European Journal of Heart Failure (2015)
doi:10.1002/ejhf.332

Signature of circulating microRNAs in patients with acute heart failure

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Received 29 April 2015; revised 11 June 2015; accepted 11 June 2015

Aims Our aim was to identify circulating microRNAs (miRNAs) associated with acute heart failure (AHF).

Methods and results Plasma miRNA profiling included 137 patients with AHF from 3 different cohorts, 20 with chronic heart failure (CHF), 8 with acute exacerbation of COPD, and 41 healthy controls. Levels of circulating miRNAs were measured using quantitative reverse transcription–polymerase chain reaction (qRT–PCR). Plasma levels of miRNAs in patients with AHF were decreased compared with CHF patients or healthy subjects, whereas no significant changes were observed between acute COPD patients and controls. Fifteen miRNAs found in the discovery phase to differ most significantly between healthy controls and patients with AHF were further investigated in an extended cohort of 100 AHF patients at admission and a separate cohort of 18 AHF patients at different time points. Out of these 15 miRNAs, 12 could be confirmed in an additional AHF validation cohort and 7 passed the Bonferroni correction threshold (miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p, all $P < 0.00005$). A further drop in miRNA levels within 48 h after AHF admission was associated with an increased risk of 180-day mortality in a subset of the identified miRNAs.

Conclusions Declining levels of circulating miRNAs were associated with increasing acuity of heart failure. Early in-hospital decreasing miRNA levels were predictive for mortality in a subset of miRNAs in patients with AHF. The discovered miRNA panel may serve as a launch-pad for molecular pathway studies to identify new pharmacological targets and miRNA-based therapies.

Keywords Heart failure • Circulating microRNAs • Biomarkers

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Introduction

MicroRNAs (miRNAs) are a class of small (~22 nucleotides in length) non-coding RNAs that are potent regulators of gene expression at the post-transcriptional level.[1,2] MiRNAs are released into the systemic circulation, are remarkably stable, and are thought to reflect miRNA expression in tissue to some extent.[3] Thus, circulating miRNAs are potential biomarkers for a variety of pathological conditions, including heart failure (HF).[3,4] Moreover, miRNAs may improve our understanding of the pathophysiology underlying HF which may aid the development of novel, targeted therapies.

Specific circulating miRNA profiles have previously been described in patients with chronic heart failure (CHF).[5–9] A few studies have examined miRNAs in acute heart failure (AHF). Two studies measured miRNA levels in patients presenting at the emergency department with acute dyspnoea, and both revealed one or more miRNAs as distinctive markers of AHF.[10,11] In addition, Corsten *et al.* described specific miRNAs involved in myocardial damage, and found miR-499 to be substantially increased in patients with AHF compared with healthy controls.[12]

However, most studies were limited by a small sample size and lack of validation. We now report a miRNA signature in a variety of independent cohorts with a larger number of AHF patients in which we additionally investigate the association between circulating miRNA levels and clinical outcome.

Methods

Study design and procedures

Study subjects originated from 5 separate cohorts in various states of HF, ranging from AHF to stable CHF, and healthy controls (Figure *1*).

Discovery phase

In the discovery phase, 30 plasma samples from 10 patients hospitalized for AHF (AHF-admission, PROTECT cohort), 10 patients with CHF, and 10 healthy controls (Telosophy cohorts) were analysed.

The AHF cohort was selected from the Placebo-controlled Randomized Study of the Selective A1 Adenosine Receptor Antagonist Rolofylline for Patients Hospitalized with Acute Decompensated Heart Failure and Volume Overload to Assess Treatment Effect on Congestion and Renal Function (PROTECT) trial.[13] Stable CHF patients and healthy control subjects originated from the Telosophy study.[14] Key exclusion criteria for healthy controls were known atherosclerotic disease, HF, or a family history of premature cardiovascular disease.

Extended cohorts

For the extended cohort, in addition to the 10 AHF PROTECT blood samples from the discovery phase, 100 PROTECT blood samples were analysed at four different time points: admission for AHF (AHF-admission), after 24 h (AHF-24h admission), after 48 h (AHF-48h admission), and day 7 after admission (AHF-7d admission). MiRNA patterns at discharge (AHF-discharge) and 6 months after hospitalization (AHF-6m follow-up) for AHF were measured in plasma samples from 18 patients from the Coordinating Study Evaluating Outcomes of Advising and Counseling in Heart Failure (COACH).[15] In addition to the discovery cohort, plasma samples from 14 age- and sex-matched healthy controls were analysed (Telosophy).

Validation cohorts

Following the analysis of the discovery and extended cohorts, the findings obtained were validated in 3 independent cohorts: AHF patients (9 samples, Wroclaw cohort), CHF patients (10 samples, Beneficial cohort), and patients with an acute exacerbation of COPD (8 samples, Paris cohort).[16] Matching controls from the same centre (8 originating from Paris, 9 from Wroclaw) were included in the validation study. In brief, the Wroclaw cohort comprised patients admitted to hospital with a diagnosis of AHF in all cases based on the presence of signs and symptoms of AHF requiring intravenous treatment (loop diuretics, nitroglycerin, and/or inotropes). Patients with acute coronary syndrome as the underlying cause of AHF were excluded.

MicroRNA profiling: isolation, cDNA synthesis, and quantitative reverse transcription–polymerase chain reaction

Plasma sample processing for all cohorts and miRNA profiling were conducted in the same laboratory, under the same conditions. In the discovery and validation phases, circulating miRNA expression profiling was performed by using a commercially available Serum/Plasma Focus microRNA PCR panel (V2.M) (Exiqon, Vedbaek, Denmark). The panel consisted of the 375 most well described and abundant human circulating miRNAs that were detected in human serum/plasma. The 15 miRNAs selected based on the results from the discovery cohort were analysed in the extended cohorts using a customized Serum/Plasma microRNA PCR panel (Exiqon).

