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Association of Vascular and Inflammatory miRNAs with Psychological stress and Heart Rate Variability Biofeedback

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Committee Chair

Association of Vascular and Inflammatory miRNAs with Psychological stress and Heart Rate Variability Biofeedback

By

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B.S.
University of Washington
2015

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An abstract of
A thesis submitted to the Faculty of the
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Abstract

Association of Vascular and Inflammatory miRNAs with Psychological stress and Heart Rate Variability Biofeedback

By Yidan Pei

Psychological stress can trigger acute cardiovascular events, especially in patients with coronary heart disease (CHD). Certain microRNAs (miRs) signal active inflammation and may serve as a biomarker to predict CHD events. We investigated the association of CHD-related miRs with acute psychological stress, as well as before/after a stress reduction intervention. Additionally, we examined which parameters predicted changes with stress. We conducted a randomized controlled pilot study of heart rate variability biofeedback (HRVB) versus waitlist control in subjects with known CAD. Of 25 patients enrolled, data were available for 22 participants, of which 13 received HRVB and 9 were placed in the waitlist control group. Stress was introduced via a three-minute mathematics test during each visit, and miRs (126-5p, 126-3p, 223) in blood samples were measured using relative expression. Hemodynamic and vascular functions were assessed and evaluated as well. The median miR levels increased with acute mental stress, although the difference was not statistically significant. HRVB was associated with decreased changes with stress in miR-126-3p ($p = 0.0002$) and miR-126-5p ($p < 0.0001$). Significant risk factors that predicted miR levels included: race, gender, waist-hip ratio (WHR), systolic blood pressure (SBP), open heart surgery, diabetes, functional capacity, and mental stress-induced myocardial ischemia (MSIMI).

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Background

Psychological stress can develop after difficulty managing or responding to mental, physical, or emotional pressure (1). About 20% of people with coronary artery disease (CAD) or heart failure (HF) suffer from depression. Depressed patients with comorbid cardiovascular disease (CVD) are 90% more likely to experience secondary coronary heart disease (CHD) events and 45% more likely to develop stroke (2). Various stress-reduction techniques have been developed to prevent recurrent events in such high-risk patients (3). For example, mindfulness meditation helped CHD patients with reduced anxiety, depression, blood pressure (BP), and body-mass index (BMI) (4, 5). Chronic stress can also lead to unhealthy behaviors such as smoking, alcohol abuse, and unhealthy diet (6). Psychological stress effects on CVD are also potentially mediated by the hypothalamic pituitary adrenal (HPA) axis and the sympathetic-adrenal medullary (SAM) systems that regulate inflammation and hemodynamics, amongst other effects (7-9).

Blood-based biomarkers are potentially useful in helping to understand the mechanism linking stress and CVD, and also risk stratify individuals who have more pathologic responses to acute mental stress (10). However, reliable measurements can be difficult because some biomarkers are unstable and degrade for various reasons. Khalaila found that some biomarkers are problematic for measuring stress levels because of insufficient sensitivity, or stability, or difficult collection procedures, or measurability (11).

Our team is investigating whether microRNAs (miRs) can function as a sensitive tool to detect psychological stress among people with CHD. miRs are a group of small (~22-

nucleotides), regulatory, and non-coding ribonucleic acids (RNAs) (12-14). Studies found that miRs regulate the immune response and inflammation (15, 16). These small regulatory RNAs in serum or plasma can be efficient biomarkers because they are stable against freezing or thawing process and are less likely to be degraded by RNase (12, 17). miRs-based therapeutic intervention has been studied as a novel treatment against multiple diseases (18). For example, the effect of anti-miR drugs on miR-122 expression regulation to block hepatitis C virus infection is currently studied via a clinic trail (18). miRs regulate post-transcription activities by inhibiting translation of mRNAs or performing message degradation, and 139 miRs have been discovered as plausible biological biomarkers for non-neoplastic diseases (12). miR-126-3p and miR-126-5p are the most abundant endothelial miRs whose precursor, miR-126, can predict psychological stress, Crohns' disease, lupus, the risk of myocardial infarction, CAD, sepsis, diabetes, heart failure, Parkinson, and end-stage renal disease (12). miR-223 is expressed in blood platelets and leukocytes and can enter vascular cells or serum as an endocrine genetic signal to protect against inflammation (14, 15). It can act as a biomarker for rheumatoid arthritis, tuberculosis, lupus, osteoarthritis, viral myocarditis, hepatitis, eosinophilic esophagitis, diabetes, myocardial infarction, and risk of myocardial infarction (12). Despite the amount of research studying miRs as biomarkers for CAD events, little is known whether miRs are associated with psychological stress (12-14, 17, 19-24) or can be modified after stress reduction intervention. We hypothesize that three miRs (126-5p, 126-3p, and 223) change in response to stress, and that management of stress with a stress-reduction intervention leads to changes in baseline levels after 2 months in a pilot

cohort of participants with CHD. Furthermore, because these biomarkers are relatively new, we also examined for other possible determinants of high or low miR levels.

Methods

Participant population

For this investigation, we conducted a randomized, controlled, and a pilot clinical trial. Participants (n = 25) were recruited in 2016-2017 from an established cohort, the Mental Stress Ischemia Mechanisms and Prognosis Study (MIPS) conducted in Emory University-affiliated clinics or hospitals, Atlanta, GA from 2009 to 2014. The recruitment methods and study protocol were previously described (25). Briefly, all participants had a stable CAD diagnosis. Baseline data from participants were accessed from MIPS and baseline questionnaires during each study visit, and included medical history, sociodemographic, physical and psychological factors, as well as hemodynamic and vascular assessments (also previously described). The study was IRB-approved and participants gave informed consent prior to entering the study. The study was also registered clinicaltrials.gov, NCT02657382.

Clinical protocol

Participants were followed for 12-18 weeks and scheduled for 3 visits with an arithmetic mental stress test at each visit. At the first visit they were randomized to heart rate variability biofeedback (HRVB) therapy or wait-list usual care (1:1) for 6 weeks. After 6-8 weeks, a second visit for repeat testing was performed. After that, participants in the wait-list control arm received biofeedback therapy as well, and then a 3rd study visit was conducted 6-8 week afterward for repeat assessments. Visit 1 and 2 occurred in the PET scanner and visit 3 occurred in a clinic room on a stretcher.

HRVB: The details of HRVB procedures were previously described (26). Participants were taught how to use biofeedback training so that they could practice at home. The 6-week training consisted of 6 sessions. The 1st, 2nd, and 6th sessions were performed by two professional biofeedback trainers at the Atlanta VA Medical Center (GA), and the other sessions (3rd, 4th, and 5th) were conducted during a phone call by an off-site HRVB trainer. All trainers had training in teaching HRVB practice. Participants were instructed to practice at least 10 minutes daily. A computerized system was used to monitor and display real-time heart rate variability (HRV) pattern, and provide feedback as participants performed focusing, resonance frequency breathing, heart focus, and positive thinking. Because HRV feedback can show patients an effect of these techniques on HRV patterns, it can reinforce these positive behaviors to improve mental health (26, 27).

Mental stress: Arithmetic mental stress was introduced during each study visit. The stressor involved serial math problems with negative feedback for wrong answers (28). At the end of the test, perceived stress was evaluated as a score from 0 (no stress) to 100 (maximum stress). Blood (20 ml) was collected 3 times: before mental stress challenge, 5 minutes after mental stress challenge, and 60 minutes after stress. Heart rate and blood pressure were measured at every minute during stress.

