ORIGINAL RESEARCH

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Circulating microRNA Profiling Needs Further Refinement Before Clinical Use in Patients With Aortic Stenosis

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Background—Aortic stenosis (AS) is a progressive condition leading to heart failure and death without treatment. No medical therapy currently exists for AS, and a major management challenge is deciding on the correct timing of aortic valve replacement. MicroRNAs (miRNAs) are short noncoding RNAs that are stable in the circulation. We wished to use miRNAs as biomarkers of disease in AS.

Methods and Results—We performed microarray-based whole miRNome profiling of 24 participants with AS and 27 control participants. After adjustment for age and multiple testing, we identified 4 miRNAs significantly different between groups. These findings were then examined using quantitative polymerase chain reaction in a larger validation cohort of 101 controls and 94 participants with AS, stratified in a prespecified analysis by presence of coexisting coronary artery disease (CAD). We obtained mixed results for miR-22-3p, miR-24-3p, miR-382-5p, and miR-451a in the validation cohort, with differing associations according to CAD status. miR-21-5p was increased in AS patients without CAD, but there was no difference between groups with CAD.

Conclusion—Despite holding great promise, circulating miRNA profiling requires further refinement before translation into clinical use as a biomarker in aortic stenosis. **(*J Am Heart Assoc.* 2015;4:e002150 doi: 10.1161/JAHA.115.002150)**

Key Words: aortic valve stenosis • biomarker • microRNA

Calcific aortic valve disease (CAVD) is a spectrum of disease that ranges from hemodynamically insignificant aortic sclerosis to end-stage aortic stenosis (AS) requiring aortic valve replacement (AVR).[1] It is highly associated with older age,[2] and so the aging of the population in high-income countries has led to projections of increasing prevalence and mortality burden over the next few decades.[3,4] Though there are strong similarities to coronary atherosclerosis,[5] and a large proportion of patients with CAVD have concomitant coronary artery disease (CAD),[6] no medical therapy has yet been developed to slow the progression of CAVD.[7,8] The mainstay of management is long-term follow-up of patients until the disease becomes severe enough that AVR is indicated.

However, whereas the initial diagnosis of CAVD by clinical examination or echocardiography is, in general, straightforward, assessment of severity is often difficult. European and American guidelines rely on a combination of symptomatic and imaging features, such as aortic valve area or transvalvular pressure gradients, as indications for surgery, but circulating biomarkers have not yet been shown to provide sufficient incremental value in risk stratification to be incorporated into these guidelines.[9,10] The most investigated of these, brain natriuretic peptide, is unlikely to be a useful biomarker owing to its strong association with left ventricular (LV) systolic function (LVSF).[11] An ideal biomarker would facilitate detection of patients before decompensation, rather than after. For example, identification of myocardial fibrosis is a major target of imaging-based techniques,[12] but the diffuse nature of fibrosis observed in AS, and the currently relatively limited availability of such advanced imaging techniques, makes this challenging.

MicroRNAs (miRNAs) are small, noncoding RNAs that act to inhibit translation of mRNA into proteins and are involved in many cardiovascular (CV) diseases (CVDs).[13] Many miRNAs are detectable in the circulation, allowing their potential use

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as circulating disease biomarkers.[14] The first step toward the use of any circulating biomarker is to demonstrate a difference between cases and controls, and this has been performed in AS with specific miRNAs, such as miR-21,[15] miR-29c,[16] miR-133,[17,18] miR-1 and miR-378,[18] miR-210 and miR-22,[19] and miR-122.[20] However, this approach of selecting specific miRNAs based on previous information potentially misses important miRNAs. We therefore performed whole miRNome profiling, followed by validation of significant miRNAs, in an effort to translate 1 or more miRNAs into biomarkers for use in CAVD. Given the established link between obstructive coronary artery disease (CAD) and altered circulating miRNA profile,[14] we prespecified separate validation of our whole miRNome findings in patients with and without CAD.

