Brief UltraRapid Communication

MiR423-5p As a Circulating Biomarker for Heart Failure

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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 on plasma of 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 reports of dyspnea, of whom 30 were diagnosed with HF and 20 were diagnosed with dyspnea attributable to non–HF-related causes. This revealed that miR423-5p was specifically enriched in blood of HF cases and receiver-operator-characteristics (ROC) curve analysis showed miR423-5p to be a diagnostic predictor of HF, with an area under the curve 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 miR423-5p is most strongly related to the clinical diagnosis of HF. These 6 circulating miRNAs provide attractive candidates as putative biomarkers for HF. (*Circ Res*. 2010;106:1035-1039.)

Key Words: MicroRNAs ■ plasma ■ heart failure ■ biomarker

Clinical management of heart failure (HF) is facilitated by circulating biomarkers like brain natriuretic peptide (BNP).[1,2] 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.[3] It was discovered only recently that miRNAs are also abundantly present in blood, where they can be detected in plasma, platelets, and erythrocytes, as well as in nucleated blood cells.[4] Aberrant expression profiles of miRNAs have been identified in blood of subjects with sickle cell anemia, prostate cancer, lung cancer and myocardial injury.[4–6] 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 reports of dyspnea, of whom 30 were diagnosed with HF (HF cases) and 20 were diagnosed to have dyspnea attributable to non-HF causes (non-HF cases). One circulating miRNA in particular, miR423-5p, 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 miR423-5p.

Methods

Human plasma samples were obtained with informed consent under a general waiver by the Academic Medical Center 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 involving 3 centers in The Netherlands. Experiments described were performed on samples obtained at the Academic Medical Center. For a detailed description of the dyspnea registry, see the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org.

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 points published by Januzzi et al.[1] In total, 50 of the 77 patients screened for the dyspnea registry fulfilled the criteria.

Experiment I: MiRNA Array

Twelve healthy volunteers were compared to 12 patients admitted to the hospital for acute HF.

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Non-standard Abbreviations and Acronyms

AUC	area under the receiver–operator characteristic curve
BNP	brain natriuretic peptide
EF	ejection fraction
HF	heart failure
miRNA	microRNA
ROC	receiver–operator characteristic

Experiment II: Validation by Real-Time PCR

Thirty-nine 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 Online Data Supplement.

Statistical analysis is described in detail in the Online Data Supplement.

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 the Table. A total of 108 miRNAs were significantly differentially expressed between HF patients and controls (Online Table I). From these, we selected 16 miRNAs based on their fold changes and probability 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 (non-HF cases, n=20), and the third group consisted of healthy controls (n=39). Baseline characteristics of these groups are displayed in the Table. In HF cases, 19 of 30 subjects had an ejection fraction (EF) lower than 45%, whereas in non-HF cases 3 of 20 subjects had an EF lower than 45%. These latter three subjects therefore had left ventricular dysfunction, but lacked the clinical and NT-proBNP criteria to diagnose HF. The expression level of the miRNAs was assessed by real-time PCR, and normalized by expression levels of miR1249, 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, miR423-5p, was found to be a significant predictor of HF diagnosis in a multivariate logistic regression model including age and sex. Figure 2 shows that miR423-5p is specifically increased in HF cases compared to both healthy controls and to dyspneic non-HF cases. MiR423-5p distinguished HF cases from healthy controls with an area under the curve (AUC) of 0.91 (95% confidence interval, 0.84 to 0.98). The predictive power of miR423-5p was also high within the dyspnea registry, when comparing HF and non-HF cases (AUC, 0.83; 95% confidence interval, 0.71 to 0.94). Circulating miR423-5p correlated with NT-proBNP and EF (Spearman correlation coefficient: 0.43, probability value: 0.002; Spearman correlation coefficient: −0.34, probability value: 0.023, respectively). The AUC for each tested miRNA is shown in Online Table II. Expression of miR423-5p was increased three-fold in failing human myocardium as compared to normal human hearts (see Online Figure I).