MicroRNAs: Purification of miRs followed the protocol from miRNeasy Mini kit (Qiagen). Blood plasma (200 ul) was mixed with 5 volumes of QiAzol lysis reagent (1000 ul) followed by 5-min incubation at RT. 5ul 5nM Syn-cel-miR-39 mimic was spiked into the samples for normalization of experimental results. Chloroform (200 ul)

was then added in the spiked samples and the mixture was incubated for another 2-3 minutes at RT. After centrifugation (12,000 g, 15 minutes, 4℃), the upper phase containing RNA was transferred and added to 100% ethanol (600 ul). 700 ul of the sample was then purified using the RNeasy Mini spin column with centrifugation (≥ 8000 g, 15 s, RT) to discard the flow-through. The centrifugation was repeated after adding 700 ul buffer RWT and 500 ul Buffer RPE to discard remaining flow-through. Plasma samples without flow-through were micro-centrifuged at a maximum speed for 2 minutes. RNA was then eluted twice with 30 ul RNase-free water by centrifugation (≥ 8000 g, 1 min, RT). miR values were defined as the relative expression to miR-39 using the comparative CT method ($\Delta\Delta C_T = C_{TmiRs} - C_{Tcel\text{-}39}$). The fold difference of targeted miRs, after normalized to the reference miR, was described as $2^{-\Delta\Delta CT}$.

Statistical analysis

Distribution of miRs at baseline in two study arms was compared using the Wilcoxon-Mann Whitney test. To access the correlation of miRs with demographical, psychological, and cardiac factors, we used the Pearson or Spearman correlation coefficient test for both continuous and categorical variables and additional Wilcoxon-Mann Whitney test for categorical variables. Bar graphs were used to visualize the change of baseline miRs and the consistency of stress-induced changes in miRs over time.

The primary analysis of changes in miRs by HRVB and stress was performed using intention-to-treat groups. The linear mixed-effects model (LMM) was used to estimate

the effect of the HRVB. Fixed effects included the intervention group, time (visit 1-2), and HRVB-time interaction, and random effects were subject and subject-time interaction. A multivariable-adjusted LMM measured the main effect of stress, time (visit 1-3), and stress-time interaction on miRs level, in which subject and subject-time interaction were included as a random effect. Covariates were determined by literature reviews and previous correlation analyses.

All statistical tests were performed using statistical software SAS (SAS version 9.4, (32)). A P-value < 0.05 was considered significant.

Results

Two participants dropped out after the first visit; in addition, several samples were corrupted or did not have sufficient volume to measure. After subtracting those samples, miR measurements were available for 14 participants at the 1st visit, 15 participants at the 2nd visit, and 18 participants at the 3rd visit. Blood samples for the same individuals across all 3 visits were available for 10 participants.

The average age of 22 participants with miR data was 60.1 (SD 5.7) years old, 68% were males, and 31.8% were women. Half of the participants were Caucasians and the rest were African Americans (Table 1). At the first visit, the distribution of miR-126-5p and miR-126-3p was comparable between two study groups, but the miR-223 level was higher in the control group (Figure 1).

Waist-to-hip ratio (WHR), history of heart surgery, mental stress-induced myocardial ischemia (MSIMI), diabetes, antidepressants, beta blockers, Duke activity status index (DASI) score, systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) were moderately or strong correlated with miR levels at the first visit only. However, this finding was not consistent with the follow-up visits (Table 2 and Figure 2). Those with higher level of miR-126-5p were less likely to have had open heart surgery ($r = -0.54$) and low DBP ($r = 0.54$), but more likely to have taken beta blockers ($r = 0.71$), and these results were statistically significant (Table 2). Similar correlations were also observed between beta blockers use and miR-126-3p level ($r = 0.67$, $p = 0.01$). Table 2 showed similar correlations of miR-126-3p and miR-126-5p with variables. Those with

higher miR-223 level were found out to have lower DASI score and higher HR and DBP, results were statistically significant but not comparable across 3 visits (Table 2). Increased miR levels (126-3p, 126-5p, 223) were observed in African Americans, females, those without open heart surgery or with MSIMI, or those who took beta blockers (Figure 2). Participants with diabetes or those who took antidepressants had higher miR-126-3p and miR-126-5p level, which was not overserved for miR-223 (Figure 2).

Figure S1 showed that baseline miRs and changes with stress were comparable between HRVB and wait-list control group at each visit. In the LMM, HRVB was not statistically associated with the unadjusted change rate of miR's relative expression ($*10^{-4}$) from visit 1 to visit 2. Baseline level and changes with stress of miR-126-5p and miR-223 increased at the 1st follow-up period and decreased at the 2nd follow-up period, while the baseline miR-126-3p level had similar distribution at visit 1 and visit 2 (Figure S1).

In the multivariable-adjusted analyses, a non-significant increasing trend after stress test was observed for all 3 miRs (Table 3).

Various risk factors were associated with changes in miR values with stress. HRVB ($p = 0.0002$) and history of open heart surgery ($p < 0.0001$) were both inversely associated with the stress-induced changes of miR-126-3p, while higher WHR ($p = 0.02$) and history with MSIMI ($p = 0.04$) were positively associated.

Variables including HRVB therapy ($p < 0.0001$), stress-induced changes in SBP ($p < 0.001$), experience with open heart surgery ($p < 0.0001$), or Caucasian race ($p < 0.0001$) were shown inversely associated with stress-induced changes in miR-126-5p. A higher DASI score ($p < 0.0001$), WHR ($p < 0.0001$), experience with MSIMI ($p < 0.0001$), diabetes ($p < 0.0001$), or male ($p = 0.02$) gender predicted an increase in changes with stress in miR-126-5p. The findings were statistically significant.

WHR ($p = 0.001$) and DASI score ($p < 0.001$), or being African Americans ($p = 0.01$), having experienced open-heart surgery ($p = 0.001$) and diabetes ($p = 0.01$) was associated with higher stress-induced changes in miR-223, and the finding was statistically significant.

Using LMM, the stress test was found to be associated with a statistically significant increase in subjective stress level ($B= 27.86$; 95% CI (16.81, 38.91), $p < 0.0001$). The effect of stress test was not significantly modified by time (study visit) with $B = 2.62$ (95% CI (-6.17, 11.42)), however.

Discussion

In this randomized controlled trial of HRVB, we found that HRVB was associated with lower stress-induced changes of miR-126-3p and miR-126-5p. To our knowledge, this is the first study to examine the effect of stress management (HRVB) on miRs. We also noted a trend towards increased levels of miR-126-3p, mir-126-5p, and miR-223 with acute stress, and associations of multiple health factors with miR levels as well.

Our study is one of the few that examine the association of psychological stress with miRs. Other studies (1 cohort, 1 experiment, 1 meta-analysis) also found that stress is associated with higher level of miR-126 (12, 29, 30). The cohort study (n = 25) conducted among young medical male students showed higher miR-126 after academic stress ($F(2,48)=6.800$, $p=0.0025$), but no change in miR-223 (29). An animal experiment study (n = 9) found a higher miR-126 level in mice vulnerable to stress than mice with higher resilience to social defeat ($r = -0.81$, $p = 0.05$) (30). A meta-analysis that utilized 104 studies covering 57 diseases also found that stress was associated with increase in miR-126 (12).

