*Clinical Study*

# MicroRNA Expression Profile in CAD Patients and the Impact of ACEI/ARB

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Coronary artery disease (CAD) is the largest killer of males and females in the United States. There is a need to develop innovative diagnostic markers for this disease. MicroRNAs (miRNAs) are a class of noncoding RNAs that posttranscriptionally regulate the expression of genes involved in important cellular processes, and we hypothesized that the miRNA expression profile would be altered in whole blood samples of patients with CAD. We performed a microarray analysis on RNA from the blood of 5 male subjects with CAD and 5 healthy subjects (mean age 53 years). Subsequently, we performed qRT-PCR analysis of miRNA expression in whole blood of another 10 patients with CAD and 15 healthy subjects. We identified 11 miRNAs that were significantly downregulated in CAD subjects ($P < .05$). Furthermore, we found an association between ACEI/ARB use and downregulation of several miRNAs that was independent of the presence of significant CAD. In conclusion, we have identified a distinct miRNA signature in whole blood that discriminates CAD patients from healthy subjects. Importantly, medication use may significantly alter miRNA expression. These findings may have significant implications for identifying and managing individuals that either have CAD or are at risk of developing the disease.

## 1. Introduction

Coronary artery disease (CAD) is a major public health problem worldwide and the single largest cause of mortality in the United States, responsible for one of every six deaths (AHA Heart Disease and Stroke Statistics, 2010). CAD is caused by atherosclerosis, which is an inflammatory disease that involves multiple cell types, including circulating cells and cells in the vessel wall [1]. Despite advances in risk factor management on an epidemiological level, many individuals continue to succumb to CAD. Various blood markers associated with increased risk for death and cardiovascular endpoints have been identified, but currently very few have been shown to have a diagnostic impact or important clinical implications that would affect patient management [2]. Therefore, there is a great need for innovative biomarkers that can assess risk for CAD, assess activity of the atherosclerotic process, and guide evaluation of therapy.

Several recent studies have suggested that circulating microRNAs could be useful as biomarkers for various human disease states, including cancer [3], acute myocardial infarction [4–7], heart failure, and chronic vascular disease [8, 9]. MicroRNAs (miRNAs) are a recently recognized class of short (19–25 nt), single-stranded, noncoding RNAs that regulate an array of cellular functions through the degradation and translational repression of mRNAs that contain complementary sequences. More than 1000 human miRNAs have been identified, and, in tissues, miRNAs regulate the expression of genes involved in critical cellular processes, including differentiation, growth, proliferation, and apoptosis [10]. Importantly, miRNAs are now regarded as rheostats that fine-tune expression of proteins involved in just about every process in human cells [11].

miRNAs have been found in tissues, whole blood, serum, plasma, and other body fluids in a stable form that is protected from endogenous RNase activity [3, 12]. Although the
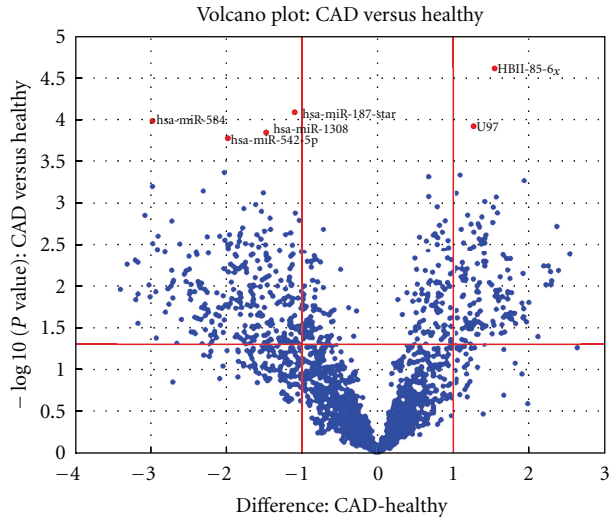
Figure 1: Volcano plot of all pairwise comparisons. Comparisons of all small RNAs assessed in microarray analysis of RNA isolated from blood of patients with CAD ($n = 5$) or healthy volunteers ($n = 5$). The volcano plot displays the relationship between fold-change and significance between the two groups, using a scatter plot view. The $y$-axis is the negative log10 of $P$ values (a higher value indicates greater significance) and the $x$-axis is the difference in expression between two experimental groups as measured in *glog2 space*. *Probes identified as significant are labeled on the plot* (FDR $t$-test <0.05). Subsequent to this analysis, miR-1308 has been removed from the Sanger database because it is a fragment of a tRNA.