Methods

Participant Selection

We enrolled participants with tricuspid aortic valves and moderate-to-severe AS, based on clinically indicated transthoracic echocardiographic findings and European Association of Echocardiography/American Society of Echocardiography recommendations for assessment of valve stenosis,[21] including any of: maximum aortic velocity ≥3.0 m/s; peak gradient ≥35 mm Hg; mean gradient ≥20 mm Hg; or calculated aortic valve area ≤1.5 cm^2. In addition, subjects were required to have normal LVSF, defined as an echocardiographic biplane ejection fraction of 50% or higher, and stable clinical features. Participants were identified through electronic search of the echocardiographic database in Dunedin Hospital, which provides secondary and tertiary cardiology and cardiac surgery services. Echocardiograms and electronic health records were reviewed by one of the investigating physicians, a European Association of Cardiovascular Imaging accredited echocardiographer, to ensure that the potential participant met the criteria. All procedures were approved by New Zealand Lower South and Multi-region ethics committees and in accord with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all participants for being included in the study.

We excluded participants with bicuspid aortic valve (BAV) owing to the additional complexities of the different BAV phenotypes and excluded patients with other congenital cardiac abnormalities or other significant structural heart diseases, such as valvular regurgitation of more than mild severity. Subjects with a past history of rheumatic heart disease or current major systemic disease (such as advanced chronic kidney disease) were also excluded. A recruitment flow chart for AS participants along with reasons for exclusion is shown in Figure S1.

Control plasma samples were obtained from 2 sources. The first was a control cohort recruited from the community by newspaper and poster advertising. They were aged predominantly 60 years and older without a history of significant CVD and in good general health—this cohort was used for both the discovery and validation phase of the study. An assumption of ≈1.3% to 2.8% population prevalence with significant AS was made,[22] and the inference drawn that such a low rate of potential false negatives would have minimal effect on the study result. Because we wished to examine the effect of CAD on our analysis, the second group consisted of randomly selected participants undergoing coronary angiography, who were enrolled in hospital preprocedurally. Clinically significant CAD was diagnosed based on either a history of previous coronary disease or a coronary revascularisation procedure or angiographic demonstration of luminal obstruction greater than 50%. A number of the participants recruited as controls (9 of 110 controls) were found to have AS on echocardiography—these were analyzed in the AS group. Lipid profiles obtained routinely were recorded for both groups.

miRNA Microarray Analysis

EDTA blood samples were centrifuged within 1 hour of collection at 3000 revolutions per minute for 8 minutes and frozen for batch processing. For those undergoing angiography, samples were obtained before administration of heparin. Total RNA, including miRNA, was extracted using miRNA purification kits (Norgen Biotek, Thorold, Ontario, Canada), according to the manufacturer's instructions. miRNA was analyzed using Affymetrix miRNA v2.0 GeneChips (Affymetrix, Santa Clara, CA). The array platform was used according to manufacturer instructions and run in an accredited service laboratory (Otago Genomics & Bioinformatics Facility, University of Otago, Dunedin, New Zealand). Array data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2785.

Quantitative Polymerase Chain Reaction (qPCR) Analysis

We examined differentially expressed miRNAs using quantitative polymerase chain reaction (qPCR). For qPCR analysis, extracted RNA was initially reverse transcribed to cDNA using the qScript microRNA cDNA Synthesis Kit (Quanta Biosciences Inc, Gaithersburg, MD). SYBR Green PCR probes (Quanta Biosciences) were used for qPCR confirmation of specific miRNAs. Any probe assay with a cycle threshold (C_T) over 35 was defined as not detected. Two normalization controls were used: miR-16-5p (based on previous use as a normalization control) and miR-151-5p (based on the

Normfinder stability score of microarray data).[23,24] Two separate runs were performed (miR-22-3p and miR-24-3p in the first run, and miR-382-3p, miR-451a, and miR-21-5p in the second), with both normalization controls measured in each run. There was excellent intrasample concordance in levels of normalization controls between runs (Figure S2).

