, i.e., a reduction in platelet activity.

On the other hand, if the anti-platelet therapy reduces both platelet microRNA shedding and platelet microparticle clearance, then the change in the levels of the microRNAs depends on the extent to which the shedding and clearance are reduced.

An increased or decreased level of a microRNA after or during the therapy means that the level of the microRNA obtained after or during the therapy is higher or lower than that obtained before the therapy, respectively.

Anti-platelet therapy can be any therapy used to reduce platelet activity. Such therapies include administering a P2Y12 inhibitor, aspirin, prasugrel, dipyrdamole, clopidogrel and ticagrelor, and combinations of such agents. It has been determined that the recited microRNAs vary in substantially the same manner irrespective of the anti-platelet therapy used. Accordingly, the recited microRNAs are suitable markers for monitoring the efficacy of any anti-platelet therapy. Preferred anti-platelet therapies that can be monitored include those recited above as well as other therapies that target the same receptor and/or have substantially the same mechanism of action.

According to a second aspect of the present invention, there is provided a method of determining platelet activity in an individual comprising determining in a sample obtained from an individual the level of at least one microRNA selected from the group consisting of miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195.

It has been found that miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195 are highly expressed in

platelets and platelet microparticles. Accordingly, an increased level of one or more of miR-191, miR-185, miR-19a, miR-106a, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195, and/or a decreased level of miR-518f, is indicative of increased platelet miRNA content and, therefore, increased platelet activity. A reduced level of one or more of miR-191, miR-185, miR-19a, miR-106a, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195, and/or an increased level of miR-518f, is indicative of decreased platelet miRNA content and, therefore, decreased platelet activity.

In response to acute I/R (ischemia/reperfusion) injury in which the compensation of platelet miroRNA shedding and platelet microparticle clearance may not occur, the levels of all the microRNAs will change as indicted above and correlate to measurements of platelet microparticles, which are markers of platelet activation.

In diseases, such as diabetes and cardiovascular disease, where a compensatory increase in clearance may actually occur, the levels of the microRNAs may decrease.

The determination of a high or low level of microRNA is based on a control level, which is typically determined from a relevant population of individuals having normal platelet activities. The relevant population can be defined based on, for example, diet, lifestyle, age, ethnic background or any other characteristic that can affect the normal levels of the markers. Once the control levels are known, the measured levels can be compared and the significance of the difference determined using standard statistical methods. If there is a substantial difference between the measured level and the control level (i.e. a statistically significant difference), then the individual from whom the levels have been measured may be considered to have abnormal platelet activity.

By determining the level of platelet activity using the method of the present invention, it is possible to predict whether an individual is at risk of developing a disease which can be characterised by an abnormal level of platelet activity (i.e., a platelet-related disorder). Abnormal platelet activity refers to a level of platelet activity that is either higher or lower than the level in a healthy individual without the disease. In addition, determining the level of platelet activity allows the assessment of the effectiveness of a therapy for such a disease.

According to a third aspect of the present invention, there is provided a method of predicting and/or diagnosing a platelet-related disorder comprising determining in a sample obtained from an individual the level of at least one microRNA selected from the group consisting of miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195, and comparing the determined level in the individual with a control level.

Since it was found that miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195 are highly expressed in platelets and that their levels vary according to the activation state of the platelets, the level of these microRNAs can be used as an indication or biomarker of the level of platelet activity of an individual, thereby predicting or diagnosing a platelet-related disorder.

The term "a platelet-related disorder" refers to a disease or condition involving abnormal platelet activity, which may be higher or lower than the level of platelet activity in a healthy individual without the disorder. Platelet-related disorders include, but are not limited to, von Willebrand disease, Bernard-Soulier syndrome, Glanzmann thrombasthenia, thrombocytopenia, Henoch-Schönlein Purpura, thrombotic thrombocytopenic purpura, hemolytic uremic syndrome, acquired aplastic anemia, Wiskott-Aldrich syndrome, grey platelet syndrome, cancer and leukemia. Preferably, a platelet-related disorder does not include diabetes, vascular diseases or cardiovascular diseases, although abnormal platelet function is known to occur.

By making the determination set out above, it is possible to determine with high specificity and sensitivity whether an individual has or is likely to develop a platelet-related disorder. Specificity is defined as the proportion of true negatives (individuals that do not have or do not develop a platelet-related disorder) identified as such in the method. Sensitivity is defined as the proportion of true positives (individuals that have or are likely to develop a platelet disorder) identified as such in the method. The method provides a highly accurate test that can be performed relatively easily using any of the biomarkers.

Based on the nature of the platelet activity (mentioned above) in a particular platelet-related disorder, the levels of the microRNAs in an individual having, or likely to develop, the platelet-related disorder may be higher or lower than a control level.

For a platelet-related disorder characterised by an abnormally high platelet miRNA content, increased platelet miRNA shedding into the circulation, decreased platelet microparticle clearance and/or enhanced thrombus resolution (i.e., increased platelet activity), an increased level of one or more of miR-191, miR-185, miR-19a, miR-106a, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195, and/or a decreased level of miR-518f, is a positive indication of the disorder or the likelihood of developing such a disorder.

For a platelet-related disorder characterised by an abnormally low platelet miRNA content, decreased platelet miRNA shedding into the circulation, increased platelet microparticle clearance and/or impaired thrombus resolution (i.e., decreased platelet activity), a decreased level of one or more of miR-191, miR-185, miR-19a, miR-106a, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195, and/or an increased level of miR-518f, is a positive indication of the disorder or the likelihood of developing such a disorder.

The method according to the third aspect of the present invention allows the identification of individuals with a platelet-related disorder. The method also allows the identification of individuals that are likely to develop a platelet-related disorder. The method therefore enables preventative action to be taken, such as changes to the diet and lifestyle of the individual, as well as medical intervention.

According to a fourth aspect of the present invention, there is provided a method of monitoring the progression of a platelet-related disorder comprising the steps of determining the level of at least one microRNA selected from the group consisting of miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195 in a sample obtained from an individual at a first time point, determining the level of the least one microRNA in a sample obtained from an individual at a second time point, and comparing the determined levels in the individual at the first and second time points.

The second time point is after the first time point and the two time points are sufficiently spaced to allow the status of the disorder to change in such a manner so as to allow progression of the disorder to be monitored.

Since it was found that the levels of miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195 can be used as an indication of the level of platelet activity in an individual, making the determination set out above allows the progression of a platelet-related disorder to be monitored.

Based on the nature of the platelet activity (mentioned above) in a particular platelet-related disorder, progression of the platelet-related disorder may be indicated by higher or lower levels of the microRNAs.

For a platelet-related disorder characterised by an abnormally high platelet miRNA content, increased shedding of platelet miRNAs into the circulation, decreased platelet microparticle clearance and/or enhanced thrombus resolution (i.e., increased platelet activity), an increased level of an increased level of one or more of miR-191, miR-185, miR-19a, miR-106a, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195, and/or a decreased level of miR-518f, is a positive indication of the progression of the platelet-related disorder.

For a platelet-related disorder characterised by an abnormally low platelet miRNA content, decreased shedding of platelet miRNAs into the circulation, increased platelet microparticle clearance and/or impaired thrombus resolution (i.e., decreased platelet activity), a decreased level of an increased level of one or more of miR-191, miR-185, miR-19a, miR-106a, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195, and/or an increased level of miR-518f, is a positive indication of the progression of the platelet-related disorder.

An increased or decreased level of a microRNA means that the level of the microRNA obtained at the second time point is higher or lower than that obtained at the first time point, respectively.

The methods according to all aspects of the present invention are performed on a sample obtained from an individual. The sample may be any suitable sample from which it is possible to measure the microRNAs mentioned above. Preferably the sample is blood, serum, plasma or other blood fractions, or a tissue sample. Most preferably the sample is a plasma sample or a serum sample.

miR-191 is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-191 is given in the NCBI protein database under accession number NR_029690.1, version GI: 262205347.

miR-185 is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-185 is given in the NCBI protein database under accession number NR_029706.1, version GI: 262205427.

miR-19a is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-19a is given in the NCBI protein database under accession number NR_029489.1, version GI: 262205639.

miR-106a is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-106a is given in the NCBI protein database under accession number NR_029523.1, version GI: 262205799.

miR-518f is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-518f is given in the NCBI protein database under accession number NR_030194.1, version GI: 262205313.

miR-335 is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-335 is given in the NCBI protein database under accession number NR_029899.1, version GI: 262205112.

miR-17 is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-17 is given in the NCBI protein database under accession number NR_029487.1, version GI: 262205631.

miR-744 is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-744 is given in the NCBI protein database under accession number NR_030613.1, version GI: 262206132.

miR-20b is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-20b is given in the NCBI protein database under accession number NR_029950.1, version GI: 262205365.

miR-130a is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-130a is given in the NCBI protein database under accession number NR_029673.1, version GI: 262205274.

miR-18a is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-18a is given in the NCBI protein database under accession number NR_029488.1, version GI: 262205635.

miR-150 is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-150 is given in the NCBI protein database under accession number NR_029703.1, version GI: 262205410.

miR-195 is a standard term well known to those skilled in the art. In particular, the sequence of the human form of miR-195 is given in the NCBI protein database under accession number NR_029712.1, version GI: 262205461.

For the avoidance of doubt the specific sequences of the markers mentioned above are defined with respect to the version present in the database at the priority date of the present application. The specific sequences of the markers are exemplary. Those skilled in the art will appreciate that polymorphic variants exist in the human population and that the identification of such polymorphic variants is standard practice to those skilled in the art.

There are numerous ways of determining the level of the microRNAs, including Northern blotting, microRNA arrays, real-time RT-PCR methods, next generation sequencing, differential display, RNA interference, RNase protection methods, etc. Such methods are well known to those skilled in the art (see for example Ach *et al.*, BMC Biotechnology, 8, 69, 2008). Preferably the levels of the microRNAs are measured using real-time RT-PCR methods.

In some embodiments, the levels of a plurality of microRNAs selected from the group consisting of miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195 are determined. For example, the levels of two, three, four or all of the microRNAs are determined.

Since these microRNAs are expressed in different cell types (i.e. not exclusively in platelets), by determining the levels of more than one microRNA and their relationship to each other, one can get more reliable information on platelet activity.

The present invention will now be described in detail by way of example only with reference to the following figures.

Figure 1 shows platelet contribution to circulating miRNAs. 377 miRNAs were assessed in PRP, 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 (Ct<35) (B). Levels of miRNAs were consistently higher in PRP compared to PPP (C) and serum (D). Differences between PPP and serum were much less pronounced (E). Black lines on x- and y-axis indicate the detection threshold, assumed to be at a Ct value of 35.

Figure 2 shows miRNAs in patients with type 2 diabetes. A panel of 28 miRNAs was assessed in PPP and serum of the same diabetic patients (n=19) by individual TaqMan qPCR assays. Levels of miR-24, miR-191, miR-197 and miR-223 were significantly higher in serum compared to 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).

Figure 3 shows 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.

Figure 4 shows 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.

Figure 5 shows 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. *Highlighted groups differ significantly according to the p values derived from linear mixed models with random intercepts comparing miRNA levels over the four time points.

Figure 6 shows 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. * Highlighted groups differ significantly in their average ΔCt value from the baseline group (tested by paired t-tests, critical P value 0.05).