Although not statistically significant, an increase in miR levels were noted with stress that warrant further investigation, as several biological mechanisms are known. Cells will restore cellular homeostasis or re-program genetic expression as an adaption to stress, which is mediated by miR activities (31). This includes modulating the amount of miRs and mRNA targets and the activation of miRNA-protein complexes. miRs expression

may be upregulated or downregulated based on the changes of the cellular environment (31). For example, a tumor suppressor protein, p53, regulates transcription and processing of miRs as a response to DNA damage (31). A transcription factor KLF2 can transcribe miR-126 to target VCAM-1 and ICAM-1, the pro-inflammatory molecules (32). NF-κB upregulates miRs to deactivate the pro-inflammatory signaling cascade in macrophages, and miR-223 was found to down-regulate this pro-inflammatory activity (33).

Strengths and Weaknesses

There are several strengths of this study. To our knowledge, this is the first RCT studying the association of miR and psychological stress in vulnerable population. The longitudinal RCT design allowed us to estimate effect of both stress management therapy and induced stress on miRs while controlling for other CHD risk factors. It was the first step to study the possibility whether miRs could be used a treatment for stress in people with chronic diseases.

Several limitations were also noted. This includes small sample size, missing data, and changes in clinic environment that occurred for visit 3. Participants would feel more relaxed at the last visit because the study clinic was different compared to previous visits, but the result for miR-126 was consistent with previous studies.

Future Directions

We noted several non-significant trends that warrant further replication in a large study, including increased miRs levels with stress and reduced stress-induced changes after HRVB therapy. Ultimately, such data may help understand the mechanisms of acute mental stress and development of miR-based therapeutics.

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Tables

Table 1. Selected baseline characteristics of the study participants (n = 22).	
	Visit 1 (n = 22)
Enrolled age, mean (SD), y	60.1 (5.7)
Race/ethnicity	
Caucasian	50.0%
African American	50.0%
Sex	
Male	68.2%
Female	31.8%
Physical exam	
BMI, mean (SD), kg/m^2	29.5 (4.8)
WHR, mean (SD)	0.9 (0.1)
Cardiac past medical history	
Open heart surgery	22.7%
Heart failure	4.6%
Diabetes	36.4%
PSIMI	68.2%
MSIMI	59.1%
Gensini score, mean (SD)	45.0 (52.9)
Troponin, mean (SD), ng/dL	8.9 (9.2)
Smoke history	
Former smoker	40.9%
Current smoker	13.6%
Never smoker	45.5%
Medications	
Antidepressant	13.6%
Beta blocker	68.2%
Psychological variables	
ETISR score, mean (SD)	5.4 (4.1)
BDI score, mean (SD)	7.1 (5.6)
Health behaviors	
DASI, mean (SD)	42.6 (11.0)
Hemodynamics	
SBP, mean (SD), mmHg	134.4 (22.1)
DBP, mean (SD), mmHg	75.3 (9.5)
HR, mean (SD), bpm	59.9 (11.5)
FMD, mean (SD), %	3.8 (1.3)

Abbreviations: BMI, body mass index; WHR, waist-hip ratio; PSIMI, physical stress-induced myocardial ischemia; MSIMI, metal stress-induced myocardial ischemia; Gensini, total amount of cardiac diseases; ETISR, early trauma inventory self-report; BDI, beck depression inventory; DASI, Duke activity status index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; FMD, flow-mediated vasodilatation.

Table 2. **Correlation of miRs and baseline characteristics at 3 visits.**

	miR-126-5p						miR-126-3P						miR-223					
Visit	1 (n = 14)		2 (n = 15)		3 (n = 18)		1 (n = 14)		2 (n = 15)		3 (n = 18)		1 (n = 14)		2 (n = 15)		3 (n = 18)	
	r^a	P*	r^a	P*	r^a	P*	r^a	P*	r^a	P*	r^a	P*	r^a	P*	r^a	P*	r^a	P*
Age	0.00	0.99	0.01	0.96	-0.17	0.51	-0.18	0.54	0.03	0.93	-0.04	0.87	-0.49	0.08	0.21	0.45	-0.32	0.19
BMI	0.05	0.88	0.37	0.18	0.14	0.57	0.10	0.73	0.40	0.14	-0.04	0.88	-0.28	0.33	**0.55**	**0.03**	-0.21	0.40
WHR	-0.42	0.13	-0.12	0.67	-0.03	0.92	-0.42	0.14	-0.10	0.73	0.13	0.61	-0.26	0.37	0.11	0.68	0.35	0.15
PSIMI	0.05	0.86	0.22	0.43	-0.27	0.27	-0.05	0.86	0.22	0.43	-0.30	0.23	-0.05	0.86	0.19	0.50	-0.23	0.36
MSIMI	0.24	0.42	0.12	0.66	0.01	0.97	0.24	0.42	0.09	0.74	-0.45	0.06	0.12	0.69	0.00	1.00	-0.05	0.83
Open heart surgery	**-0.54**	**0.05**	-0.32	0.25	-0.13	0.61	-0.45	0.10	-0.32	0.25	0.19	0.46	-0.19	0.51	-0.32	0.25	-0.07	0.78
Diabetes	0.48	0.08	0.13	0.64	0.03	0.90	0.34	0.24	0.07	0.82	0.12	0.63	-0.02	0.95	0.03	0.91	0.10	0.70
Troponin	-0.41	0.18	0.19	0.52	-0.09	0.76	-0.18	0.57	0.18	0.55	-0.35	0.22	0.24	0.44	-0.01	0.97	-0.12	0.69
Gensini score	0.04	0.90	-0.17	0.55	0.14	0.57	-0.03	0.91	-0.21	0.46	0.33	0.18	-0.18	0.56	-0.13	0.65	0.11	0.68
Smoking history	-0.04	0.89	-0.28	0.31	0.11	0.67	0.07	0.81	-0.28	0.31	0.00	1.00	0.06	0.85	-0.13	0.65	0.03	0.89
Antidepressant	0.10	0.73	-0.12	0.68	-0.24	0.33	0.15	0.60	0.00	1.00	-0.22	0.39	0.00	1.00	-0.15	0.58	-0.27	0.27
Beta blocker	**0.71**	**<.01**	-0.26	0.35	-0.33	0.18	**0.67**	**0.01**	-0.26	0.35	-0.33	0.18	0.28	0.33	-0.33	0.23	-0.45	0.06
ETISR	-0.20	0.50	0.33	0.24	-0.11	0.69	0.05	0.87	0.33	0.24	0.05	0.85	-0.10	0.75	0.31	0.29	0.00	1.00
BDI	-0.25	0.38	-0.10	0.74	-0.03	0.89	-0.23	0.43	-0.04	0.88	0.28	0.26	0.10	0.74	0.03	0.92	0.11	0.66
DASI	-0.52	0.08	0.25	0.45	0.30	0.31	-0.55	0.07	0.19	0.58	0.32	0.29	**-0.69**	**0.01**	0.21	0.54	0.45	0.12
SBP	0.44	0.12	-0.03	0.93	0.27	0.30	0.50	0.07	-0.06	0.84	0.36	0.15	-0.15	0.62	0.10	0.72	0.37	0.14
DBP	**0.54**	**0.05**	-0.01	0.97	0.27	0.30	0.51	0.06	-0.06	0.83	0.41	0.10	0.17	0.56	0.04	0.89	**0.50**	**0.04**
HR	0.52	0.06	-0.39	0.16	0.05	0.86	0.35	0.21	-0.36	0.21	0.34	0.18	**0.54**	**0.05**	-0.39	0.17	0.19	0.46
FMD	-0.04	0.90	-0.24	0.39	-0.02	0.93	-0.11	0.73	-0.17	0.54	0.10	0.69	0.14	0.65	-0.10	0.74	0.20	0.43

Notes: miR, microRNA; BMI, body mass index; WHR, waist-hip ratio; PSIMI, physical stress-induced myocardial ischemia; MSIMI, metal stress-induced myocardial ischemia; Gensini, total amount of cardiac diseases; ETISR, early trauma inventory self-report; BDI, beck depression inventory; DASI, Duke activity status index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; FMD, flow-mediated vasodilatation.

