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Prospective Study on Circulating MicroRNAs and Risk of Myocardial Infarction

Anna Zampetaki, PhD,* Peter Willeit, MD, MPhil,†‡ Lindsey Tilling, MD,* Ignat Drozdov, MPhil,*§ Marianna Prokopi, PhD,* Jean-Marie Renard, PhD,‖¶ Agnes Mayr, MD,# Siegfried Weger, MD,** Georg Schett, MD,†† Ajay Shah, MD, FMedSci,* Chantal M. Boulanger, PhD,‖¶ Johann Willeit, MD,† Philip J. Chowienczyk, MD,* Stefan Kiechl, MD,† Manuel Mayr, MD, PhD*

London and Cambridge, United Kingdom; Innsbruck, Austria; Paris, France; Bruneck, Italy; and Erlangen, Germany

Objectives This study sought to explore the association between baseline levels of microRNAs (miRNAs) (1995) and incident myocardial infarction (1995 to 2005) in the Bruneck cohort and determine their cellular origin.

Background Circulating miRNAs are emerging as potential biomarkers. We previously identified an miRNA signature for type 2 diabetes in the general population.

Methods A total of 19 candidate miRNAs were quantified by real-time polymerase chain reactions in 820 participants.

Results In multivariable Cox regression analysis, 3 miRNAs were consistently and significantly related to incident myocardial infarction: miR-126 showed a positive association (multivariable hazard ratio: 2.69 [95% confidence interval: 1.45 to 5.01], $p = 0.002$), whereas miR-223 and miR-197 were inversely associated with disease risk (multivariable hazard ratio: 0.47 [95% confidence interval: 0.29 to 0.75], $p = 0.002$, and 0.56 [95% confidence interval: 0.32 to 0.96], $p = 0.036$). To determine their cellular origin, healthy volunteers underwent limb ischemia-reperfusion generated by thigh cuff inflation, and plasma miRNA changes were analyzed at baseline, 10 min, 1 h, 5 h, 2 days, and 7 days. Computational analysis using the temporal clustering by affinity propagation algorithm identified 6 distinct miRNA clusters. One cluster included all miRNAs associated with the risk of future myocardial infarction. It was characterized by early (1 h) and sustained activation (7 days) post–ischemia-reperfusion injury and consisted of miRNAs predominantly expressed in platelets.

Conclusions In subjects with subsequent myocardial infarction, differential co-expression patterns of circulating miRNAs occur around endothelium-enriched miR-126, with platelets being a major contributor to this miRNA signature. (J Am Coll Cardiol 2012;60:290–9) © 2012 by the American College of Cardiology Foundation

MicroRNAs (miRNAs) are small noncoding RNAs that modify gene expression at the post-transcriptional level. They bind to the 3′-untranslated regions of target messenger RNAs and induce degradation or reduce translation efficiency of specific transcripts (1,2). By now, miRNAs have emerged as key regulators of cardiac growth, vascular development, and angiogenesis (3,4). Recent studies also uncovered the presence of circulating miRNAs (5,6). Altered levels have been reported in patients with heart failure, coronary artery disease, and diabetes (7–12).

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The aim of this study was to identify changes in circulating miRNAs that might precede subsequent cardiac

From the *King's British Heart Foundation Centre, King's College London, London, United Kingdom; †Department of Neurology, Medical University Innsbruck, Innsbruck, Austria; ‡Department of Public Health and Primary Care, University of Cambridge, Cambridge, United Kingdom; §Centre for Bioinformatics, School of Physical Sciences and Engineering, King's College London, London, United Kingdom; ‖Paris Centre de Recherche Cardiovasculaire à l'HEGP, INSERM U970, Paris, France; ¶Université Paris Descartes, Sorbonne Paris Cité, UMR-S970, Paris, France; #Department of Laboratory Medicine, Bruneck Hospital, Bruneck, Italy; **Department of Internal Medicine, Bruneck Hospital, Bruneck, Italy; and the ††Department of Internal Medicine III and Institute for Clinical Immunology, University of Erlangen-Nuremberg, Erlangen, Germany. Dr. Mayr is a Senior Fellow of the British Heart Foundation. Drs. Zampetaki, Kiechl, J. Willeit, and M. Mayr and Mr. Drozdov filed a patent application related to circulating miRNAs as cardiovascular biomarkers. Dr. Chowienczyk is a shareholder in Cennon Diagnostics (blood pressure measurement technology unrelated to the present study). All other authors have reported that they have no relationships relevant to the contents of this paper to disclose. This work was supported by Diabetes Research UK (BDA 10/0004115), the Juvenile Diabetes Research Foundation (17-2011-658), and the Department of Health via a National Institute for Health Research (NIHR) Biomedical Research Centre award to Guy's and St. Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust. Drs. Zampetaki and P. Willeit contributed equally to this study and are shared first authors.

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events. A total of 19 miRNAs were screened by quantitative (real-time) polymerase chain reaction (qPCR) in the Bruneck cohort, a large prospective, population-based study on cardiovascular disease. Baseline levels of 3 miRNAs (1995 evaluation) were associated with the incidence of myocardial infarction (MI) over a 10-year observation period (1995 to 2005). Additional miRNA measurements were performed in healthy volunteers after thigh cuff-induced ischemia-reperfusion injury (I/R). In this interventional study, the miRNAs associated with the risk of future MI formed a distinct cluster that predominantly originated from platelets.

Abbreviations and Acronyms

AIC = Akaike information criterion
Ct = cycle threshold
I/R = ischemia-reperfusion
MI = myocardial infarction
miRNA = microRNA
MP = microparticle
PMP = platelet-derived microparticle
qPCR = quantitative polymerase chain reaction

Methods

Study subjects. The Bruneck study is a prospective, population-based survey initiated in 1990 as a sex- and age-stratified random sample of all inhabitants of Bruneck 40 to 79 years old (125 women and 125 men in their fifth to eighth decades) (13). The current analysis focuses on the 1995 evaluation. Circulating miRNA levels were available in 820 of the 826 participants (99.3%). Follow-up in 2000 and 2005 was 100% complete for clinical endpoints and >90% complete for repeated laboratory examinations. Fatal and nonfatal MIs were ascertained following the World Health Organization criteria for definite disease status (14) and did not rely on hospital discharge codes or the patient's self-report but on a careful review of the medical records provided by general practitioners, death certificates, and Bruneck Hospital files. Diabetes was coded present if the individual had a fasting glucose ≥140 mg/dl, a 2-h glucose level ≥200 mg/dl after 75-g glucose load, or a pre-established, record-confirmed diagnosis of diabetes. Systolic and diastolic blood pressure was taken with a standard mercury sphygmomanometer after at least 10 min of rest (the mean of 3 independent measurements). Low-density lipoprotein cholesterol was assessed using the Friedewald equation (15). For the interventional study, healthy volunteers (n = 11, 19 to 51 years old) underwent limb I/R generated by thigh cuff inflation. Plasma samples were taken at baseline, 10 min, 1 h, 5 h, 2 days, and 7 days after injury. The protocols of both studies were approved by the appropriate ethics committees, and all study subjects gave their written informed consent before entering the study.

Taqman qPCR assay. MiRNAs were extracted using the miRNeasy kit (Qiagen, Hilden, Germany) (12). A fixed volume of 3 μl of the 25 μl RNA eluate was used as input for reverse transcription reactions as described previously (12). In brief, miRNAs were reversely transcribed using Megaplex Primer Pools (Human Pools A version 2.1, Applied Biosystems, Darmstadt, Germany). Reverse transcription reaction products were further amplified using the Megaplex PreAmp Primers (Primers A v2.1). Both reverse transcription and PreAmp products were stored at −20°C. Taqman miRNA assays were used to assess the expression of individual miRNAs. Diluted pre-amplification product (0.5 μl) was combined with 0.25 μl Taqman miRNA assay (20×) (Applied Biosystems) and 2.5 μl Taqman Universal PCR Master Mix No AmpErase UNG (2×) 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 s and 60°C for 1 min. All samples were run in duplicates. Relative quantification was performed using the software SDS2.2 (Applied Biosystems). U6 expression was used for normalization purposes. In a second round, the average cycle threshold (Ct) value of all miRNAs was applied as an additional normalization control. This commonly used approach is expected to result in weaker associations because levels of individual miRNAs and their clusters contribute to the Ct average.

miRNA screening. Platelets, platelet-derived microparticles (PMPs), and peripheral blood mononuclear cells were isolated from healthy subjects as previously described (16). The pellet was lysed in Qiazol reagent, and the extracted RNA was eluted in 25 μl of nuclease free H_2O. Total RNA (20 ng) was used for reverse transcription. The expression profile of platelets and PMPs was assessed using the Human Taqman miRNA Array Card A (Applied Biosystems) (12). Human umbilical vein endothelial cells were purchased from Cambrex (Verviers, Belgium) and cultured as previously described (16).

Cellular origin of plasma microparticles. Platelet-free plasma was analyzed using a Guava 8HT flow cytometer (Merck-Millipore, Billerica, Massachusetts), as described previously (17). Microparticles (MPs) were characterized as events with a diameter of 0.1 to 1.0 μm identified using Megamix beads (0.5 to 3 μm, Biocytex, Marseille, France) and positively labeled by specific antibodies: anti-CD41-PC7 (0.05 μg/test) from Beckman-Immunotech (Marseille, France); anti-CD62E-FITC (0.2 μg/test) from R&D Systems (Minneapolis, Minnesota); anti-CD144-PE (0.2 μg/test) from eBioscience (San Diego, California); or anti-CD235a-APC (0.05 μg/test) from BD Biosciences (Le Pont de Claix, France). Control experiments were performed using their respective isotypic fluorescent immunoglobulins. MPs expressing phosphatidylserine were labeled using AnnexinV-APC (5 μl/test; BD Pharmingen) in presence of 2.5 mmol/l $CaCl_2$. MP plasma concentration was assessed using Flowcount calibrator beads (Beckman Coulter, Villepinte, France). The following specific MP subpopulations were defined: CD235a+ MPs (erythrocyte derived); CD144+ or CD62E+ MPs (endothelial origin); and CD41+ MPs (platelet derived).

Statistical analysis. Data were analyzed using the statistical packages SPSS version 15.0 (SPSS Inc., Chicago, Illinois) and STATA version 10.1 (StataCorp, College Station, Texas). Normally distributed continuous variables

were presented as mean ± SD, variables with a skewed distribution as median (interquartile range) and dichotomous variables as numbers and percentages. The Student *t* test and Fisher exact test were used to analyze differences in participant characteristics between those in whom MI developed during follow-up and those in whom it did not. Log_e-transformed miRNAs were used for all computations to approximate a Gaussian distribution. Correlations between miRNAs were assessed using Pearson correlation coefficients with Bonferroni-adjusted p values. Cox proportional hazard regression models were fitted to assess the association between log_e-transformed miRNA levels and incident MI. To identify the subset or pattern of miRNA with the highest prognostic ability for future MI, 2 distinct approaches were used. 1) The first was a 2-step procedure. To reduce the number of candidate miRNAs and subsequent computational requirements, forward and backward stepwise Cox regression analyses with relaxed inclusion and exclusion criteria ($p_{entry} = 0.15$ and $p_{removal} = 0.20$; adjusted for age, sex, and previous cardiovascular disease) were fitted. Seven miRNAs consistently detectable in the circulation were selected in either or both of the analyses (miR-24, miR-126, miR-140, miR-150, miR-197, miR-223, and miR-486) and considered eligible for the second best subset step. Cox regression models of all combinations of eligible miRNAs were computed and compared according to the models' Akaike information criterion (AIC) that is based on the maximized log-likelihood and imposes a penalty for increasing the number of parameters in the model. Lower values of AIC indicate the preferred model, which is the one with the few parameters still providing adequate fit (trade-off between accuracy and complexity). 2) The second approach used the technique called L_1 penalization, implementing the least absolute shrinkage and selection operator algorithm for all 19 miRNAs. L_1-penalized methods shrink the estimates of the regression coefficients toward 0 relative to the maximum likelihood estimates. The technique has been used to generate gene signatures from microarray data to avoid overfitting arising from both collinearity and high dimensionality. The amount of shrinkage is determined by the tuning parameter λ_1, which is progressively increased to the value that shrinks all regression coefficients to 0. Plots of fitted regression coefficients (y-axis) versus λ_1 (x-axis) were generated using the package 'penalized' of R statistical software (18). The least absolute shrinkage and selection method allows assessment of the relevance and robustness of individual explanatory variables but produces biased estimates for the regression coefficients. Accordingly, risk estimates for the 3 miRNAs finally selected (both approaches identified the same miRNA signature) were computed by standard Cox regression analysis and adjusted for age and sex, smoking status (ever vs. never smokers), systolic blood pressure, low-density lipoprotein cholesterol, diabetes, and history of cardiovascular disease (multivariable model 1) plus other miRNAs (multivariable model 2) plus body mass index, waist-to-hip ratio, high-density lipoprotein cholesterol, log_e C-reactive protein, and fibrinogen (multivariable model 3). The incremental predictive value of selected miRNAs over the Framingham Risk Score for hard coronary heart disease as an endpoint was assessed by calculating: 1) Harrell's C-indices (19), a measure of discrimination for censored time-to-event data; 2) the net reclassification index (20); and 3) the integrated discrimination improvement (20). The net reclassification index was calculated across the categories of predicted 10-year risk of <10%, 10% to 20%, and >20%. In the interventional study, miRNA expression profiles were clustered using the temporal clustering by affinity propagation algorithm (21). Two-sided p values <0.05 were considered significant.

