MicroRNAs in cardiac diseases: The devil is in the details
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CHAPTER 9
MIR-423-5P AS A CIRCULATING BIOMARKER FOR HEART FAILURE

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ABSTRACT

Rationale
Aberrant expression profiles of circulating microRNAs (miRNAs) have been described in various diseases and provide high sensitivity and specificity. We explored circulating miRNAs as potential biomarkers in patients with heart failure (HF).

Objective
The goal of this study was to determine whether miRNAs allow to distinguish clinical HF not only from healthy controls, but also from non-HF forms of dyspnea.

Methods and results
A miRNA array was performed in 12 healthy controls and 12 HF patients. From this array we selected 16 miRNAs for a second clinical study in 39 healthy controls, and in 50 cases with complaints of dyspnea of whom 30 were diagnosed with HF and 20 were diagnosed to have dyspnea due to non-HF causes. This revealed that miR-423-5p was specifically enriched in blood of HF-cases and receiver-operator-characteristics (ROC) curve analysis showed miR-423-5p to be a diagnostic predictor of HF, with an area under the curve (AUC) of 0.91 (p<0.001). Five other miRNAs were elevated in HF-cases, but also slightly increased in non-HF dyspnea cases.

Conclusion
We identify 6 miRNAs that are elevated in patients with HF, among which miR-423-5p is most strongly related to the clinical diagnosis of HF. These six circulating miRNAs provide attractive candidates as putative biomarkers for HF.
INTRODUCTION
Clinical management of heart failure (HF) is facilitated by circulating biomarkers like Brain Natriuretic peptide (BNP). Still there is a need for simple and reliable measurement of circulating biomarkers as objective measures of HF.

Recent studies have unveiled powerful and unexpected roles for microRNAs (miRNAs) in cardiovascular diseases, including HF. There are estimated to be more than 1000 different miRNAs, many of which are expressed in a tissue and cell-specific manner. It was discovered only recently that miRNAs are also abundantly present in blood, where they can be detected in plasma, platelets, erythrocytes as well as in nucleated blood cells. Aberrant expression profiles of miRNAs have been identified in blood of subjects with sickle cell anaemia, prostate cancer, lung cancer and myocardial injury. This led us to hypothesize that miRNA profiling can also be used for diagnostic approaches in HF.

Here we explored whether circulating miRNAs can be used as biomarkers in patients with HF. We first performed miRNA arrays on RNA isolated from plasma and selected 16 miRNAs expressed differentially in HF patients. Next, we evaluated these miRNAs in a second group of patients, consisting of 50 cases with complaints of dyspnea, of whom 30 were diagnosed with HF (HF-cases) and 20 were diagnosed to have dyspnea due to non-HF causes (non-HF-cases). One circulating miRNA in particular, miR-423-5p, was able to distinguish HF-cases from non-HF-cases. In conclusion, we demonstrate a number of miRNAs as putative biomarkers for HF, in particular miR-423-5p.

MATERIALS AND METHODS
Human plasma samples were obtained with informed consent under a general waiver by the Academic Medical Center (AMC) institutional review board for the proper secondary use of human material. For the dyspnea registry, plasma samples were obtained as part of a multicenter effort that runs in three centers in the Netherlands. Experiments described were done on samples obtained at the AMC. For detailed description of the dyspnea registry see supplemental methods.

Definition of HF diagnosis
Subjects were classified as HF-cases when they met the Framingham criteria for the diagnosis and if circulating NT-proBNP was above 1000 ng/L. Subjects were classified as non-HF-cases if clinical diagnosis excluded HF and the circulating NT-proBNP was below the age-related cutoff point published by Januzzi et al. In total 50 of the 77 patients screened for the dyspnea registry fulfilled these criteria.
Experiment I: miRNA array
12 healthy volunteers were compared to 12 patients admitted for acute HF.

Experiment II: Validation by real-time PCR
39 healthy controls and 50 subjects of the dyspnea registry were studied. Of the 50 patients, 20 subjects were diagnosed not to have HF and 30 subjects were diagnosed to have HF.

Blood processing, miRNA arrays and real-time PCR on plasma and human myocardial tissue are described in the supplemental methods.

Statistical analysis is described in detail in the supplemental methods.