RNA was isolated from 200 µL of plasma using the miRCURY RNA isolation kit–Biofluids (Exiqon). Reverse transcription reactions were performed using the Universal cDNA Synthesis Kit (Exiqon). For each reaction, 4 µL of RNA was used. A total of 226 out of 375 miRNAs were detected in qRT–PCR analysis of the plasma samples. The remaining miRNAs were below the detection level. All the procedures were performed according to the manufacturer's instructions. See the Supplementary material online for further details.

Statistical methods

Differences in miRNA expression between different groups were determined by a two-tailed unpaired *t*-test. Bonferroni correction was applied to *P*-values to adjust for multiple testing. The Bonferroni correction for *P*-values sets the significance cut-off at P/n, where P is 0.05 and n is the number of tests. The significance threshold was set to a change of two-fold or more with a corrected *P*-value $\leq$0.00022 (discovery and validation cohorts, data set with 226 detected miRNAs) or $\leq$0.0033 (extended cohorts, data set with 15 detected miRNAs) for the comparison of plasma miRNA expression between studied conditions and time points.

Cox proportional hazards regression was performed to examine associations with outcome. Survival analysis included Harrell's C-index calculation. The exact binomial test was used to estimate the likelihood of the occurrence of multiple miRNAs being significant predictors of outcome, by calculating whether the amount of significant observations is more than expected by chance. *P*-values $\leq$0.05 were considered significant. All statistical analyses where performed using GenEx

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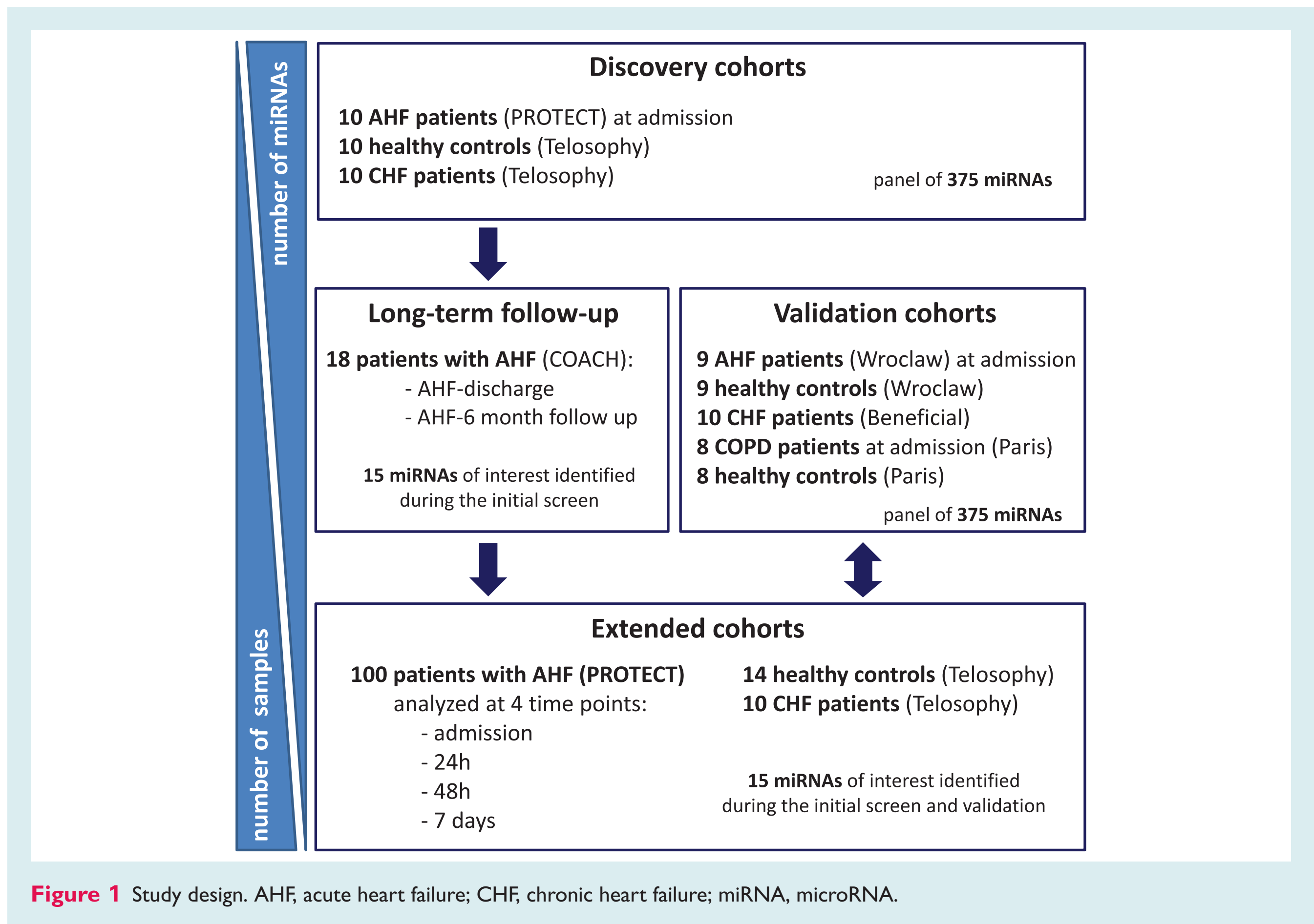

Figure 1 Study design. AHF, acute heart failure; CHF, chronic heart failure; miRNA, microRNA.

Professional version and R: a language and environment for statistical computing, version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Patient characteristics

Table 1 shows the demographic and clinical characteristics of all cohorts used in this study. Plasma NT-proBNP concentrations differed markedly between the cohorts in the discovery and extended study, being lowest in healthy controls and highest in patients with AHF at the time of admission (*Table 1*).