Results

Circulating miRNAs in the Bruneck Study. Baseline demographic, clinical, and laboratory characteristics of the 820 participants in the 1995 evaluation are shown in Online Table I. All subjects were of Caucasian origin. A total of 47 participants experienced MI over the 10-year follow-up period, corresponding to an incidence rate of 5.3 (95% confidence interval: 4.1 to 7.2) per 1,000 person-years. For the initial screening, Human Taqman miRNA arrays (CardA version 2.1 and CardB version 2.0, Applied Biosystems) covering 754 small noncoding RNAs were applied to 8 pooled samples, consisting of subjects with and without atherosclerotic vascular disease matched for different cardiovascular risk factors (hypercholesterolemia, smoking, hypertension, diabetes), as described previously (12). Of the 148 miRNAs with Ct values <36, 130 miRNAs were detected using fluidic CardA, and therefore all further analysis focused on this dataset. Our network analysis revealed 7 miRNAs as promising targets that were reliably detectable in the circulation (12). These were selected and measured in the entire Bruneck cohort (n = 820). An additional 12 miRNAs were quantified as part of an ongoing project on osteoarthritis (n = 820). miRNA levels were strongly correlated with each other, with some reaching almost perfect correlation (e.g., miR-24 and miR-223, r = 0.939). The complex dependency of circulating miRNAs was further scrutinized as miRNA-miRNA correlation profiles (Online Fig. I).

Association with incident MI. miRNAs associated with incident MI were selected using 2 different approaches. 1) Stepwise Cox regression with comparison of AIC, a criterion considering both goodness of fit and the number of parameters in the model, identified 2 preferred combinations of miRNAs: miR-126/-197/-223 and miR-126/-197/-24 (AIC ~562 each with 6 degrees of freedom). 2) In L_1-penalized Cox regression analysis, miR-126, miR-197, and miR-223 showed the strongest associations with incident MI (Online Fig. II), whereas miR-24 performed worse. Accordingly, we gave preference to the miR-126/-197/-223 combination excluding miR-24. MiR-126 was positively associated with incident MI, whereas miR-197

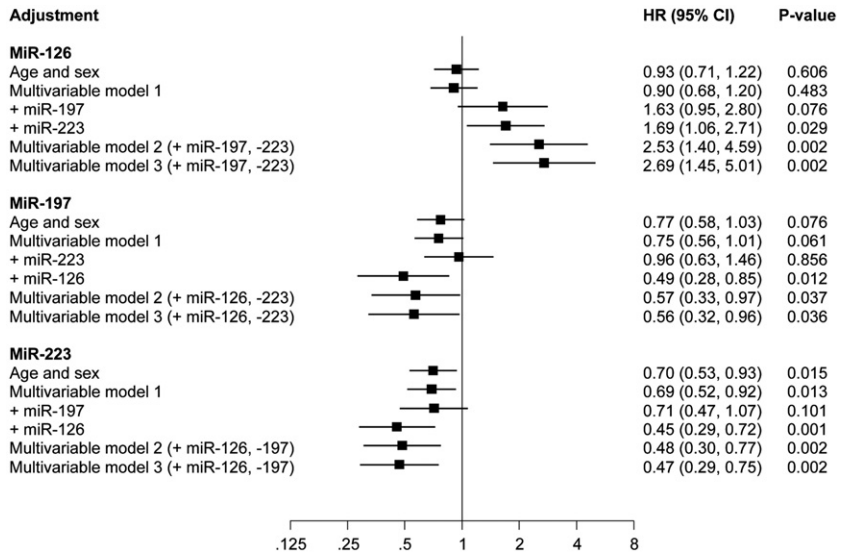

Figure 1 **Risk Estimates for the 3 miRNAs Most Consistently Associated With MI**

The 3 miRNAs were identified by Akaike Information Criterion–based models and the technique of least absolute shrinkage and selection operator. HR (95% CI) were derived from standard Cox regression models with progressive adjustment (for details, see Methods). Expression levels were normalized to U6 expression (for normalization to cycle threshold average, see Online Figure III). CI = confidence interval; HR = hazard ratio; MI = myocardial infarction.

and miR-223 were inversely related to disease risk (Fig. 1). Similar results were obtained after normalization to the average Ct value of all assessed miRNAs (Online Fig. III). There was no effect modification by sex or pre-existing cardiovascular disease (Online Table II). When the other miRNAs were individually added to the multivariable model already including miR-126/-197/-223, none achieved statistical significance (Online Fig. IV). In subanalysis, miR-223 showed a stronger association with fatal than nonfatal MI (Fig. 2A) but was equally associated with early (1995 to 2000) and late (2000 to 2005) events (Fig. 2B). In contrast, miR-126 and miR-197 were predominantly associated with early events (Fig. 2B). These findings were similar for normalization to U6 or the average Ct value of all miRNAs (Online Fig. V) and replicated when the time period was divided into smaller intervals (1995 to 1997, 1997 to 2000, 2000 to 2005; Online Fig. VI). Findings for cardiovascular death and less so for all-cause death were similar to those for MI (Online Fig. VII). Findings for stroke, however, were different, possibly due to its heterogeneous origin (cardiac embolism and microangiopathy vs. atherothrombosis).

MI risk reclassification. Addition of the 3 miRNAs to a base model of the Framingham Risk Score for hard coronary heart disease as an endpoint increased the C-index from 0.763 (95% confidence interval [CI]: 0.702 to 0.824) to 0.800 (95% CI: 0.743 to 0.856) (Δ0.037, p = 0.171). In comparison, the gain for $\log_e$-transformed high-sensitivity C-reactive protein levels was only Δ0.005. The net reclassification improvement was 16.86% (−1.99% to 35.71%) (p = 0.080). The integrated discrimination improvement was 0.047 (95% CI: 0.005 to 0.089) (p = 0.029). Details are given in Tables 1 and 2. In subjects with incident MI (n = 47), 14 were correctly reclassified to a higher risk category and 6 were reclassified to a lower category (p = 0.074). In participants who had no MI (n = 628), 38 were correctly reclassified to a lower risk category and 39 were reclassified to a higher category (p = 0.909) (10-year risk categories <10%, 10% to 20%, and >20%).

Interventional study. To explore the cellular origin of circulating miRNAs, healthy volunteers (n = 11) underwent limb I/R generated by cuff inflation. Their clinical characteristics are shown in Online Table III. Samples were collected at baseline and 10 min, 1 h, 5 h, 2 days, and 7 days after injury (Fig. 3A). Thirty miRNAs, including the miRNAs associated with MI in the Bruneck study, were assessed by qPCR. Computational analysis using the temporal clustering by an affinity propagation algorithm based on average miRNA expression at baseline and over time identified 6 distinct miRNA clusters (Fig. 3B). Cluster 2 was of particular interest: all miRNAs associated with future MIs (miR-126, miR-223, miR-197, and miR-24) were part of this cluster characterized by an early increase at 1 h and sustained elevation until 7 days post I/R injury (Fig. 4). MiR-21 was also included. Results were robust with different methods of normalization (exogenous spike-in cel-miR-39 control and unadjusted Ct values). The response of the other miRNAs to I/R injury is shown in Online Fig. VIII.

Platelet contribution. I/R injury induces platelet activation (22,23), and platelets were recently shown to contain miRNAs (24,25). To test for a potential platelet contribu-

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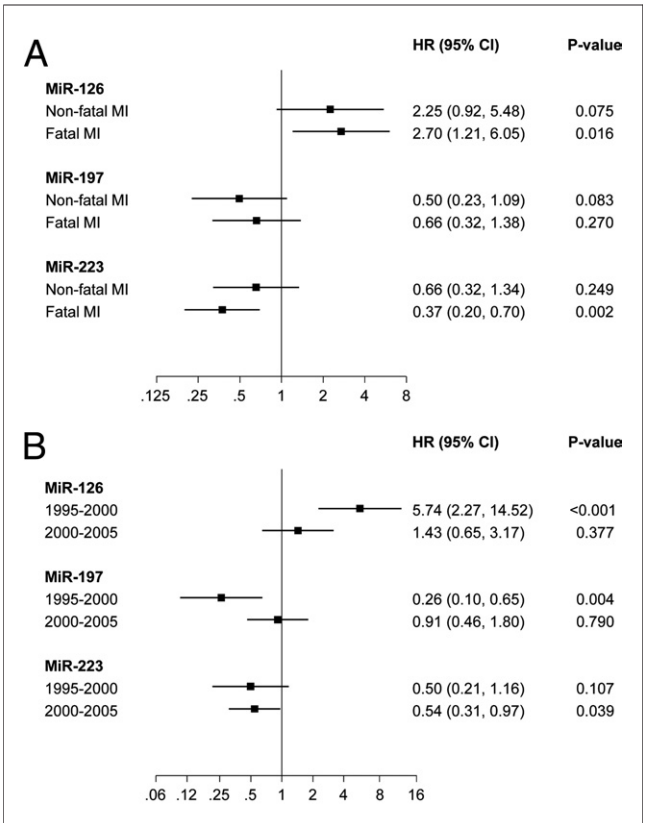

Figure 2 Subanalysis for Cases of MI

Subanalysis for fatal versus nonfatal MI **(A)** and early (1995 to 2000) versus late (2000 to 2005) events **(B)**. HR (95% CI) were derived from multivariable Cox regression model 2, including miR-126, miR-197, and miR-223 (for details, see Methods). During 10 years of follow-up (1995 to 2005), there were 26 fatal and 21 nonfatal MIs. There were 17 MIs during follow-up period 1995 to 2000 (incidence rate, 3.7 [range, 2.2 to 5.9] per 1,000 person-years) and 30 MIs during the period 2000 to 2005 (incidence rate, 7.4 [range, 5.2 to 10.6] per 1,000 person-years). Expression levels were normalized to U6 expression (for normalization to Ct average, see Online Figure V). Abbreviations as in Figure 1.

tion to the observed miRNA changes after I/R, miRNA screening was performed in preparations of platelets and PMPs derived from thrombin-activated platelets. The miRNAs constituting our cluster of interest (miR-223, -126, -24, -21, and -197) were among the miRNAs highly expressed in platelets (Fig. 5A) and PMPs (Fig. 5B). A comparison with peripheral blood mononuclear cells and endothelial cells confirmed that miR-223 was predominantly found in platelets, whereas miR-197 and miR-24 were also present in endothelial cells. In contrast, miR-126 and miR-21 were highly enriched in endothelial cells, but detectable in platelets, albeit at much lower concentrations (Fig. 5C, upper panel). The platelet origin of these circulating miRNAs was further supported by a significant correlation (except miR-21) with PMP counts 2 days after I/R injury (Fig. 5C, lower panel). Hence, adjustment for platelet-derived miRNAs may refine the endothelial contribution to the miR-126 content in the circulation. This is in agreement with our results from the Bruneck study, in which adjustment for miR-197 and miR-223 exposed the opposing directionality of changes in miR-126 in participants with subsequent MI (Fig. 1).

Table 1 Reclassification of Study Participants Into MI Risk Groups by Addition of miR-126, miR-197, and miR-223 to a Model With the FRS for Hard Coronary Heart Disease

	Model With FRS Score Plus miR-126, miR-197, and miR-223			
	<10% Risk	10%–20% Risk	>20% Risk	Total
Model With FRS	No. (Row Percentage)			
Participants who experienced an MI event, % risk				
<10	20 (69.0)	8 (27.6)	1 (3.4)	29
10–20	3 (27.3)	3 (27.3)	5 (45.4)	11
>20	1 (14.3)	2 (28.6)	4 (57.1)	7
Total	24	13	10	47
Participants who did not experience an MI event, % risk				
<10	522 (94.4)	28 (5.1)	3 (0.5)	553
10–20	31 (52.5)	20 (33.9)	8 (13.6)	59
>20	2 (12.5)	5 (31.2)	9 (56.3)	16
Total	555	53	20	628

Framingham Risk Score for the hard coronary heart disease endpoint; 145 participants were not included in the analysis because they were censored before 10 years of follow-up not due to a myocardial infarction.
FRS = Framingham Risk Score; MI = myocardial infarction.