RESULTS
Expression profiles of miRNAs in plasma of HF patients.
MiRNA arrays (Illumina beadchip, human v2 miRNA panel) were performed on RNA from plasma of 12 HF patients and 12 healthy controls. The baseline characteristics of this population are displayed in Table 1. 108 miRNAs were significantly differentially expressed between HF patients and controls (Supplemental table 1, which can be found on the website of circulation research). From these we selected 16 miRNAs based on their fold changes and p-values for further validation in experiment II (Figure 1).

Validation of candidate miRNAs in an independent population
In experiment II we validated the expression of 16 candidate miRNAs in three novel groups of subjects. The first group consisted of subjects from the dyspnea registry who were diagnosed with HF (HF-cases, n=30), the second group of subjects were also obtained from the dyspnea registry but clinical diagnostics established them to be free of HF (nonHF-cases, n=20), and the third group consisted of healthy controls (n=39). Baseline characteristics of these groups are displayed in Table 1. In HF-cases, 19 out of 30 subjects had an ejection fraction (EF) lower than 45%, while in nonHF-cases 3 out of 20 subjects had an EF lower than 45%. These latter three subjects therefore had left ventricular dysfunction, but lacked the clinical and BNP criteria to diagnose HF. The expression level of the miRNAs was assessed by real-time PCR, and normalized by expression levels of miR-1249, a miRNA that was found to be unchanged in the arrays. The fold changes of miRNA levels for the HF-cases versus healthy controls are shown in Figure 1. Of the 14 miRNAs that were significantly upregulated in experiment I, 7 miRNAs were confirmed to be significantly upregulated also in experiment II.

Diagnostic accuracy of candidate miRNAs
One miRNA, miR-423-5p, was found to be a significant predictor of HF diagnosis in a multivariate logistic regression model including age and sex. Figure 2 shows that miR-423-5p is specifically increased in HF-cases compared to both healthy controls and to dyspeptic nonHF-cases. MiR-423-5p distinguished HF-cases from healthy controls with an AUC of 0.91 (95%CI 0.84-0.98). The predictive power of miR-423-5p was also high within the dyspnea registry, when comparing
MIRNA-423-5p as a circulating biomarker for heart failure

HF- and nonHF-cases (AUC: 0.83, 95% CI 0.71-0.94). Circulating miR-423-5p correlated with NT-proBNP and EF (Spearman correlation coefficient 0.43, p-value 0.002 and -0.34, p-value 0.023, respectively). The AUC for each tested miRNA is shown in Supplemental table 2. Expression of miR-423-5p was increased three-fold in failing human myocardium as compared to normal human hearts (see Supplemental figure 1).

Besides miR-423-5p, six other miRNAs (miR-18b*, miR-129-5p, miR-1254, miR-675, HS_202.1 and miR-622) were found to be increased in HF-cases, of which miR-18b* and miR-675 are depicted in Figure 2. However, within the dyspnea population, miR-18b* is also slightly

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Table 1. Characteristics of the subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MiRNA array</th>
<th>Real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy controls (n=12)</td>
<td>HF patients (n=12)</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>57 (1.5) range 52-66</td>
<td>72 (3.0)* range 57-86</td>
</tr>
<tr>
<td>Sex‡</td>
<td>Men 12 (100)</td>
<td>12 (100)</td>
</tr>
<tr>
<td></td>
<td>Women 24 (61.5)</td>
<td>11 (55)</td>
</tr>
<tr>
<td>Ejection Fraction%</td>
<td>&gt; 45%</td>
<td>7 (58.3)</td>
</tr>
<tr>
<td></td>
<td>&lt; 45%</td>
<td>32.3 (3.4) range 20-41</td>
</tr>
<tr>
<td>NT-proBNP (ng/L)*</td>
<td>79.4 (6.8) range 53-193</td>
<td>98.9 (7.2) range 48-193</td>
</tr>
<tr>
<td>Creatinine (μmol/L)*</td>
<td>330</td>
<td>range 49-1691</td>
</tr>
<tr>
<td>β-blockers‡</td>
<td>4 (20.0)</td>
<td>17 (56.7)</td>
</tr>
<tr>
<td>ACE-inhibitors‡</td>
<td>5 (25)</td>
<td>19 (63.3)</td>
</tr>
<tr>
<td>Diuretics‡</td>
<td>7 (35.0)</td>
<td>20 (66.7)</td>
</tr>
<tr>
<td>Lung- medication‡</td>
<td>4 (20)</td>
<td>4 (13.3)</td>
</tr>
</tbody>
</table>

