Multivariate miRNA signatures as biomarkers for non-ischaemic systolic heart failure

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Aims
Non-ischaemic heart failure is one of the today’s most prevalent cardiovascular disorders. Since modern pharmacotherapy has proved to be very effective in delaying disease progression and preventing death, imaging modalities and molecular biomarkers play an important role in early identification and clinical management as well as risk assessment of patients. The present study evaluated for the first time whole peripheral blood miRNAs as novel biomarker candidates for non-ischaemic heart failure with reduced ejection fraction (HF-REF).

Methods and results
We assessed genome-wide miRNA expression profiles in 53 HF-REF patients and 39 controls. We could identify and validate several miRNAs that show altered expression levels in non-ischaemic HF-REF, discriminating cases from controls both as single markers or when combined in a multivariate signature. In addition, we demonstrate that the miRNAs of this signature significantly correlate with disease severity as indicated by left ventricular ejection fraction.

Conclusion
Our data further denote that miRNAs are potential biomarkers for systolic heart failure. Since their detection levels in whole blood are also related to the degree of left ventricular dysfunction, they may serve as objective molecular tools to assess disease severity and prognosis.

Keywords
Non-ischaemic heart failure • HF-REF • miRNA • Biomarker

Introduction
Non-ischaemic heart failure is among the most prevalent causes for heart transplantation and sudden cardiac death. It can be the result of a variety of underlying conditions, including genetic and inflammatory cardiomyopathy, hypertension, metabolic diseases, or toxic injury.1–3 Often, affected individuals suffer from a progressive impairment of cardiac contractile function accompanied by dilation of both ventricles. Therapeutic management of these patients may be challenging and treatment strategies in current guidelines depend on the symptoms of the patient as well as objectively assessed disease severity. Therefore, imaging modalities and molecular biomarkers are needed to not only identify heart failure in early stages of the disease, but also to facilitate clinical management and risk assessment in patients.

Small non-coding miRNAs modulate the expression of target genes on the posttranscriptional level, thereby regulating a multitude of physiological and pathological processes.4–7 Since miRNAs also represent biologically extraordinarily stable molecules as they are protected through the formation of complexes from being degraded by RNAses, several studies have started to elucidate their role as biomarkers for various human disorders including acute myocardial infarction and heart failure.8–15 In the present work, we assessed miRNA expression profiles in whole peripheral blood of patients with non-ischaemic heart failure with reduced ejection fraction (HF-REF) on a genome-wide level using microfluidic primer extension arrays. We found several miRNAs that might contribute to the biomarker-guided detection and risk assessment of heart failure.

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Methods

Patient recruitment
The present study has been approved by the local Ethics Committee and participants have given written informed consent. In the screening cohort, we included 53 patients with new diagnosis of systolic heart failure, which had undergone diagnostic coronary angiography. Non-ischaemic HF-REF was defined according to ESC guidelines as impairment of left-ventricular ejection fraction (LVEF < 50%) and signs or symptoms of HF (e.g. breathlessness, reduced walking capacity, ankle swelling, or pulmonary crackles) in the absence of significant coronary artery disease (CAD).1 Patients with relevant CAD, valvular heart disease, acute myocarditis, or a history of cardio-toxic chemotherapy were excluded. The controls (n = 39), in whom coronary angiography was performed for exclusion of an underlying cardiovascular disease due to—in retrospect—atyypical symptoms, did not suffer from LV systolic impairment (LVEF ≥ 50%) or relevant CAD. Left-ventricular ejection fraction was quantitatively assessed in all patients and controls of the screening stage during diagnostic angiography. The centreline method was applied to analyse dynamic wall motion of the ventricle. At first, end-diastolic and end-systolic contours of the left ventricle had to be defined on the retrieved angiographic movies. A centreline was then drawn midway between these contours. Finally, 100 chords were constructed perpendicular to the centreline. The length and position of the chords allowed estimation of wall motion and hence contractility. Results were normalized for heart size by dividing the length of the chords by the length of the end-diastolic contour.

To validate selected miRNAs in whole blood, we recruited 14 non-ischaemic HF-REF patients and eight controls complying with the same criteria as mentioned earlier. For validation of miRNAs in serum samples, we recruited 10 non-ischaemic HF-REF patients and 10 controls.