Discussion

This is the first prospective study on the association of circulating miRNAs and incident MI with miR-223 and miR-197 showing negative associations and miR-126 showing a positive association with subsequent MIs.

Prediction of cardiovascular events. There are currently few biomarkers that could be used to identify subjects who are at risk of the development of acute manifestations of cardiovascular disease. Despite extensive studies and development of several risk prediction models, traditional risk factors fail to predict cardiovascular events in a large group of cases. Inflam-

Table 2 Summary Statistics of MI Risk Reclassification by Addition of miR-126, miR-197, and miR-223 to a Model With the FRS for Hard Coronary Heart Disease

Statistic	Estimate	95% CI	p Value
Net reclassification improvement			
Controls	−0.16%	−2.90% to 2.58%	0.909
Cases	17.02%	−1.63% to 35.67%	0.074
Overall	16.86%	−1.99% to 35.71%	0.080
Integrated discrimination improvement			
Controls	0.003331	0.000364–0.006297	0.028
Cases	0.043967	0.001742–0.086191	0.041
Overall	0.047297	0.004969–0.089626	0.029

CI = confidence interval; other abbreviations as in Table 1.

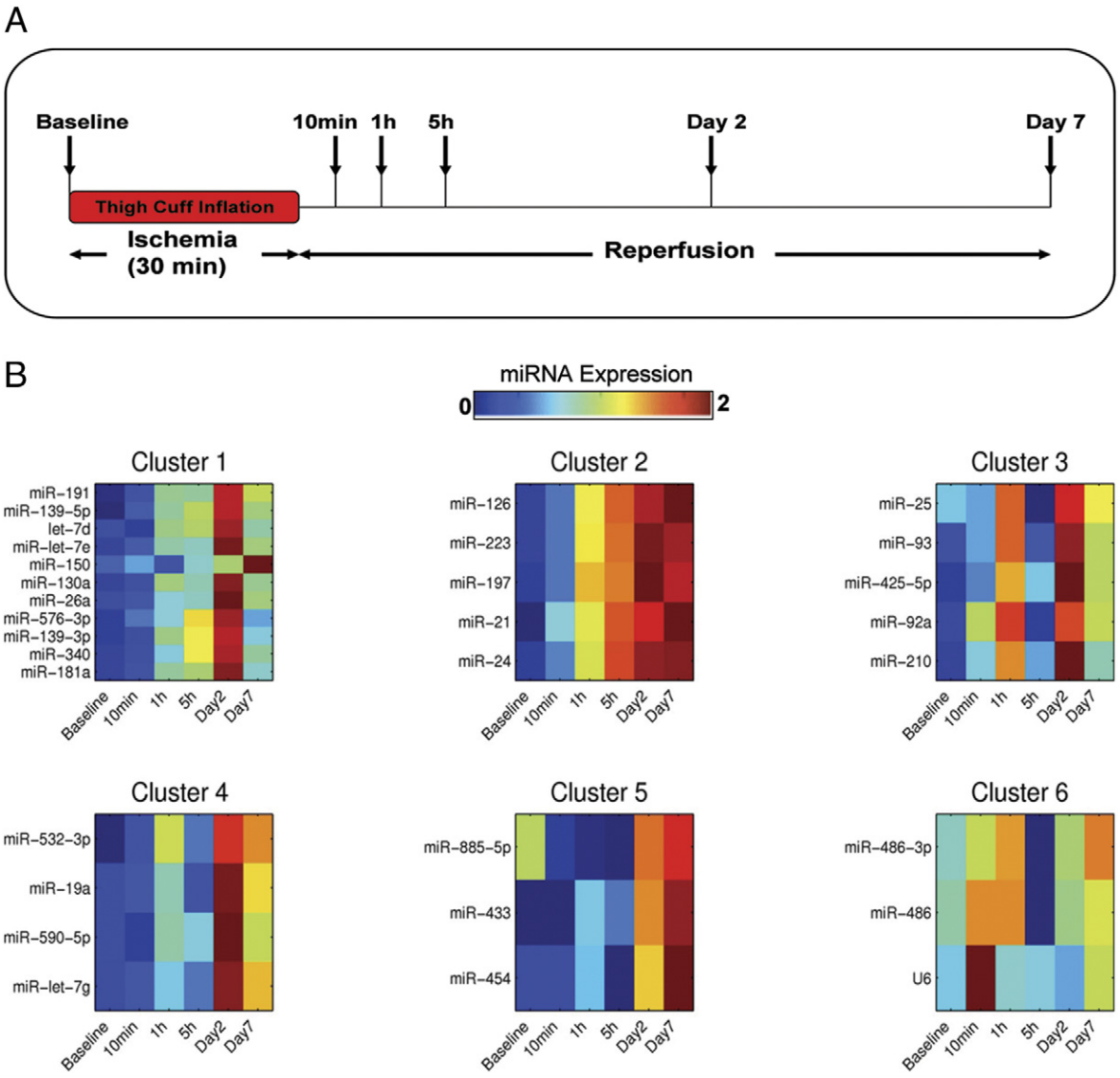

Figure 3 **Interventional Study to Identify miRNA Clusters**

(A) Model of limb ischemia-reperfusion injury in healthy volunteers. **Arrows** indicate time points of sample collection.
(B) miRNA clusters. Temporal clustering by affinity propagation grouped the 30 miRNAs into 6 distinct clusters. miRNA = microRNA.

matory markers such as high-sensitivity C-reactive protein lack specificity for the vasculature. Advanced imaging techniques are expensive and not suitable for population-wide screening. In addition, atherosclerosis is a diffuse disorder with various local and systemic manifestations. Thus, there is a clinical need for cardiovascular biomarkers that could complement the assessment of traditional risk factors.

miRNAs as biomarkers for MI. In our analysis, we considered 7 miRNAs that emerged as promising targets for cardiovascular disease in the pre-screening and displayed unique network topology (12). Three of these miRNAs formed part of a signature for MI: miR-126, miR-197, and miR-223. Our findings were independent of classic vascular risk factors, stable in subgroups (men and women, participants with and without previous cardiovascular disease) and robust when using distinct statistical approaches. Another 12 miRNAs were not related to atherosclerotic vascular disease in the pre-screening and fell short of significance in the main analysis. miR-223 is considered to be a myeloid-specific miRNA that acts as a fine tuner of granulocyte production and the inflammatory response (26–28). miR-197 has been reported as differentially expressed in tumors (29,30). In contrast, miR-126 is highly enriched in endothelial cells and facilitates vascular endothelial growth factor signaling (31). However, little is known about the cellular origin of these miRNAs in the circulation.

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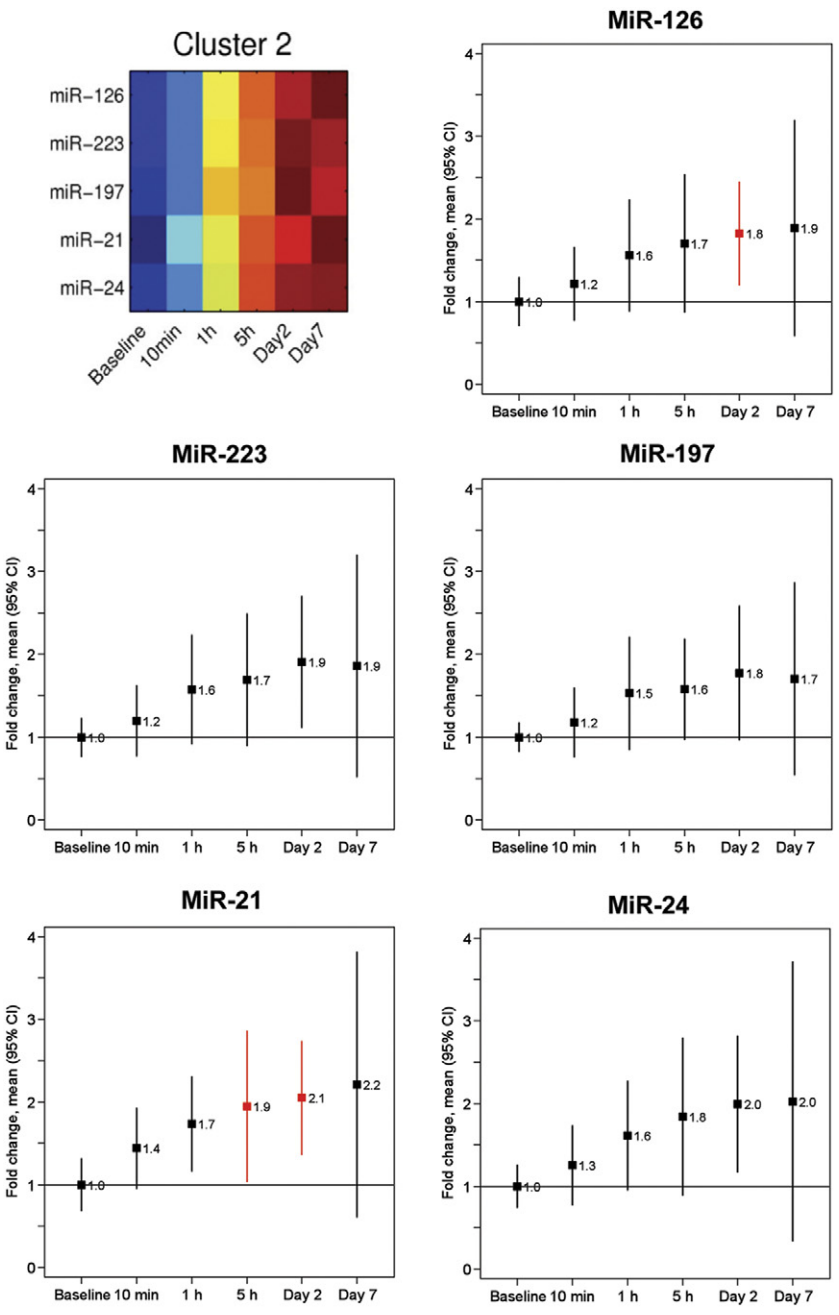

Figure 4 **miRNA Cluster Associated With MI**

Cluster 2 contained all miRNAs associated with MI. Temporal changes were assessed by quantitative polymerase chain reaction. Exogenous spike-in cel-miR-39 was used as a normalization control. **Red bars** indicate statistically significant differences ($p < 0.05$) compared with baseline. Abbreviations as in Figures 2 and 3.

miRNA response after I/R injury. Based on expression profiles after I/R injury, miR-126, miR-197, and miR-223 were part of 1 cluster that also included miR-21 and miR-24. All these miRNAs are highly expressed in platelets and PMPs. This is, to our knowledge, the first time that the contribution of a specific cell type to circulating miRNAs has been defined by a controlled intervention. The present findings extend our previous observations in patients with diabetes (12) and raise the possibility that the observed loss of several miRNAs, including miR-126, miR-197, miR-223, miR-24, and miR-21, may reflect abnormal platelet function in diabetic patients. A reduction of platelet miRNAs could reflect a decrease in the miRNA content of platelets, increased clearance of PMPs by inflammatory cells, or impaired thrombus resolution. In contrast, changes in miR-126 in participants with future MI did not follow