Circulating microRNA profiling in acute heart failure patients

Of the 226 miRNAs detected during the discovery phase, 40 remained significantly different in AHF patients compared with healthy controls after Bonferroni correction (Supplementary material online, *Table S1*). *Figure 2A* provides an overview of the initial screening data depicted in a volcano plot. A panel of 15 miRNAs with a greater than four-fold change was selected for further analysis, and included miR-423-5p as one of the miRNAs reported most consistently with different levels in HF patients.[6,8,10]

Differential microRNA levels in various stages of heart failure

The association of the selected 15 miRNAs with AHF was further supported by a highly consistent pattern of decreased miRNA levels with increased acuity of HF (*Figure 2A*). The lowest levels of miRNAs were observed in patients from admission for AHF to day 7 (PROTECT-extended cohort). The miRNAs of the panel gradually increased in COACH AHF patients at discharge (AHF-discharge) and converged at 6 months (AHF-6m follow-up) towards the CHF (Telosophy) levels. A significant trend over the different time points was observed for all miRNAs (all $P < 0.001$), as shown in *Figure 2B*. The results were confirmed using unsupervised hierarchical cluster analysis, which showed a clear separation of patients with AHF from the healthy controls and CHF patients (Supplementary material online, *Figure S1*).

Circulating microRNA profiles in the validation cohorts

Plasma levels of 12 miRNAs out of 15 that were identified during the discovery phase were also shown to be significantly decreased in AHF (Wroclaw) compared with CHF (Beneficial) patients and healthy controls (Supplementary material online, *Tables S2* and *S3*), of which 7 crossed the false discovery rate settled to correct for

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Table 1 Clinical characteristics of the cohorts

	Discovery and extended study			
	PROTECT cohort AHF at admission (*n* = 100)	**COACH cohort AHF at discharge (*n* = 18)**	**Telosophy cohort Stable CHF (*n* = 10)**	**Healthy controls (*n* = 24)**
Demographics				
Age (years)	68.9 ± 11.4	69.6 ± 9.9	67 ± 6.1	65.4 ± 6.6
Sex (% male)	50 (50)	55.5 (10)	70 (7)	83.3 (20)
Measurements				
LVEF (%)	34.1 ± 12.6	30.3 ± 9.1	27.6 ± 7	
Systolic blood pressure (mmHg)	119.4 ± 17.2	111.4 ± 19.1	112.9 ± 13.3	133.1 ± 17.8
Diastolic blood pressure (mmHg)	71.3 ± 11.8	66.2 ± 15.8	66 ± 7.4	80.3 ± 10.1
Heart rate (b.p.m.)	78.7 ± 15.6	81.3 ± 10.9	67.6 ± 8.1	66.9 ± 9.3
NYHA class (%)				
II	7 (7)	61.1 (11)	50 (5)	–
III	35 (35)	38.9 (7)	50 (5)	–
IV	56 (56)	0 (0)	0 (0)	–
Medical history (%)				
Myocardial infarction	49 (49)	55.5 (10)	100 (10)	0 (0)
Hypertension	83 (83)	50 (9)	10 (1)	20.8 (5)
Diabetes mellitus	44 (44)	27.8 (5)	20 (2)	4.2 (1)
Ischaemic heart disease	73 (73)	–	60 (6)	–
Atrial fibrillation	58 (58)	33.3 (6)	40 (4)	0 (0)
COPD	15 (15)	16.7 (3)	0 (0)	–
Laboratory values				
BNP (pg/mL)	382.7 (247.3–640.7)	456 (197–911)	–	–
NT-proBNP (pg/mL)	3000 (3000–5779.2)	2070.1 (1466.3–4443)	1153.5 (231–1792)	52 (35–63)
Creatinine (mg/dL)	1.4 (1.2–1.9)	1.15 (0.9–1.4)	–	–
Blood urea nitrogen (mg/dL)	30 (25–45.2)	–	–	–
eGFR (mL/min/1.73 m^2)	45.3 (35.1–62.2)	59.8 (51.9–71.3)	–	–

	Validation study				
	Wroclaw cohort AHF at admission (*n* = 9)	**Healthy controls (*n* = 9)**	**Beneficial cohort Stable CHF (*n* = 10)**	**Paris cohort AECOPD (*n* = 8)**	**Healthy controls (*n* = 8)**
Demographics					
Age (years)	68.5 ± 8.5	68.2 ± 7.8	68.9 ± 4.9	69.1 ± 10.4	71.0 ± 8.7
Sex (% male)	55.6 (5)	44.4 (4)	50 (5)	62.5 (5)	75 (6)
Measurements					
LVEF (%)	32.2 ± 14.4	–	33.5 ± 9.1	–	–
Systolic blood pressure (mmHg)	119.2 ± 19.4	123.1 ± 13.1	119.6 ± 19.1	130.3 ± 28.1	–
Diastolic blood pressure (mmHg)	71.6 ± 12.1	73.8 ± 10.6	74.2 ± 9.2	79.9 ± 14.3	–
Heart rate (b.p.m.)	88.2 ± 19.3	73.2 ± 10.1	67.3 ± 15.4	108.1 ± 22.6	–
NYHA class (%)					
II	0 (0)	–	70 (7)	–	–
III	44.4 (4)	–	30 (3)	–	–
IV	55.6 (5)	–	0 (0)	–	–
Medical history (%)					
Myocardial infartion	44.4 (4)	–	80 (8)	0 (0)	0 (0)
Hypertension	44.4. (4)	–	40 (4)	0 (0)	75 (6)
Diabetes mellitus	55.6 (5)	–	0 (0)	0 (0)	12.5 (1)
Ischaemic heart disease	66.7 (6)	–	–	0 (0)	0 (0)
Atrial fibrillation	22.2 (2)	–	–	25 (2)	12.5 (1)
COPD	11.1 (1)	–	–	75 (6)	0 (0)
Laboratory values					
NT-proBNP (pg/mL)	7714 (2666.8–15 443.3)	–	344 (80.0–525.3)	–	–

AECOPD, acute exacerbation of COPD; AHF, acute heart failure; CHF, chronic heart failure; eGFR, estimated glomerular filtration rate.