Statistical Analysis

Demographic and clinical variables were compared between groups using the *t* test for continuous variables and the chi-square test or Fisher's exact test for categorical variables. Microarray results were normalized using Robust multiarray averaging. Normalized values were loaded into Qlucore Omics Explorer (version 2.3; Qlucore AB, Lund, Sweden) and analyzed using principal components analysis (PCA) and unsupervised hierarchical clustering. The raw *P* value was adjusted for multiple testing using the Benjamini-Hochberg method. Age was adjusted for using linear regression with Qlucore Omic Explorer's built-in factor elimination.

Statistical analysis of qPCR results was conducted using Stata/SE (v12.1; StataCorp LP, College Station, TX), with $P<0.05$ considered to be significant. Expression levels were normalized to the mean of the normalization controls using the delta C_T method.[25] Because qPCR distributions were not normally distributed, we used nonparametric tests (for continuous variables: Mann–Whitney U test; for categorical variables: Fisher's exact test). Spearman's correlation was used to examine associations with echocardiographic measures in participants with AS and with age in controls. To show the main body of data without log transformation, figures with qPCR data had extreme outliers removed, but all qPCR data were used for statistical testing. Given previously demonstrated differences in miRNA levels in different age groups,[26] we used Spearman's rank-correlation coefficient to look for statistically significant associations between age and circulating levels of these miRNAs in control participants.

Results

Demographics of Participants in Microarray Analysis

The plasma miRNA profile of 24 participants with AS was compared to 27 healthy elderly controls using microarray. Despite attempts to use an elderly control group, the AS participants were still significantly older (mean age, 77.9 years in AS group compared to 67.0 years in controls; *t* test, $P<0.001$). There was no significant gender difference between groups (37% female in AS group compared to 50% female in control group; Fisher's exact $P=0.40$). Participants with AS had severe disease, with mean aortic valve maximum velocity of 4.2 m/s (SD, 0.6 m/s), mean pressure gradient 46 mm Hg (SD, 13 mm Hg), and calculated aortic valve area 0.8 cm^2 (SD, 0.3 cm^2).

Microarray Results

In EDTA plasma samples, 16 miRNAs were significantly differentially expressed between the AS and control groups (adjusted $P<0.05$). PCA and hierarchical clustering showed partial clustering according to disease status using these miRNAs (Figure 1). Two upregulated miRNAs, miR-451a and