To assess a potential prognostic value of selected miRNAs, we retrospectively analysed cardiovascular death, heart transplantation, hospitalization due to worsening of HF, and stroke in the patients of the screening cohort. Of the 53 patients in the screening cohort, 10 examinees were lost for follow-up.

miRNA expression profiling from peripheral whole blood samples
Five millilitres of peripheral blood was collected according to a standardized protocol in two PAXgene Blood RNA tubes (BD, USA) for each participant. The tubes were immediately inverted five times and stored for a maximum of 3 days at 4°C until total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Germany).16 Quality of the samples was checked by spectrophotometric analysis (Nanodrop) and by Bioanalyzer 2100 RNA nano kit measurements. For random samples, we also performed Bioanalyzer 2100 small RNA kit measurements to directly visualize mature miRNA content. For serum samples, an 8 mL aliquot of blood samples from each participant was collected directly into serum collection tubes (Sarstedt Monovette). The serum samples were allowed to stand for 1 h at room temperature before being centrifuged at 3000 rpm for 10 min. Then the serum supernatant was recovered and stored at −80°C until further analysis. The RNA was isolated from a 100 μL aliquot using the miRNeasy Serum/Plasma Kit according to the manufacturer’s protocol (Qiagen, Germany). Personnel blinded to patient characteristics performed miRNA expression profiling including data acquisition and normalization. Samples for microarray experiments were analysed with the Geniom Real-time Analyser (febit, Germany) using the Geniom Biochip miRNA Homo sapiens. Each array contained seven replicates of 883 miRNAs and miRNA star sequences as annotated in the Sanger miRBase 12.0. Sample labelling with biotin was carried out by microfluidic-based enzymatic on-chip labelling of miRNAs (MPEA) as described before.17,18 After hybridization for 16 h at 42°C, the biochip was washed, signals were measured, and the resulting images were evaluated using the Geniom Wizard Software (febit, Germany). For each array, the median signal intensity of all features was extracted from the raw data file. Hence, seven intensity values were calculated for each miRNA corresponding to each replicate on the array. Finally, after background correction, the seven replicate intensity values of each miRNA were summarized by their median value. To normalize the data across different arrays, quantile normalization was applied. All further analyses were based on these normalized and background subtracted data.

For miRNA expression quantification in different white blood cells of a healthy individual, we first separated CD3-, CD14-, CD15-, CD19-, and CD56- positive cells. Total RNA was extracted as described earlier. All signature miRNAs were measured using the Agilent miRNA microarray except for miR-520d-5p, which is not described in the Sanger miRBase.

Table 1  Patient characteristics of screening cohort

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 39)</th>
<th>HF-REF patients (n = 53)</th>
<th>P-value</th>
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</thead>
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<tr>
<td>Age (years)</td>
<td>63 ± 13</td>
<td>60 ± 16</td>
<td>0.3</td>
</tr>
<tr>
<td>Male/female (n/n)</td>
<td>23/16</td>
<td>44/9</td>
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</tr>
<tr>
<td>Current smoking (n%)</td>
<td>12 (31)</td>
<td>12 (23)</td>
<td>0.7</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>57 ± 5</td>
<td>33 ± 12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NYHA functional class</td>
<td>n.a.</td>
<td>LVEF 35–50%:</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.9 ± 0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LVEF &lt; 35%:</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>2.3 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Previous hospitalizations due to HF, n (%)</td>
<td>0</td>
<td>7 (13)</td>
<td>0.003</td>
</tr>
<tr>
<td>NT-proBNP (ng/L)</td>
<td>146 ± 92</td>
<td>2399 ± 3395</td>
<td>0.003</td>
</tr>
<tr>
<td>Rales/signs of pulmonary oedema, n (%)</td>
<td>—</td>
<td>6 (11)</td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>124 ± 17</td>
<td>127 ± 18</td>
<td>0.4</td>
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<tr>
<td>DBP (mmHg)</td>
<td>72 ± 12</td>
<td>77 ± 12</td>
<td>0.053</td>
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<tr>
<td>DM, n (%)</td>
<td>12 (31)</td>
<td>11 (21)</td>
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<tr>
<td>Hypertension, n (%)</td>
<td>28 (72)</td>
<td>36 (68)</td>
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<tr>
<td>HDL (mmol/L)</td>
<td>1.3 ± 0.5</td>
<td>1.1 ± 0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.6 ± 0.8</td>
<td>2.1 ± 1</td>
<td>0.14</td>
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<tr>
<td>WBC (×10³)</td>
<td>8.5 ± 3</td>
<td>8.5 ± 2.5</td>
<td>0.9</td>
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<tr>
<td>Creatinine (μmol/L)</td>
<td>97 ± 80</td>
<td>133 ± 168</td>
<td>0.2</td>
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<tr>
<td>Urea (mmol/L)</td>
<td>5.8 ± 2.3</td>
<td>7.2 ± 4.3</td>
<td>0.2</td>
</tr>
<tr>
<td>ICD, n (%)</td>
<td>—</td>
<td>5 (9)</td>
<td></td>
</tr>
<tr>
<td>CRT</td>
<td>—</td>
<td>—</td>
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</tbody>
</table>