Figure 7 shows the response to anti-platelet therapy in healthy individuals. Following screening of 92 miRNAs using custom-made qPCR plates, a panel of 8 miRNAs plus U6 was assessed by individual Taqman qPCR assays in PPP from all healthy volunteers (n=9) participating in a dose-escalation study for anti-platelet therapy. P values are from linear mixed models with random intercepts comparing miRNA levels over the four time points.

Figure 8 shows the response to anti-platelet therapy in patients. Following screening of 92 miRNAs using custom-made qPCR plates, a panel of 8 miRNAs plus U6 was assessed in patients with symptomatic carotid atherosclerosis (n=33) who were on 75mg aspirin (ASA) at baseline. Samples were taken 48h after initiation of dual anti-platelet therapy with either dipyridamole or clopidogrel. *Highlighted groups differ significantly in their average ΔCt value from the baseline group (tested by paired t-tests, critical P value 0.05).

EXAMPLES

Material 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). *(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). *(c)* Findings in healthy volunteers were corroborated by miRNA measurements in 33 patients 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. Treatment efficiency was evaluated using transcranial Doppler detection of embolic signals and platelet aggregometry.

MiRNA screening in platelets and PMPs. Platelets were isolated from three healthy volunteers. 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 MgCl2, 5 mmol/L glucose, pH 7.3) at a concentration of 4 x 108/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 H2O. 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 (Zampetaki et al., 2010).

RNA isolation, reverse transcription and pre-amplification. MiRNAs were extracted using the miRNeasy kit (Qiagen) as described previously (Zampetaki et al., 2010; Zampetaki et al., 2012). 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. 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.

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 (Zampetaki et al., 2010).

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.

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 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 $P<0.05$.

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, PRP and PPP of healthy individuals. Consistent with the expression data from platelets and PMPs (Figure 3), miR-223 was the most differentially expressed miRNA in PRP (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 highest efficiency of prediction (Figure 2B). Other miRNAs present in platelets, i.e. 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 surrogate marker of efficacy of anti-platelet therapy. Healthy young males (<40yrs, n=9) were given 10mg prasugrel (week 1), followed by a combination therapy with low dose aspirin (75mg, week 2) and higher dose aspirin (300mg, week 3). This dose escalation of aspirin in combination with prasugrel resulted in increasing platelet inhibition. As reported previously (Leadbeater et al., 2011), platelet function was assessed by 96-well plate aggregometry and the formation of thromboxane A2 (measured as thromboxane B2) 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 (Figure 4). Using custom designed qPCR plates preplated by the TaqMan custom plating service, 92 miRNAs were tested in PPP of a subset of six 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 four time points (Figure 5). To confirm the effect of dual anti-platelet therapy, selected miRNAs were quantified in all participants by individual TaqMan qPCR assays (Figure 7). Correlation coefficients with custom-made qPCR plates exceeded 0.9. Notably, U6, a non-coding RNA frequently used for normalisation, was affected by anti-platelet 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 stenosis (n=33) participating in a randomized trial to determine whether treatment with dipyridamole or clopidogrel, in addition to aspirin, was more effective at reducing embolization. Both treatment regimens had similar efficacy in reducing embolization, as evaluated using transcranial Doppler detection of cerebral embolic signals (King et al., 2011). Twelve patients were randomly selected for miRNA analysis. All patients were on 75mg of aspirin at baseline. Eight were randomised and received dipyridamole and four clopidogrel in addition to aspirin. After 48h, effects on miRNAs were assessed in PPP using custom-designed miRNA qPCR plates based on Exiqon's LNA™ technology to further ensure robustness of data independent of the technological platform (Figure 6). Selected miRNAs were quantified in all patients (n=33) by individual TaqMan qPCR assays (Figure 8). 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 anti-platelet agents. In comparison, changes in *ex vivo* measurements of peak aggregation to ADP and collagen were less pronounced.

Platelet activity measurements. Data has been generated comparing the levels of some of the microRNA markers of the present invention with commercial platelet function tests (VerifyNow and Platelet VASP Test). The levels of the microRNA markers correspond with the level of platelet function as expected. Data not shown.

Discussion

The present study identified circulating platelet miRNAs that are responsive to anti-platelet therapy. Particular strengths of our study are: 1) The repeated measurements in healthy individuals; 2) the additional investigation in patients on dual anti-platelet therapy; 3) the large number of miRNAs that were assessed by using custom-designed miRNA qPCR plates based on two different technologies (LNA™ and TaqMan) (Zampetaki et al., 2012). The identified miRNAs are good markers of the effectiveness of anti-platelet therapy.

Circulating platelet miRNAs. Platelets represent the second most abundant cell type in blood. Although their miRNA content is low compared to 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 upon activation and reduced microparticle shedding upon platelet inhibition is likely to be responsible for the observed decrease in plasma miRNAs. Anti-platelet therapy was probably a confounding factor in previous case-control studies reporting a loss of miRNAs in patients with coronary artery disease (Fichtlscherer et al., 2010). Similarly, the transcoronary concentration gradients of non-cardiac miRNAs may be related to platelet adhesion in the coronary circulation (De Rosa et al., 2011). Given our limited knowledge about circulating miRNAs (Engelhardt et al., 2012), 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 anti-platelet therapy. Anti-platelet 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 anti-platelet efficacy (Gremmel et al., 2011). Importantly, the combination therapy of aspirin plus one or more P2Y12 inhibitors is associated with a significant bleeding risk, which can be life-threatening in a small but significant number of patients (Wallentin et al.,2009). 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. P2Y12 inhibitors such as clopidogrel, prasugrel, and ticagrelor act by inhibiting adenosine diphosphate receptors. Thus, their mechanisms are complementary but variability in response to clopidogel has been associated with gastrointestinal absorption, drug interactions and P450 isoenzyme activity (Price et al., 2012). There is currently no one measure or standard

definition in tests for antiplatelet efficacy. The study provides proof-of concept that platelet miRNAs should be explored as a point-of-care test for tailoring anti-platelet therapies. These findings could become even more important as new potent anti-platelet agents are currently developed and tested in clinical trials.

MicroRNA analysis has also been performed on plasma samples obtained from 32 matched patients 30 days post acute coronary syndrome and following treatment with different anti-platelet therapies, namely: 1. Aspirin only; 2. Clopidogrel and Aspirin; 3. Prasugrel and Aspirin; and 4. Ticagrelor and Aspirin. The results show that for the miRNAs tested (miR-126, MiR-197, miR-223, miR-24, miR-21, miR-150, miR-191 and miR-20b) there is no substantial variation between the different treatment groups, i.e., the miRNAs are good markers of all the anti-platelet therapies tested. Data not shown.

To further confirm the value of the presently claimed microRNAs the ability of the mircoRNAs to monitor the effectiveness of anti-platelet therapy was compared with a commercially available method of determining anti-platelet therapy. In particular, 125 patients with a history of acute coronary syndrome, were tested to determine the level of the circulating miRNAs of the present invention. The results were then compared to the current commercially available test, namely the Platelet VASP test from Lancet Laboratories. The results from our miRNAs markers correlate well to the Platelet VASP test confirming that the miRNAs provide an accurate indication of anti-platelet efficacy. Data not shown. It is important to note that the use of miRNAs provides advantages over the Platelet VASP test, e.g., they are easier to measure by qPCR (the Platelet VASP test requires measurement by flow cytometry). Moreover, miRNAs can be measured in frozen samples, whereas the Platelet VASP test has to be done on fresh samples.

Conclusions

Repeated measurements in healthy volunteers and patients on anti-platelet therapy provided proof-of-concept that miRNAs can serve as novel biomarkers of platelet activation and co-diagnostic for efficacy of anti-platelet therapy. Our findings also highlight that anti-platelet therapy is a potential confounding factor for miRNA measurements in case-control studies of cardiovascular disease.

All references cited above are herein incorporated by reference.

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Claims

1. A method of monitoring the efficacy of an anti-platelet therapy, the method comprising the steps of determining the level of at least one microRNA selected from the group consisting of miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195 in a sample obtained from an individual before the therapy, determining the level of the least one microRNA in a sample obtained from an individual during or after the therapy, and comparing the determined levels in the individual before and during or after the therapy.

2. A method of determining platelet activity in an individual comprising determining in a sample obtained from an individual the level of at least one microRNA selected from the group consisting of miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195.

3. A method of predicting and/or diagnosing a platelet-related disorder comprising determining in a sample obtained from an individual the level of at least one microRNA selected from the group consisting of miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195, and comparing the determined level in the individual with a control level.

4. A method of monitoring the progression of a platelet-related disorder comprising the steps of determining the level of at least one microRNA selected from the group consisting of miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195 in a sample obtained from an individual at a first time point, determining the level of the at least one microRNA in a sample obtained from an individual at a second time point, and comparing the determined levels in the individual at the first and second time points.

5. A method according to any preceding claim, wherein the levels of a plurality of microRNAs selected from the group consisting of miR-191, miR-185, miR-19a, miR-106a, miR-518f, miR-335, miR-17, miR-744, miR-20b, miR-130a, miR-18a, miR-150 and miR-195 are determined.