All miRs are described by $2^{-\Delta\Delta CT.}$

*P values from Pearson or Spearman correlation test.

[a]Correlation coefficient, statistically significant values ($p < 0.05$) are in bold fonts.

Table 3. Multivariable-adjusted estimate of miRs change rate by induced stress[a].

microRNA ($2^{-\Delta\Delta CT}*10^{-4}$)	β[b]	95% Confidence Interval		P value[c]
		lower bound	upper bound	
miR-126-3p				
At 5 minutes[e]	16.92	-0.87	34.71	0.06
At 60 minutes[f]	11.63	-3.62	26.88	0.13
miR-126-5p				
At 5 minutes[e]	5.80	-6.22	17.81	0.33
At 60 minutes[f]	1.69	-8.92	12.31	0.75
miR-223				
At 5 minutes[e]	7.92	-14.63	30.46	0.48
At 60 minutes[f]	8.00	-11.94	27.94	0.42

Notes: miR, microRNA.

[a]All miRs ($2^{-\Delta\Delta CT}*10^{-4}$) are measured from blood samples collected at baseline, 5 minutes, and 60 minutes after induced-stress during three visits.

[b]The change rate of miR after stress, controlling for race, sex, heart rate variability biofeedback (HRVB), study visit, waist-hip ratio (WHR), metal stress-induced myocardial ischemia (MSIMI), open heart surgery, diabetes, beck depression inventory (BDI) score, Duke activity status index (DASI), systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), flow-mediated vasodilatation (FMD), beta blockers.

[c]P value from t test for miR levels after stress compared to baseline miR levels.

[e]Changes in miRs at 5 minutes after stress compared to the baseline (reference)

[f]Changes in miRs 60 minutes after stress compared to the baseline (reference)

Table S1. Unadjusted estimate of the effect of stress test on miR levels at each visit[a].

	Visit 1				Visit 2				Visit 3			
		95% Confidence Interval				95% Confidence Interval				95% Confidence Interval		
miR ($2^{-\Delta\Delta CT}*10^{-4}$)	β[b]	lower bound	upper bound	P[c]	β[b]	lower bound	upper bound	P[c]	β[b]	lower bound	upper bound	P[c]
miR-126-3p												
At 5 minutes[e]	**16.87**	**0.02**	**33.72**	**0.05**	9.80	-14.59	34.19	0.29	0.53	-14.08	15.14	0.89
At 60 minutes[f]	4.26	-1.77	10.30	0.15	**5.38**	**0.54**	**10.21**	**0.03**	4.30	-0.23	8.82	0.06
miR-126-5p												
At 5 minutes[e]	5.94	-2.94	14.81	0.17	10.40	-5.81	26.61	0.13	0.20	-8.37	8.76	0.93
At 60 minutes[f]	1.44	-2.24	5.12	0.41	**5.69**	**0.01**	**11.36**	**0.05**	0.62	-0.36	1.59	0.20
miR-223												
At 5 minutes[e]	8.79	-4.06	21.63	0.16	9.41	-41.69	60.50	0.60	5.99	-9.82	21.80	0.24
At 60 minutes[f]	4.73	-2.38	11.84	0.17	**13.76**	**0.90**	**26.63**	**0.04**	**4.25**	**1.16**	**7.35**	**0.01**

Abbreviations: miR, microRNA.

[a]All miRs ($2^{-\Delta\Delta CT}*10^{-4}$) are measured from blood samples collected at baseline, at 5 minutes, and 60 minutes after induced-stress during three visits.

[b]The change rate of miR after stress at each visit, statistically significant values ($p < 0.05$) are in bold fonts.

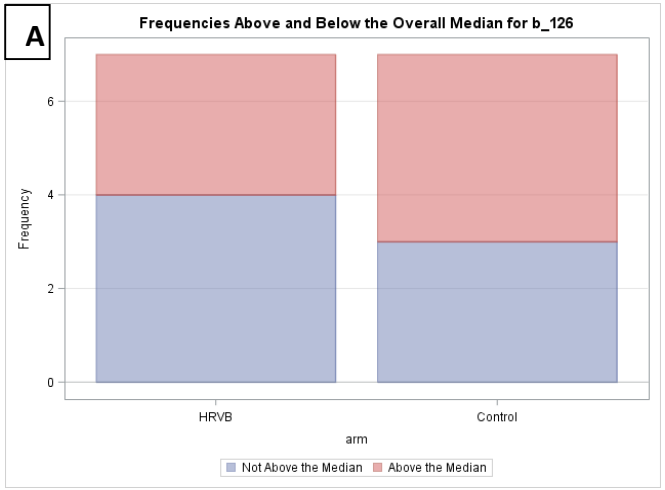
[c]P value from t test for miR levels after stress test compared to baseline miR levels.

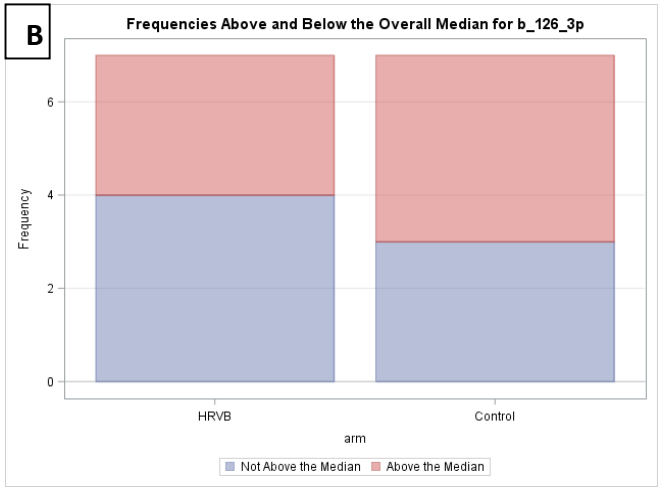
[e]Changes in miRs at 5 minutes after stress compared to the baseline (reference)

[f]Changes in miRs 60 minutes after stress compared to the baseline (reference)

Figures and Figure Legends

Figure 1. Distribution of the median of 3 miRs at baseline in two study arms in the BIMI study.
Figure A-C, (A) miR-126-5P; (B) miR-126-3p; (C) miR-223.