LVEF, left-ventricular ejection fraction, NYHA, New York Heart Association, HF, heart failure, SBP, systolic blood pressure, DBP, diastolic blood pressure, DM, diabetes mellitus, TC, total cholesterol, HDL, high-density lipoprotein, LDL, low-density lipoprotein, WBC, white blood cell count, ICD, internal cardioverter/defibrillator, CRT, cardiac resynchronization therapy. n.a., not applicable.
cohort cohorts using quantitative real-time PCR. Additionally, serum samples from 10 HF-REF patients and 10 controls were measured. To this end, custom miRNA primer assays were synthesized by Qiagen (Hilden, Germany) and expression levels of miR-519e, miR-520d-5p, miR-1231, miR-200b, miR-622, miR-122b, miR-551b, miR-345, miR-122b, miR-558, and mir-let-7g were assessed as described previously. For whole blood, the small nuclear RNA RNU6B-2 served as reference and for serum, miR-16 was used.

Statistical analysis

All statistical analyses of array experiments have been carried out using the normalized expression intensities. After verifying approximated normal distribution using Shapiro–Wilk test, we detected differentially regulated miRNAs with relevant diagnostic information content by carrying out two-tailed unpaired t-tests. For each miRNA, we computed the area under the receiver operator characteristics curve (AUC value).

To transform the high-dimensional miRNA data sets of the screening stage to a two-dimensional space in order to graphically represent potential differences between healthy controls, mild–moderate (35% ≤ LVEF < 50%), and severe (LV-EF < 35%) systolic HF patients, we carried out a principal component analysis. Having computed all principal components, we calculated for each sample the first and second component, corresponding to orthogonal components with highest variance. For each of the three cohorts, the mean value of these two components was plotted. For principal component analysis, the prcomp function was used. Linear models have been computed using the lm function of R.

To identify potential multivariate non-ischaemic HF signatures, we applied linear kernel basis Support Vector Machines (SVM) with a stepwise forward filter feature selection technique. The classification has been evaluated using 25 repetitions of 10-fold cross-validation. To test for over-training, we repeated the same classification with 25 permutation tests, i.e. we used the same miRNA expression profiles but randomly permuted class labels. As for the single markers, we computed ROC curves and AUC values for each of the classification results. For classifications, the e1071 work-package of R has been applied. For Kaplan–Meier analysis R has been used calculating P-values using non-parametric log-rank test.

To compute pairwise correlation values between LVEF and the eight miRNAs and in order to determine significance of correlations, we used the freely available R software (version 2.13.0). The software is available for download at http://cran.r-project.org/ (last accessed June 21, 2013). To calculate the Pearson correlation coefficient, we removed missing data and applied the ‘cor’ function. To assess the significance of the correlations, the test statistic based on Pearson’s product moment is calculated, which follows a t distribution with (number of samples − 2) degrees of freedom since the samples approximate two independent

Figure 1 Overall, miRNA expression levels in systolic heart failure patients highly correlate with control subjects. (A) Matrix plot showing high linear correlation of the mean expression of miRNAs in heart failure patients (n = 53) vs. controls (n = 39). (B) The left histogram represents raw P-values for the t-test for all screened miRNAs. The vertical blue line in the first histogram denotes the significance threshold of 0.05. The right graph shows the AUC values over all miRNAs. AUC, area under the curve.
normal distributions. To calculate a regression line, a standard least square regression has been carried out. The regression line is shown in red in the respective plots. \( r \) is the correlation coefficient.