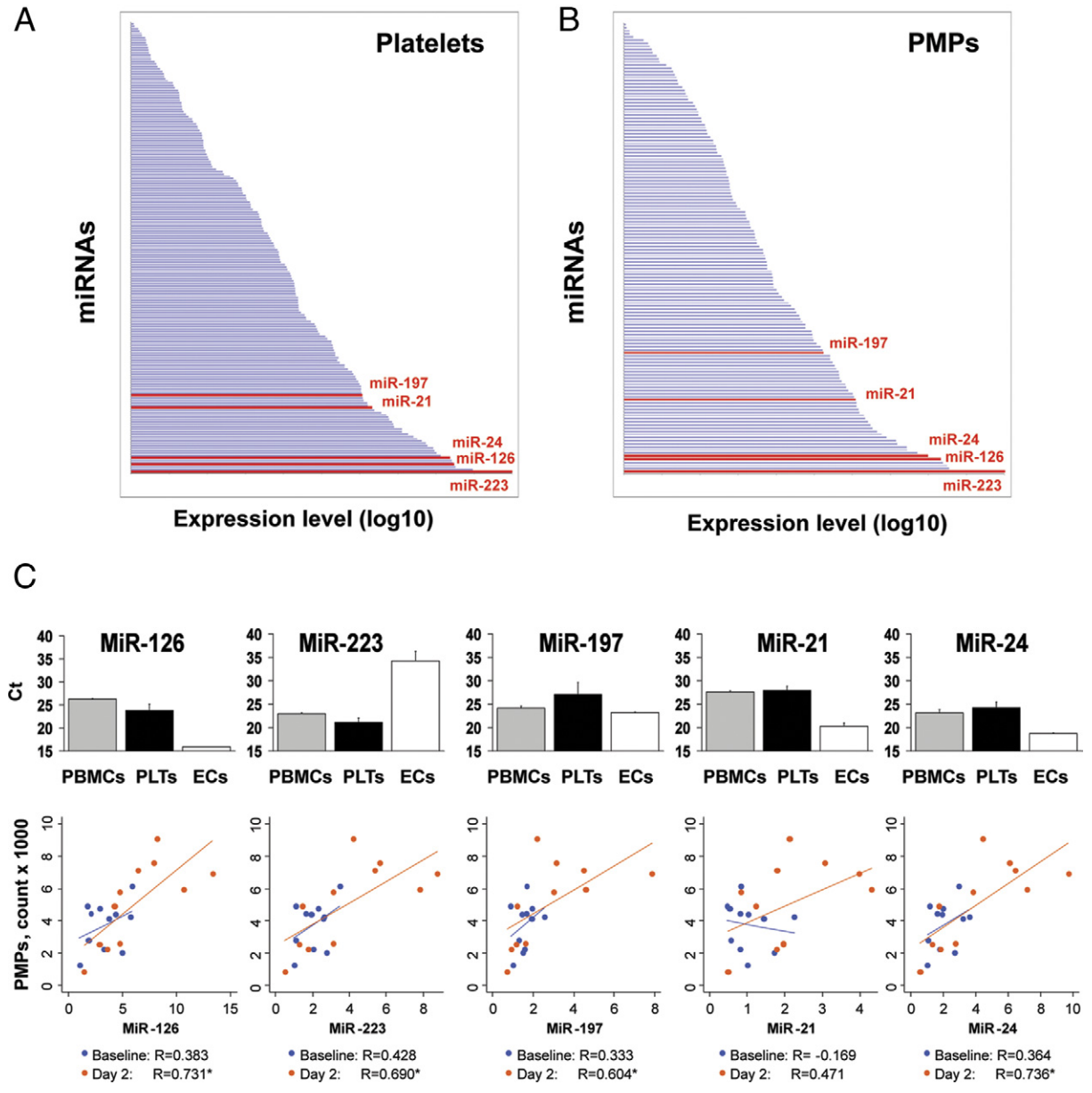

Figure 5 **Platelet Contribution to miRNA Signature for MI**

(A) miRNA profile of platelets, as determined by microarray screening. The average Ct value was used as a normalization control. Expression levels (2^{-DCt}) were log transformed. **(B)** Corresponding miRNA profile of PMPs. miRNAs associated with myocardial infarction (MI) were abundant in platelets and PMPs. **(C)** Cell type–specific miRNA expression. Comparison of miR-126, miR-223, miR-197, miR-21, and miR-24 expression in peripheral blood mononuclear cells (PBMCs), platelets (PLTs), and endothelial cells (ECs) using quantitative polymerase chain reaction **(upper panel)**. Ct values are provided as mean ± SD derived from 3 independent preparations. Correlation of miRNA levels with PMP counts **(lower panel)** at baseline **(blue dots)** and 2 days post I/R injury **(red dots)**. R values are Pearson correlation coefficients (n = 11). *$p < 0.05$. Ct = cycle threshold; miRNA = microRNA; PMPs = platelet-derived microparticles.

the same directionality as miR-197 and miR-223, arguing against a common platelet origin. Higher levels of circulating miR-126 could be in line with previous reports implicating endothelial MPs as biomarkers for vascular damage and increased cardiovascular risk (32). Regardless, our findings advise caution in interpreting individual miRNA changes in isolation. Because circulating miRNAs are highly correlated, global patterns of expression should be studied by representing miRNA data as coexpression networks. miRNA signatures rather than individual miRNAs may be more reliable biomarkers for cardiovascular events.

The underexplored role of miRNAs in platelets. It is noteworthy that several of the most abundant platelet miRNAs have previously been implicated in cardiovascular pathologies: miR-126 as a master regulator of endothelial homeostasis and vascular integrity (33), miR-21 as a medi-

ator of cardiac fibrosis (34), although this is contested by others (35), and miR-24 as an inducer of endothelial apoptosis after myocardial infarction (36). Although the functional importance of platelet miRNAs is currently unknown, it is conceivable that systemic inhibition of miRNAs, which are also abundant in platelets, may alter platelet function and contribute to the observed cardiovascular phenotypes. The presence of these miRNAs within platelets should be taken into account in future studies. Also, care must be taken in the design of case-control studies for biomarker analysis. Comparisons of circulating miRNAs between patients with manifest cardiovascular disease and healthy controls, for example, are likely to be confounded by medication, in particular, antiplatelet therapy.

Study limitations. Our findings await confirmation in independent cohorts. Thus, their exploratory/hypothesis-generating nature has to be emphasized. Two conservative approaches were used for reliable miRNA selection. Both methods returned the same 3 miRNAs. A model including these 3 miRNAs plus age and sex captured the entire information (5 variables for 47 endpoints). Additional adjustment for other cardiovascular risk factors had almost no effect on risk estimates and little effect on the respective standard errors. Nonetheless, a risk of overfitting of data remains and risk relationships emerged only after adjustment, which highlights one of the challenges in miRNA research (37). Also, causality cannot be inferred from associations of biomarkers in population studies.

Conclusions

This study highlights the importance of platelets as a major contributor to the circulating miRNA pool and suggests that endothelial-enriched miR-126 is part of a miRNA signature associated with incidence of MI in the general population. Future studies will need to address whether endothelial and platelet miRNAs can serve as novel biomarkers for clinical decision making.

Reprint requests and correspondence: Dr. Manuel Mayr, King's British Heart Foundation Centre, King's College London, 125 Coldharbour Lane, London SE59NU, United Kingdom. E-mail: manuel.mayr@kcl.ac.uk.

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Key Words: biomarker ■ endothelium ■ microRNA ■ myocardial infarction ■ platelets.

APPENDIX

For supplemental tables, please see the online version of this article.

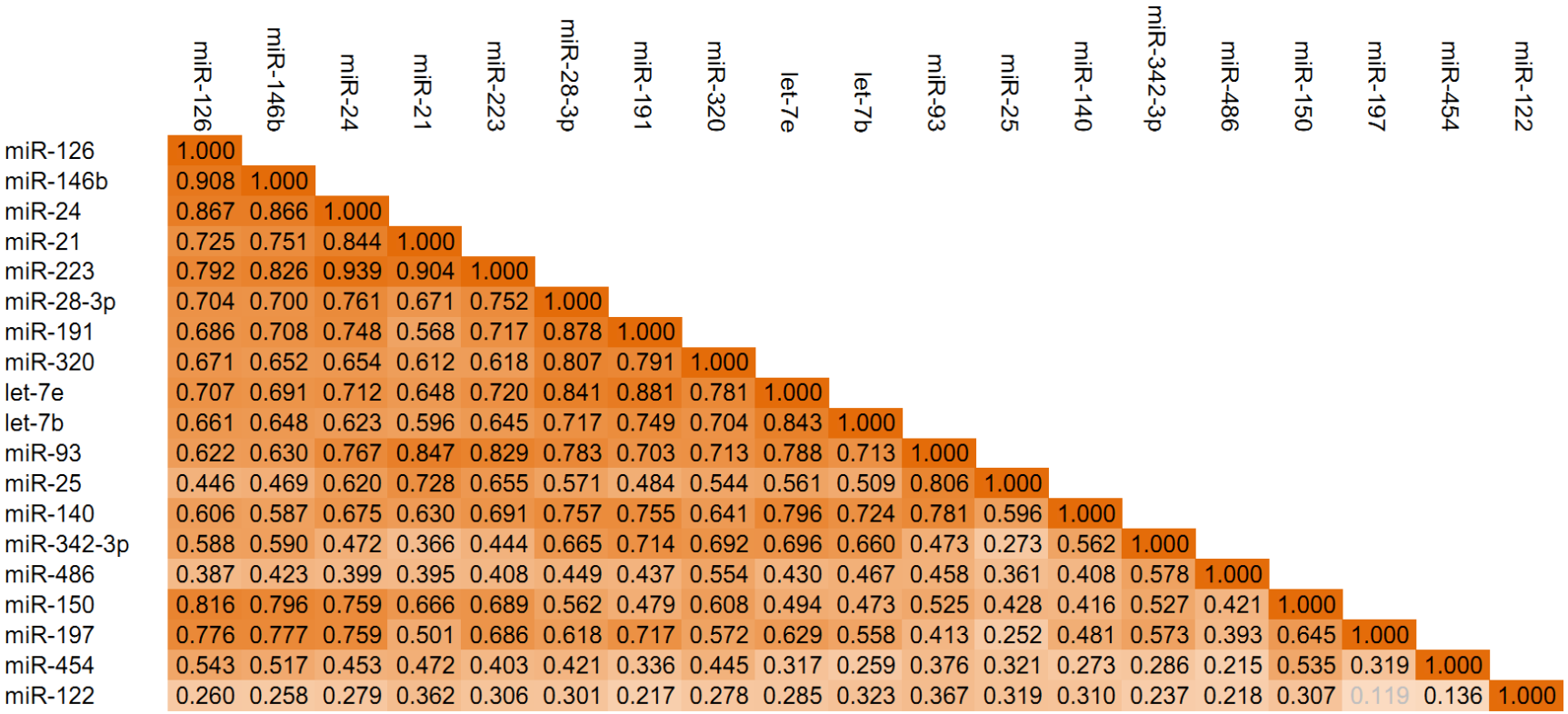
Supplemental material

Prospective Study on Circulating MicroRNAs and Risk of Myocardial Infarction.

Anna Zampetaki*, Peter Willeit*, Lindsey Tilling, Ignat Drozdov, Marianna Prokopi, Jean-Marie Renard, Agnes Mayr, Siegfried Weger, Georg Schett, Ajay Shah, Chantal Boulanger, Johann Willeit, Philip.J. Chowienczyk, Stefan Kiechl, Manuel Mayr.