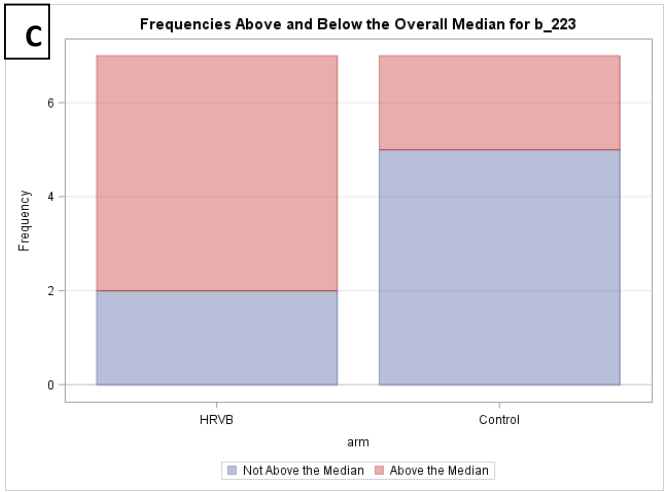


Figure 2. Distribution of Wilcoxon scores of baseline miRs by each characteristic including race, sex, open heart surgery, MSIMI, diabetes, antidepressants, and beta blockers.

Figure A-G, values in each box plot from left to right are **(**A) race, Caucasians and African Americans; (B) sex, males and females; (C) open heart surgeries, no and yes; (D) mental stress-induced myocardial ischemia, yes and no; (E) diabetes, yes and no; (F) antidepressants, no and yes; (G) beta blockers, yes and no. Wilcoxon scores from Wilcoxon-Mann Whitney test.

A

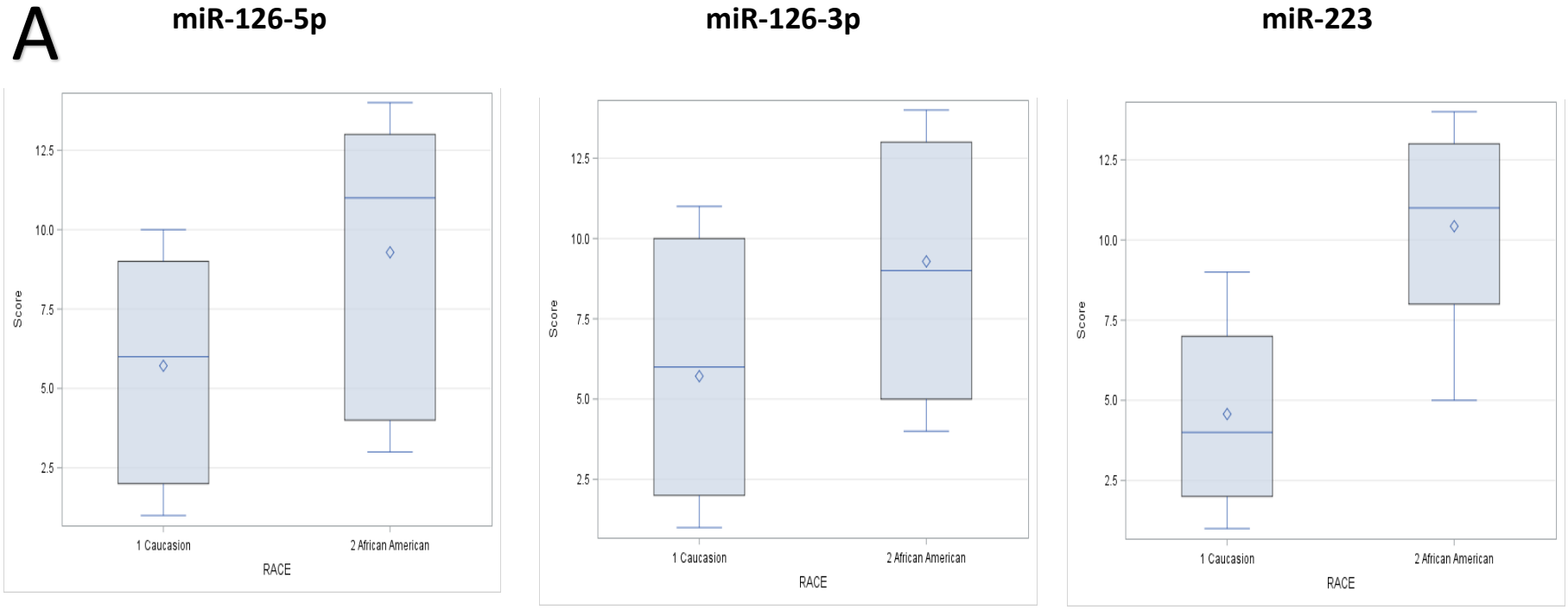


Figure 2 (continued). Distribution of Wilcoxon scores of baseline miRs by each characteristic including race, sex, open heart surgery, MSIMI, diabetes, antidepressants, and beta blockers.
Figure (B) sex, males and females.

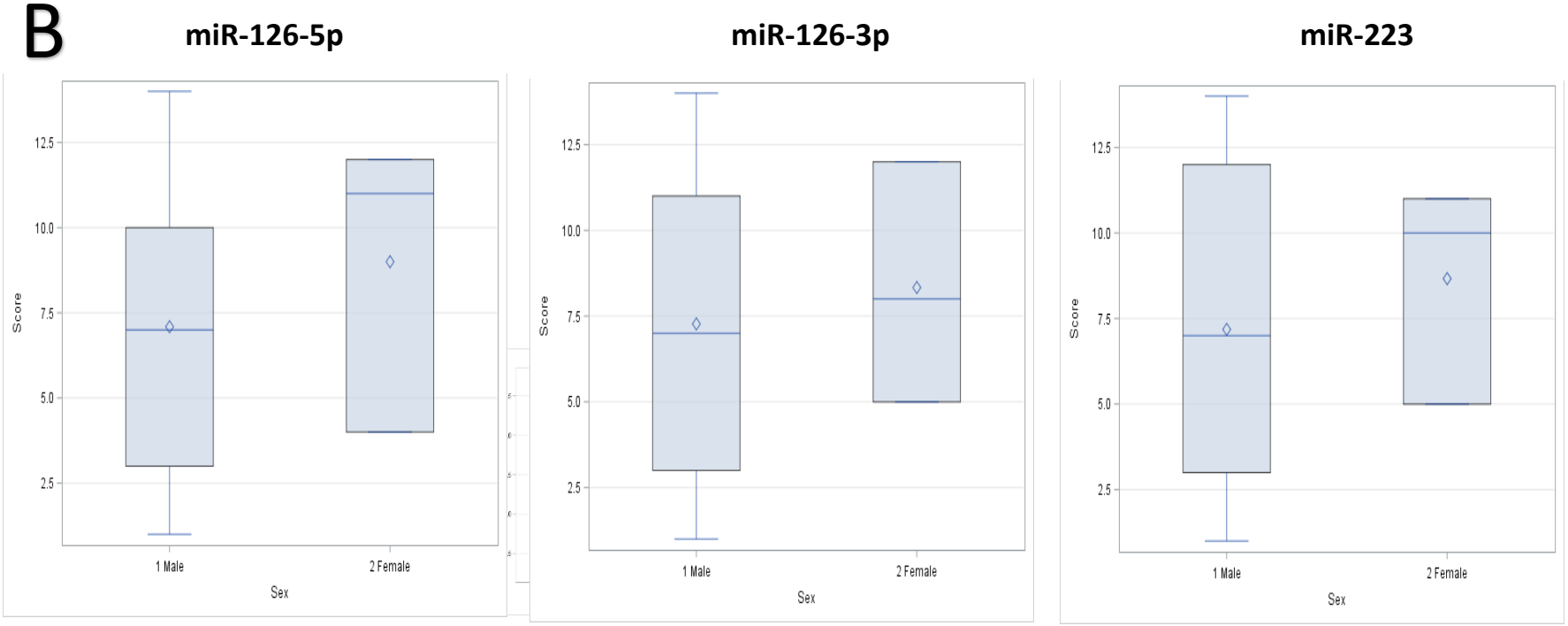


Figure 2 (continued). Distribution of Wilcoxon scores of baseline miRs by each characteristic including race, sex, open heart surgery, MSIMI, diabetes, antidepressants, and beta blockers.