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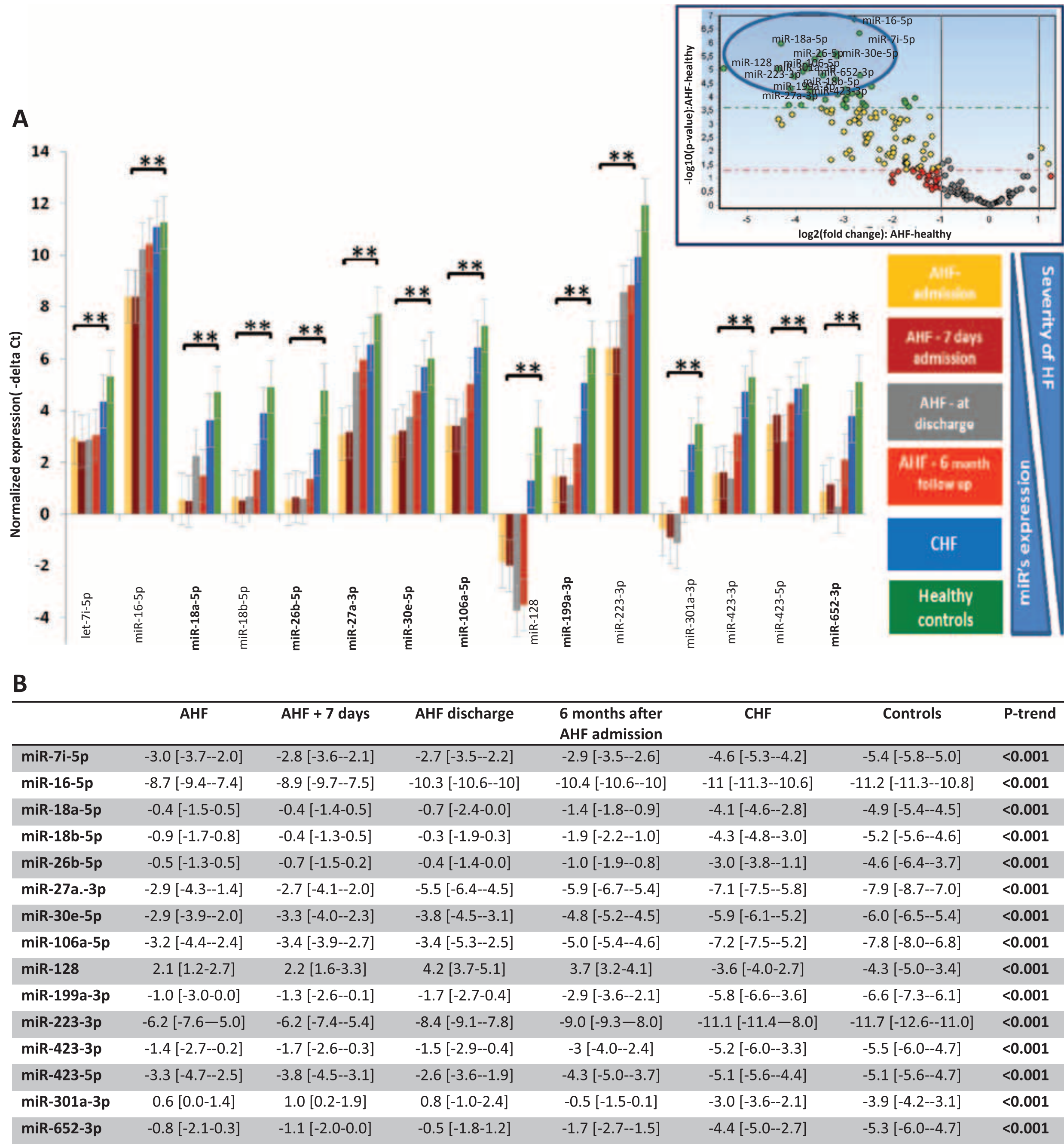