biological function of miRNA is yet to be fully understood, tissue levels of specific miRNAs have been shown to correlate with pathological development of different diseases [13]. MiRNAs function as managers in gene regulatory networks, and they are distinct from other biomarkers because they have a pathogenic role in the disease process and are not merely byproducts of the disease state. This feature of miRNAs also enhances their attractiveness as therapeutic targets. Thus, miRNA expression signatures in tissues and blood have a potential role in the diagnosis, prognosis, and assessment of therapy.

In this study, we sought to compare miRNA expression in whole blood of patients with angiographically significant CAD to that of healthy aged-matched controls. We performed an initial exploratory microarray analysis in 5 cases and controls and then further examined the most highly expressed miRNAs in an additional 15 cases and controls.

## 2. Materials and Methods

### 2.1. Study Population

*2.1.1. CAD Subjects.* Study participants were recruited as part of the Emory Cardiology Biobank, consisting of 3492 consecutive patients enrolled prior to undergoing elective or emergent cardiac catheterization across three Emory Healthcare sites, between 2003 and 2008. Patients aged 20–90 years were interviewed to collect demographic characteristics, medical history, and lifestyle habits. Risk factor prevalence

was determined by physician diagnosis and/or treatment for hypertension, hyperlipidemia, and diabetes.

Coronary angiograms were evaluated independently by two operators, who made visual estimation of luminal narrowing in multiple segments based on a modified form of the AHA/ACC classification of the coronary tree. Using these data, significant CAD was defined as at least one major epicardial vessel with >50% stenosis, assessed by quantitative coronary angiography. Additionally, we collected whole blood samples from patients with ≥2 risk factors for CAD, but did not have angiographically significant CAD. The study was approved by the Institutional Review Board at Emory University, Atlanta, GA, USA. All subjects provided written informed consent at the time of enrollment.

*2.1.2. Healthy Controls.* The healthy subject cohort was a random sample of employees from Emory University who were fully employed, productive people. These subjects were aged 18 and older, not taking medications, and had not been hospitalized for at least one year prior to participation. All subjects provided written informed consent at the time of enrollment.

*2.2. RNA Isolation.* Venous blood samples were drawn via antecubial venipuncture into PAXgene Blood RNA Tubes and stored at $-20°C$ within 24 hours before RNA extraction. miRNA was isolated using the PreAnalytiX PAXgene miRNA isolation kit (Qiagen) according to the manufacturer's protocol.

*2.3. Microarray Analysis.* miRNA microarray analysis was performed by Asuragen, Inc., which uses the Affymetrix manufactured GeneChip miRNA arrays. Quality assessment of the samples was done using TaqMan assays. The microRNA targets were biotinylated using an Asuragen developed direct labeling procedure. Once labeled, the miRNA targets were hybridized overnight onto the microarrays following which the arrays were washed and stained using Streptavidin-Phycoerythrin (SAPE). Arrays were processed using the GCOS software and scanned using the Affymetrix Scanner. Expression analysis was performed for all small RNAs for Homo sapiens (e.g., miRNA, small nuclear RNAs like snoRNA and scaRNA) and separately for Sanger miRNA registry content (miRBase 11.0, http://microrna.sanger.ac.uk/) for Homo sapiens.