6. A method according to any preceding claim, wherein the sample is a tissue sample, blood, serum or plasma.

7. A method according to claim 6, wherein the sample is plasma or serum.

FIGURE 1

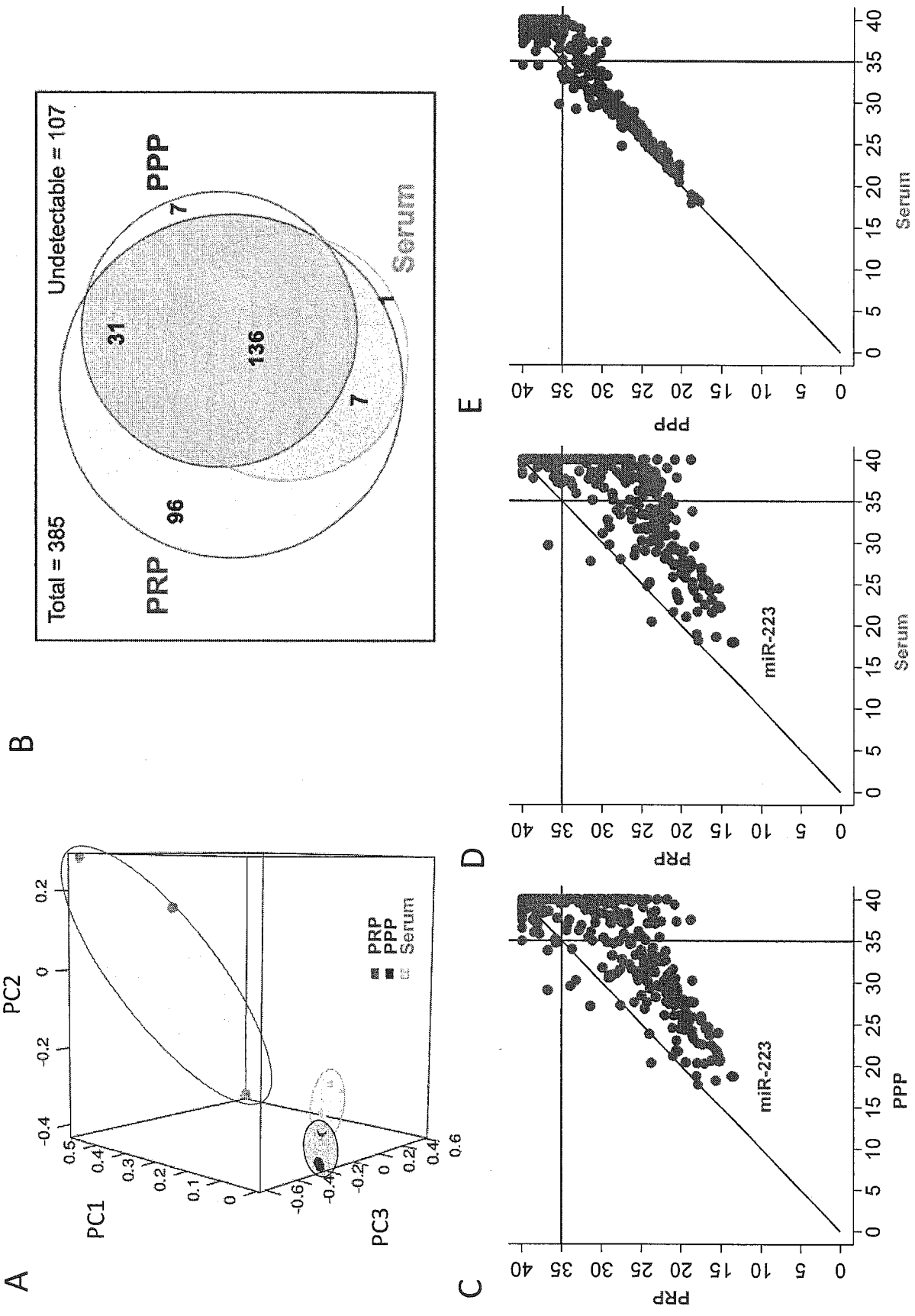

A
PC2
PC1
PC3
PRP
PPP
Serum
B
Total = 385
Undetectable = 107
PRP
PPP
Serum
96
31
136
7
7
1
C
PRP
PPP
miR-223
D
PRP
Serum
miR-223
E
PPP
Serum

FIGURE 2

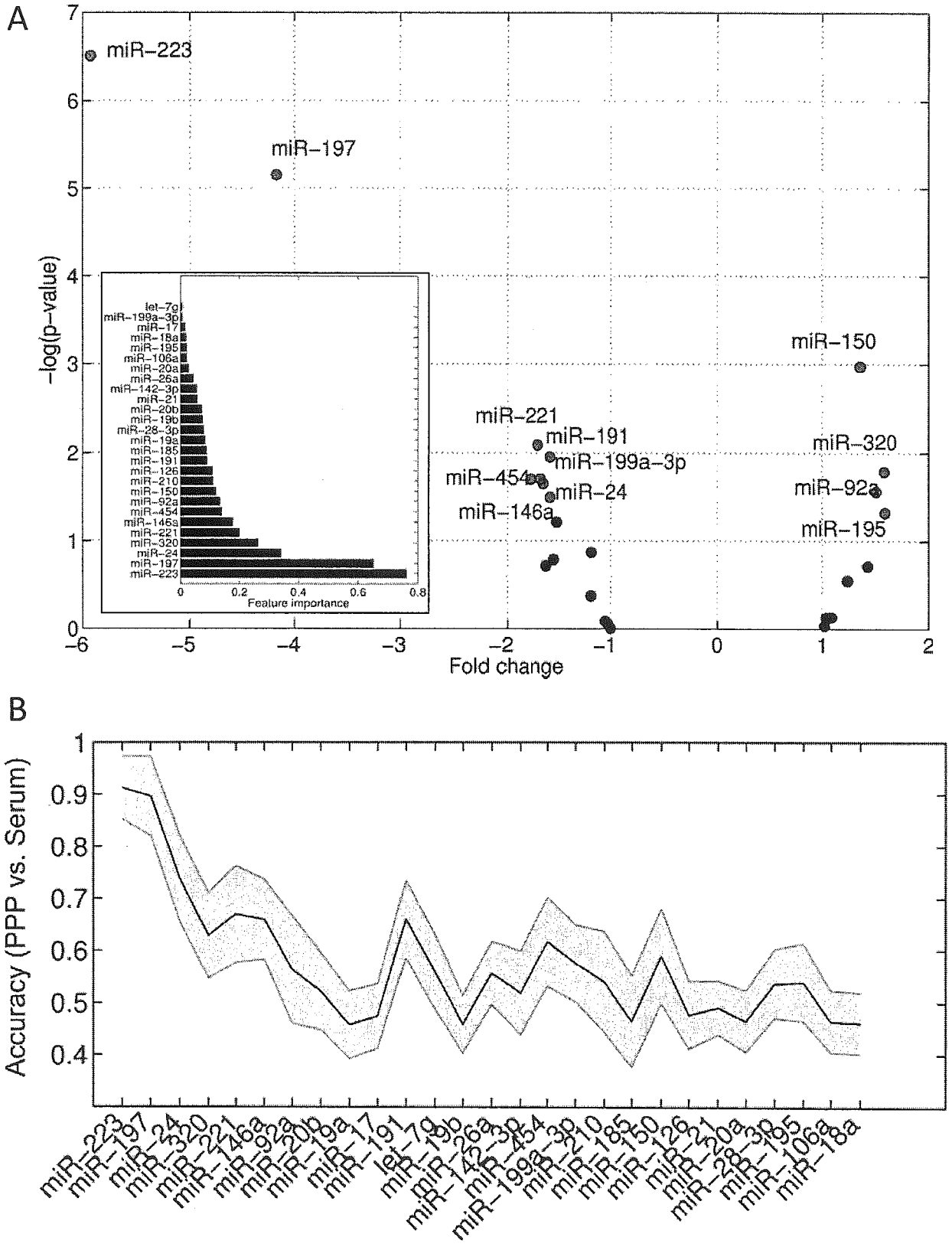

A
miR-223
miR-197
miR-150
miR-221
miR-191
miR-199a-3p
miR-454
miR-24
miR-146a
miR-320
miR-92a
miR-195
−log(p-value)
Fold change
Feature importance
B
Accuracy (PPP vs. Serum)

FIGURE 3

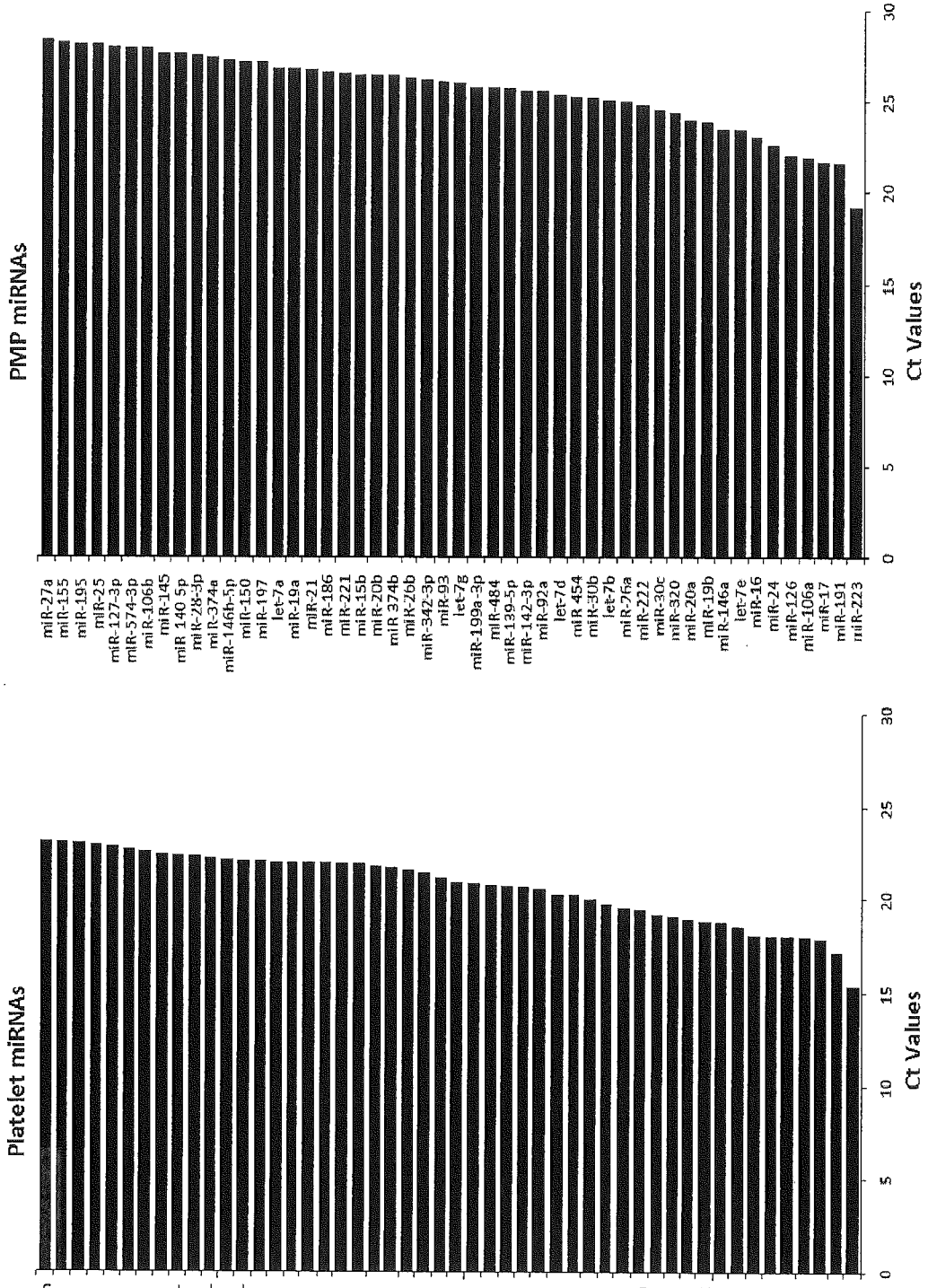

Platelet miRNAs
miR-177-3p
miR-423-5p
miR-652
miR-19a
miR-374a
miR-331-3p
miR-26b
miR-574-3p
miR-146b-
miR-15U
miR-199a-
miR-28-3p
miR-125a-
miR-139-5p
let-7g
miR-145
miR-20b
miR-21
miR-374b
miR-103
miR-15b
miR 744
miR-197
miR-328
miR-186
let-7a
let-7b
miR-93
miR-221
miR-342-3p
miR-26a
let-7d
miR-30c
miR-30b
miR-142-3p
miR-20a
miR-92a
miR-19b
miR-484
miR-16
miR-222
miR-320
miR-146a
miR-126
miR-106a
miR-17
miR-24
let 7c
miR-191
miR-223
0
5
10
15
20
25
30
Ct Values
PMP miRNAs
miR-27a
miR-155
miR-195
miR-25
miR-127-3p
miR-574-3p
miR-106b
miR-145
miR 140 5p
miR-28-3p
miR-374a
miR-146b-5p
miR-150
miR-197
let-7a
miR-19a
miR-21
miR-186
miR-221
miR-15b
miR-20b
miR 374b
miR-26b
miR-342-3p
miR-93
let-7g
miR-199a-3p
miR-484
miR-139-5p
miR-142-3p
miR-92a
let-7d
miR 454
miR-30b
let-7b
miR-76a
miR-222
miR-30c
miR-320
miR-20a
miR-19b
miR-146a
let-7e
miR-16
miR-24
miR-126
miR-106a
miR-17
miR-191
miR-223
0
5
10
15
20
25
30
Ct Values