## Results

### Study design and patient characteristics

miRNAs are known to be dysregulated in the course of different cardiovascular disorders. To further elucidate their expression patterns in patients with non-ischaemic systolic heart failure, we examined whole blood samples from affected patients with different degrees of disease severity. In all patients, a relevant CAD was ruled out by coronary angiography. In the screening stage, we included 53 patients with HF-REF (LVEF \( \leq 50\% \)) and 39 individuals with normal LVEF (\( \geq 50\% \)). For detailed patient characteristics, please refer to Table 1.

Non-ischaemic heart failure leads to alterations in miRNA expression patterns

miRNA expression profiles were assessed in all individuals of the screening cohort using microfluidic primer extension assays on a microarray platform. For most miRNAs, we found a high correlation between HF-REF patients and controls as depicted in the correlation plot in Figure 1A. As shown in the given histograms (Figure 1B), raw \( P \)-values for the \( t \)-test for most of the screened miRNAs are above the significance threshold of 0.05, whereas the AUC values over all miRNAs centre around 0.5, as expected. However, some miRNAs were specifically dysregulated in systolic HF (Supplementary material online, Figure S2 for the miRNAs with highest abundance in whole blood). Figure 2 gives an overview on the 20 miRNAs that are up- (A) or down-regulated (B) sorted according to the highest AUC values when comparing HF-REF patients against the control group.

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**Figure 2** Several miRNAs are significantly dysregulated in heart failure patients with reduced ejection fraction. Bar graphs represent the up- (A) and down-regulated (B) miRNAs according to the highest AUC in HF patients. Median relative expression values are given for HF-REF patients normalized to the corresponding values in the control group. Error bars indicate standard deviations. AUC, area under the curve.
**miRNAs as potential biomarkers for systolic heart failure**

To evaluate whether specific miRNAs might be capable of discriminating HF-REF patients from controls, we applied receiver operating characteristic (ROC) analysis for all miRNAs. The best performance as single markers showed miR-558, miR-122*, and miR-520d-5p. As shown in Figure 3A, the AUC values of these three miRNAs ranged from 0.7 to 0.71 with miR-520d-5p displaying the highest discriminatory power as a single marker, exhibiting a sensitivity of 64%, specificity of 74%, and accuracy of 68%.

Although these single miRNAs thus may predict systolic heart failure with already good sensitivity and specificity, we evaluated whether miRNA signatures derived from supervised classification may improve test sensitivity and specificity. We therefore combined the information content of eight selected miRNAs (miR-520d-5p, miR-558, miR-122*, miR-200b*, miR-622, miR-519e*, miR-1231, and miR-1228*). The ROC analyses for all eight miRNAs as single

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**Figure 3** Single miRNAs predict heart failure with reduced ejection fraction. Receiver operating characteristic analyses are given for miR-558, miR-122*, and miR-520d-5p (A), which were found to discriminate heart failure from control patients with highest statistical power. Combining the statistical information of these three miRNAs with five additional miRNAs (miR-200b*, miR-622, miR-519e*, miR-1231, and miR-1228*) even improves sensitivity and specificity as visualized in the corresponding ROC analysis (B). On the right side, red bars indicate accuracy, specificity, and sensitivity of 25 repeated cross-validation runs, while blue bars denote randomized permutation tests serving as negative control. (C) Accuracy, specificity, and sensitivity are given as bar graphs for NT-proBNP and each of the miRNAs in the signature. AUC, area under the curve.
markers for HF-REF are given in Figure 3A and Supplementary material online, Figure S1. Next, we applied statistical learning techniques as SVM with different kernel implementations. The best results for a combined signature were obtained using a radial basis function SVM. The cross-validation procedure was carried out 25 times to gain additional statistical significance. On average, the eight miRNA signature reached an accuracy of 70%, a specificity of 66%, and a sensitivity of 74%. The best AUC for a signature comprising the eight miRNAs was 0.81 (Figure 3B), representing a significant improvement in comparison to each single miRNA marker.