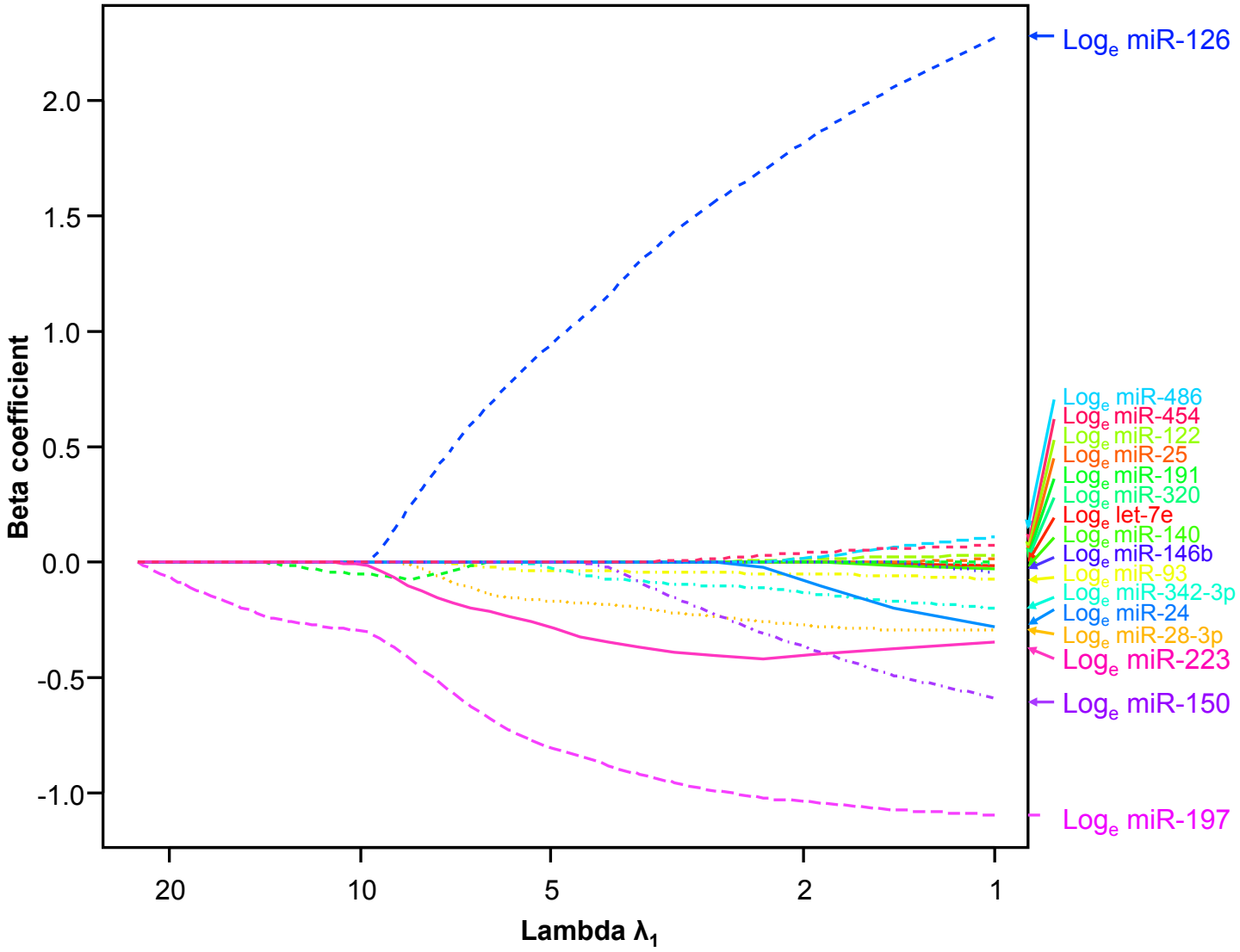
Supplemental figures and figure legends

Online Figure I. Correlation patterns of circulating miRNAs. Values of miRNA levels were $\log_e$-transformed before calculation of correlation coefficients. Correlation coefficients with Bonferroni-adjusted P-values ≤0.05 are shown in black, those with P values >0.05 are shown in grey.

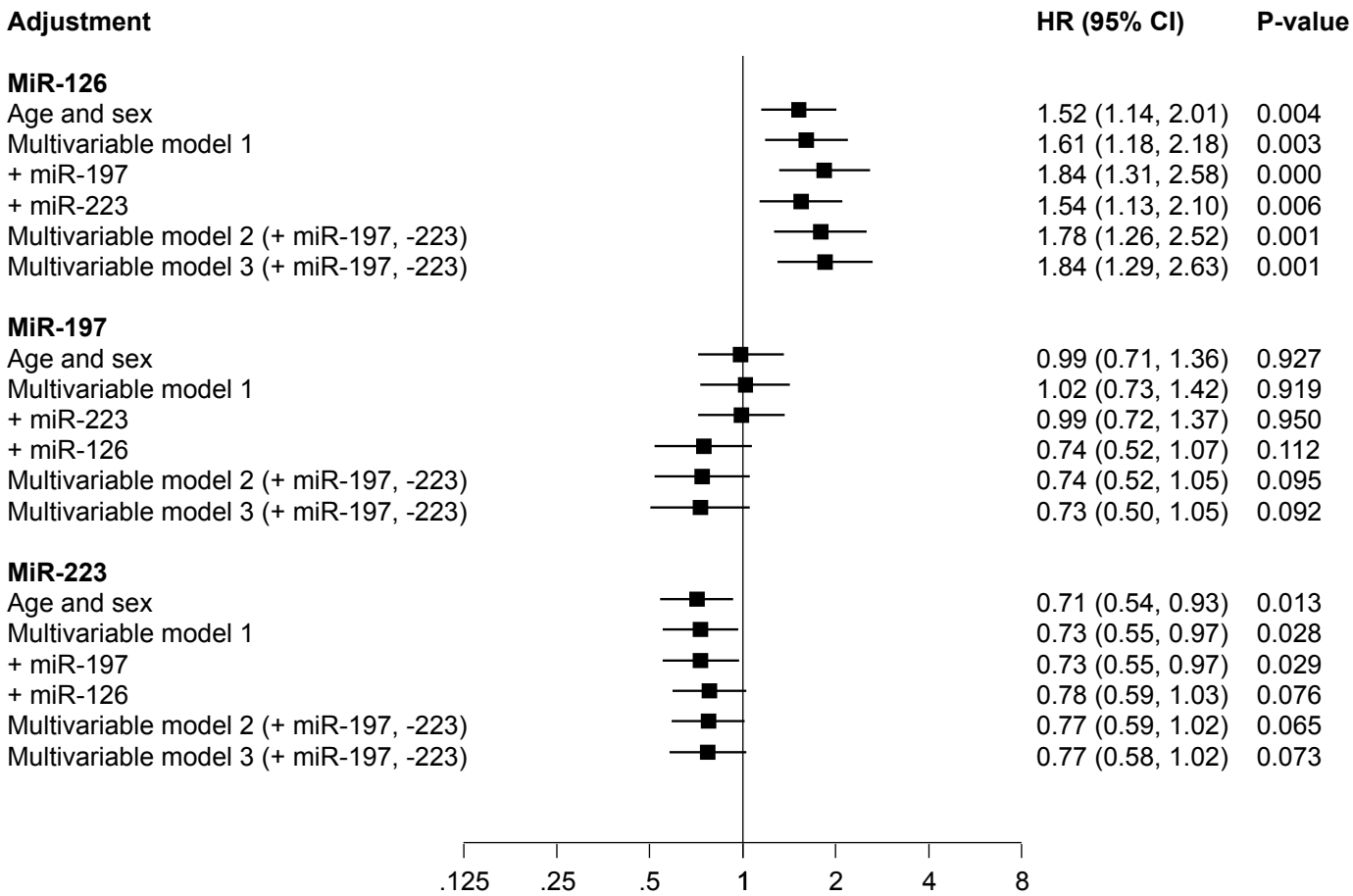


	miR-126	miR-146b	miR-24	miR-21	miR-223	miR-28-3p	miR-191	miR-320	let-7e	let-7b	miR-93	miR-25	miR-140	miR-342-3p	miR-486	miR-150	miR-197	miR-454	miR-122
miR-126	1.000																		
miR-146b	0.908	1.000																	
miR-24	0.867	0.866	1.000																
miR-21	0.725	0.751	0.844	1.000															
miR-223	0.792	0.826	0.939	0.904	1.000														
miR-28-3p	0.704	0.700	0.761	0.671	0.752	1.000													
miR-191	0.686	0.708	0.748	0.568	0.717	0.878	1.000												
miR-320	0.671	0.652	0.654	0.612	0.618	0.807	0.791	1.000											
let-7e	0.707	0.691	0.712	0.648	0.720	0.841	0.881	0.781	1.000										
let-7b	0.661	0.648	0.623	0.596	0.645	0.717	0.749	0.704	0.843	1.000									
miR-93	0.622	0.630	0.767	0.847	0.829	0.783	0.703	0.713	0.788	0.713	1.000								
miR-25	0.446	0.469	0.620	0.728	0.655	0.571	0.484	0.544	0.561	0.509	0.806	1.000							
miR-140	0.606	0.587	0.675	0.630	0.691	0.757	0.755	0.641	0.796	0.724	0.781	0.596	1.000						
miR-342-3p	0.588	0.590	0.472	0.366	0.444	0.665	0.714	0.692	0.696	0.660	0.473	0.273	0.562	1.000					
miR-486	0.387	0.423	0.399	0.395	0.408	0.449	0.437	0.554	0.430	0.467	0.458	0.361	0.408	0.578	1.000				
miR-150	0.816	0.796	0.759	0.666	0.689	0.562	0.479	0.608	0.494	0.473	0.525	0.428	0.416	0.527	0.421	1.000			
miR-197	0.776	0.777	0.759	0.501	0.686	0.618	0.717	0.572	0.629	0.558	0.413	0.252	0.481	0.573	0.393	0.645	1.000		
miR-454	0.543	0.517	0.453	0.472	0.403	0.421	0.336	0.445	0.317	0.259	0.376	0.321	0.273	0.286	0.215	0.535	0.319	1.000	
miR-122	0.260	0.258	0.279	0.362	0.306	0.301	0.217	0.278	0.285	0.323	0.367	0.319	0.310	0.237	0.218	0.307	0.119	0.136	1.000

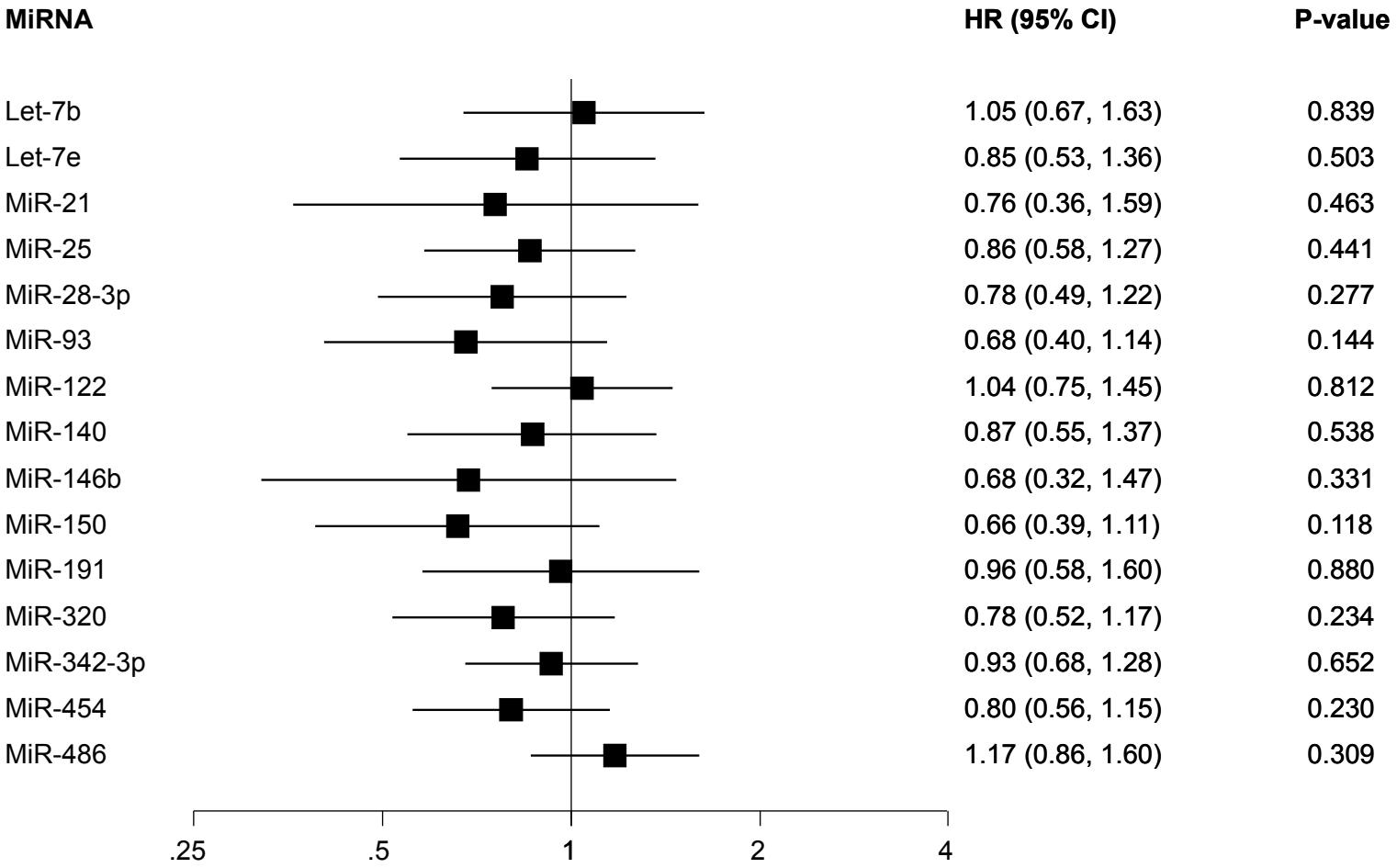
Online Figure II. MiRNA signature for incident MI. L_1-penalized Cox regression analysis (least absolute shrinkage and selection operator method). The graph shows Cox regression coefficients of miRNAs (y-axis) for different levels of penalization (the tuning parameter λ_1 escalates beyond 20). Variables withstanding shrinkage up to high λ_1 values are those most relevant for disease prediction.