Table. Characteristics of the Subjects

			Real-Time PCR		
	MiRNA Array			Dyspnea Registry	
Characteristic	Healthy Controls (n=12)	HF Patients (n=12)	Healthy Controls (n=39)	Non-HF Cases (n=20)	HF Cases (n=30)
Age, yr*	57 (1.5) (range, 52–66)	72 (3.0)§ (range, 57–86)	55.7 (0.7) (range, 50–69)	65.5 (3.7) (range 33–88)	68.2 (2.5)§ (range, 44–89)
Sex‡					
Men	12 (100)	12 (100)	15 (38.5)	9 (45)	16 (53.3)
Women			24 (61.5)	11 (55)	14 (46.7)
Ejection fraction‡					
>45%				13 (65)	10 (33.3)¶
<45%		7 (58.3)		3 (15)	19 (63.3)
Ejection fraction, %*		32.3 (3.4) (range 20–41)		55.9 (3.3) (range 25–75)	37.1 (3.2)¶ (range 14–70)
NT-proBNP, ng/L†				330 (range 49–1691)	3388¶ (range 1092–28568)
Creatinine, μmol/L*				79.4 (6.8) (range 53–193)	98.9 (7.2) (range 48–193)
β-blockers‡				4 (20.0)	17 (56.7)¶
ACE-inhibitors‡				5 (25)	19 (63.3)¶
Diuretics‡				7 (35.0)	20 (66.7)¶
Lung-medication‡				4 (20)	4 (13.3)

*Mean (SEM); †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 non-HF cases.