FIGURE 4

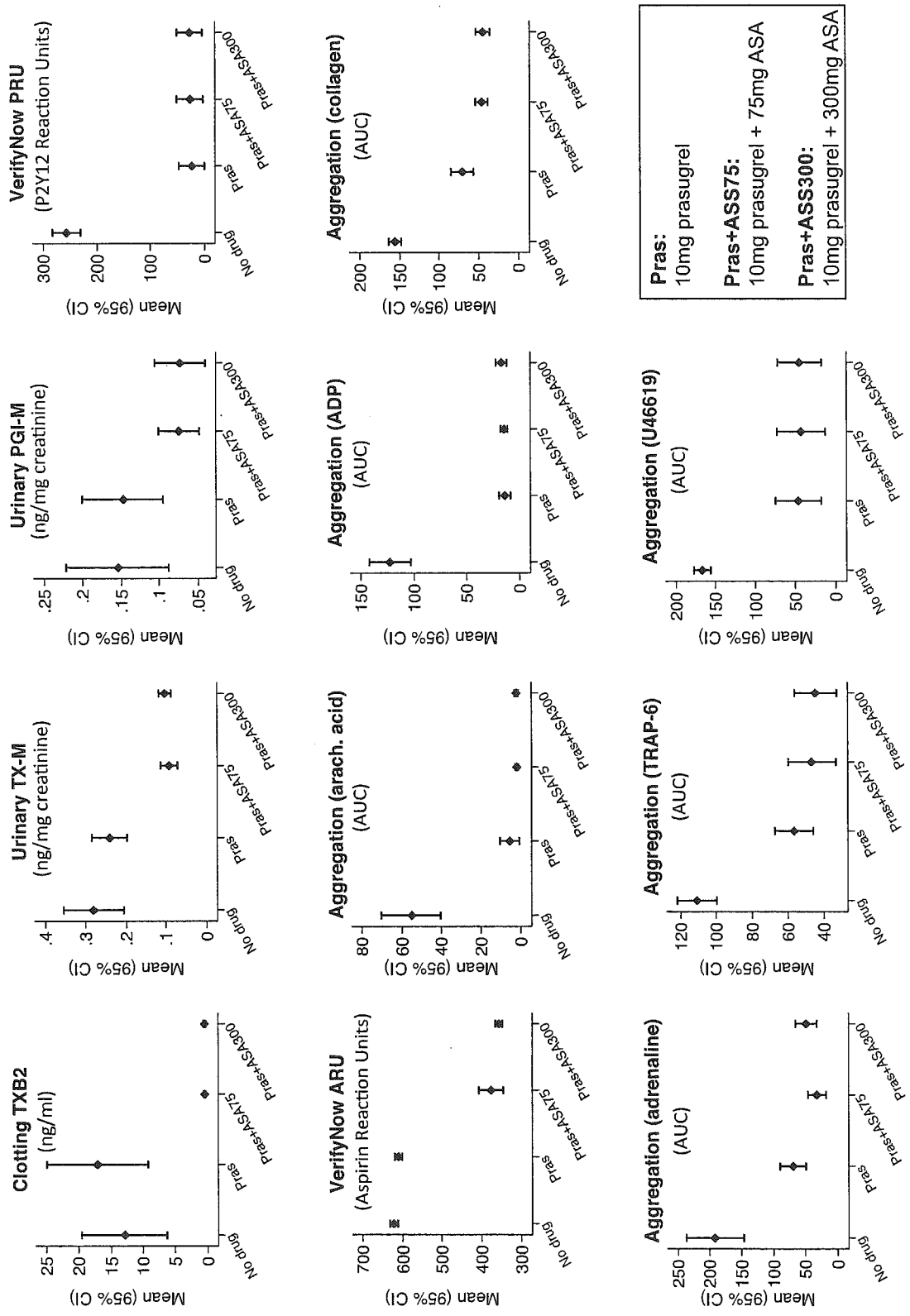
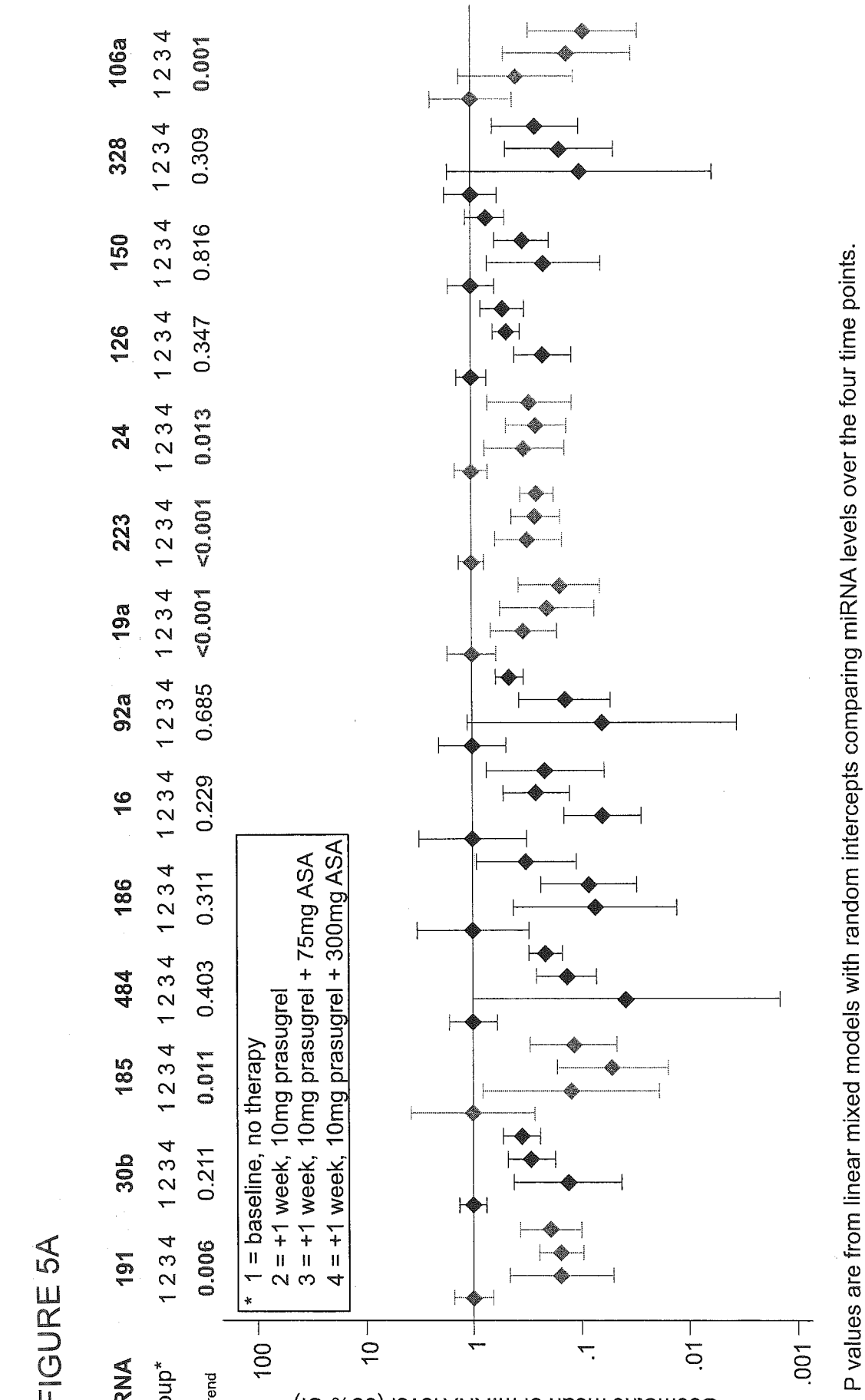
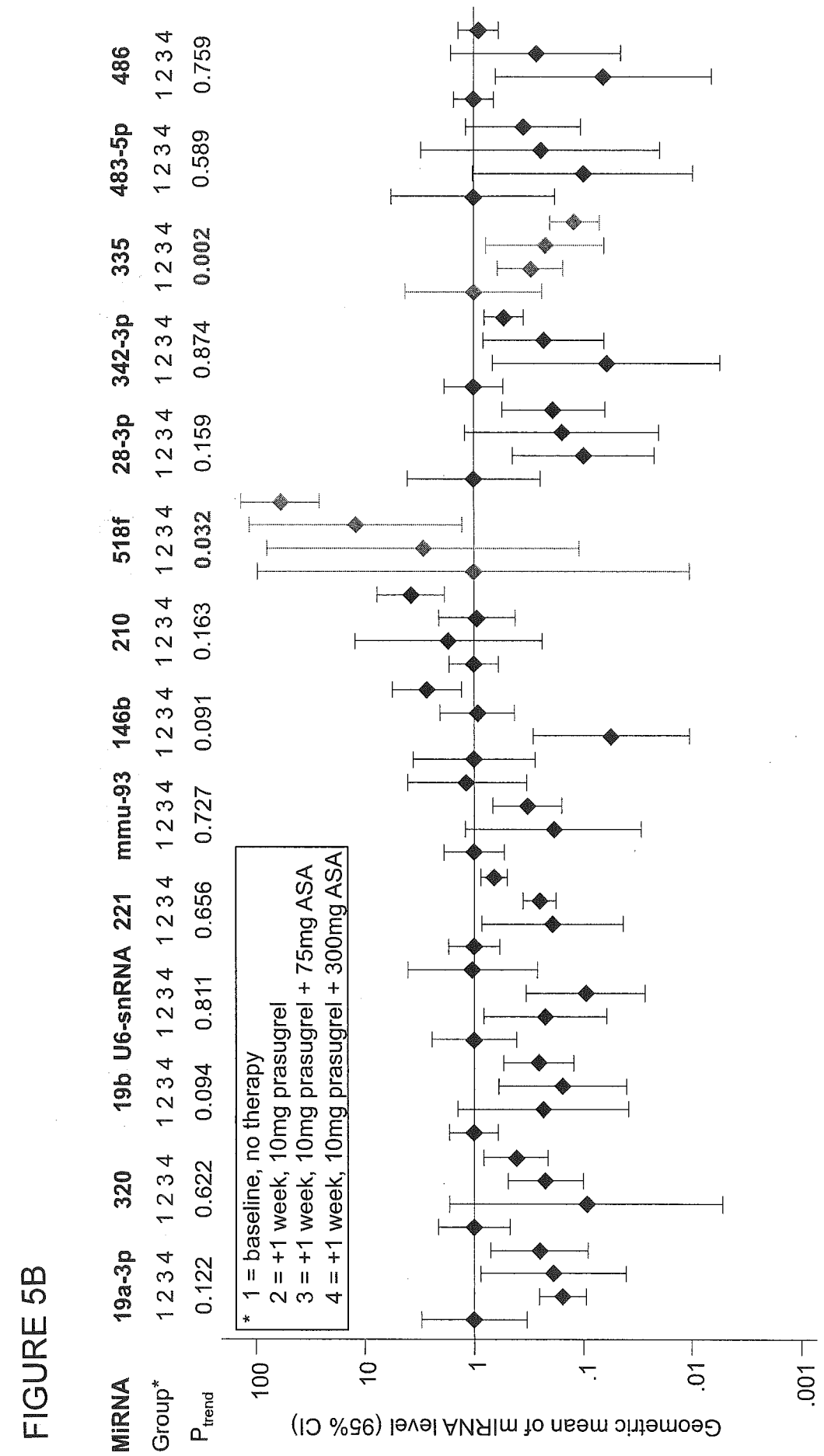