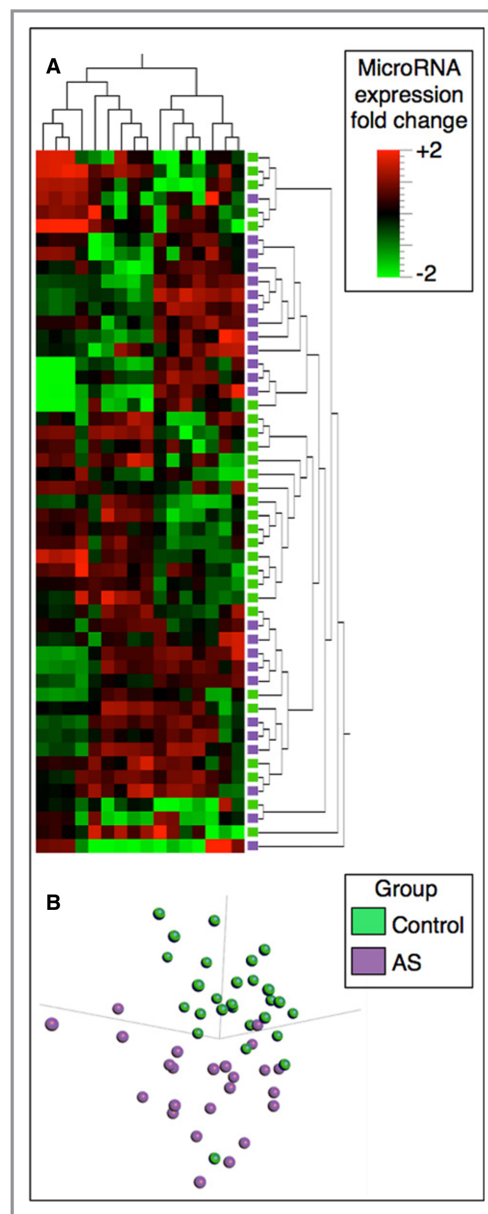

Figure 1. Plasma microarray analysis using microRNAs differentially expressed at adjusted $P<0.05$ showed some, but not complete, clustering on heatmap (A) and principal components analysis (B). AS indicates aortic stenosis.

Table 1. Differentially Expressed microRNAs in Plasma of Participants With Aortic Stenosis Compared to Control Participants (Adjusted *P*<0.05, After Adjustment for Age and Multiple Testing)

microRNA	*P* Value	Adjusted *P* Value	Fold Change
Upregulated in aortic stenosis			
miR-451a	<0.0001	0.004	1.94
miR-22-3p	0.0002	0.011	1.38
Downregulated in aortic stenosis			
miR-24-3p	0.0011	0.037	0.84
miR-382-5p	0.0003	0.014	0.38

miR indicates microRNA.

miR-22-3p, and 2 downregulated miRNAs, miR-24-3p and miR-382-5p remained significantly different after adjusting for age, leading to incomplete clustering according to disease status (Table 1 and Figure 2).

Demographics of Participants in qPCR Validation

After quality control, plasma samples from a total of 195 participants were used for validation qPCR analysis, consisting of 101 controls and 94 with AS. Those with AS were older and had a greater proportion of CV comorbidities, including higher body mass index, diabetes, and hypertension (Table 2). Participants with AS had moderate (n=60) or severe (n=34) disease, with mean aortic valve maximum velocity of 3.7 m/s (SD, 0.7 m/s), mean pressure gradient 35 mm Hg (SD, 13 mm Hg), and calculated aortic valve area 1.0 cm^2 (SD, 0.3 cm^2).

qPCR Validation

The 4 differentially expressed miRNAs on microarray analysis and miR-21-5p, chosen based on previous research findings, were then examined in plasma using qPCR, stratified according to the presence or absence of significant CAD (Figure 3). In participants with no CAD, miR-22-3p was reduced in those with AS, compared to controls (*P*=0.02), but there was no difference in miR-24-3p, miR-382-3p, or miR-451a levels between groups (*P*=0.83, 0.55, and 0.06 respectively). As expected from previous literature, miR-21-5p was increased in those with AS (*P*=0.002). In participants with CAD, both miR-22-3p and miR-24-3p were increased in those with AS (*P*=0.04 and 0.007, respectively), whereas miR-382-3p was reduced (*P*=0.04). There were no differences in levels of miR-451a and miR-21-5p between groups (*P*=0.82 and 0.30, respectively).

When compared with echocardiographic measures of disease in participants with AS, miR-21-5p and miR-382-5p levels showed a statistically significant correlation with maximum transvalvular velocity (Spearman's rho=0.35, *P*=0.0005, and $\rho=-0.23$, *P*=0.02, respectively; Figure S3) and mean gradient ($\rho=0.32$, *P*=0.002, and $\rho=-0.31$, *P*=0.003), but not LV mass index ($\rho=0.14$, *P*=0.19, and $\rho=0.05$, *P*=0.67). miR-22-3p, miR-24-3p, and miR-451a did not have any statistically significant correlation with these measures.

In summary, compared to microarray findings, only miR-22-3p and miR-382-5p had the expected results on qPCR, and these were observed only in those with CAD. miR-21-5p levels were higher in those with AS without CAD, but showed no difference between groups in those with CAD. Only miR-21-5p and miR-382-5p levels correlated weakly with measures of disease severity.