	AHF	AHF + 7 days	AHF discharge	6 months after AHF admission	CHF	Controls	P-trend
miR-7i-5p	-3.0 [-3.7--2.0]	-2.8 [-3.6--2.1]	-2.7 [-3.5--2.2]	-2.9 [-3.5--2.6]	-4.6 [-5.3--4.2]	-5.4 [-5.8--5.0]	**<0.001**
miR-16-5p	-8.7 [-9.4--7.4]	-8.9 [-9.7--7.5]	-10.3 [-10.6--10]	-10.4 [-10.6--10]	-11 [-11.3--10.6]	-11.2 [-11.3--10.8]	**<0.001**
miR-18a-5p	-0.4 [-1.5-0.5]	-0.4 [-1.4-0.5]	-0.7 [-2.4-0.0]	-1.4 [-1.8--0.9]	-4.1 [-4.6--2.8]	-4.9 [-5.4--4.5]	**<0.001**
miR-18b-5p	-0.9 [-1.7-0.8]	-0.4 [-1.3-0.5]	-0.3 [-1.9-0.3]	-1.9 [-2.2--1.0]	-4.3 [-4.8--3.0]	-5.2 [-5.6--4.6]	**<0.001**
miR-26b-5p	-0.5 [-1.3-0.5]	-0.7 [-1.5-0.2]	-0.4 [-1.4-0.0]	-1.0 [-1.9--0.8]	-3.0 [-3.8--1.1]	-4.6 [-6.4--3.7]	**<0.001**
miR-27a.-3p	-2.9 [-4.3--1.4]	-2.7 [-4.1--2.0]	-5.5 [-6.4--4.5]	-5.9 [-6.7--5.4]	-7.1 [-7.5--5.8]	-7.9 [-8.7--7.0]	**<0.001**
miR-30e-5p	-2.9 [-3.9--2.0]	-3.3 [-4.0--2.3]	-3.8 [-4.5--3.1]	-4.8 [-5.2--4.5]	-5.9 [-6.1--5.2]	-6.0 [-6.5--5.4]	**<0.001**
miR-106a-5p	-3.2 [-4.4--2.4]	-3.4 [-3.9--2.7]	-3.4 [-5.3--2.5]	-5.0 [-5.4--4.6]	-7.2 [-7.5--5.2]	-7.8 [-8.0--6.8]	**<0.001**
miR-128	2.1 [1.2-2.7]	2.2 [1.6-3.3]	4.2 [3.7-5.1]	3.7 [3.2-4.1]	-3.6 [-4.0-2.7]	-4.3 [-5.0--3.4]	**<0.001**
miR-199a-3p	-1.0 [-3.0-0.0]	-1.3 [-2.6--0.1]	-1.7 [-2.7-0.4]	-2.9 [-3.6--2.1]	-5.8 [-6.6--3.6]	-6.6 [-7.3--6.1]	**<0.001**
miR-223-3p	-6.2 [-7.6—5.0]	-6.2 [-7.4--5.4]	-8.4 [-9.1--7.8]	-9.0 [-9.3—8.0]	-11.1 [-11.4—8.0]	-11.7 [-12.6--11.0]	**<0.001**
miR-423-3p	-1.4 [-2.7--0.2]	-1.7 [-2.6--0.3]	-1.5 [-2.9--0.4]	-3 [-4.0--2.4]	-5.2 [-6.0--3.3]	-5.5 [-6.0--4.7]	**<0.001**
miR-423-5p	-3.3 [-4.7--2.5]	-3.8 [-4.5--3.1]	-2.6 [-3.6--1.9]	-4.3 [-5.0--3.7]	-5.1 [-5.6--4.4]	-5.1 [-5.6--4.7]	**<0.001**
miR-301a-3p	0.6 [0.0-1.4]	1.0 [0.2-1.9]	0.8 [-1.0-2.4]	-0.5 [-1.5-0.1]	-3.0 [-3.6--2.1]	-3.9 [-4.2--3.1]	**<0.001**
miR-652-3p	-0.8 [-2.1-0.3]	-1.1 [-2.0-0.0]	-0.5 [-1.8-1.2]	-1.7 [-2.7--1.5]	-4.4 [-5.0--2.7]	-5.3 [-6.0--4.7]	**<0.001**

Figure 2 MicroRNA (miRNA) levels in plasma samples of acute heart failure (AHF) patients at various time points. (*A*) Circulating levels of miRNAs of interest in plasma samples were quantified by quantitative reverse transcription–polymerase chain reaction (qRT–PCR) assays. Values are plotted as geometrical mean ± SD, $^{**}P < 0.001$. The intercept shows a volcano plot illustrating a cluster of the 15 circulating miRNAs that changed most significantly between AHF patients and healthy controls. Log2 ratio of fold change (*x*-axis) is plotted against statistical significance based on −log10 (*y*-axis) for each miRNA. MiRNAs plotted in green passed the Bonferonni correction (based on $P \leq 0.00022$; represented by a green dashed line) and changed more than two-fold (represented by two black vertical lines). MiRNAs plotted in yellow did not pass Bonferroni correction but changed more than two-fold compared with controls. Statistically insignificant miRNAs but with a change of more than two-fold are plotted in red. Biologically and statistically insignificant miRNAs are plotted in grey. (*B*) MiRNA levels per cohort (median [interquartile range]) including *P* for trend. MiRNAs shown to be significantly changed for both discovery and validation cohorts are highlighted in bold. CHF, chronic heart failure.

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multiple testing; miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p. The validation results are summarized in *Figure 3*. In the AHF validation cohort, the levels of these seven miRNAs were significantly lower in AHF patients compared with healthy controls (*Figure 3A* and *B*). There were no differences in miRNA levels between patients admitted with an acute exacerbation of COPD (Paris) and healthy controls (*Figure 3C* and *D*). The differentiation between AHF patients from both the discovery and validation cohorts and the healthy controls is clearly shown by the result of the principal component analysis (PCA) depicted in *Figure 3E*.

Circulating microRNAs related to clinical outcome

With univariable Cox proportional hazards analysis, the prognostic value of the miRNA panel in the 100 AHF patients (PROTECT-extended cohort) was assessed. After admission, a further decrease in miRNA levels after 48 h in a subset of 7 out of 15 miRNAs (let-7i-5p, miR-18a-5p, miR-18b-5p, miR-223-3p, miR-301a-3p, miR-423-5p miR-652-3p) was found to be predictive for 180-day mortality (*Table* 2). Two out of the seven miRNAs (miR-18a-5p and miR-652-3p) passed Bonferroni correction in the AHF validation cohort. The result of the exact binomial test was highly significant ($P < 3.518 \times 10^{-6}$); thus, the likelihood of the occurrence of multiple miRNAs predicting outcome by chance is highly improbable. Harrell's C-indexes were calculated to obtain the discriminative value of our models to predict outcome, which ranged in the significant miRNAs from 0.63 to 0.70. Furthermore, the directionality of the hazard ratios of all 15 miRNAs ($HR > 1$) supports the consistency of these findings.

Discussion

In the present study we identified a panel of 15 circulating miRNAs associated with AHF that were consistently decreased in patients with AHF compared with both CHF patients and healthy controls. Moreover, decreasing levels of circulating miRNAs were associated with increasing acuity of HF. A further drop in 7 out of 15 miRNAs early during hospitalization was associated with an increased risk of mortality within 180 days. Validation in an independent cohort of patients with AHF confirmed this panel of miRNAs and led to seven AHF-specific miRNAs, of which miR-18a-5p and miR-652-3p were predictive for 180-day mortality. Another cause of acute breathlessness (acute exacerbation of COPD) was not related to a change in circulating miRNA profiles compared with controls, implying that our results were not a consequence of general respiratory distress.