Next, to compare each of the signature miRNAs against established biomarkers of HF, we calculated the diagnostic performance in the individuals in whom NT-proBNP levels were assessed (in total n = 36). If we apply a cut-off of 125 ng/L, we find in our screening cohort a sensitivity of NT-proBNP of 78% and specificity of 44% (Figure 3C). To make a meaningful comparison of the miRNAs with NT-proBNP and avoid overestimation of the performance of the miRNAs, we calculated a cut-off for each miRNA so that we obtained the same specificity as for natriuretic peptides (44%). As shown in Figure 3C, we find a similar or even slightly improved performance of miR-622, miR-520d-5p, miR-519e*, miR-520d, and miR-200b* when compared with NT-proBNP. Only miR-1228*, miR-122*, and miR-1231 showed to be less sensitive markers.

Dysregulation of miRNAs correlates with disease severity in patients with heart failure with reduced ejection fraction

As shown earlier, we find novel miRNAs to be potential diagnostic tools for systolic heart failure. Next, we sought to test if they also correlate to measures of disease severity. Hence, to segregate the informational content of the high-dimensional miRNA data sets with disease severity, we first grouped the cases according to their systolic function into mild–moderate (35 ≤ LVEF < 50%) and severe cases (LVEF < 35%). We then computed the Principal Components of these different data sets. As shown in Figure 4, the expression levels of miRNAs in the control group (LVEF ≥ 50%) are distant from those in the HF-REF groups. Furthermore, the two severity groups within the cases also show a significant distance to each other, together indicating that miRNAs might correlate with different disease stages or severities.

To further evaluate whether expression levels of the signature miRNAs correspond to quantitative aspects of disease severity or clinical symptoms of heart failure, we performed linear correlation analyses between miRNAs and the LVEF or NYHA functional class. As shown in Figures 5 and 6, we find significant correlations with LVEF (P < 0.05) for miR-622, miR-520d-5p, miR-519e*, miR-200b*, and miR-122*, and miR-558, miR-1231 and miR-1228* showed no significant correlation. Concerning NYHA functional class, we could not find a significant correlation for any of the eight miRNAs.

Finally, we performed for all six miRNAs showing significant correlation with disease severity Kaplan–Meier analyses of event-free survival in 43 HF-REF patients with complete follow-up. The combined endpoint of this retrospective analysis included hospitalization due to worsening of HF (six events), heart transplantation (one event), stroke (0 events), or cardiovascular death (three events). The follow-up was up to 1222 days (median 924 days). As shown in Figure 7, we find after correction for multiple testing using the Benjamini–Hochberg method a highly significant separation of event-free survival curves for miR-519e*, indicating that at least this miRNA might not only correlate with disease severity but also provides prognostic information. The estimated cut-off value for miR-519e* was 157 normalized fluorescence intensity.

Replication of miRNAs in independent serum and whole blood samples

To technically and biologically validate the miRNA markers from the screening stage in an independent cohort, we performed quantitative real-time PCR measurements in 14 HF-REF patients and eight controls, referred to as replication cohort (see Table 2 for detailed characteristics). Of the nine tested miRNAs that were found to perform well as single diagnostic markers in the screening stage, we find concordant dysregulation for miR-519e*, miR-520d-5p, miR-1231, miR-200b*, miR-622, and miR-1228 in the replication cohort using quantitative real-time PCR. The AUC values derived from the ROC analyses of the individual miRNAs ranged from 0.56 (miR-200b* and -345) to 0.83 (miR-1228). Regarding the expression changes, miR-622 and miR-1228 also reached statistical significance when comparing cases vs. controls, while miR-520d showed trend to significance (P = 0.07). The AUC values and relative expression changes are given in Figure 8A.

Because it is currently not known how the detected miRNAs are distributed over different cell types, we next sorted white peripheral blood cells for the surface markers CD3 (T cells), CD14 (Monocytes), CD15 (Granulocytes), CD19 (B cells), and CD56 (NK cells). As shown in Figure 8B, we find that the expression profiles over the different cell types align towards granulocytes (CD15+ for miR-519*, -622, and -1228*). In contrast, for miR-200b*, we observe alignment towards CD19+ B cells.

