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Signature of circulating microRNAs in patients with acute heart failure

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Running title: Circulating miRNAs profile in acute heart failure

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ABSTRACT

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 chronic obstructive pulmonary disease (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 to 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 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 hours 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.

Key Words: Heart failure, circulating microRNAs, biomarkers
INTRODUCTION

MicroRNAs (miRNAs) are a class of small (approximately 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 some extent reflect miRNA expression in tissue (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) (5,6-9). 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 dyspnea 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 to 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.

MATERIALS AND 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.

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 analyzed.
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, additionally to the 10 AHF PROTECT blood samples from the discovery phase, 100 PROTECT blood samples were analyzed at four different time points: admission for AHF (AHF-admission), after 24 hours (AHF-24h admission), 48 hours (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) study (15). In addition to the discovery cohort 10 plasma samples of healthy controls and 10 samples of CHF patients, plasma samples from 14 age and sex matched healthy controls were analyzed (Telosophy).

Validation cohorts:

Following the analysis of the discovery and extended cohorts, obtained findings 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 center (8 originating from Paris, 9 from Wroclaw) were included into the validation study. Briefly, the Wroclaw cohort comprised patients admitted to the 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 underlying cause of AHF were excluded (Figure 1).

MiRNA profiling: isolation, cDNA synthesis and qRT-PCR.
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 commercially available Serum/Plasma Focus microRNA PCR panel (V2.M) (Exiqon, Vedbaek, Denmark). The panel consists 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 analyzed in the extended cohorts using customized Serum/Plasma microRNA PCR panel (Exiqon, Vedbaek, Denmark).

RNA was isolated from 200 μl of plasma using the miRCURY RNA isolation kit – Biofluids (Exiqon, Vedbaek, Denmark). Reverse transcription reactions were performed using the Universal cDNA Synthesis Kit (Exiqon, Vedbaek, Denmark). 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 detection level. All the procedures were performed according to the manufacturer’s instructions. See Supplementary Information 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-value sets the significance cut-off at p/n, where P is 0.05 and n number of tests. The significance threshold was set to a ≥2-fold change with a corrected P-value ≤0.00022 (discovery and validation cohorts, dataset with 226 detected miRNAs) or ≤0.0033 (extended cohorts, dataset 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 if the amount of significant observations is more than expected by chance. P-values ≤0.05 were considered significant. All statistical analyses were performed using GenEx Professional version and R: A language and
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 miRNA profiling in acute heart failure patients

Of the 226 miRNAs detected during the discovery phase, 40 remained significantly different in AHF patients compared to healthy controls after the Bonferroni correction (Table S1). Figure 2A provides an overview of the initial screening data depicted in a volcano plot. A panel of 15 miRNAs with a >4 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 miRNA 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 Figure S1).
Circulating miRNAs 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 to CHF (Beneficial) patients and healthy controls (Table S2, Table S3), of which 7 crossed the false discovery rate settled to correct for multiple testing; miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p, respectively. The validation results are summarized in Figure 3. In the AHF validation cohort, the levels of these 7 miRNAs were significantly lower in AHF patients compared to healthy controls (Figure 3A, B). There were no differences in miRNA levels between patients admitted with an acute exacerbation of COPD (Paris) and healthy controls (Figure 3C, D). The differentiation between AHF patients from both discovery and validation cohorts and healthy controls is clearly shown by the result of the principal component analysis (PCA) depicted in Figure 3E.

Circulating miRNAs related to clinical outcome

With univariable Cox proportional hazards analysis, the prognostic value of the miRNA panel in the 100 AHF (PROTECT-extended cohort) patients was assessed. After admission, a further decrease in miRNA levels after 48 hours in a subset of 7 out of 15 miRNAs (let-7i-5p, miR-18a-5p, miR-18b-5p, miR-223-3p, miR-301a-5p, 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*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 to both patients with CHF 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 7 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 to controls, implying that our results were not a consequence of general respiratory distress.