Involvement of microRNAs in heart failure

Recent evidence suggests that miRNAs play an important role in cardiac development and are involved in the pathogenesis of cardiovascular diseases, including HF.[17–19] A relationship between miRNAs and cardiac tissue development was shown by deleting the miRNA-processing enzyme DICER in cardiomyocytes and epicardium tissue in mice. These mutations cause profound cardiac defects and lead to either embryonic or neonatal death.[17–19] Other studies reported that the hearts of these knock-out mice developed hypertrophy, fibrosis, and ventricular dilatation, resulting in HF.[17] The discovery that miRNAs are secreted and extracellularly measurable in blood and other body fluids has stimulated research on circulating miRNAs. The ongoing results suggested a differential diagnostic utility of circulating miRNAs in HF, and several studies investigated the diagnostic potential of circulating miRNAs in HF.[8,20] Furthermore, a more accurate discrimination between HF with preserved ejection fraction (HFpEF) and HF with reduced ejection fraction (HFrEF) has been proposed by using circulating miRNAs.[21–23] However, most of the studies on circulating miRNAs in HF either have been conducted in small numbers of patients or only include patients with CHF.[5–8] Circulating miRNA patterns related to AHF are not well described.

The HF-specific panel of seven circulating miRNAs identified in our study overlaps with published results of other related investigations. Although several investigators report an up-regulation of miRNAs, more recent publications describe lower levels of miRNAs to be associated with cardiac disease, in concordance with our study.[6,24–26] For example, a study of patients with symptomatic HF showed that 24 circulating miRNAs were decreased compared with control subjects, including miR-26b-5p, miR-223-3p, miR-16-5p, miR-30e-5p, miR-423-5p, miR-27a-3p, and miR-18a-5p,[6] and Watson *et al.* also found that all of the 5 identified circulating miRNAs were reduced in HF patients.[21]

The identified miRNAs have been shown to play a role in important pathophysiological mechanisms implicated in HF development, such as cardiomyocyte proliferation, myocardial matrix remodelling, and cardiac hypertrophy (Supplemetnary material online, *Table S4*).

The miRNA miR-199a-3p is able to promote cardiomyocyte proliferation in the heart of mice and stimulates cardiac regeneration after induced myocardial infarction.[27] A relationship between hypoxic conditions and miRNA expression has been described for miR-199a, with possible cardiac involvement of the miR-199a-214 cluster, inducing the shift from fatty acid utilization to glucose metabolism in HF.[28] Dysregulation of miR-18a-5p, another member of our panel, is involved in vascular smooth muscle cell differentiation by targeting Syndecan4 in a carotid artery injury model.[29] The expression levels of miR-26a and miR-26b were reduced in cardiomyocytes of human and canine subjects with AF.[30] Several miRNAs in our panel were associated with cardiac hypertrophy. MiR-18b, miR-26b, miR-27a, and miR-199a might play an important role in the development of cardiac hypertrophy,[31–34] while overexpression of miR-26b and miR-27a *in vitro* resulted in fewer hypertrophic cardiomyocytes.[35,36] Bernardo *et al.* reported the effect of silencing miR-652 in a hypertrophic mouse model, which improved cardiac function and attenuated hypertrophy and adverse remodelling.[37]

Circulating miRNAs have the potential to serve as novel prognostic markers. Pilbrow *et al.* reported that plasma concentrations

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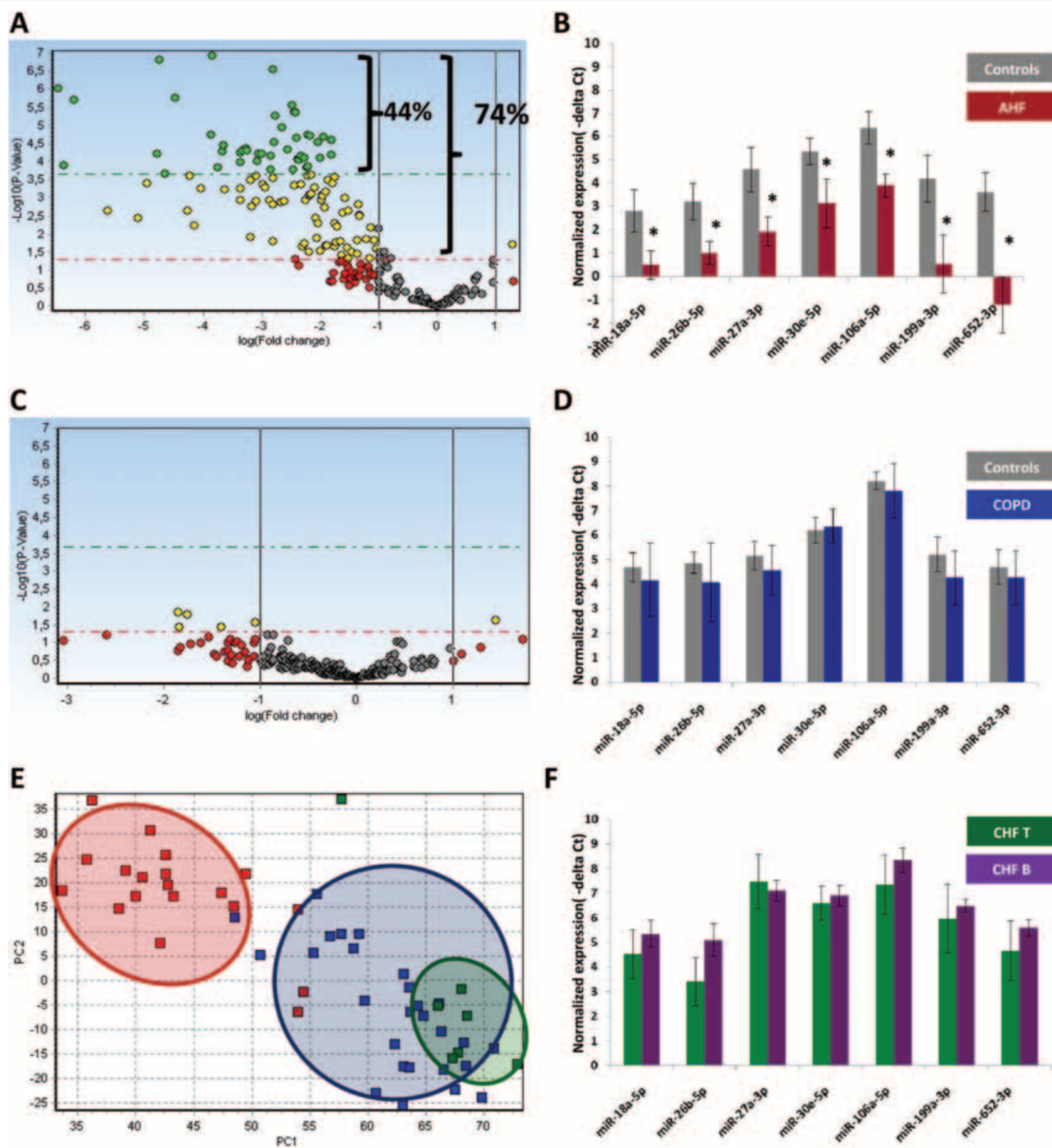