Association Between Age and Circulating miRNA Levels

No statistically significant association was observed between age and any of the circulating miRNAs examined by qPCR in

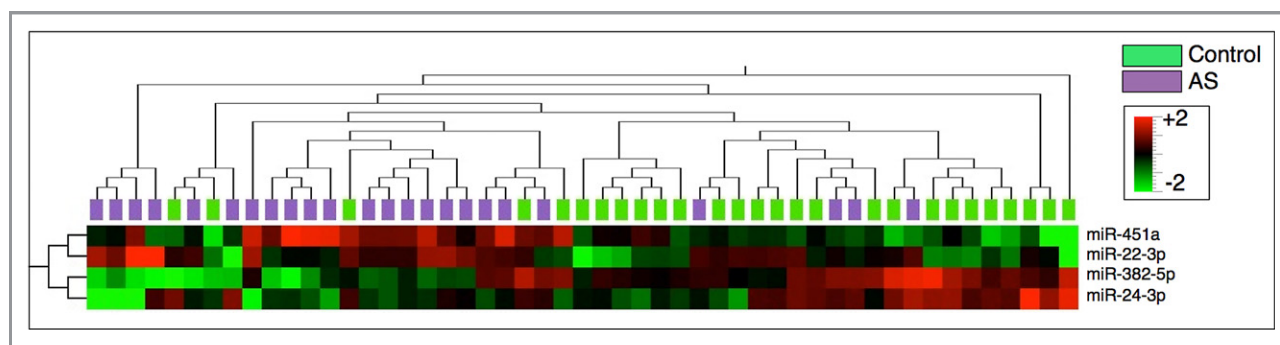

Figure 2. Heatmap of differentially expressed (adjusted *P*<0.05, after age adjustment) microRNAs in plasma of participants with and without AS, showing some, but not complete, clustering according to disease status. AS indicates aortic stenosis; miR, microRNA.

Table 2. Demographics and Baseline Characteristics of Participants in Plasma Polymerase Chain Reaction Analysis

	Controls	Aortic Stenosis	*P* Value
n	101	94	
Age, mean (sd)	72.4 (7.1)	78.8 (7.5)	<0.001
Percent female (%)	12 (12)	47 (50)	<0.001
Body mass index (sd)	26.4 (4.2)	28.2 (4.9)	0.005
History (%)			
Diabetes mellitus	14 (14)	21 (22)	0.14
Dyslipidaemia	39 (39)	50 (53)	0.045
Hypertension	42 (42)	62 (66)	0.001
Coronary artery disease	31 (31)	37 (39)	0.23
Medications (%)			
Statins	39 (39)	59 (63)	0.001
ACE inhibitors	29 (29)	41 (44)	0.037
Angiotensin receptor blockers	4 (4)	10 (11)	0.10
Aldosterone antagonists	3 (3)	3 (3)	1.0
Aspirin	38 (38)	53 (56)	0.01
Beta-blockers	28 (28)	37 (39)	0.10
Calcium-channel blockers	12 (12)	27 (29)	0.004
Fibrates	1 (1)	2 (2)	0.61
Nitrates	18 (18)	13 (14)	0.56
Warfarin	0 (0)	3 (3)	0.11
Laboratory findings mean (sd)			
Total cholesterol, mmol/L	4.9 (1.0)	4.6 (1.0)	0.09
Low-density lipoprotein, mmol/L	2.9 (0.9)	2.4 (0.7)	<0.001
High-density lipoprotein, mmol/L	1.4 (0.4)	1.5 (0.5)	0.02
Triglycerides, mmol/L	1.3 (0.6)	1.4 (0.9)	0.50

Statistical tests were performed using the *t* test for continuous variables and the chi-square test or Fisher's exact test for categorical variables. ACE indicates angiotensin-converting enzyme.

participants without aortic stenosis (n=101; age range, 54 to 89 years; Figure 4; Table S1).