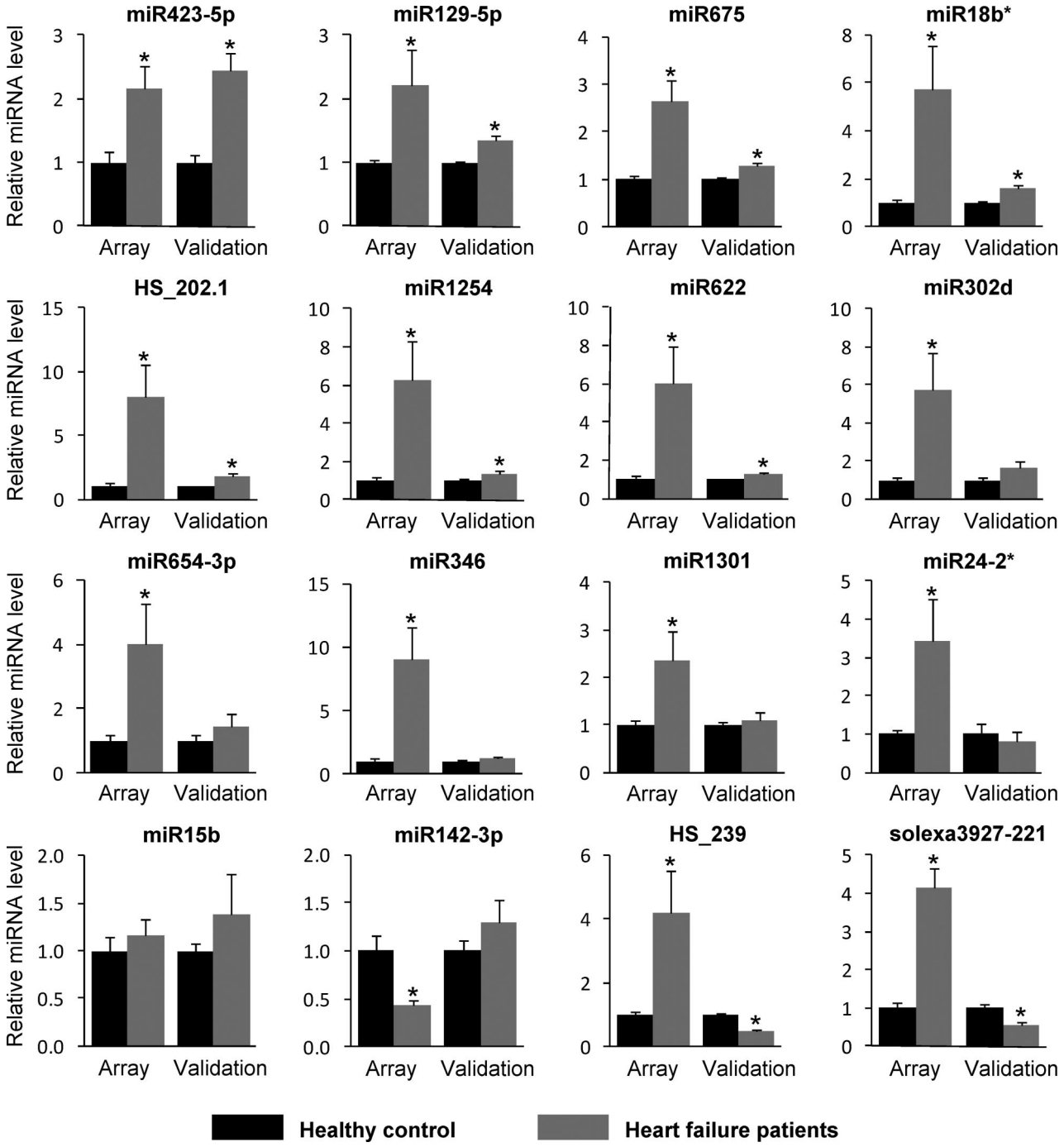

Figure 1. Expression profiles of 16 candidate miRNAs in plasma of HF patients and control subjects. Left 2 bars of each **panel** show miRNA levels determined by miRNA array in 12 healthy controls and 12 HF patients. **Right 2 bars** show levels of the same miRNA now validated by real-time PCR in separate subjects (30 HF cases and 39 healthy controls). Data are presented as means±SEM. *$P<0.05$ compared to healthy controls.

Besides miR423-5p, six other miRNAs (miR18b*, miR129–5p, miR1254, miR675, HS_202.1 and miR622) were found to be increased in HF cases, of which miR18b* and miR675 are depicted in Figure 2. However, within the dyspnea population, miR18b* is also slightly elevated in non-HF cases, whereas miR675 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 NT-proBNP can be slightly increased with pulmonary disease attributable to right ventricular overload,[2] so that miRNAs like miR18b* may still be valuable biomarkers of HF.

To investigate whether candidate miRNAs relate to disease severity or etiology, we categorized HF patients according to their EF, NYHA class or underlying etiology. Levels of circulating miR423-5p and miR18b* were higher in subjects with EF of ≤45%, compared to subjects with EF of >45%, but this