Since the PAXgene approach includes miRNAs from cellular and non-cellular fractions of the whole blood sample, we next also evaluated RNA from dedicated serum samples for the abundance and
Figure 5 miRNA expression levels correlate with disease severity. Matrix plots visualize the correlation of miRNAs from the signature with cardiac systolic function and with NYHA functional class. MiR-622, miR-520d-5p, miR-519e* and miR-200b* significantly correlate with left-ventricular ejection fraction ($P < 0.05$). No significant correlation could be found between miRNA expression levels and the corresponding NYHA class, although a trend can be observed.
potential dysregulation of these miRNAs. While miR-520d-5p, -588, -122*, and -519e* could not be detected in serum, miR-200b, -622, and -1228* were significantly higher represented in HF-REF than in controls (Figure 8C).

Discussion

Elucidating the potential role of miRNAs as biomarkers for cardiovascular disorders is of major interest. In the present study, we evaluated the changes of the human miRNome related to non-ischaemic systolic heart failure. To do so, we assessed whole blood samples from HF-REF patients with varying disease severity and found several miRNAs to be dysregulated. These miRNAs can aid in the distinction of affected individuals from healthy controls and their expression levels also correlate with disease severity.

Tijsen et al. recently assessed expression levels of miRNAs in plasma samples from patients with heart failure and identified six miRNAs to be significantly dysregulated. One of these was miR-622, which is in parallel elevated in myocardium of patients with non-ischaemic HF. We also find miR-622 to be significantly enriched in response to systolic HF in our whole blood approach, showing good sensitivity in detecting HF as well as correlating to LVEF. Besides miR-622, Tijsen et al. showed that miR-423-5p is significantly correlated with disease severity (Figure 6).
Various immune active cell types, cytokines, and chemokines are involved in the cascades triggering maladaptive pathways of the myocardium, supporting remodelling and failure. 

Part of this aggregate information is also present in plasma or serum, since we find three of our miRNAs to be significantly elevated in serum. This might point to the so-called convergence of serum and cellular miRNAs, meaning that miRNAs released from tissue can be taken up by different cell types and vice versa. 

A whole blood approach may therefore be a valuable tool to capture all the important biological information in a single assay.

The pathophysiological mechanisms that lead to the dysregulation of the detected miRNAs remain elusive. To date, it is even unknown for most of the here-described miRNAs if they are ubiquitously expressed or in a tissue-specific manner. In a miRNA-mRNA pathway analysis of our eight miRNA signature, a significant over-representation of MAPK signalling (P = 0.02), cardiac muscle contraction (P = 0.01), Notch pathway (P = 0.01), and cardiac muscle fibre development (P = 0.007) are found, hinting to a potential role of the here identified miRNA biomarkers in cardiac function. Secondary effects of HF on non-cardiovascular tissues and organ systems might also promote miRNA dysregulation, potentially reflecting heart failure as a syndrome. Andersson et al. 

evaluated miRNA expression levels in a porcine cardiogenic shock model and found miR-122 to be significantly up-regulated in blood, with hypothermia reducing its levels. Its minor form, miR-122* was also among the most significantly up-regulated miRNAs in the present study with good discriminative power as a single marker for systolic HF. Interestingly, in the described study, they speculated that miR-122 originates from hepatic cells in response to diminished cardiac output and resulting liver congestion.

Potential limitations of the current study are the overrepresentation of male HF-REF patients and the statistically not significant decrease in kidney function of affected individuals. However, we analysed the expression patterns of the eight identified miRNAs comprising our diagnostic signature in regard to their gender specificity in a cohort of seven male and 13 female individuals and did not find any gender-specific deviations (P-values ranged from 0.21 to 0.95). Another limitation is the relatively small size of our replication cohort. This may have led to the fact that we could only achieve significance for a part of the studied miRNAs. Finally, we could benchmark miRNAs against NT-proBNP only in a subgroup of individuals and quantification of LVEF was routinely done by LV angiography, not by echocardiography.

Different options in heart failure treatment are applied in a stepwise manner according to disease severity. As mentioned earlier, this should not be solely based on the patients’ symptoms that may be subjective or inconsistent, but should be complemented by quantitative measures based on cardiac imaging or molecular biomarkers. Despite their tremendous clinical importance in disease detection and risk prediction, even well-established HF biomarkers such as natriuretic peptides may show relatively moderate AUC values of 0.71–0.83 depending on the cohorts studied. 