Discussion

In this study, we have shown that circulating whole miRNome profiles discriminate, albeit incompletely, between participants with AS and those without. Age-adjusted analysis identified 4 miRNAs that were differentially expressed between the 2 groups. However, on attempted validation according to CAD status, a mixed picture emerged. Only miR-22-3p and miR-382-5p had levels expected from microarray analysis. Even these 2 miRNAs had different results according to CAD status, with miR-22-3p being increased in AS participants with CAD, but decreased in those without.

Previous studies have looked at specific circulating miRNAs in AS, but only 1 examined those we identified as suitable for qPCR validation.[19] No difference was found in miR-22-3p between groups in that study.[19] This is in contrast to our study, but it should be noted that 67% of the Røsjø et al. AS group had CAD, and their control group was relatively small. In addition to those miRNAs identified by microarray, we also examined circulating levels of miR-21-5p, based on previous research showing that circulating miR-21-5p is increased in patients with AS, and correlates with both myocardial miR-21-5p and degree of myocardial fibrosis.[15] Furthermore, there is strong preclinical evidence that pressure overload leads to increased myocardial miR-21-5p in both animal and human models.[27,28] Whereas we found that miR-21-5p was increased in AS participants without CAD, there was no difference between groups in those with CAD. Two other studies, using different analysis methods (qPCR using an exogenous control and a PCR array), have also found no difference in this miRNA between AS and control groups.[16,20] Therefore, despite the modest correlation with disease severity, it is unlikely that miR-21-5p will provide sufficient discrimination between heterogeneous groups of patients.

Our discrepant findings lead us to suggest that miRNAs are unlikely to be useful as biomarkers in the near future. The AS group had a number of different clinical features compared to the non-AS group, such as having more males and more participants taking statin medications (Table 2). Another possibility is that the miRNA profile changes little until ventricular decompensation occurs—all of our participants with AS had preserved ejection fraction. However, the recent study by Derda et al. showed no difference in miR-21-5p levels, despite 41% of AS participants having reduced ejection fraction.[16] It is possible that confounding factors such as these make the already noisy signal from circulating miRNA profiles harder to detect. Even within the non-AS group, there were large variations in miRNA levels assessed by qPCR (Figure 4). Alternative techniques for examining circulating miRNAs may improve the signal-to-noise ratio. These include the use of platelet-poor plasma,[29] examining Argonaute or high-density lipoprotein-bound miRNAs,[30,31] or, perhaps most promisingly, focusing on miRNAs contained within circulating microparticles.[32]

This study highlights the difficulties translating miRNAs into clinically useful biomarkers. The wide variation in levels, even among participants without significant disease, means that finding differences between groups is difficult. These findings are not exclusive to AS, with, for example, a previous review noting that miRNAs were not yet suitable for clinical use in CAD owing to, among other issues, the inherent variability in qPCR.[14] A well-recognized limitation when performing miRNA qPCR is the lack of agreed normalization controls, with