*2.4. miRNA qRT-PCR.* miRNA reverse transcription was performed using the TaqMan microRNA Reverse transcription Kit (Applied Biosystems) at $16°C$ for 30 min, $42°C$ for 30 min, and denaturation of the enzyme at $85°C$ for 5 min. The RT reaction was performed at $37°C$ for 1 hour followed by 5 min at $95°C$. TaqMan microRNA assays (Applied Biosystems) were performed using the 7500 Fast Real-Time PCR System at the 9600 emulation run mode. Ct values were converted into copy numbers (copy no. $= 2^{(-Ct)}$) and normalized to RNU44.

*2.5. Statistical Analysis.* Statistical analyses were performed using GraphPad Prism software. Values are expressed as

means ± SEM. Unpaired Student's *t*-tests were used to compare data. A *P* value <.05 (two sided) was considered significant. For analysis of microarray expression data, a two-sample *t*-test was carried out for every gene, followed by multiplicity correction to control the false discovery rate (FDR) at .05.

## 3. Results

We initially performed expression profiling of all small RNAs (1770 genes, Figure 1) in the whole blood samples. Three miRNAs passed the FDR cutoff of 0.05: miR-584 (7.9-fold higher in healthy versus CAD patients, $P$ = .000103, *t*-test), miR-542-5p (3.9-fold higher in healthy versus CAD pts, $P$ = .000168, *t*-test), and miR-187* (2.77-fold higher in healthy versus CAD pts, $P = 8.1 \times 10^{-5}$, *t*-test). However, signal intensities for miR-542-5p and miR-187* were very low and not detected in most samples. Separately, we performed an analysis of Sanger registry miRNAs (848 for Homo sapiens) in the whole blood samples, but none of the miRNAs passed the FDR cutoff of 0.05. Ten miRNAs passed a FDR cutoff of 0.10, but only one (miR-129-5p, 1.57 fold higher in healthy patients, $P$ = .000044) of these miRNAs had consistently detectable signal across the 10 blood samples. miR-584 was not among these 10 miRNAs. Although we were able to detect some differences in whole blood miRNA levels between healthy subjects and CAD patients (miR-584, in particular), our microarray data suggest that, similar to other reports [14], levels of miRNAs in the blood are low and microarrays may lack the sensitivity to adequately identify miRNAs that might serve as vascular disease biomarkers. Interestingly, among the miRNAs that tended to show consistent differences between healthy and CAD blood, miRNA expression was generally higher in the blood of healthy subjects, a finding previously observed by others [8].

Next, we performed qRT-PCR on RNA isolated from whole blood of another 10 patients with angiographically significant CAD and 15 healthy subjects. We evaluated the levels of miRNAs that, based on our microarray data, were highly expressed in blood and tended to have different levels of expression between the two groups. This analysis included miR-150, miR-584, miR-21, miR-24, miR-126, miR-92a, miR-34a, miR-19a, miR-145, miR-155, miR-222, miR-378, miR-29a, miR-30e-5p, miR-342, and miR-181d. Among these we found that miR-19a, miR-584, miR-155, miR-222, miR-145, miR-29a, miR-378, miR-342, miR-181d, miR-150, and miR-30e-5p were significantly downregulated in the blood of patients with CAD compared to healthy subjects (Figure 2). Furthermore, we also assessed expression of these 11 miRNAs in a cohort of patients who had ≥2 risk factors for CAD, but did not have angiographically significant CAD. We found that there was no difference in whole blood miRNA expression of this latter group compared to that in patients with significant CAD (not shown), suggesting that these miRNAs are markers for vascular inflammation rather than markers specific for significant CAD.