Figure (C) open-heart surgeries, no and yes.

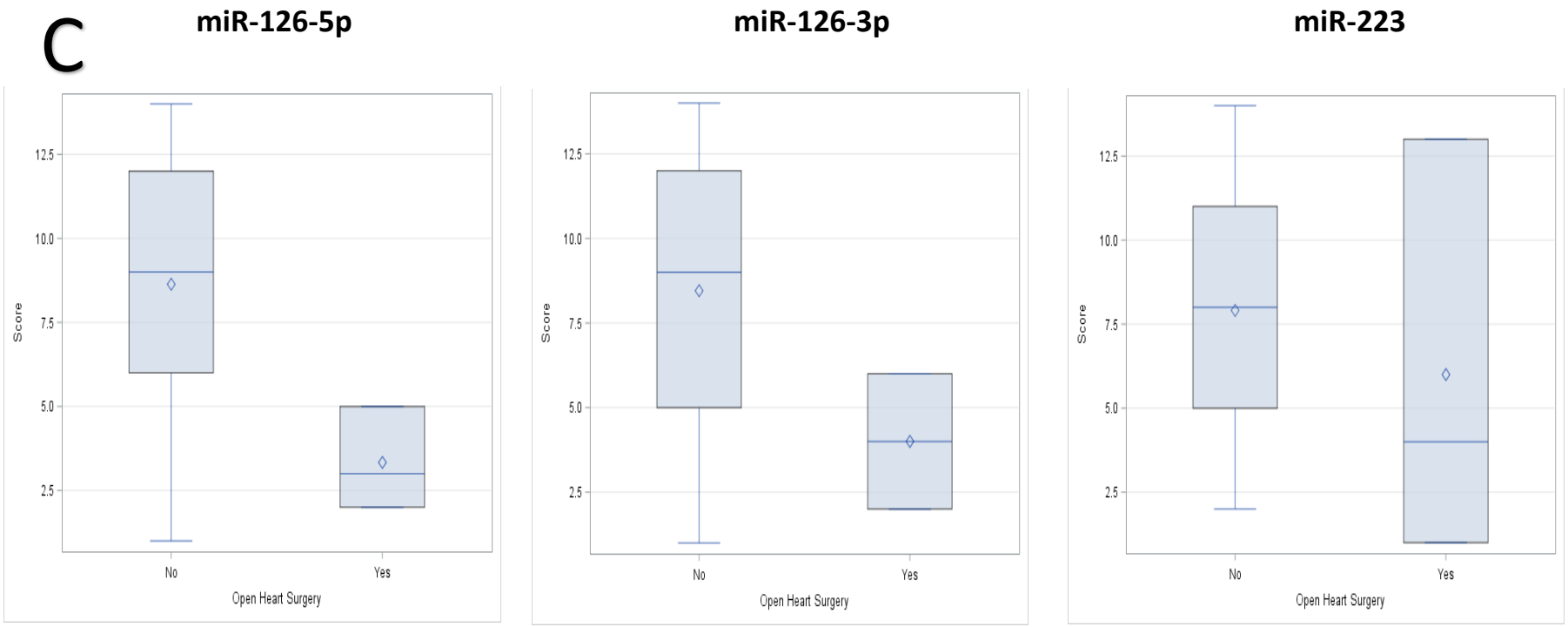


Figure 2 (continued). Distribution of Wilcoxon scores of baseline miRs by each characteristic including race, sex, open heart surgery, MSIMI, diabetes, antidepressants, and beta blockers.

Figure (D) mental stress-induced myocardial ischemia, yes and no.

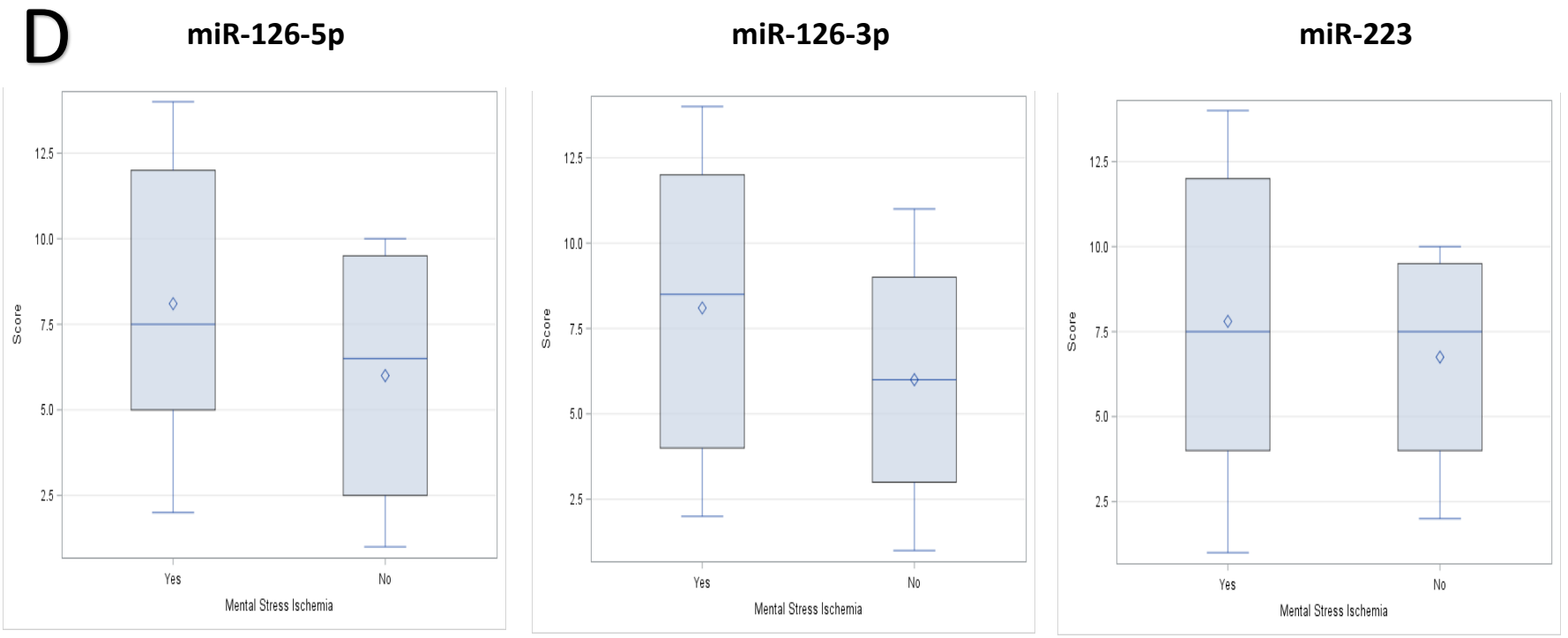


Figure 2 (continued). Distribution of Wilcoxon scores of baseline miRs by each characteristic including race, sex, open heart surgery, MSIMI, diabetes, antidepressants, and beta blockers.