* Mean (standard error of the mean).
† Median of not normally distributed variables.
‡ N (percentages), in one case ejection fraction was missing
§ p<0.05 compared to healthy controls
|| p<0.05 compared to nonHF-cases

HF- and nonHF-cases (AUC: 0.83, 95% CI 0.71-0.94). Circulating miR-423-5p correlated with NT-proBNP and EF (Spearman correlation coefficient 0.43, p-value 0.002 and -0.34, p-value 0.023, respectively). The AUC for each tested miRNA is shown in Supplemental table 2. Expression of miR-423-5p was increased three-fold in failing human myocardium as compared to normal human hearts (see Supplemental figure 1).

Besides miR-423-5p, six other miRNAs (miR-18b*, miR-129-5p, miR-1254, miR-675, HS_202.1 and miR-622) were found to be increased in HF-cases, of which miR-18b* and miR-675 are depicted in Figure 2. However, within the dyspnea population, miR-18b* is also slightly
Figure 1. Expression profiles of 16 candidate miRNAs in plasma of HF patients and control subjects. Left two bars of each Figure show miRNA levels determined by miRNA array in 12 healthy controls and 12 HF patients. Right two bars show levels of the same miRNA now validated by real-time PCR in separate subjects (30 HF-cases and 39 healthy controls). Data are presented as mean ± SEM and *p<0.05 compared to healthy controls.
Figure 2. Diagnostic accuracy of miRNAs. A-D, G show miRNA levels in healthy controls, non-HF-cases and HF-cases. Data are shown as mean ± SEM. * p<0.05 compared to healthy controls and $p<0.05$ compared to non-HF-cases. E-E, H show ROC-curves and AUC regarding diagnostic power to distinguish HF- from non-HF-cases. F-F, I, show Spearman correlation between proBNP and miRNA, which is significant for miR-423-5p (p=0.002) and borderline significant for miR-18b* (p=0.049). Omission of the single outlier did not affect correlation of miR-423-5p but reduced that of miR-18b*.
elevated in non-HF-cases, while miR-675 is even upregulated in non-HF-cases to the same level as HF-cases. This exemplifies that some miRNAs initially identified by comparing HF-cases to healthy controls, actually appear also to be elevated when dyspnea is not caused by HF, and therefore are less specific for HF. However, it is known that also BNP can be slightly increased with pulmonary disease due to right ventricular overload so that miRNAs like miR-18b* may still be valuable biomarkers of HF.

To investigate if candidate miRNAs relate to disease severity or etiology, we categorized HF patients according to their EF, NYHA class or underlying etiology. Levels of circulating miR-423-5p and miR-18b* were higher in subjects with EF<45%, compared to subjects with EF>45%, but this did not reach statistical significance. Circulating levels of miR-423-5p, and also other miRNAs like miR-18b* increased with increasing NYHA class (see Supplemental Figures 2 and 3, for EF and NYHA class respectively). Finally, some candidate miRNAs (miR-423-5p and miR-675, but not miR-18b*) were higher in atherosclerotic forms of HF as compared to non-atherosclerotic forms of HF (see Supplemental Figure 4).

**DISCUSSION**

Recent evidence suggests that circulating miRNAs might be useful as stable blood-based biomarkers in cancer. Two cardiac miRNAs, miR-1 and miR-208 are elevated in plasma following myocardial injury, which is suggested to be caused by release of these miRNAs from damaged cardiac cells. In this study we present circulating miRNAs that are altered specifically in HF. In particular, we show that circulating levels of miR-423-5p are increased only in subjects with clinical HF, and that miR-423-5p levels are related to NT-proBNP and NYHA classification. We excluded subjects with recent cardiac ischemia or infarction, so that results are less likely to be influenced by major cardiac cell loss. Indeed, we did not find increased miR-1 and miR-208 in HF-cases. Therefore, miR-423-5p may be an attractive novel miRNA-biomarker specific for HF.

miR-423-5p has been reported in array studies to be upregulated in human failing myocardium. Here we confirm this upregulation by real-time PCR in human failing myocardium. This cardiac upregulation suggests that increased circulating miR-423-5p is derived from the myocardium. However, that is still uncertain. In this regard, other miRNAs known to be locally expressed at high levels in failing myocardium are not found in this study. It remains therefore elusive whether increased miR-423-5p is caused by increased myocardial production and subsequent release from the heart, or whether other mechanisms elevate miR-423-5p.