Clotting TXB2
(ng/ml)
Urinary TX-M
(ng/mg creatinine)
Urinary PGI-M
(ng/mg creatinine)
VerifyNow PRU
(P2Y12 Reaction Units)
VerifyNow ARU
(Aspirin Reaction Units)
Aggregation (arach. acid)
(AUC)
Aggregation (ADP)
(AUC)
Aggregation (collagen)
(AUC)
Aggregation (adrenaline)
(AUC)
Aggregation (TRAP-6)
(AUC)
Aggregation (U46619)
(AUC)
Mean (95% CI)
No drug
Pras
Pras+ASA75
Pras+ASA300
Pras:
10mg prasugrel
Pras+ASS75:
10mg prasugrel + 75mg ASA
Pras+ASS300:
10mg prasugrel + 300mg ASA

FIGURE 5A



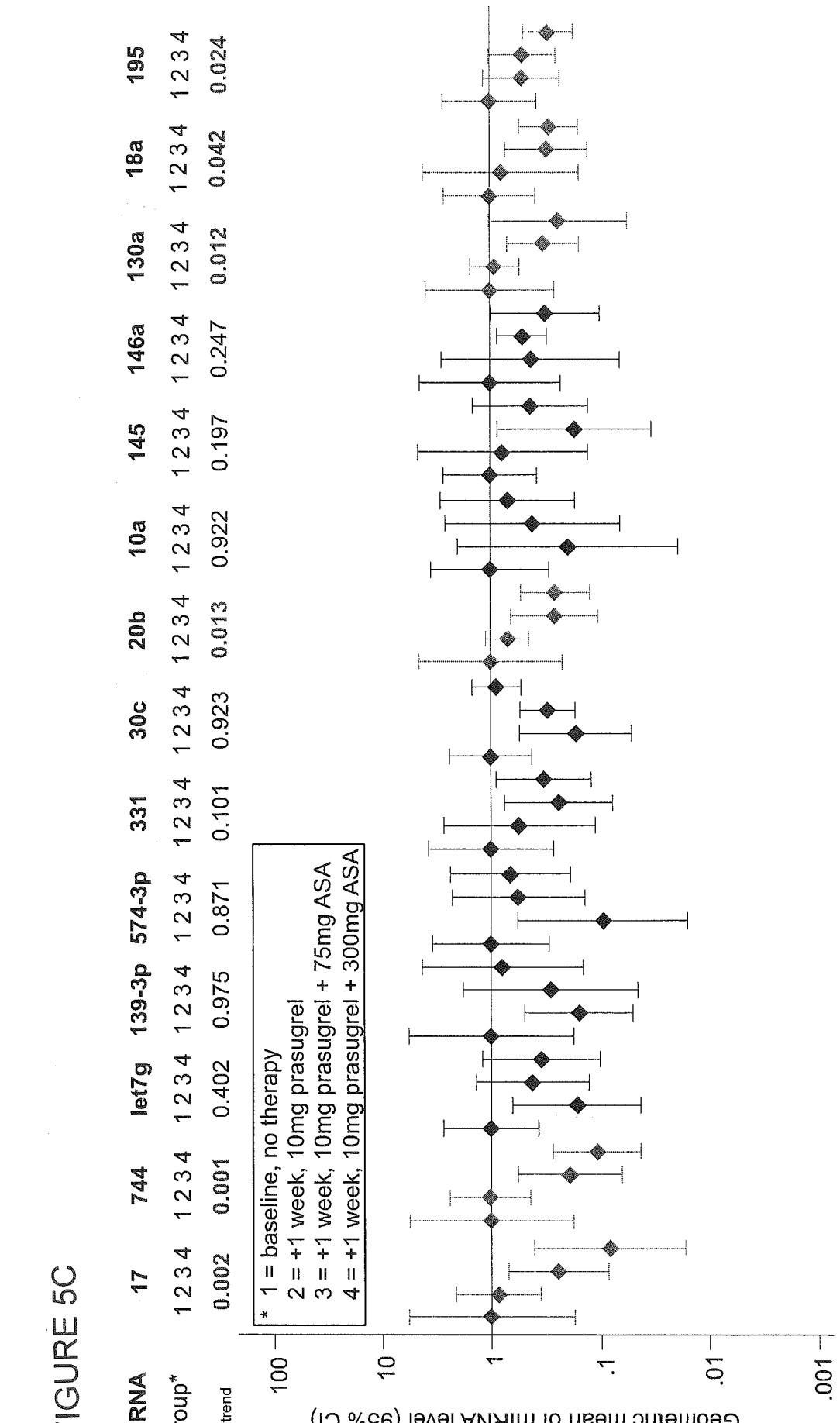
P values are from linear mixed models with random intercepts comparing miRNA levels over the four time points.

FIGURE 5B



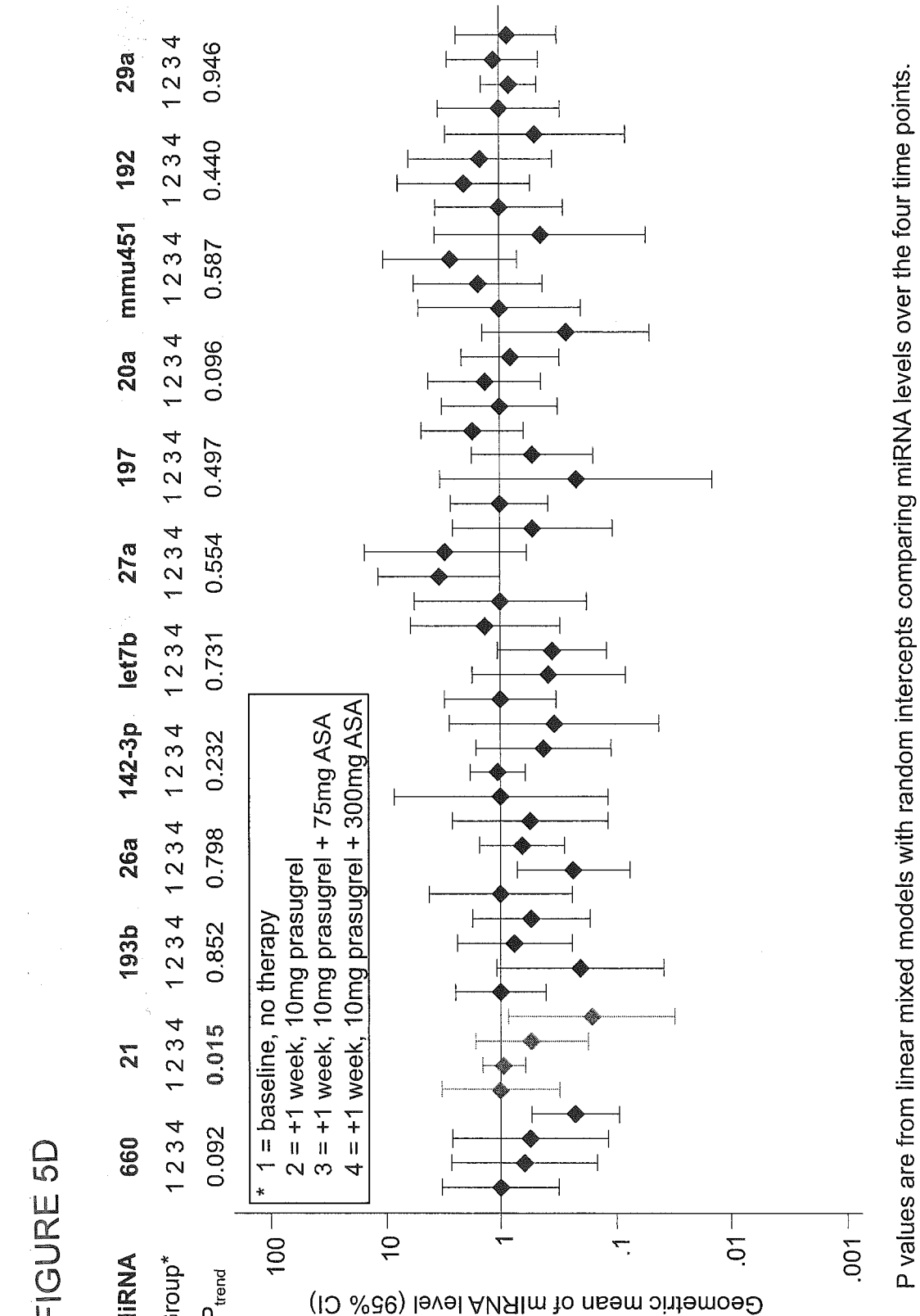
P values are from linear mixed models with random intercepts comparing miRNA levels over the four time points.

FIGURE 5C



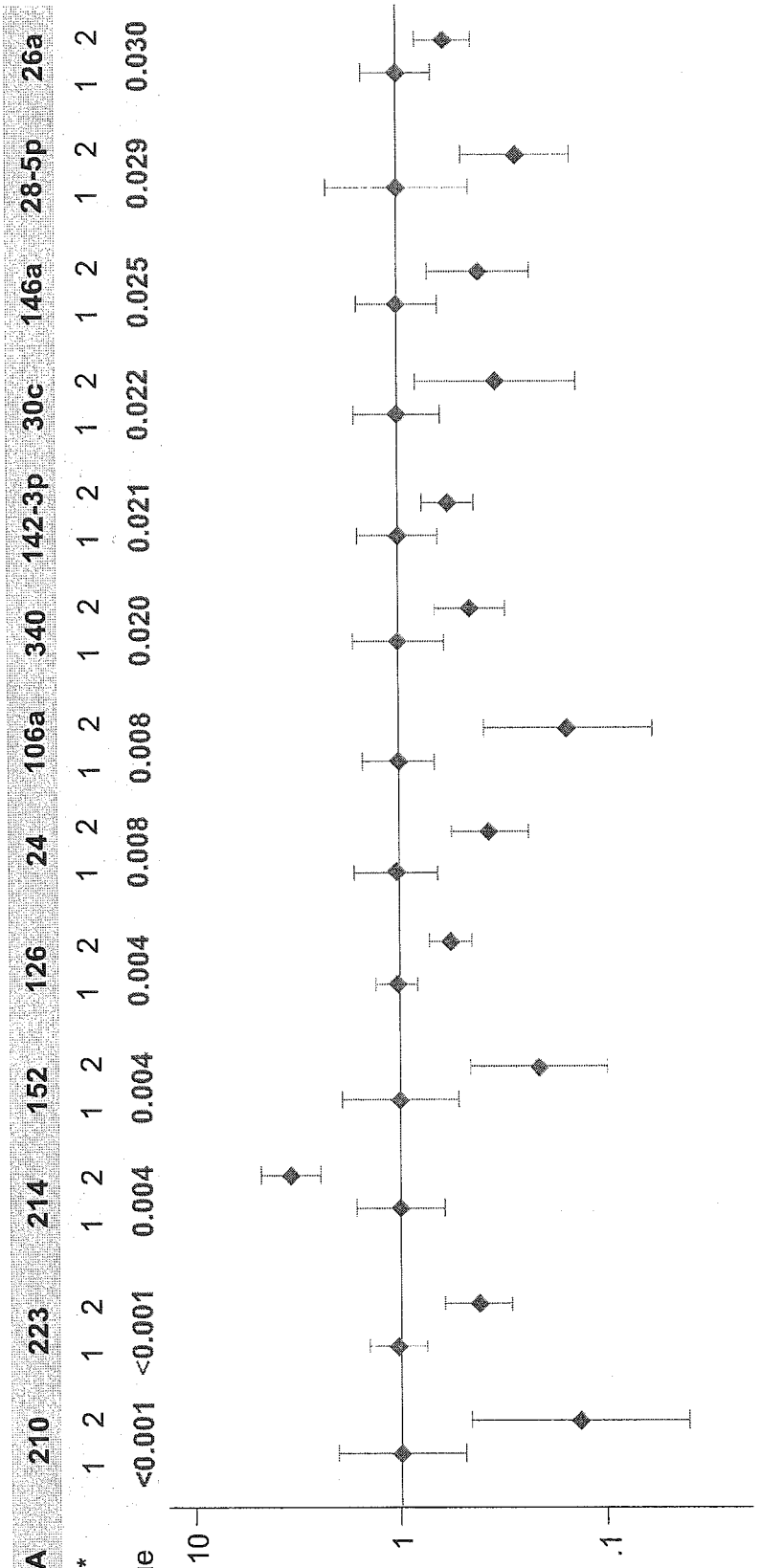
P values are from linear mixed models with random intercepts comparing miRNA levels over the four time points.