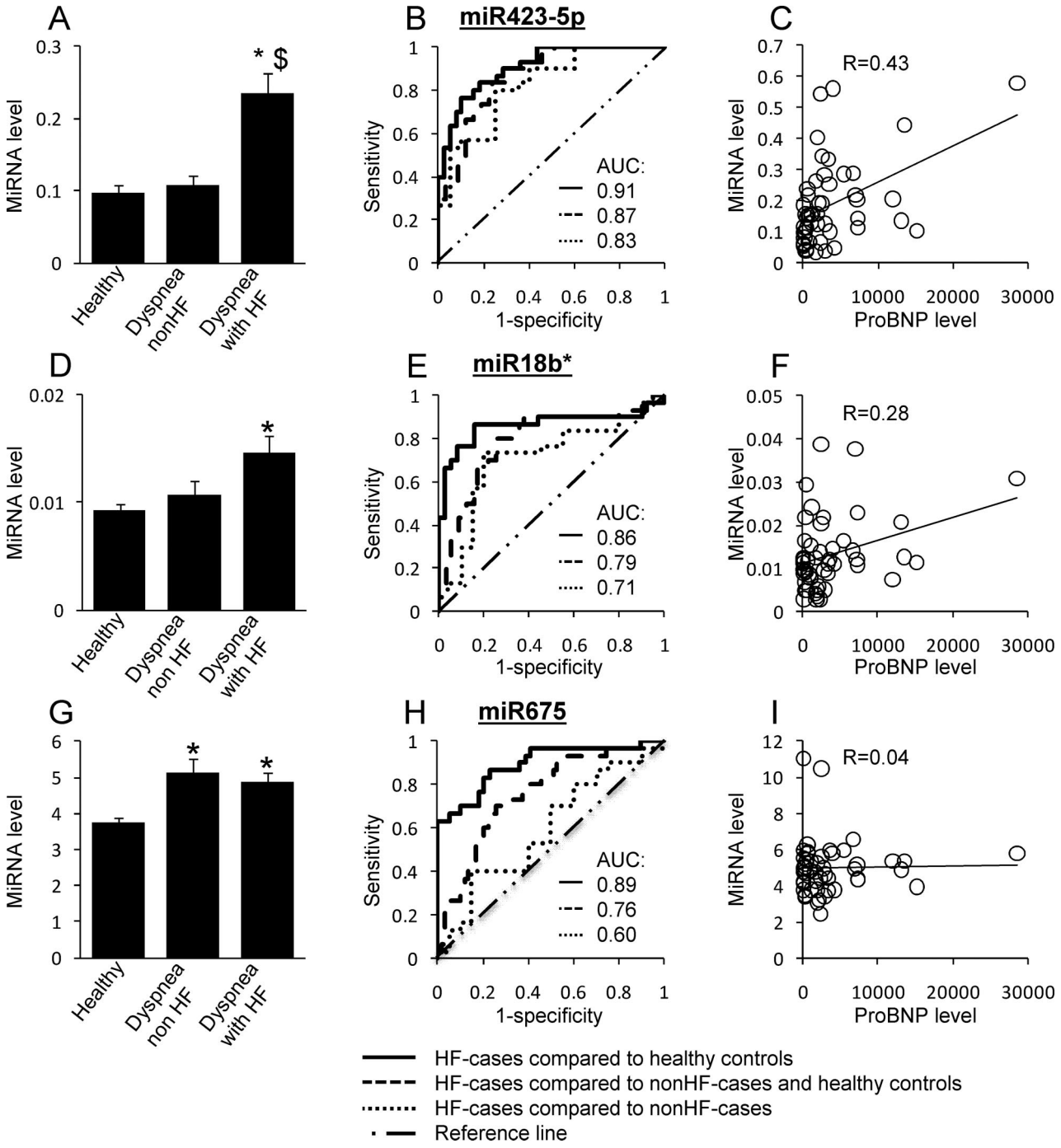

Figure 2. Diagnostic accuracy of miRNAs. A, **D**, and **G** show miRNA levels in healthy controls, non-HF cases, and HF cases. Data are shown as means±SEM. *$P<0.05$ compared to healthy controls; $: $P<0.05$ compared to non-HF cases. **B**, **E**, and **H** show ROC curves and AUC regarding diagnostic power to distinguish HF from non-HF cases. **C**, **F**, and **I** show Spearman correlation between proBNP and miRNA, which is significant for miR423-5p ($P=0.002$) and borderline significant for miR-18b* ($P=0.049$). Omission of the single outlier did not affect correlation of miR423-5p but reduced that of miR18b*.

did not reach statistical significance. Circulating levels of miR423-5p, and also other miRNAs like miR18b* increased with increasing NYHA class (see Online Figures II and III, for EF and NYHA class, respectively). Finally, some candidate miRNAs (miR423-5p and miR675, but not miR18b*) were higher in atherosclerotic forms of HF as compared to nonatherosclerotic forms of HF (see Online Figure IV).

Discussion

Recent evidence suggests that circulating miRNAs might be useful as stable blood-based biomarkers in cancer.[4] Two cardiac miRNAs, miR1 and miR208, are elevated in plasma following myocardial injury, which is suggested to be caused by release of these miRNAs from damaged cardiac cells.[5,6] In this study, we present circulating miRNAs that are altered specifically in HF.

In particular, we show that circulating levels of miR423-5p are increased only in subjects with clinical HF, and that miR423-5p 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 miR1 and miR208 in HF cases. Therefore, miR423-5p may be an attractive novel miRNA-biomarker specific for HF.

MiR423-5p has been reported in array studies to be upregulated in human failing myocardium.[7] Here we confirm this upregulation by real-time PCR in human failing myocardium. This cardiac upregulation suggests that increased circulating miR423-5p 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 miR423-5p is caused by increased myocardial production and subsequent release from the heart or whether other mechanisms elevate miR423-5p.

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 miR675. 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 (miR129–5p, miR18b*, HS_202.1, miR622, and miR1254) 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 symptom 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.

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Disclosures

A.J.T., E.E.C., and Y.M.P. filed a patent application, owned by the Academic Medical Center, that details claims related to described circulating miRNAs as biomarkers for HF.

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Novelty and Significance

What Is Known?

- Circulating biomarkers are used clinically to detect and monitor disease.
- MicroRNAs (miRNAs), important novel molecular regulators, can be measured in the circulation and are potential biomarkers.
- Measurement of circulating miRNAs provides important novel information in diseases like cancer.

What New Information Does This Article Contribute?

- A number of circulating miRNAs provide diagnostic information in patients with heart failure.
- Increased levels of miR423-5p are robustly related to heart failure diagnosis and severity.
- Newly identified miRNAs like miR423-5p and miR18b* are reliably measurable in routine blood samples and provide attractive novel biomarkers for heart failure patients.

Biomarkers allow monitoring of disease. Circulating miRNAs are emerging as novel biomarkers in various diseases. Here we have identified circulating miRNAs as biomarkers of heart failure. MiR423-5p and miR18b* were most strongly related to heart failure and its severity. We excluded myocardial infarction and compared heart failure patients not only with healthy controls but also with subjects with other causes of dyspnea, which enabled us to identify miRNAs highly specific for heart failure, avoiding miRNAs attributable to cardiac injury or general distress. This first identification is an important step toward using circulating miRNAs as novel biomarkers in these patients.