Figure 3 Profiling of circulating microRNAs (mRNAs) in the validation cohorts. (*A*) Volcano plot showing miRNAs levels in plasma samples of acute heart failure (AHF) patients compared with healthy controls. Forty-four per cent of the significantly lowered miRNAs detected in the validation cohort overlapped with the decreased miRNAs found in the discovery phase. Log2 fold change in miRNAs levels is plotted on the *x*-axis and false discovery rate-adjusted significance (Bonferroni correction, $P \leq 0.00022$; represented by a green dashed line) is plotted on the *y*-axis (−log10 scale). MiRNAs plotted in green passed the Bonferonni correction and changed more than two-fold. MiRNAs plotted in yellow did not pass Bonferroni correction but changed more than two-fold compared with controls. Statistically insignificant miRNAs but with a change of more than two-fold are plotted in red. Biologically and statistically insignificant miRNAs are plotted in grey. (*B*) Seven miRNAs out of 15 were consistently and significantly changed in the discovery and validation cohorts. (*C*) Volcano plot showing miRNA levels in plasma samples of COPD patients compared with healthy controls. Log2 fold change in miRNAs level is plotted on the *x*-axis and false discovery rate-adjusted significance (Bonferroni correction, $P \leq 0.00022$; represented by a green dashed line) is plotted on the *y*-axis (−log10 scale). MiRNAs plotted in yellow did not pass Bonferroni correction but changed more than two-fold compared with controls. Statistically insignificant miRNAs but with a change of more than two-fold are plotted in red. Biologically and statistically insignificant miRNAs are plotted in grey. (*D*) There were no differences in miRNAs level between COPD patients and healthy subjects. (*E*) Graph of principal components analysis (PCA), showing partial differentiation between patients with AHF from both the discovery and validation cohorts (depicted in red), COPD patients (depicted in green), and healthy subjects (depicted in blue). Each square represents one patient. (*F*) MiRNA levels in plasma samples of chronic heart failure (CHF) patients of the discovery phase (CHF T, Telosophy, discovery cohort) compared with CHF patients of the validation phase (CHF B, Beneficial, validation cohort).

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Table 2 Cox analysis for 180-day mortality

Delta miRNA	Hazard ratio (95% CI)	*P*-value	Harrell's C-index
let-7i-5p	**1.958 (1.197–3.203)**	**0.007**	**0.657**
miR-16-5p	1.499 (0.938–2.394)	0.091	0.617
miR-18a-5p	**1.616 (1.104–2.365)**	**0.014**	**0.664**
miR-18b-5p	**1.851 (1.138–3.010)**	**0.013**	**0.669**
miR-26b-5p	1.090 (0.714–1.665)	0.069	0.515
miR-27a-3p	1.379 (0.914–2.079)	0.125	0.596
miR-30e-5p	1.547 (0.976–2.451)	0.063	0.609
miR-106a-5p	1.499 (0.989–2.274)	0.057	0.638
miR-128	1.253 (0.803–1.956)	0.032	0.576
miR-199a-3p	1.262 (0.833–1.911)	0.273	0.572
miR-223-3p	**1.557 (1.035–2.343)**	**0.034**	**0.649**
miR-301a-3p	**1.782 (1.197–2.652)**	**0.004**	**0.697**
miR-423-3p	1.349 (0.878–2.073)	0.173	0.585
miR-423-5p	**1.681 (1.085–2.604)**	**0.002**	**0.064**
miR-652-3p	**1.541 (1.002–2.369)**	**0.049**	**0.633**

Univariable proportional hazards Cox regression analysis was performed to assess associations of microRNA (miRNA) changes during the first 48 h of hospitalization for acute heart failure (AHF) and 180-day mortality.
Delta miRNA indicates change of miRNA levels at 48 h compared with baseline.
The hazard ratio is depicted per SD change of the delta miRNA.
Predictive performance was quantified with the C-index.
MiRNAs depicted in grey represent the two miRNAs validated in the additional independent cohorts.
CI, confidence interval.

of miR-652 in the lowest tertile were associated with readmission for HF in patients with an acute coronary syndrome.[38] Cakmak *et al.* found that miR-182 could serve as a significant independent predictor for cardiovascular mortality in systolic heart failure.[39] Earlier, Fukushima *et al.* found that the level of miR-126 in plasma of patients with congestive HF negatively with disease severity, assessed with the NYHA functional classification.[40] Furthermore, the dynamic change in plasma-derived miR-133b can reflect early myocardial injury after heart transplantation.[41] In our study the subset of miRNAs were found at lower levels in patients with AHF compared with healthy individuals. Moreover, for the first time, early in-hospital changes of miR-18a-5p and miR-652-3p were described as significant independent predictors of 180-day mortality in AHF patients. The analysis had some limitations, however, such as a lack of validation of prognostic value in other independent cohorts and a relatively small sample size.