There has been one report that miRNA expression in blood may be influenced by medications [8]. Indeed, one
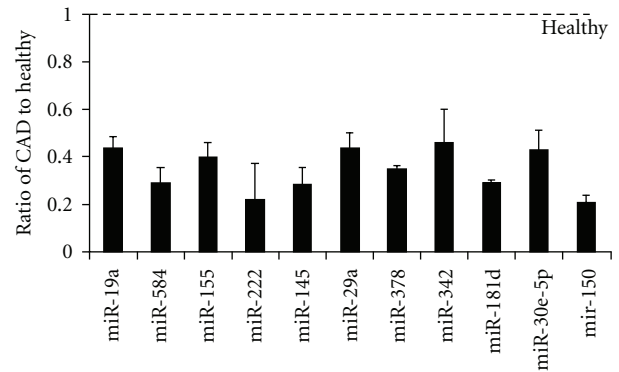


Figure 2: Identification of miRNAs that are decreased in CAD patients compared to healthy subjects. TaqMan miRNA assays were performed on RNA isolated from blood obtained from 2 different groups: patients with angiographically significant CAD ($n$ = 15) and healthy volunteers ($n$ = 5). $P$ values: miR-19a = .012, miR-584 = .036, miR-155 = .002, miR-222 = .001, miR-145 = .008, miR-29a = .012, miR-378 = .001, miR-342 = .001, miR-30e-5p = .02, and miR-150 = .006.

of the miRNAs that we found to be downregulated in the blood of patients with CAD is miR-155, which is known to target the AT1 receptor [15]. Therefore, we assessed the impact of medications on expression of miR-155, miR-145, miR-181d, miR-222, miR-19a, miR-342, miR-29a, miR-30e-5p, and miR-378. We compared the miRNA expression levels in the blood of patients with angiographically significant CAD or ≥2 risk factors for CAD but not on ACEI or ARB to that in blood of similar patients who were taking at least one of medications. Interestingly, levels of miR-155, miR-19a, miR-145, miR-222, miR-342, miR-30e-5p, and miR-378 (Figure 3) were significantly decreased in patients taking ACEI or ARB compared to those who were not, suggesting that these medications may directly modulate expression of these miRNAs, or they may influence inflammatory factors that otherwise regulate their expression. Importantly, we did not find that statin use had a significant effect on the levels of miRNAs identified in this study (not shown).

## 4. Discussion

Several recent studies have indicated that there is a potential role for circulating miRNA levels as valuable biomarkers for different disease processes, including cancer, cardiomyopathy, and acute myocardial infarction. In this study, we wanted to address the hypothesis that miRNA expression levels in blood could predict the presence of significant coronary artery disease in human subjects. We identified 11 miRNAs whose expression was significantly downregulated in patients with angiographic evidence of significant atherosclerosis compared to healthy subjects that were matched for age and gender. In addition, our data suggest that inhibition of the renin angiotensin system by ACEI or ARB use further influences miRNA expression in blood. This study confirms previous reports showing differences in circulating miRNA levels of patients with CAD compared to those of healthy subjects [8, 16]. Our study differs from the other studies in
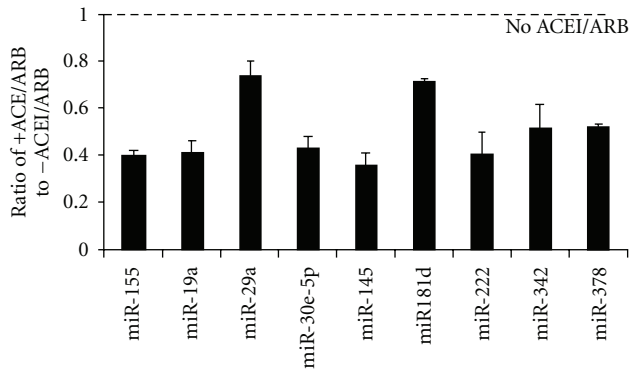
Figure 3: Medications influence the circulating levels of specific miRNAs. Expression data obtained from the patients (angiographically significant CAD or ≥2 CAD risk factors but no significant CAD lesions) on ACEI or ARB compared to similar patients not on ACEI or ARB. Circulating levels of miR-155, miR-19a, miR-378, miR-222, miR-342, miR-145, and miR-30e-5p were decreased in patients who were taking these medications. $P$ values: miR-155 = .0007, miR-19a = .035, miR-29a = NS, miR-30e-5p = .011, miR-145 = .003, miR-181d = NS, miR-222 = .002, miR-342 = .0036, and miR-378 = .0004.

the content of the group of miRNAs that were downregulated in patients with CAD.