We explicitly chose to compare HF-cases not only to controls, but also to dyspneic patients who were free of clinical HF. This enabled us to distinguish miRNAs that are upregulated in clinical HF from miRNAs that are upregulated more in general with dyspnea. An example of this is miR-675. This miRNA seemed an attractive candidate when only comparing HF-cases to fully healthy controls, but appears to be generally upregulated in dyspnea, and not specific for HF. A second group of circulating miRNAs (miR-129-5p, miR-18b*, HS_202.1, miR-622, and miR-1254) were slightly upregulated in non-HF-cases so that the upregulation of this group of
miRNAs was only statistically significant when compared to the healthy controls and not when compared to non-HF-cases. Our results clearly show that solely comparing circulating miRNA levels in patients with HF to fully healthy controls fails to address changes induced by other causes of dyspnea. Therefore, the comparison of HF patients to subjects with non-HF dyspnea more reliably addresses the clinical challenge to distinguish underlying causes in patients with a complex and distressing complaint like dyspnea.

This study is limited by the relatively small number of patients. However, it does allow to propose circulating miRNAs that may be of clinical importance in HF, but larger studies are needed to confirm the diagnostic capacity of identified miRNAs.
REFERENCES


2. van Kimmenade RR, Pinto YM, Januzzi JL. Importance and interpretation of intermediate amino-terminal pro-B-type natriuretic peptide concentrations. Am J Cardiol 2008;101:39-42.


SUPPLEMENTAL METHODS

Dyspnea registry

Objective

Primary objective of the present registry was to develop a clinically directly applicable, easy to use scheme to stratify short-term (60-days) risk in patients presenting with dyspnea. This scheme should optimally combine the most powerful known independent prognostic markers in order to determine the individual short term outcome of patients presenting with dyspnea on the first heart aid.

Registry

All patients presenting with dyspnea were eligible for this registry. Several diagnostic and prognostic markers were measured at the first heart aid in all patients presenting with dyspnea. These markers encompass NT-proBNP, cTNT, hsCRP, Cystatin-C, hemoglobin and serum electrolytes. Informed consent was asked to store excess of serum samples (maximum of 5 ml) for future analysis of new prognostic markers for heart failure. 90-day follow-up data were collected via chart review, data from the electronic patient chart, and if necessary data from the general practitioner. All procedures according to blood storage and data collection were performed according to the guidelines of the Dutch federation of Biomedical Scientific Societies.

Patients were excluded from the current study when there were signs of a recent acute myocardial infarction, when they were known with renal insufficiency and when unwilling to provide informed consent. Blood samples were obtained within 12 hours after presentation with dyspnea, and clinical data was collected of 77 consecutive patients.

Statistical Analysis

Analysis of experiment i:

The array was analyzed using the beadarray package in the statistical software package R. Differential expression was assessed using a moderated t-test.

Analysis of experiment ii:

miRNA expression levels between cases and controls were compared using the students t-test or the Mann-Whitney-U-test. Analyses were conducted using logistic regression analysis. Receiver-operator-characteristics (ROC) curves were made across various cut-off levels of the predicted probabilities of the logistic regression model in differentiating HF-cases from nonHF-cases or healthy controls. The area under the ROC curve (AUC) was estimated to assess the diagnostic accuracy of miRNAs. All analyses were performed using SPSS and all statistical tests were two-sided. For all analyses, p-values<0.05 were considered statistically significant.