FIGURE 5D



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

FIGURE 6A

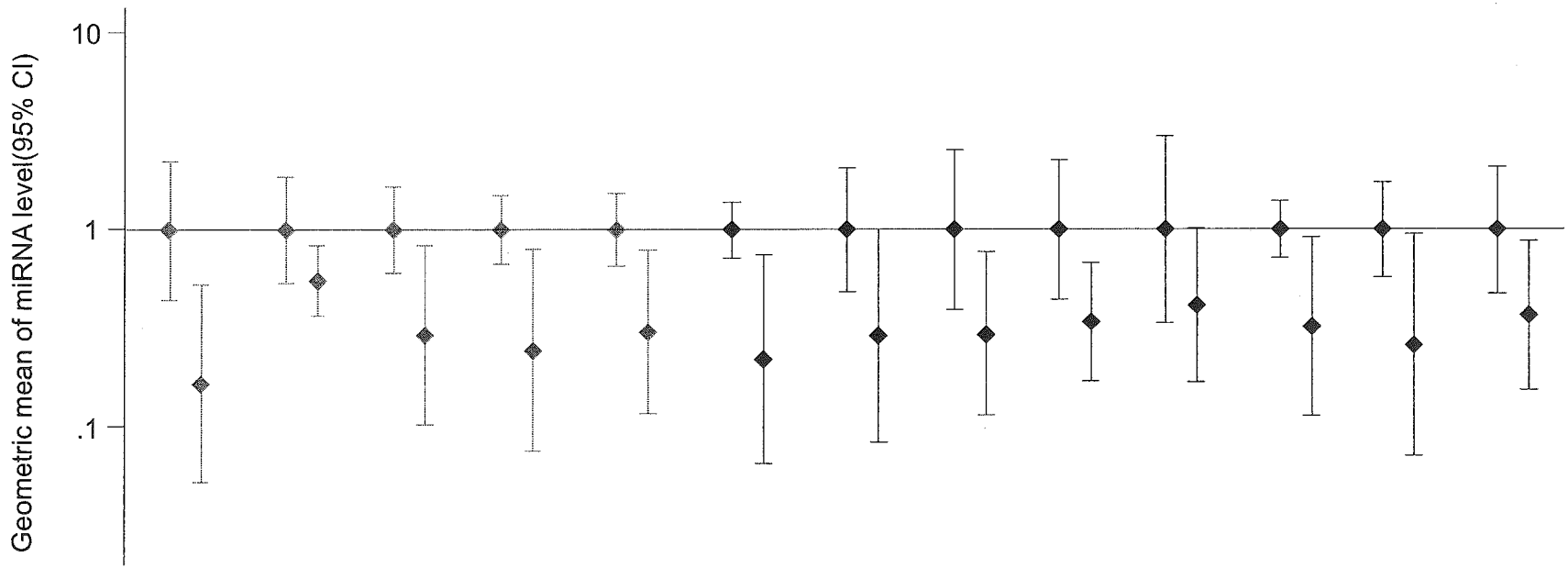


* 1 = baseline, 75 mg ASA
2 = +48 h, 75 mg ASA + dipyridamole 200 mg bd. or
75 mg ASA + 300 mg clopidogrel loading dose followed by 75 mg od.

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

FIGURE 6B

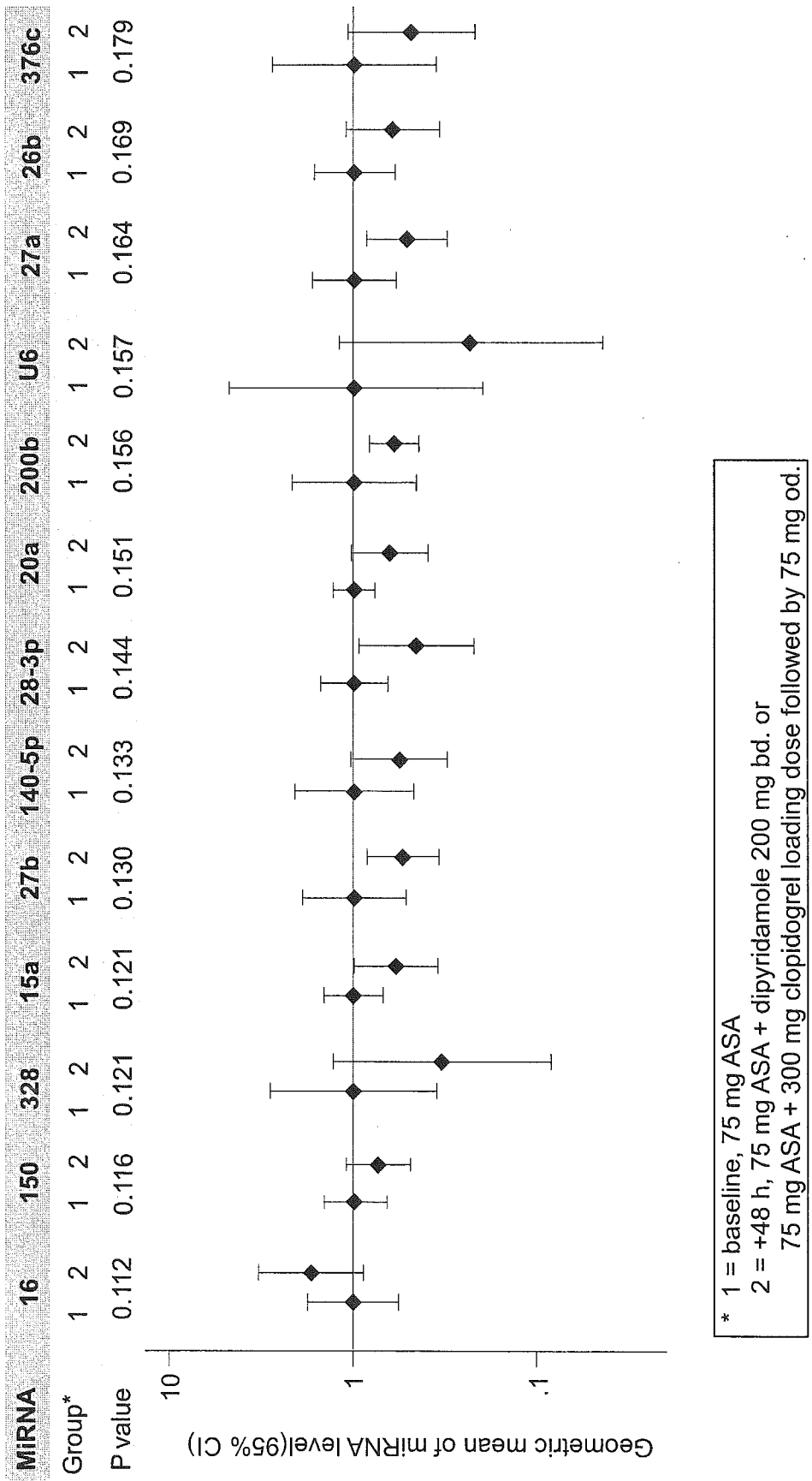
MiRNA	590-5p	574-3p	19b	103	199a-3p	93	885-5p	375	454	130b	107	335	17
Group*	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2
P value	0.032	0.038	0.042	0.045	0.049	0.051	0.053	0.064	0.072	0.079	0.086	0.097	0.109



* 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.

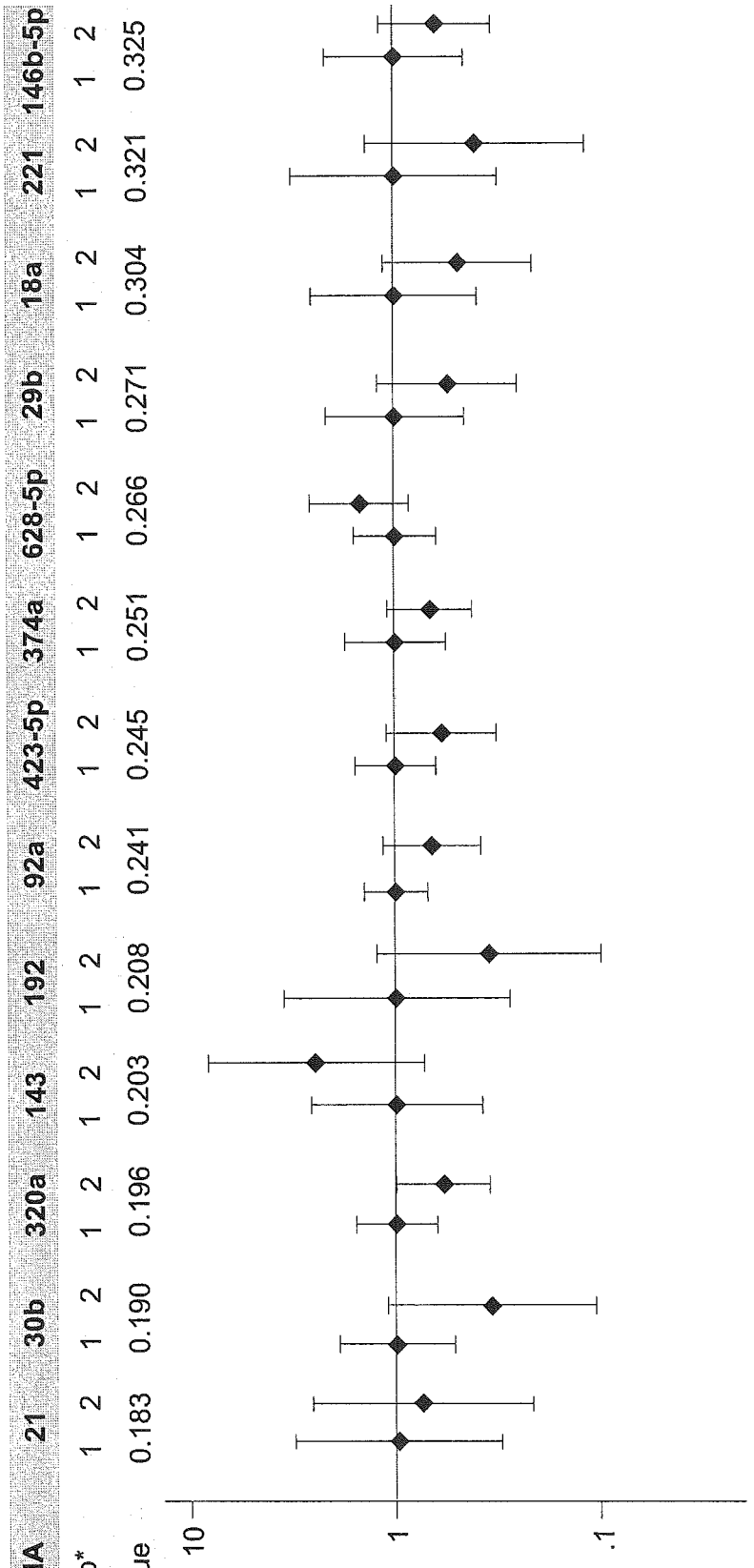
P values are from paired t-tests comparing miRNA levels at baseline (75mg ASA) and 48 hours later.