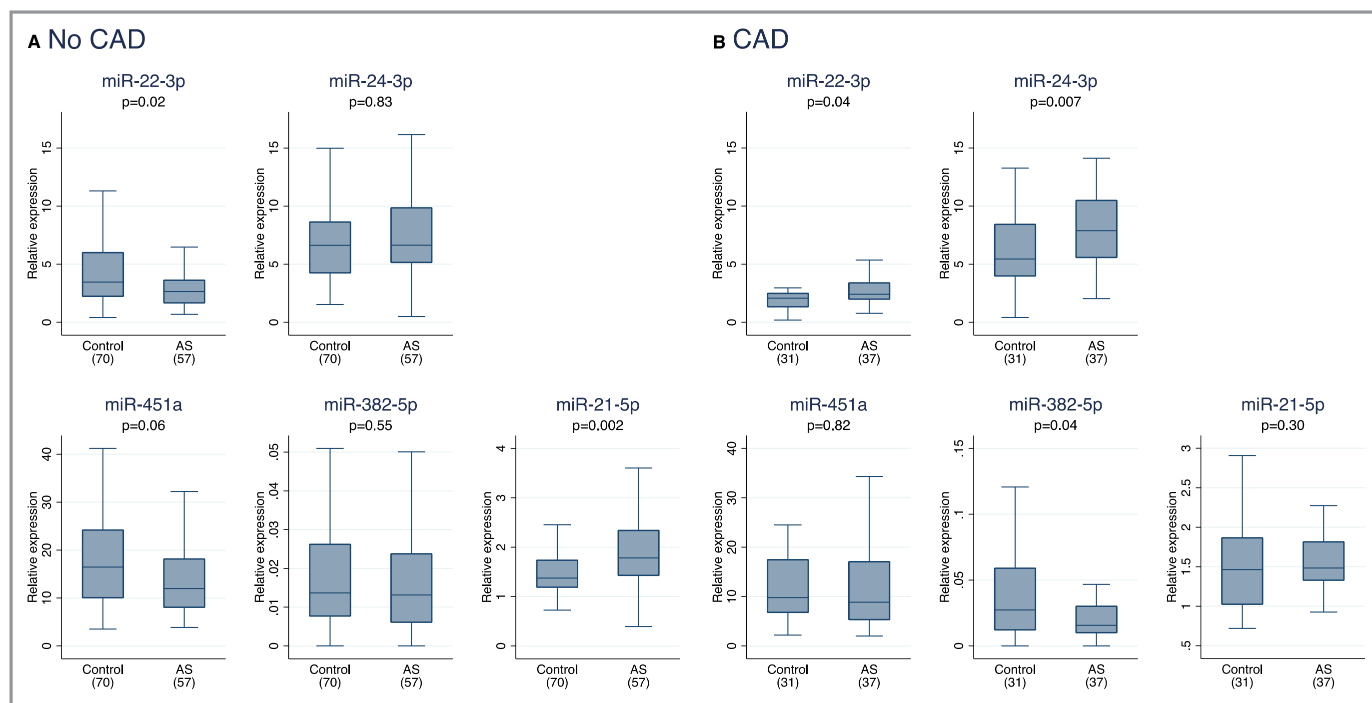

Figure 3. Plasma microRNA expression as measured by qPCR in participants (A) without and (B) with coronary artery disease. The top and bottom of the boxes indicate the 25th to 75th percentiles, respectively, and bands within boxes indicate the median of the data. Whiskers indicate the Tukey upper and lower adjacent values, that is, the highest data point within 1.5 times the interquartile range from the 75th percentile and the lowest data point within 1.5 times the interquartile range from the 25th percentile, respectively. Statistical testing was with the Mann–Whitney U test, with numbers in each group shown in parentheses. In participants without CAD, miR-22-3p was decreased, whereas miR-21-5p was increased, in the AS group. In participants with CAD, miR-22-3p and miR-24-3p were increased, whereas miR-382-3p was decreased. Other microRNA levels were similar between groups. AS indicates aortic stenosis; CAD, coronary artery disease; miR, microRNA; qPCR, quantitative polymerase chain reaction.

exogenous miRNAs, endogenous miRNAs, and endogenous RNAs of similar size all being used previously.[33] Exogenous miRNAs provide information primarily on miRNA extraction efficiency (rather than amplification) and generate relative, rather than absolute, results. The use of non-miRNA RNA (such as RNU6) as endogenous “normalizers” has also been proposed, but this marker has been shown to differ in different disease conditions, including inflammatory disease.[34] We therefore used 2 endogenous miRNAs, identified from both previous literature and our own microarray experiment, as neutrally expressed normalization markers. A different approach, using an endogenous control that most closely follows the mean intrasample expression (rather than the intersample expression assessed by NormFinder) may be a superior method.[35] We had considered using this method, but the most suitable miRNA identified, miR-103a-3p, has previously been investigated for heart failure diagnosis,[36] making its use as a normalization control contentious in this case.