Figure (E) diabetes, yes and no.

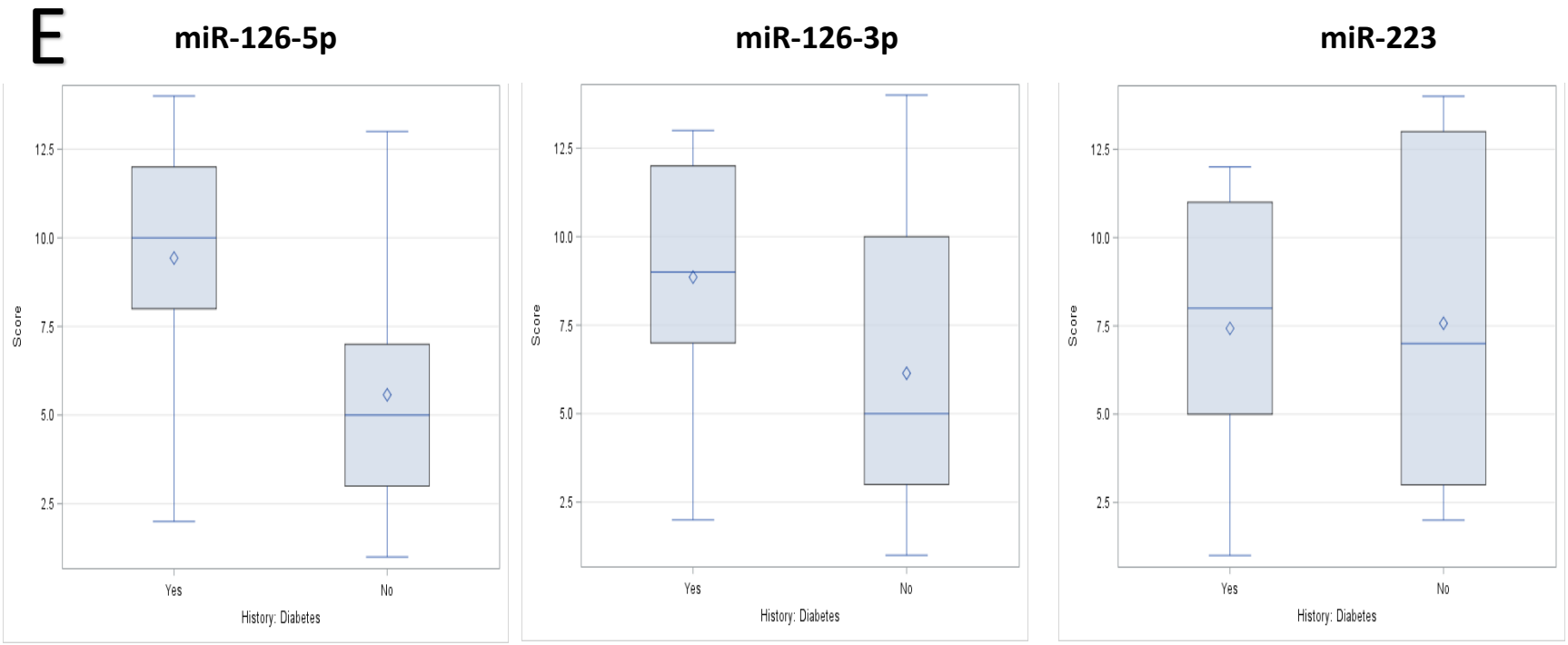
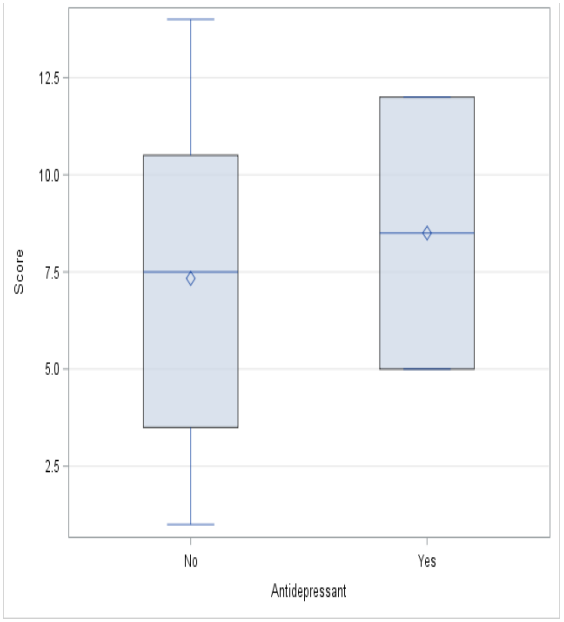


Figure 2 (continued). Distribution of Wilcoxon scores of baseline miRs by each characteristic including race, sex, open heart surgery, MSIMI, diabetes, antidepressants, and beta blockers.

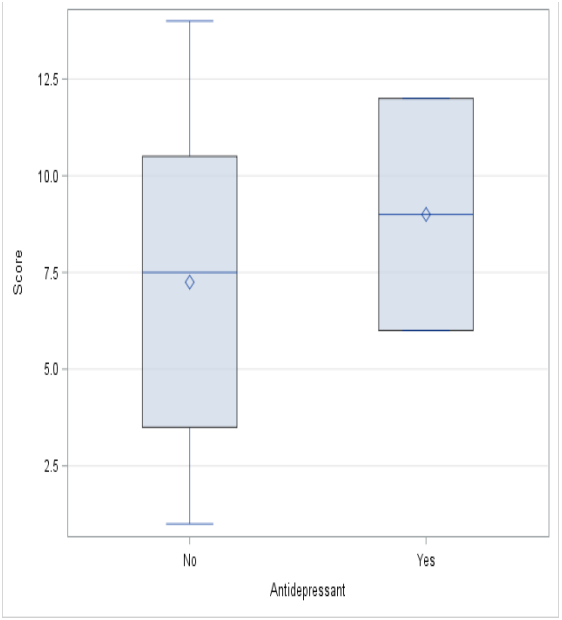
Figure (F) antidepressants, no and yes.

F

miR-126-5p



miR-126-3p



miR-223

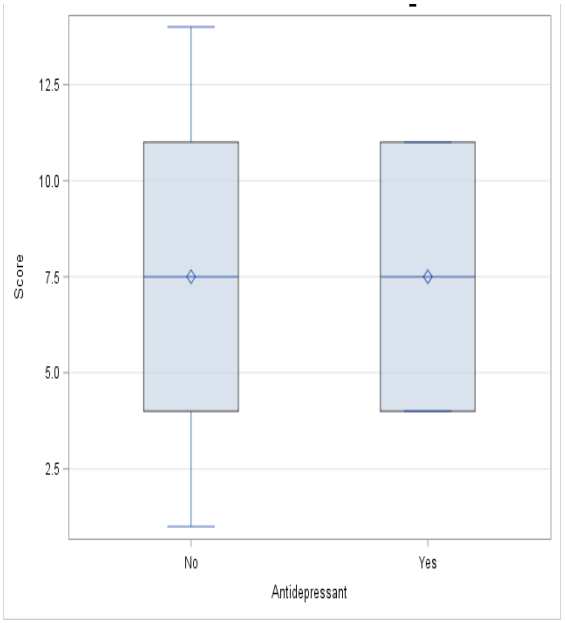


Figure 2 (continued). Distribution of Wilcoxon scores of baseline miRs by each characteristic including race, sex, open heart surgery, MSIMI, diabetes, antidepressants, and beta blockers.