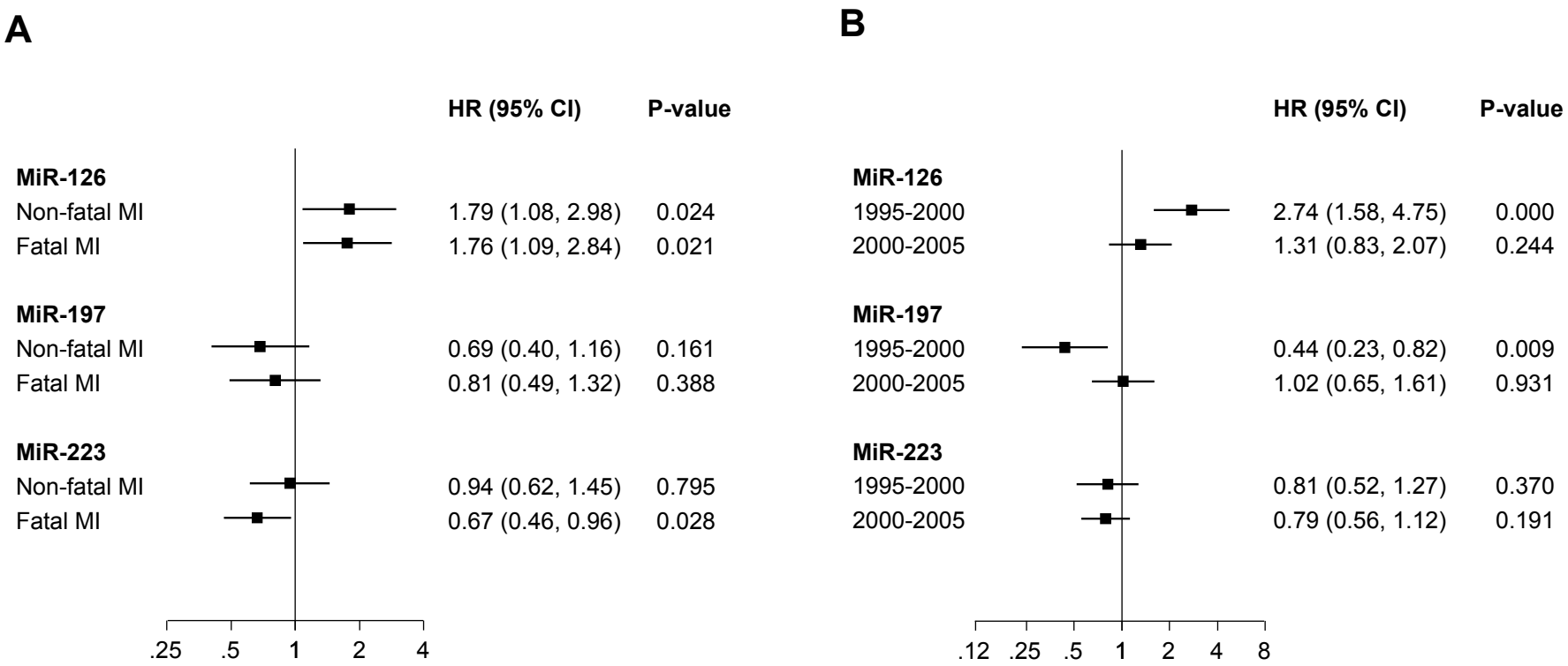
Online Figure III. Risk estimates for the three miRNAs most consistently associated with MI. The three miRNAs were identified by AIC-based models and the technique of least absolute shrinkage and selection operator. Hazard ratios (95% CI) were derived from standard Cox regression models with progressive adjustment (for details, see Methods). Expression levels were normalised to Ct average.



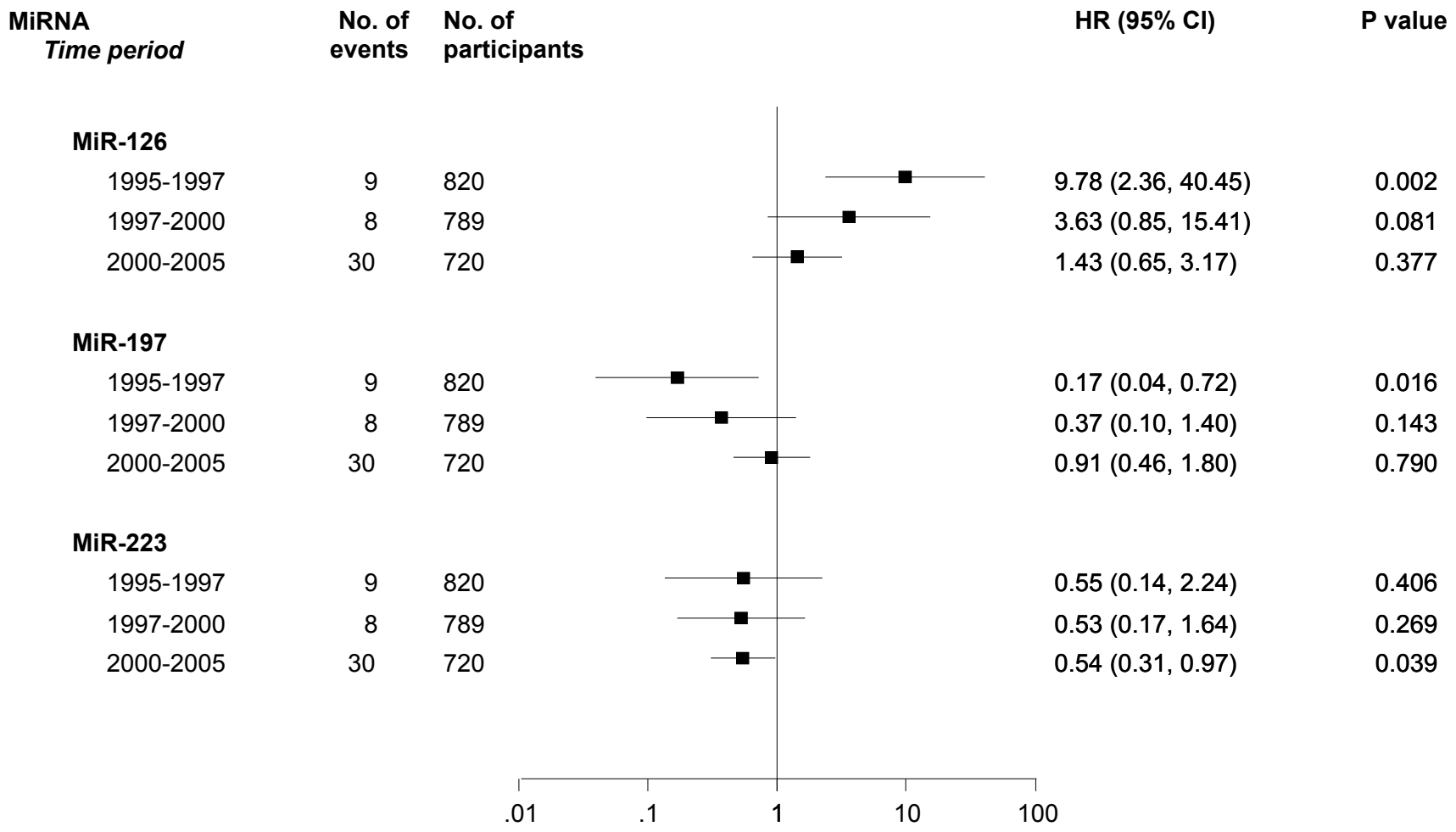
Online Figure IV. Risk estimates for the other miRNAs. Hazard ratios (95% CI) were derived from the multivariable Cox regression model 2, including miR-126, miR-197 and miR-223 (for details, see methods).



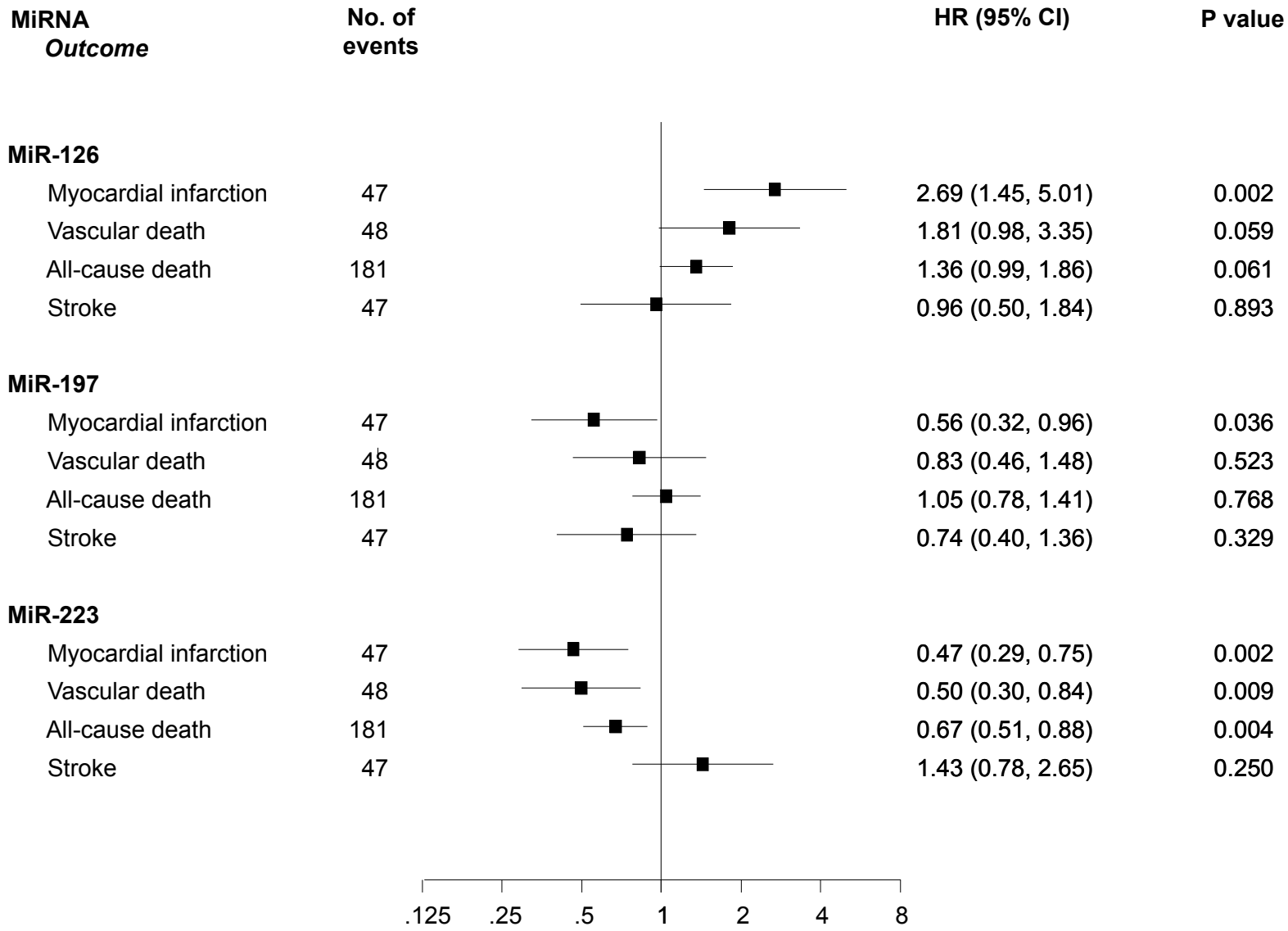
Online Figure V. Subanalysis for fatal versus non-fatal MI (A) and early (1995-2000) versus late (2000-2005) events (B). Hazard ratios (95% CI) were derived from the multivariable Cox regression model 2, including miR-126, miR-197 and miR-223 (for details, see methods). During ten years of follow-up (1995-2005), there were 26 fatal and 21 non-fatal MI events. There were 17 MI events during follow-up time 1995-2000 (incidence rate, 3.7 [2.2-5.9] per 1000 person-years) and 30 MI events during 2000-2005 (7.4 [5.2-10.6] per 1000 person-years). Expression levels were normalised to Ct average.



Online Figure VI. Subanalysis for very early (1995-1997), early (1997-2000) versus late (2000-2005) events. Hazard ratios (95% CI) were derived from the multivariable Cox regression model 2, including miR-126, miR-197 and miR-223 (for details, see methods).