Another limitation is that we were not able to enroll a group of control participants with a mean age of almost 79 years and without significant comorbidity, in part influenced by the high population prevalence of aortic sclerosis and AS in such age groups.[2,22] Circulating miRNA levels are known to alter with age, and indeed our microarray results showed a number of differentially expressed miRNAs losing statistical significance once adjusted for age. However, in those without aortic stenosis, despite a wide age range, none of those miRNAs examined by qPCR showed an association with age. Finally, as with any observational study, there is a potential for recall bias, in particular in misclassification of the major confounder, CAD.

Conclusion

In conclusion, this study identified different circulating miRNA profiles in participants with AS, compared to those without. However validation of these miRNAs gave a mixed picture. These results indicate that translating circulating miRNAs into useful prognostic biomarkers for AS will be a challenging process requiring considerable further refinement.

Sources of Funding

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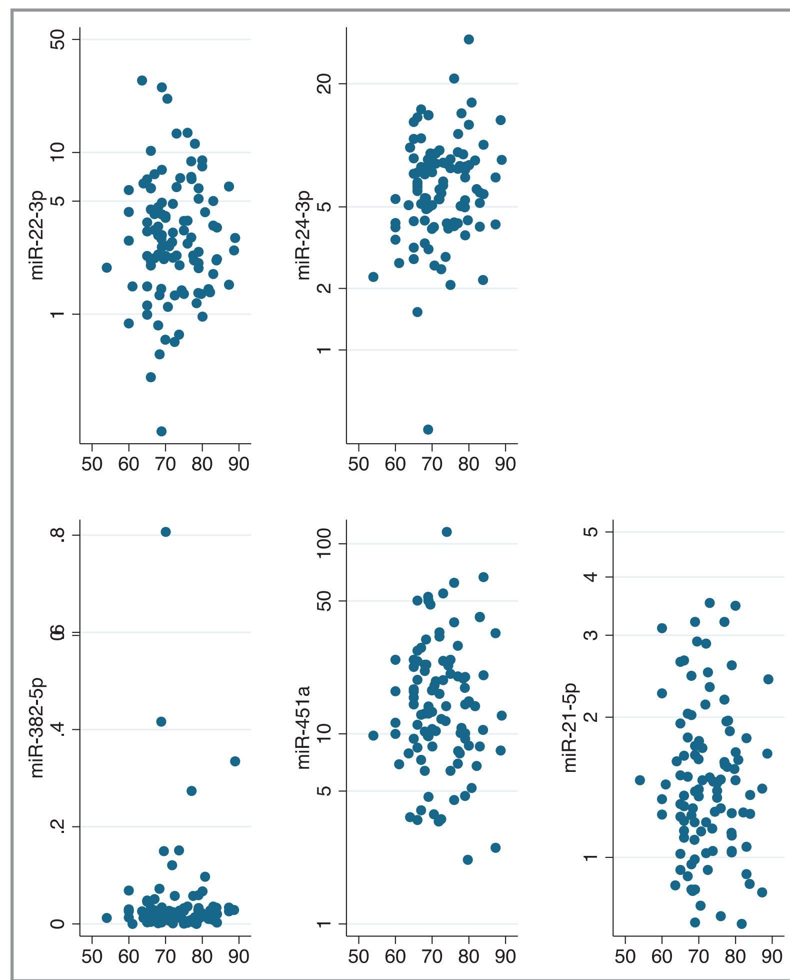

Figure 4. No association between age and circulating microRNA levels was observed in control participants (n=101). Age is shown on the *x*-axis, and expression relative to mean of the normalization controls is shown on the *y*-axis. Outside of miR-382-5p, the *y*-axis scale is log-transformed. Circulating levels were assessed using quantitative polymerase chain reaction. miR indicates microRNA.

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Disclosures

None.

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