**Involvement of miRNAs 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 either to 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) have been proposed by using circulating miRNAs ((21-23). However, most of the studies on circulating miRNAs in HF have been conducted either 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 7 circulating miRNAs identified in our study overlaps with published results of other related investigations. Although several investigators report about an upregulation 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 to 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 also Watson et al. found that all of the 5 identified 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 remodeling and cardiac hypertrophy (Table S4).

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). Human and canine cardiomyocytes expression levels of miR-26a and miR-26b were reduced in subjects with atrial fibrillation (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 less hypertrophic cardiomyocytes (35,36). Bernardo et al. reported about the effect of silencing miR-652 in a hypertrophic mouse model, which improved cardiac function and attenuated hypertrophy and adverse remodeling (37).

Circulating miRNAs have the potential to serve as novel prognostic markers. Pilbrow et al. reported that plasma concentrations 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 level of miR-126 in plasma of patients with congestive heart failure correlated negatively with disease severity, assessed with the New York Heart Association Functional Classification (NYHA) (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 in lower levels in patients with AHF compared to 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 7 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 miRNA in cell-to-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 pathophysiologic conditions (e.g. prolonged hypoxia or stretch), cells do not function properly, which could lead to reduced miRNA production and excretion. Once the pathophysiologic 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 of 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.

**Translational perspectives:**

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 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.

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peptide), Roche Diagnostics Nederland BV, Venlo, the Netherlands (N-terminal prohormone brain natriuretic peptide), and Novartis Pharma BV, Arnhem, the Netherlands.

Conflicts of Interest

Dr. Pinto has a minor stake (<5%) in University spin-off which commercializes IP in the field of biomarkers and miRNAs. Dr. Berezikov is co-founder and member of scientific advisory board of InteRNA Technologies B.V., which develops microRNA therapeutics for cancer. No other conflicts were reported.
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FIGURE LEGENDS

Figure 1. Study design

Figure 2. MiRNA levels in plasma samples of AHF patients at various time points

(A) Circulating levels of miRNAs of interest in plasma samples were quantified by qRT-PCR assay. Values are plotted as geometrical mean ±SD, **P<0.001. Intercept shows 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 ≤ 0.00022; represented by green dash line) and changed more than 2-fold (represented by two black vertical lines). MiRNAs plotted in yellow did not pass Bonferroni correction but changed more than 2-fold compared to controls. Statistically insignificant miRNAs but with a change of more than 2-fold are plotted in red. Biologically and statistically insignificant miRNAs are plotted in grey.

Figure 3. Profiling of circulating miRNAs in validation cohorts

(A) Volcano plots showing miRNAs levels in plasma samples of AHF patients compared to healthy controls. Forty-four percent 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 level is plotted on the x-axis and false discovery rate-adjusted significance (Bonferroni-correction, p ≤ 0.00022; represented by green dash line) is plotted on the y-axis (–log10 scale). MiRNAs plotted in green passed the Bonferonni correction and changed more than 2-fold. MiRNAs plotted in yellow did not pass Bonferroni correction but changed more than 2-fold compared to controls. Statistically insignificant miRNAs but with a change of more than 2-fold are plotted in red. Biologically and statistically insignificant miRNAs are plotted in grey. (B) 7 miRNAs out of 15 were consistently and significantly changed in the discovery and validation cohorts. (C) Volcano plots showing miRNA levels in plasma samples of COPD patients compared to healthy controls. Log2 fold change in miRNAs level is plotted on the x-axis and false discovery rate-adjusted significance (Bonferroni-correction, p ≤ 0.00022; represented by green dash line) is plotted on the y-axis (–log10 scale). MiRNAs plotted in yellow did not pass Bonferroni correction but changed more than 2-fold compared to controls. Statistically insignificant miRNAs but with a change of more than 2-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 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 CHF patients of the discovery phase (CHF T, Telosophy, discovery cohort) compared to CHF patients of the validation phase (CHF B, Beneficial, validation cohort).