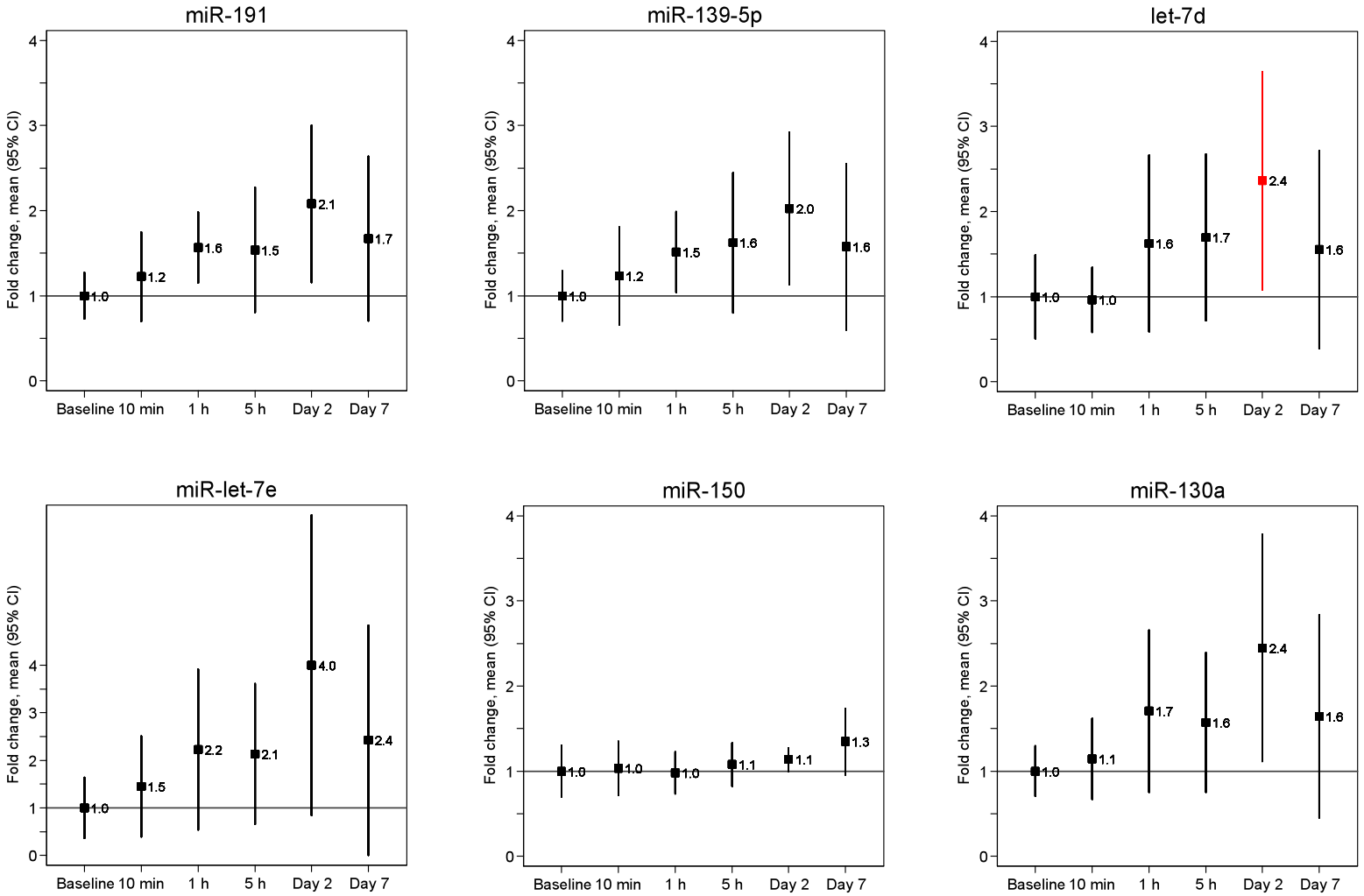


Online Figure VII. Association with different vascular endpoints and all-cause death. Hazard ratios (95% CI) were derived from the multivariable Cox regression model 2, including miR-126, miR-197 and miR-223 (for details, see methods).



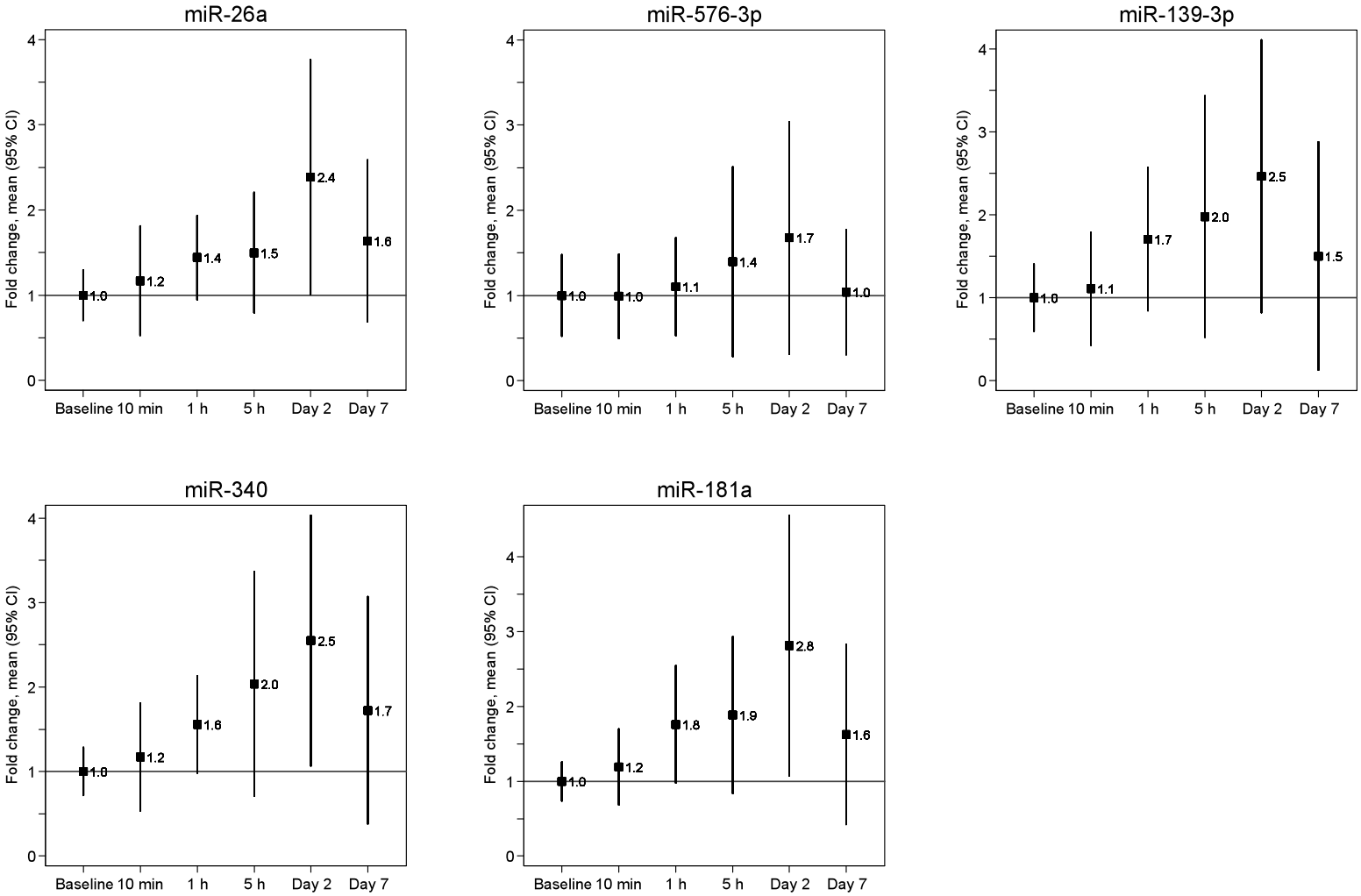
Online Figure VIII. MiRNA expression in the IR cohort at baseline and at different time points, as assessed by QPCR. Exogenous spike-in cel-miR-39 was used as a normalization control. Red bars indicate statistically significant differences ($p<0.05$) compared to baseline.

Cluster 1



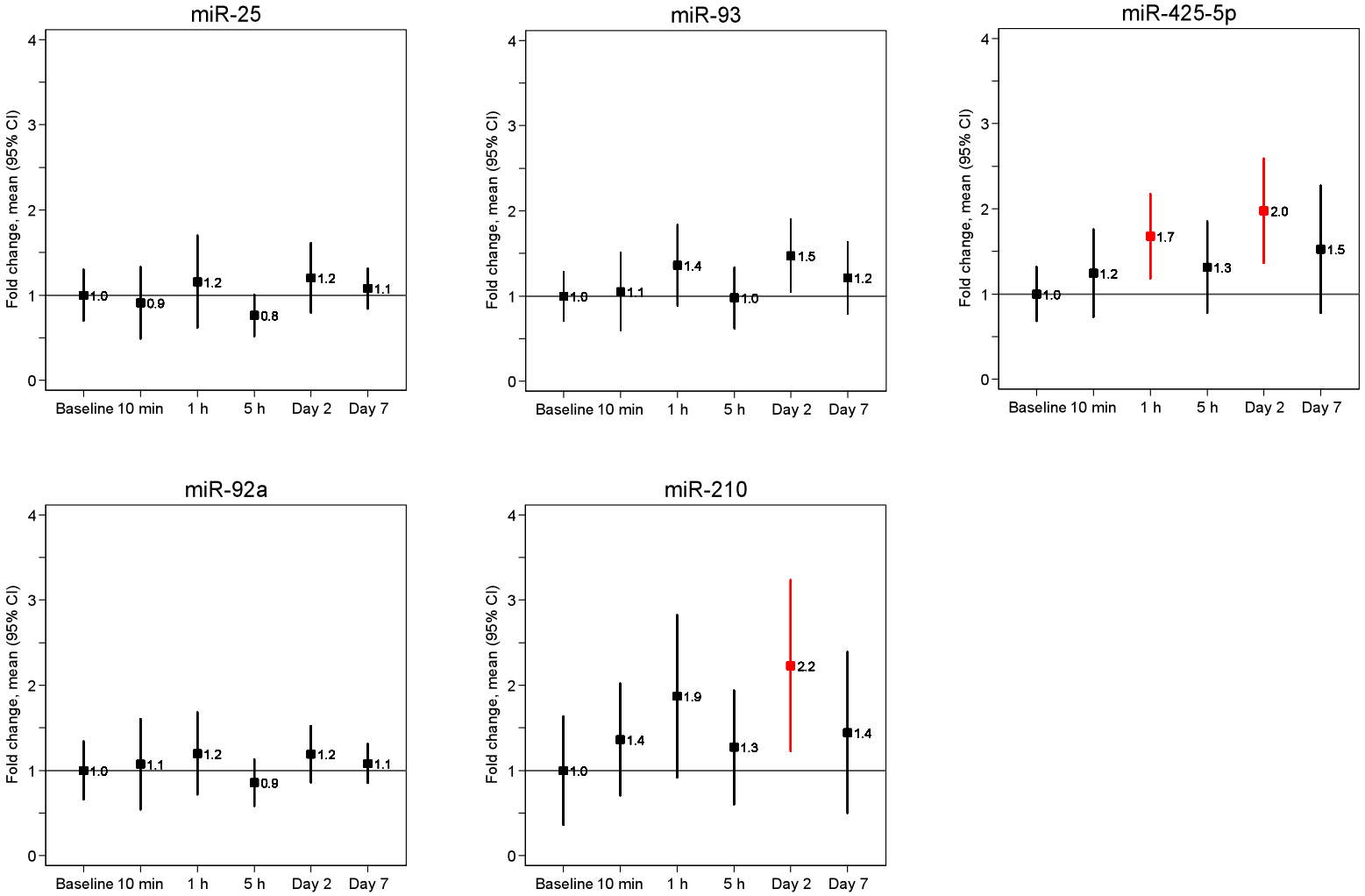
Online Figure VIII.

Cluster 1 (continued)



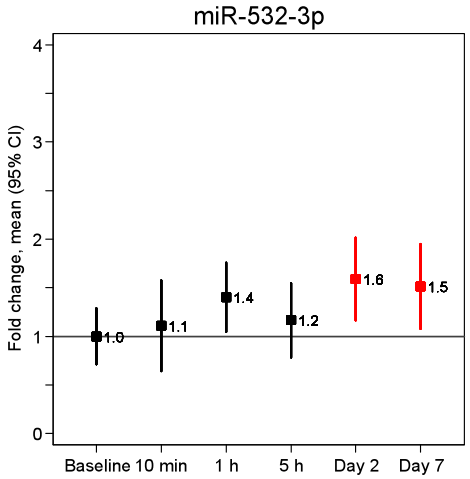
Online Figure VIII.