Myocardial tissue

We obtained post-mortem myocardial samples from subjects who had died due to idiopathic DCM and myocardial samples from subject who had died due to non-cardiac causes. Total RNA was extracted using Trizol (Invitrogen). RNA quality was assured and subsequent real-time PCR was used to assess expression levels of miR-423-5p. This was normalized for expression of U6 to evaluate the relative myocardial expression of miR-423-5p.
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Blood processing
Blood of all subjects was collected via a direct venous puncture into 2.7 ml sodium citrate containing tubes (BD Biosciences 363048). From the subjects for the miRNA array 10.8 ml blood was drawn and for real-time PCR we collected 5.4 ml blood.

All blood was processed for isolation of plasma within 4 hours of collection. Blood was processed by spinning at 1550 x g for 10 minutes at room temperature. Plasma was carefully transferred to a fresh RNAse/DNAse free tube and stored at -80ºC.

RNA isolation
For the miRNA array we isolated RNA of 4.4 ml plasma and for the real-time PCR we isolated RNA of 500 μl plasma. Plasma was thawed on ice and RNA was isolated using the miVana PARIS kit (Ambion) according to the manufacturer's protocol for liquid samples. The protocol was modified such that samples were extracted twice with an equal volume of acid-phenol chloroform and the column was dried for 3 minutes after the last washing step and before elution.

miRNA array
From the eluted samples for the miRNA array 50 μl was concentrated to 12 μl and from each sample 5 μl was used for the array. The Illumina human v2 miRNA expression profiling was performed on these samples. The Illumina miRNA beadchip probes 1146 miRNAs and other small RNAs predicted to be miRNAs. Raw data were pre-processed, summarized, log-transformed, and quantile normalized using the beadarray package (version 1.12.1) in the statistical software package R (version 2.9.0). Differential expression was assessed using a moderated t-test using the limma package (version 2.18.3). MicroRNAs were considered significantly differentially expressed if the p-values were less than 0.05.

Real-Time PCR
For plasma samples a fixed volume of 8 μl of the eluate from the RNA isolation and for myocardial tissue samples 1 μg of input RNA was used as input in the reverse transcription reaction. Input RNA was reverse transcribed using the miScript reverse transcription kit (Qiagen) according to the manufacturer's protocol.

The real-time PCR was performed using High Resolution Melting Master (Roche). MgCl2 was used in an end-concentration of 2.5 mmol/L and 2 μl of 8 times diluted cDNA was used in a total volume of 10 μl. The forward primers had the same sequence as the mature miRNA sequence with all Us changed into Ts and the sequence of the reverse primer was 5’-GAATCGAGCACCAGTTACGC-3’, which is complementary to the adapter sequence of the RT-primer (part of the miScript reverse transcription kit) used to create cDNA of miRNAs. Real-time PCR reactions were performed on a LightCycler480 system II (Roche) using the following program: 10 minutes pre-incubation at 95ºC and 40 cycle of 45 seconds of denaturation at 95ºC, 45 seconds of annealing at 55ºC, and 45 seconds of elongation at 72ºC.

Data were analyzed using LinRegPCR quantitative PCR data analysis software, version 11.3. The starting concentration of miRNAs estimated by this software were corrected for the estimated starting concentration of miR-1249, an endogenous miRNA stably expressed in our miRNA array.
REFERENCES


Supplemental figure 1. miRNA expression in postmortem myocardial tissue from subjects who had died due to dilated cardiomyopathy (DCM) compared to subjects who had died due to non-cardiac causes (control). Real-time PCR for miR-423-5p was normalized for expression of U6. *p<0.05 compared to controls.