We performed this analysis using the PAXgene Blood RNA system because it provides a way to stabilize RNA immediately after sample collection and facilitates storage of the samples for a relatively long period of time without compromising RNA integrity [17, 18]. Importantly, mRNA profiling of whole blood or peripheral mononuclear cells has been previously applied to cardiovascular disease [19, 20], and a relationship has been identified between mRNA levels in whole blood and extent of coronary artery disease. Our study extends this work by providing insight into the whole blood expression of miRNAs that are potentially involved in regulating these CAD genes.

In our qRT-PCR analysis of whole blood samples from CAD patients, we found similar changes in expression of miR-155 and miR-145 as what has been reported previously [8]. However, we failed to detect changes in other miRNAs that were described in this previous report, namely miR-126 and miR-92a. There are several possible explanations for this. First, we studied whole blood samples, whereas plasma was studied previously. Thus our miRNA profile likely reflects intracellular and extracellular miRNAs levels in contrast to exclusively extracellular miRNAs that would be detected in plasma. Another difference in our study is that we normalized miRNA expression to expression of RNU44, a small nucleolar RNA that, based on our microarray analysis, we found to be highly and consistently expressed across blood samples from CAD patients and controls.

This study confirmed the observation made by others regarding the difficulty of using microarray analysis to profile miRNA expression in blood samples [14]. Undoubtedly, this is in part due to the limitation of a relatively small sample size for our microarray analysis, as well as reduced sensitivity of the microarray method compared to qRT-PCR. In addition, principal component analysis of our data

suggested that two of the miRNA profiles for healthy subjects were actually similar to that of the CAD patients, suggesting that these individuals may in fact have subclinical vascular inflammation. This finding is in the process of being further investigated. Despite this, we believe that careful selection of patients and well-matched control subjects from a larger group of well-characterized individuals reduced some of the variability in our qRT-PCR analysis.

We can only speculate as to why expression of circulated miRNAs is generally decreased in patients with CAD. It has been hypothesized that levels of circulating miRNAs are decreased in vascular disease because they have been taken up into atherosclerotic lesions [8]. The levels of circulating miRNAs may be affected by multiple factors, including transcription, processing and stability of the miRNAs within circulating cells, as well as the ability of these cells to release miRNAs into the plasma. Circulating miRNAs may be delivered to cells in the heart or blood vessels through microvesicles, exosomes, or apoptotic bodies [21]. Because our study assessed miRNA expression in whole blood, our findings are likely more reflective of changes in miRNA transcription or processing rather than release from the circulating cells.

It remains to be determined whether downregulation of miRNAs in CAD patients is directly involved in inflammation or a compensatory response to this process. Based on our observed changes in miRNA expression in response to ACEI/ARB therapy, we believe that circulating miRNA levels reflect a compensatory response to inflammation. Further experimental studies are necessary to explore the mechanisms by which CAD and therapies affect tissue versus circulating miRNA levels.

In summary, the present study provides insight into whole blood levels of miRNAs in patients with CAD compared to healthy subjects and demonstrates their potential utility as biomarkers for vascular disease. Validation of the changes in miRNA expression observed here in larger studies will be a necessary step to confirm their candidacy as biomarkers and therapeutic targets. We believe that further elucidation of the role that these miRNAs play in the pathogenesis and progression of chronic CAD will contribute to our understanding of the disease process and lead to new therapeutic and preventative strategies.

## Acknowledgment

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