FIGURE 6C



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

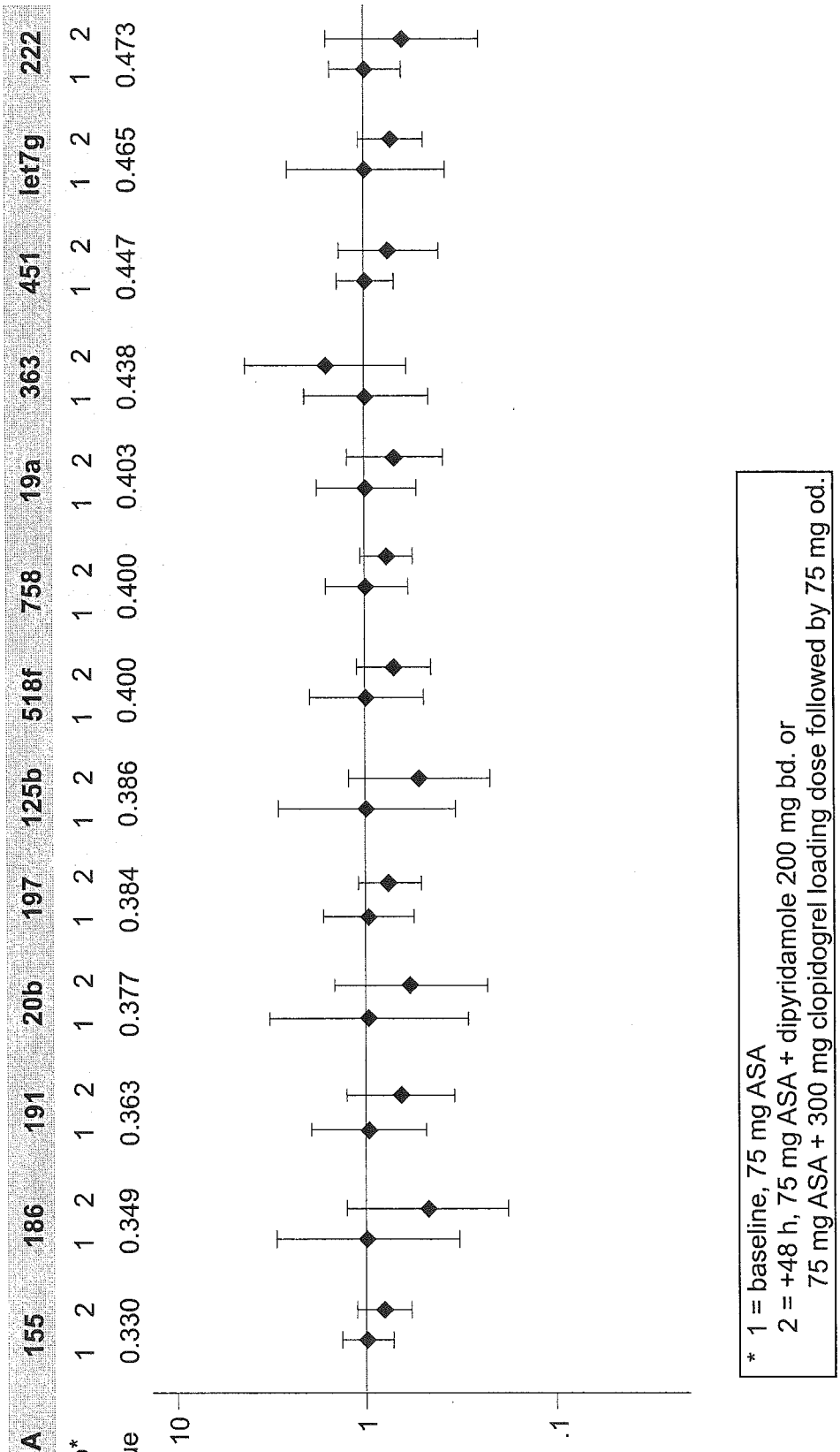
FIGURE 6D



* 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.

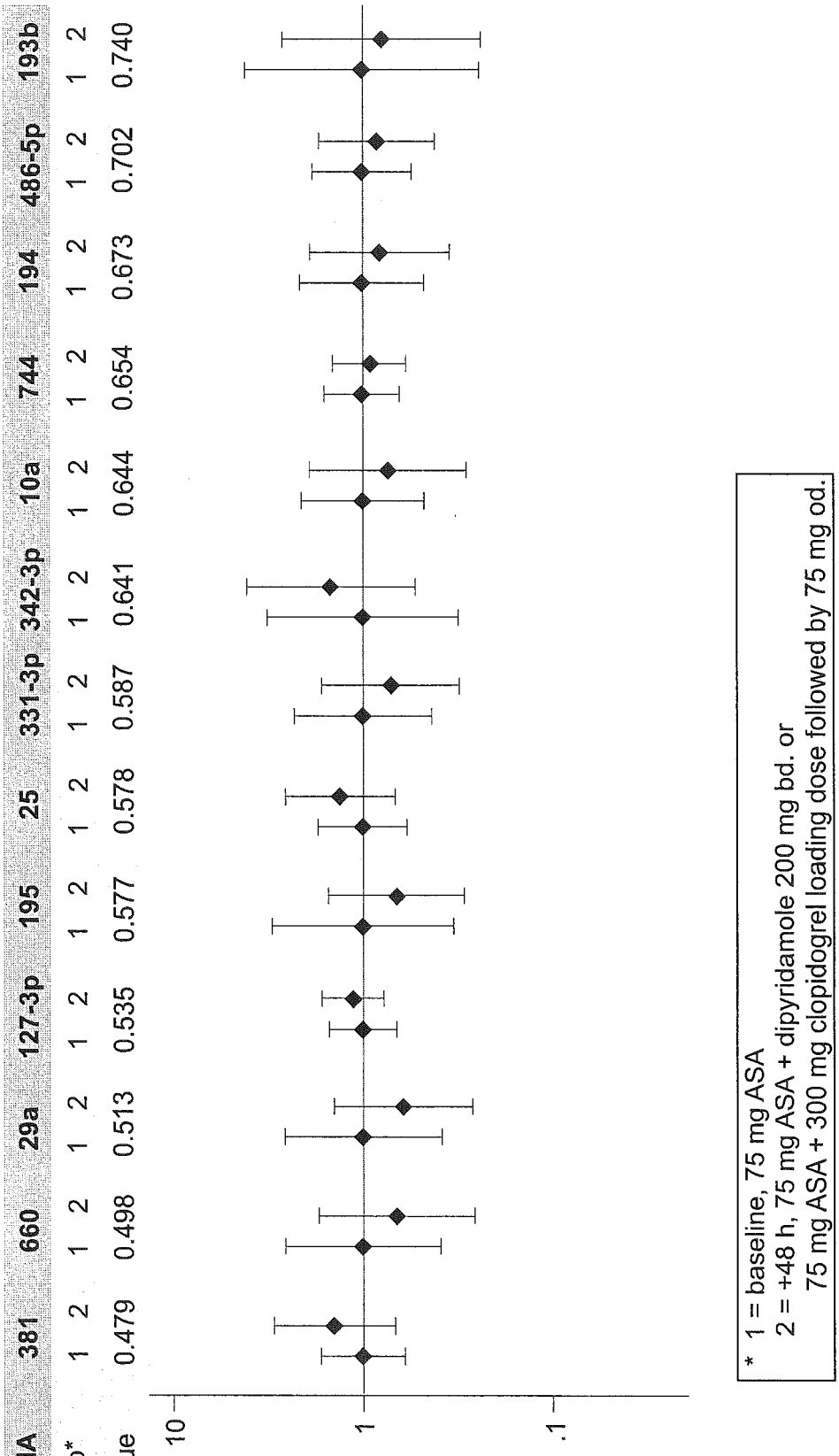
P values are from paired t-tests comparing miRNA levels at baseline (75mg ASA) and 48 hours later.

FIGURE 6E



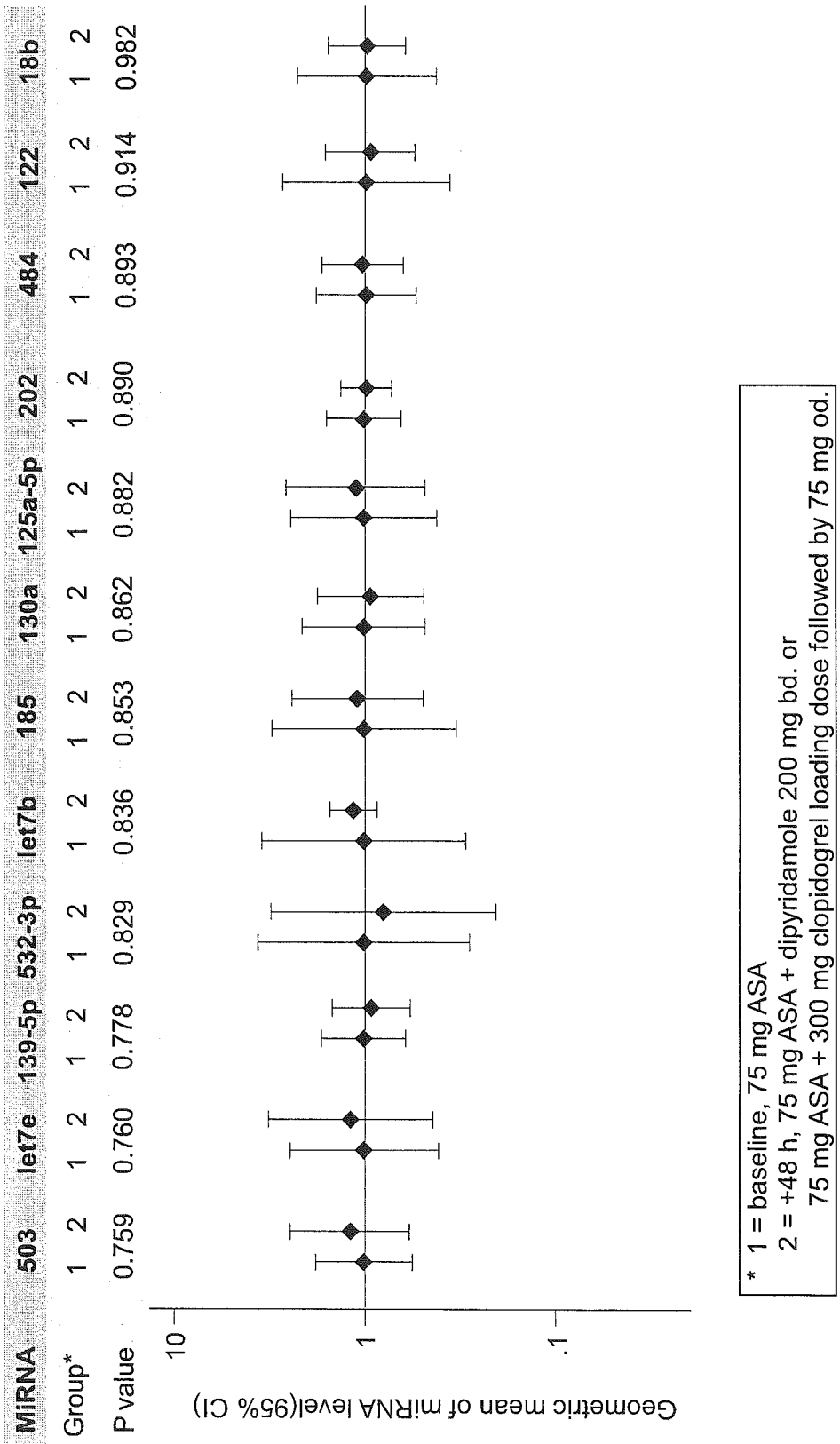
P values are from paired t-tests comparing miRNA levels at baseline (75mg ASA) and 48 hours later.