Supplemental figure 2. Levels of circulating miRNAs in healthy controls compared to HF-cases with either EF<45% (n=19) or EF>45% (n=10) In one patient EF was missing. * p<0.05 compared to healthy controls.
Supplemental figure 3. Circulating levels of the 16 candidate miRNAs according to NYHA classification. It is important to note that the studied HF patients all presented to the first aid with complaints of dyspnea so that none of the subjects were in NYHA class I. We compared the subjects in NYHA class II-III to the NYHA class IV patients. Number of subjects per group controls n=39, NYHA class II-III n=23 and NYHA group IV n=5. *p<0.05 compared to NYHA class II-III. In two patients NYHA classification was missing.
Supplemental figure 4. Levels of circulating miRNAs in subjects with atherosclerotic versus non-atherosclerotic forms of heart failure. To compare levels of circulating miRNAs according to the underlying cause of heart failure, we distinguished atherosclerotic forms of HF and non-atherosclerotic forms of HF. It is important to stress here that in this study we excluded patients who showed actual signs of myocardial ischemia, so that active ischemia was excluded as a cause of HF. 12 patients out of 30 had a history of chronic extensive coronary atherosclerotic disease leading to HF. These subjects with atherosclerotic disease showed significantly higher circulating levels of miR-423-5p and miR-675, but not of miR-18b*, compared to the patients with non-atherosclerotic HF (n=18). *p<0.05 compared to non-atherosclerotic forms.
### Supplemental table 2. Diagnostic accuracy of the miRNAs.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>HF-cases compared to healthy controls</th>
<th>HF-cases compared to nonHF-cases and healthy controls</th>
<th>HF-cases compared to nonHF-cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-423-5p</td>
<td>0.91 (0.84-0.98)</td>
<td>0.87 (0.79-0.94)</td>
<td>0.83 (0.71-0.94)</td>
</tr>
<tr>
<td>miR-129-5p</td>
<td>0.90 (0.82-0.98)</td>
<td>0.80 (0.71-0.90)</td>
<td>0.67 (0.52-0.83)</td>
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<tr>
<td>HS_239</td>
<td>0.90 (0.81-0.98)</td>
<td>0.77 (0.67-0.88)</td>
<td>0.62 (0.45-0.79)</td>
</tr>
<tr>
<td>miR-675</td>
<td>0.89 (0.81-0.97)</td>
<td>0.76 (0.66-0.86)</td>
<td>0.60 (0.43-0.76)</td>
</tr>
<tr>
<td>miR-18b*</td>
<td>0.86 (0.76-0.97)</td>
<td>0.79 (0.69-0.90)</td>
<td>0.71 (0.56-0.86)</td>
</tr>
<tr>
<td>H5_202.1</td>
<td>0.86 (0.76-0.96)</td>
<td>0.78 (0.68-0.89)</td>
<td>0.63 (0.47-0.80)</td>
</tr>
<tr>
<td>solexa3927-221</td>
<td>0.85 (0.76-0.95)</td>
<td>0.73 (0.62-0.84)</td>
<td>0.74 (0.60-0.88)</td>
</tr>
<tr>
<td>miR-622</td>
<td>0.84 (0.75-0.94)</td>
<td>0.77 (0.67-0.88)</td>
<td>0.63 (0.47-0.79)</td>
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<tr>
<td>miR-302d</td>
<td>0.84 (0.73-0.94)</td>
<td>0.73 (0.62-0.85)</td>
<td>0.59 (0.43-0.75)</td>
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<tr>
<td>miR-1254</td>
<td>0.82 (0.71-0.93)</td>
<td>0.76 (0.65-0.87)</td>
<td>0.71 (0.56-0.86)</td>
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<tr>
<td>miR-1301</td>
<td>0.80 (0.69-0.92)</td>
<td>0.72 (0.60-0.84)</td>
<td>0.59 (0.43-0.75)</td>
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<tr>
<td>miR-15b</td>
<td>0.80 (0.68-0.92)</td>
<td>0.75 (0.63-0.87)</td>
<td>0.71 (0.57-0.86)</td>
</tr>
<tr>
<td>miR-346</td>
<td>0.79 (0.67-0.91)</td>
<td>0.73 (0.62-0.85)</td>
<td>0.63 (0.46-0.79)</td>
</tr>
<tr>
<td>miR-142-3p</td>
<td>0.79 (0.66-0.91)</td>
<td>0.76 (0.65-0.87)</td>
<td>0.76 (0.63-0.89)</td>
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<tr>
<td>miR-24-2*</td>
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<td>miR-654-3p</td>
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<td>0.72 (0.61-0.84)</td>
<td>0.59 (0.42-0.75)</td>
</tr>
<tr>
<td>Sex and age</td>
<td>0.78 (0.65-0.90)</td>
<td>0.72 (0.60-0.84)</td>
<td>0.58 (0.42-0.75)</td>
</tr>
</tbody>
</table>

This table shows the AUC (95% confidence interval), which assesses the diagnostic power of the miRNAs to distinguish HF- from nonHF-cases.