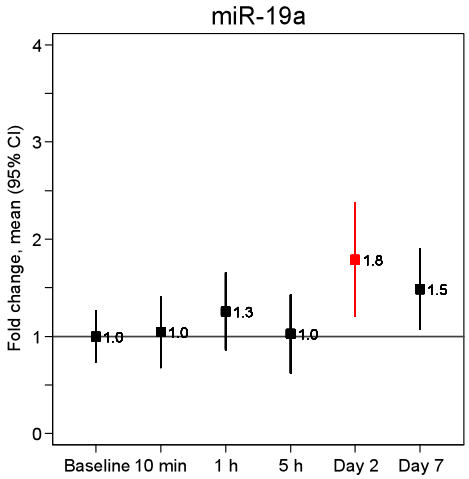
Cluster 3

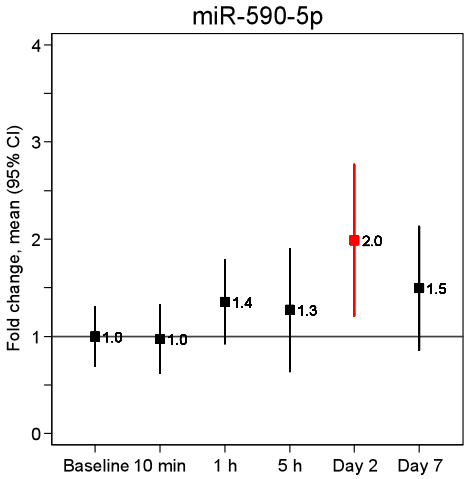


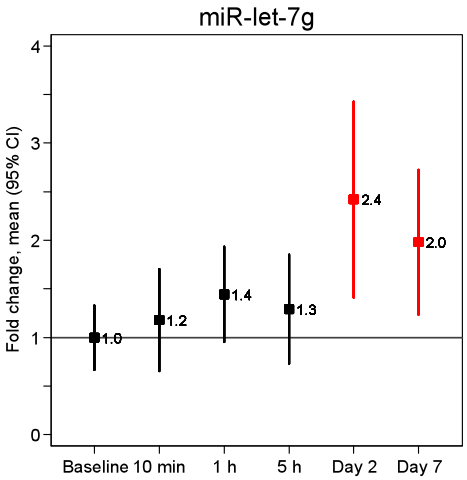
Online Figure VIII.

Cluster 4



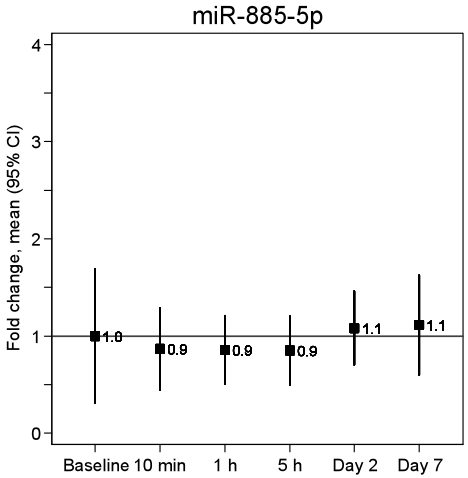


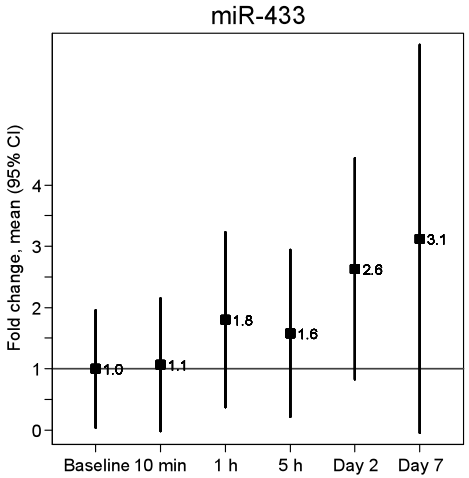


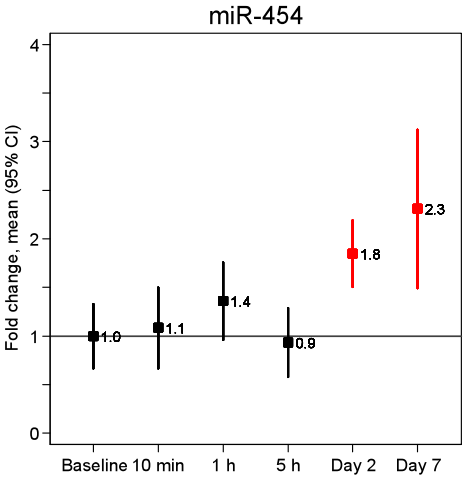


Online Figure VIII.

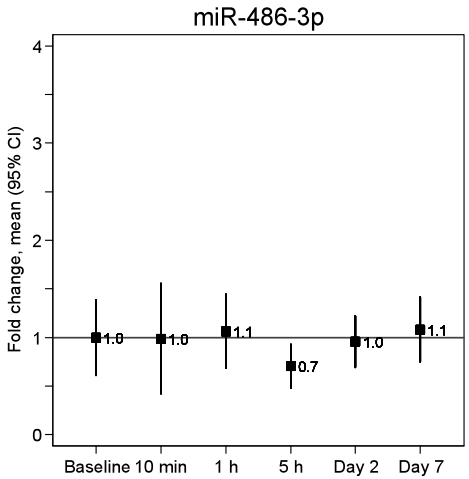
Cluster 5

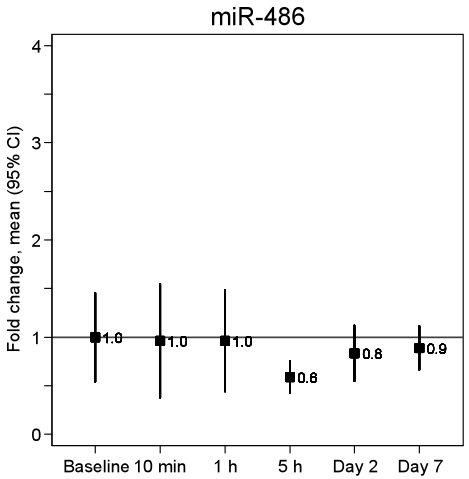


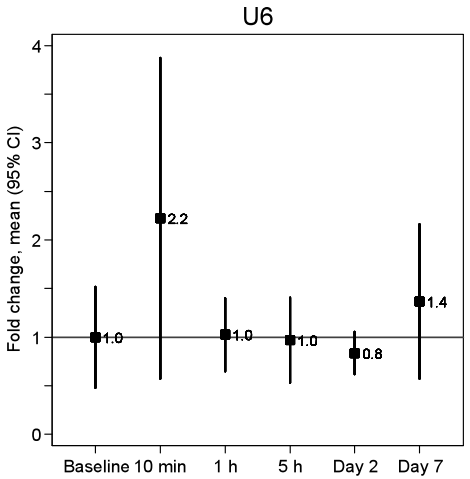




Cluster 6







Supplemental Tables and supporting information

Online Table I. Baseline characteristics of the Bruneck Study population.

Variables	All participants	Incident myocardial infarction		P value
	(n=820)	Yes (n=47)	No (n=773)	
Demographics and life-style				
Age, years	62.9 (11.1)	70.0 (9.6)	62.4 (11.1)	<0.001
Female sex, n (%)	409 (49.9)	17 (36.2)	392 (50.7)	0.070
Current/ex-smoker, n (%)	372 (45.4)	23 (48.9)	349 (45.2)	0.652
Alcohol consumption, g/d	23.8 (31.2)	25.0 (34.1)	23.7 (31.0)	0.781
Physical examination				
Body mass index, kg/m^2	25.7 (3.9)	26.4 (4.9)	25.6 (3.8)	0.165
Waist-hip ratio, cm/cm	0.9 (0.1)	1.0 (0.1)	0.9 (0.1)	0.012
Systolic blood pressure, mmHg	148.2 (20.6)	155.9 (22.9)	147.7 (20.4)	0.008
Diastolic blood pressure, mmHg	87.1 (9.1)	87.7 (8.9)	87.0 (9.2)	0.646
Lipid markers				
Total cholesterol, mmol/L	6.0 (1.1)	6.4 (1.4)	5.9 (1.1)	0.003
HDL cholesterol, mmol/L	1.5 (0.4)	1.5 (0.5)	1.5 (0.4)	0.762
LDL cholesterol, mmol/L	3.8 (1.0)	4.1 (1.3)	3.7 (1.0)	0.033
Triglycerides, mmol/L	1.3 [0.9-1.8]	1.4 [1.0-1.9]	1.3 [0.9-1.8]	0.223
Apolipoprotein A-I, g/L	1.7 (0.3)	1.7 (0.3)	1.7 (0.3)	0.926
Apolipoprotein B, g/L	1.2 (0.3)	1.3 (0.4)	1.2 (0.3)	0.004
Inflammatory markers				
Leukocytes, $x10^9/L$	6.5 (1.7)	6.9 (1.5)	6.5 (1.7)	0.111
C-reactive protein, nmol/L	16.2 [8.6-31.4]	20.6 [11.4-47.6]	15.2 [7.9-30.5]	0.070
Fibrinogen, g/L	2.9 (0.8)	3.1 (0.6)	2.9 (0.8)	0.023
Diabetes				
Type 2 Diabetes, n (%)	82 (10.0)	6 (12.8)	76 (9.8)	0.457
HbA1c, %	5.8 (3.7)	6.7 (7.5)	5.7 (3.4)	0.074
Previous diseases				
Cardiovascular diseases, n (%)	57 (7.0)	12 (25.5)	45 (5.8)	<0.001

Values are means (SD), medians [interquartile range] or numbers (percentages).

Online Table II. Interaction analysis of the association between miRNAs and incident myocardial infarction.

Subgroups	No. of MI events	Incidence rate (per 1000 person-years)	Hazard ratio (95% CI) for myocardial infarction per 1 SD increase of $\log_e$ miRNA*		
			MiR-126	MiR-197	MiR-223
Diagnosis of diabetes					
Yes	6	7.9 (3.6-17.6)	4.07 (1.63-10.13)	0.85 (0.32-2.25)	0.70 (0.26-1.85)
No	41	5.2 (3.8-7.0)	2.35 (1.28-4.30)	0.55 (0.32-0.94)	0.47 (0.29-0.75)
			$P_{interaction}$=0.174	$P_{interaction}$=0.337	$P_{interaction}$=0.405
Sex					
Male	30	7.2 (5.0-10.3)	2.61 (1.40-4.88)	0.64 (0.36-1.12)	0.52 (0.31-0.88)
Female	17	3.7 (2.3-6.0)	2.36 (1.12-4.96)	0.42 (0.21-0.85)	0.42 (0.23-0.77)
			$P_{interaction}$=0.755	$P_{interaction}$=0.206	$P_{interaction}$=0.491
History of cardiovascular disease					
Yes	12	25.0 (14.2-44.0)	2.45 (1.34-4.47)	0.81 (0.37-1.80)	0.47 (0.29-0.76)
No	35	4.3 (3.1-5.9)	3.25 (1.28-8.25)	0.52 (0.30-0.90)	0.55 (0.24-1.23)
			$P_{interaction}$=0.490	$P_{interaction}$=0.252	$P_{interaction}$=0.711

All Cox models included an interaction term of diabetes, sex or history of cardiovascular disease with the miRNA of interest, plus the variables age, sex, smoking status (ever vs. never smokers), systolic blood pressure, LDL cholesterol, diabetes, history of cardiovascular disease, miR-126, miR-197 and miR-223 (multivariable model 2).

Online Table III. Clinical characteristics of the healthy volunteers (n=11).

Male, n (%)	9 (82%)
Age, years	30±12.2
Sodium, mmol/L	141.36±2.06
Potassium, mmol/L	4.65±0.35
Urea, mmol/L	5.58±0.73
Creatinine, µmol/L	76.28±29.43
Calcium, mmol/L	2.23±0.08
Albumin, g/L	42.83±13.66
Alkaline phosphatase, IU/L	56±21.82
Bilirubin, mmol/L	10.81±9.67
Alanine transaminase, IU/L	25.72±17.77
Gamma-glutamyl transpeptidase, IU/L	24.27±13.61
Glucose, mmol/L	5.12±0.81
INR	1.02±0.06
Total cholesterol, mmol/L	4.43±1.27
HDL, mmol/L	1.56±0.6
LDL, mmol/L	2.5±0.98
Trigycerides, mmol/L	0.83±0.53
C-reactive protein, µmol/L	<0.5

INR indicates International Normalized Ratio