FIGURE 6F



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

FIGURE 6G



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

FIGURE 7

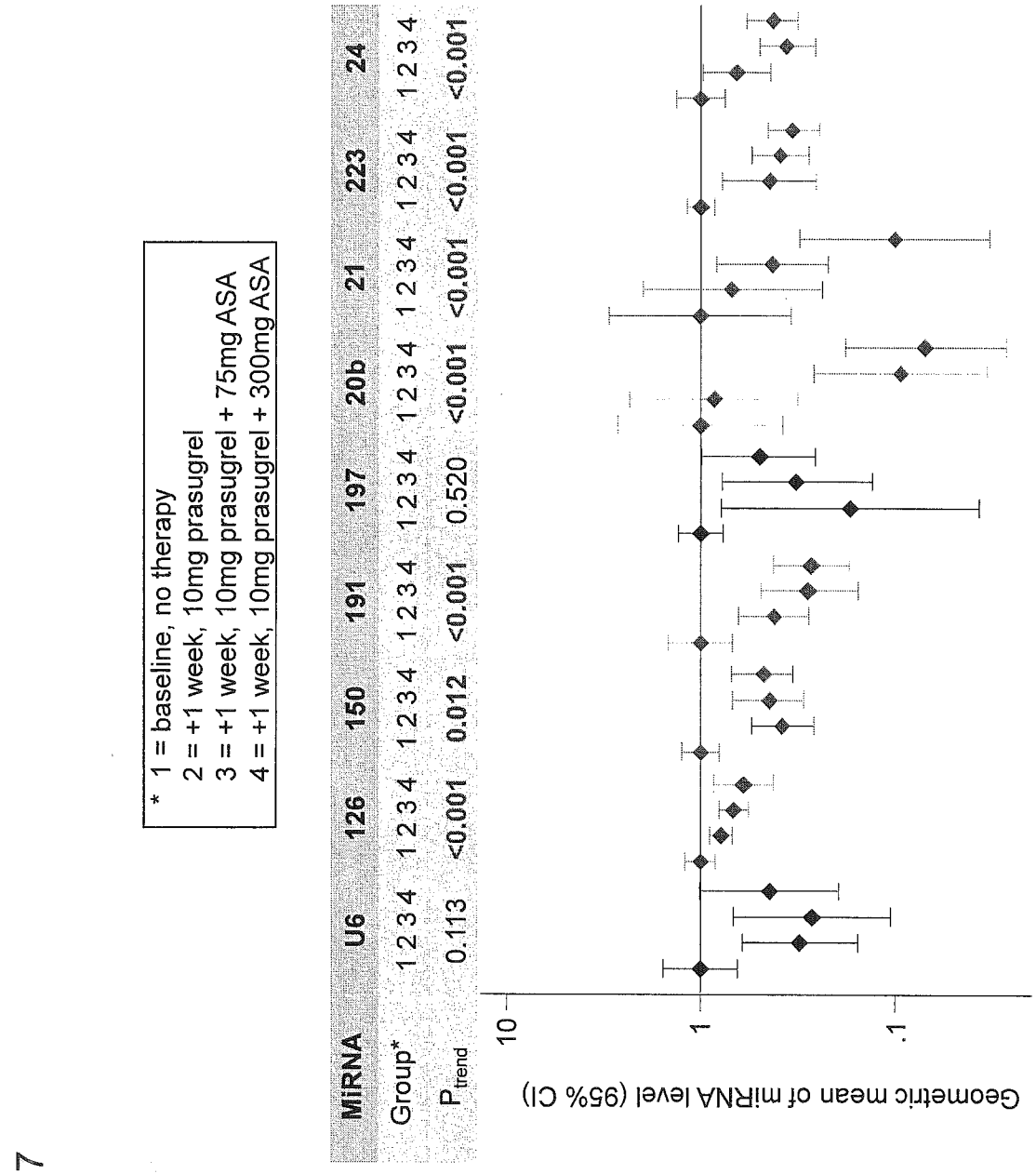
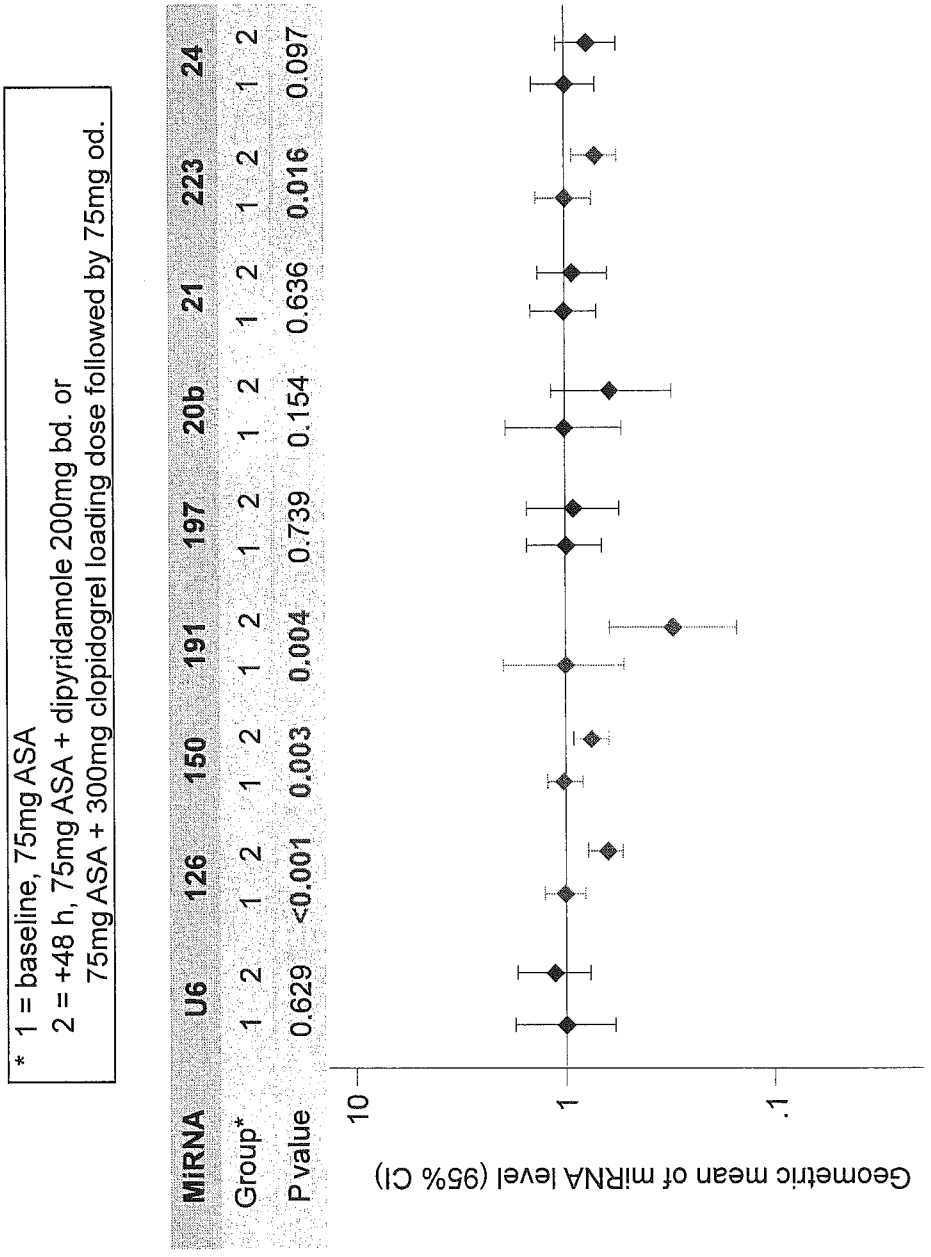

* 1 = baseline, no therapy
2 = +1 week, 10mg prasugrel
3 = +1 week, 10mg prasugrel + 75mg ASA
4 = +1 week, 10mg prasugrel + 300mg ASA
MiRNA U6 126 150 191 197 20b 21 223 24
Group* 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
P trend 0.113 <0.001 0.012 <0.001 0.520 <0.001 <0.001 <0.001 <0.001
Geometric mean of miRNA level (95% CI)
10
1
.1

FIGURE 8


* 1 = baseline, 75mg ASA
2 = +48 h, 75mg ASA + dipyridamole 200mg bd. or
75mg ASA + 300mg clopidogrel loading dose followed by 75mg od.
MiRNA U6 126 150 191 197 20b 21 223 24
Group* 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2
P value 0.629 <0.001 0.003 0.004 0.739 0.154 0.636 0.016 0.097
Geometric mean of miRNA level (95% CI)
10
1
.1

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/053417

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/133036 A2 (ACADEMISCH MEDISCH CT BIJ DE UNIVERSITEIT VAN AMSTERDAM [NL]; CREEMERS) 27 October 2011 (2011-10-27) the whole document	1-7

X	STRATZ C ET AL.: "Micro-array profiling exhibits remarkable intra-individual stability of human platelet micro-RNA", THROMBOSIS AND HEAMOSTASIS, vol. 107, no. 4, 4 February 2001 (2001-02-04), pages 634-641, XP002722077,	1,2,5-7
Y	the whole document	3,4

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 March 2014	02/04/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Knehr, Michael

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	L. C. EDELSTEIN ET AL: "MicroRNAs in platelet production and activation", BLOOD, vol. 117, no. 20, 19 May 2011 (2011-05-19) , pages 5289-5296, XP55046384, ISSN: 0006-4971, DOI: 10.1182/blood-2011-01-292011	2,5-7
Y	the whole document	3,4

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X	TANRIVERDI KAHRAMAN ET AL: "Platelet MicroRNA is altered by thrombin-induced aggregation", CIRCULATION, LIPPINCOTT WILLIAMS & WILKINS, US, vol. 114, no. 18, Suppl, 1 October 2006 (2006-10-01), pages 27-28, XP009166639, ISSN: 0009-7322 abstract	2,5-7

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/053417

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANNA ZAMPETAKI ET AL: "Prospective Study on Circulating MicroRNAs and Risk of Myocardial Infarction", JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY, ELSEVIER, NEW YORK, NY, US, vol. 60, no. 4, 12 March 2012 (2012-03-12) , pages 290-299, XP028427529, ISSN: 0735-1097, DOI: 10.1016/J.JACC.2012.03.056 [retrieved on 2012-05-26] the whole document -----	2-7

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2013/053417

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