The origin and role of circulating miRNAs is currently under investigation. Some studies have suggested that plasma concentrations of miRNAs might reflect concentrations of these miRNAs in organs and tissue, for example in the heart.[12,42,43] Notably, all seven miRNAs validated in our plasma screen (miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p) are expressed in cardiac tissue.

It has been postulated that tissue cells try to compensate for miRNA deficiency by extracting them from the circulation.[44] However, the role of miRNAs in cell–cell communication is poorly understood, and whether circulating miRNAs are sufficient to exert physiological effect in cells remains to be proven. We hypothesize that under pathophysiological conditions (e.g. prolonged hypoxia or stretch), cells do not function properly, which could lead to reduced miRNA production and excretion. Once the pathophysiological conditions improve, miRNA production and excretion may recover and lead to restored plasma concentrations.

Strengths and limitations

We performed a relatively large and robust study in order to identify a novel miRNA signature in AHF. This is the first study to show gradual changes in circulating miRNA levels depending on the acuity of HF. However, our study has several limitations. Although this is the largest AHF cohort reported to date, its size remains modest. Furthermore, we did not perform any functional investigation of the identified miRNAs. The miRNA panel used for the initial screening consisted of only 375 well characterized miRNAs and might not include miRNAs with low circulating levels in healthy patients. Further, due to variability in expression levels and strict statistical correction, we may have missed other miRNAs related to AHF.

Conclusion

We identified a distinct panel of circulating miRNAs decreased in AHF. The association of these miRNAs with AHF was further supported by a highly consistent pattern of increased miRNA levels with decreasing acuity of HF. A further drop in a subset of miRNAs early after hospital admission for AHF was associated with increased mortality through 180 days. A better understanding of the role of these miRNAs in AHF may provide insights into underlying disease mechanisms, potentially leading to better and more targeted management and therapies.

Circulating miRNAs might become new biomarkers in HF. Expression levels of our selected panel of miRNAs was distinctly and consistently lower in patients with AHF than in patients with CHF, acute exacerbation of COPD, and control subjects, suggesting the diagnostic value of these miRNAs. We also demonstrated a possible association between mortality and a number of miRNAs. This suggests that these miRNAs might predict those AHF patients at risk for a poorer outcome. Moreover, it remains to be established whether the identified miRNA set is able to predict not only outcome but also response to treatment, and further prospective studies should explore its performance in guiding therapy in AHF. Finally, these miRNAs may become targets for therapy once we learn more about their role in the pathology and progression of AHF.

Acknowledgements

We acknowledge support from the Netherlands CardioVascular Research Initiative: the Dutch Heart Foundation, Dutch Federation of University Medical Centres, the Netherlands Organisation for Health Research and Development, and the Royal Netherlands Academy of Sciences.

Supplementary Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Heat map of miRNA profiles.

Table S1 List of all significantly changed circulating miRNAs in plasma of AHF patients at admission (PROTECT) compared with healthy controls.

Table S2 List of all significantly changed circulating miRNAs in plasma of AHF patients at admission (Wroclaw, validation study) compared with healthy controls.

Table S3 List of 15 circulating miRNAs with reduced levels in plasma samples of AHF patients at admission (PROTECT) compared with healthy controls.

Table S4 Functional clustering of the validated miRNAs in relation to cardiac biology and pathophysiology.

Funding

This study was supported by a Grant from the Dutch Heart Foundation: Approaching Heart Failure By Translational Research of RNA Mechanisms (ARENA). The PROTECT trial was supported by NovaCardia, a subsidiary of Merck. COACH was supported by grant 2000Z003 from the Netherlands Heart Foundation and by additional unrestricted grants from Biosite France SAS, Jouy-en-Josas, France (BNP), Roche Diagnostics Nederland BV, Venlo, The Netherlands (NT-proBNP), and Novartis Pharma BV, Arnhem, The Netherlands.

Conflicts of interest: E.S.O., D.S., E.L.V., J.M.T.M., M.A.E.V., L.C.Y., P.V.D.H., R.A.D.B., S.M., A.M., S.L. and P.V.D.M. have nothing to disclose. Y.M.P. has a minor stake (<5%) in a University spin-off which commercializes IP in the field of biomarkers and miRNAs. J.R.T. has received research funds and consulting fees from Actelion, Amgen, Cytokinetics, Merck, and Novartis. C.M.O.C. is a consultant to Merck. M.M. has received consulting incomes from Abbot Vascular, Bayer, Novartis and Servier. G.C. and B.D. are employees of Momentum Research Inc, which was contracted to perform work on the project by Merck & Co, Inc. D.M.B. is an employee of Merck. J.G.C. was on the Steering Committee of the study, served on the Advisory Board for MSD, and received payments for both. M.M.G. has received institutional research support and served on a scientific Advisory Board for Merck. P.P. has received honoraria from Merck. D.J.V.V. has received Board Membership fees from Amgen, Biocontrol, Johnson & Johnson, Novartis, Sorbent and Vifor. A.A.V. was a member of the Steering Committee of PROTECT. E.B. is a co-founder and member of the scientific advisory board of InteRNA Technologies B.V., which develops miRNA therapeutics for cancer. A.A.V., E.B., E.S.O. and P.V.D.M. are patent holders of the circulating miRNAs in this manuscript. No other conflicts were reported.

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