Figure (G) beta blockers, yes and no.

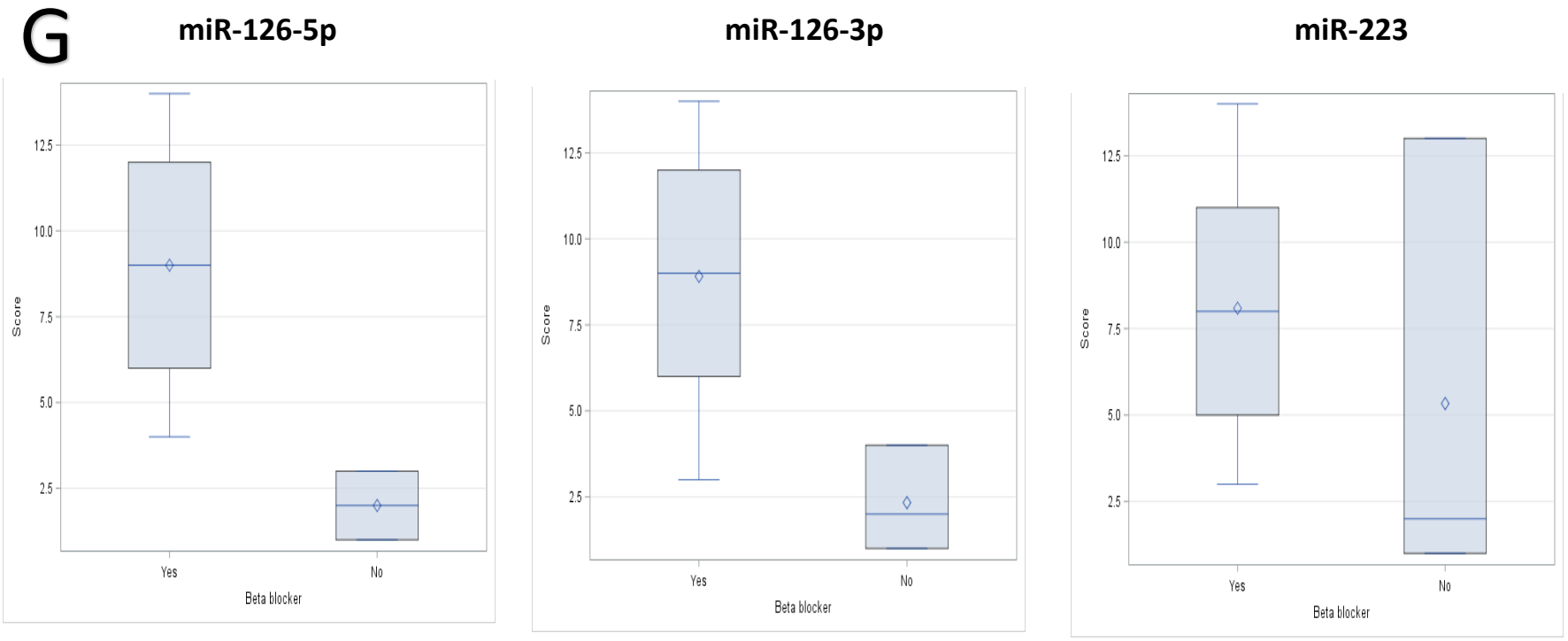


Figure S1. Distribution of relative expression ($2^{-\Delta\Delta CT}*10^{-4}$) of miRs at baseline and at 60 minutes (compared to baseline) by study visit and intervention.

Abbreviation: miR, microRNA.

Figure A-B, values in each bar graph from left to right: visit 1, visit 2, and visit 3. Blue bar represents HRVB and red bar represents wait-list control. (A) baseline miRs; (B) stress-induced changes of miRs at 60 minutes.

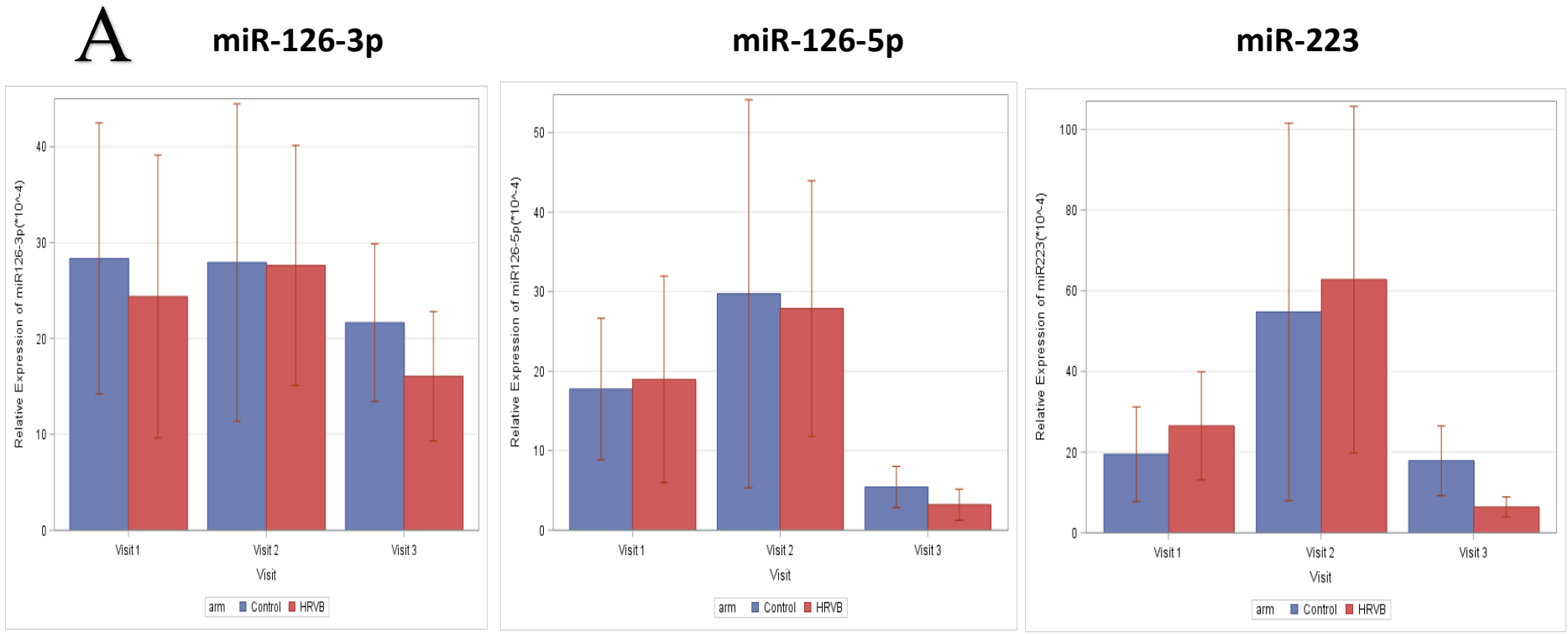


Figure S1 (continued). Distribution of relative expression ($2^{-\Delta\Delta CT}*10^{-4}$) of miRs at baseline and at 60 minutes (compared to baseline) by intervention and study visit. miR, microRNA. Figure (B) stress-induced changes of miRs at 60 minutes.

B