In our low risk cohort with only three cardiovascular deaths within a median follow-up period of 924 days, we find a better sensitivity for four miRNAs compared with NT-proBNP, making them attractive candidates for further translation into clinical application. Additionally, the miRNAs show a good correlation to disease severity as judged by LVEF and in case of

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Table 2  Patient characteristics of replication cohort

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 8)</th>
<th>HF-REF patients (n = 14)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
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<td>Age (years)</td>
<td>59 ± 12</td>
<td>59 ± 8</td>
<td>0.8</td>
</tr>
<tr>
<td>Male/female (n/n)</td>
<td>4/4</td>
<td>8/6</td>
<td>0.8</td>
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<td>Current smoking, n (%)</td>
<td>3 (38)</td>
<td>4 (28)</td>
<td>0.7</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>55 ± 3</td>
<td>55 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NYHA functional class</td>
<td>n.a.</td>
<td>LVEF 35–50%:</td>
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<td>1.8 ± 1</td>
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<tr>
<td></td>
<td></td>
<td>LVEF &lt; 35%:</td>
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<td>2.6 ± 1.2</td>
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<tr>
<td>Previous hospitalizations due to HF, n (%)</td>
<td>0</td>
<td>3 (21)</td>
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<tr>
<td>Rales/signs of pulmonary oedema, n (%)</td>
<td>—</td>
<td>2 (14)</td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>123 ± 20</td>
<td>128 ± 25</td>
<td>0.6</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>70 ± 13</td>
<td>74 ± 15</td>
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<td>DM, n (%)</td>
<td>3 (38)</td>
<td>4 (29)</td>
<td>0.7</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>5 (63)</td>
<td>8 (57)</td>
<td>0.8</td>
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<tr>
<td>HDL (mmol/L)</td>
<td>1.3 ± 0.1</td>
<td>0.9 ± 0.7</td>
<td>0.4</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.7 ± 0.3</td>
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<td>WBC (×10⁹/L)</td>
<td>8.2 ± 2</td>
<td>8.2 ± 1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>88 ± 28</td>
<td>82 ± 22</td>
<td>0.6</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>5.1 ± 2.4</td>
<td>5.4 ± 2.3</td>
<td>0.8</td>
</tr>
<tr>
<td>ICD, n (%)</td>
<td>—</td>
<td>1 (7)</td>
<td></td>
</tr>
<tr>
<td>CRT</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

LVEF, left-ventricular ejection fraction; NYHA, New York Heart Association; HF, heart failure; SBP, systolic blood pressure; DBP, diastolic blood pressure; DM, diabetes mellitus; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; WBC, white blood cell count; ICD, internal cardiovertor/defibrillator; CRT, cardiac resynchronization therapy.

n.a., not applicable.

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most significantly related to the clinical diagnosis of heart failure. In our study cohort, miR-423-5p was also up-regulated in HF-REF patients, but without reaching statistical significance. Very interestingly, Tijssen et al. measured miRNAs from plasma samples, while we assessed whole peripheral blood. In contrast to plasma or serum samples, our approach not only accounts for tissue-released miRNAs but comprises miRNAs from all circulating cells. Hence, we further investigated in which cell types the different miRNAs are expressed most abundantly. Interestingly, we find high expression of most of the here-described miRNAs if they are ubiquitously expressed or in a tissue-specific manner. In a miRNA-mRNA pathway analysis of our eight miRNA signature, a significant over-representation of MAPK signalling (P = 0.02), cardiac muscle contraction (P = 0.01), Notch pathway (P = 0.01), and cardiac muscle fibre development (P = 0.007) are found, hinting to a potential role of the here identified miRNA biomarkers in cardiac function. Secondary effects of HF on non-cardiovascular tissues and organ systems might also promote miRNA dysregulation, potentially reflecting heart failure as a syndrome. Andersson et al. evaluated miRNA expression levels in a porcine cardiogenic shock model and found miR-122 to be significantly up-regulated in blood, with hypothermia reducing its levels. Its minor form, miR-122* was also among the most significantly up-regulated miRNAs in the present study with good discriminative power as a single marker for systolic HF. Interestingly, in the described study, they speculated that miR-122 originates from hepatic cells in response to diminished cardiac output and resulting liver congestion.

Potential limitations of the current study are the overrepresentation of male HF-REF patients and the statistically not significant decrease in kidney function of affected individuals. However, we analysed the expression patterns of the eight identified miRNAs comprising our diagnostic signature in regard to their gender specificity in a cohort of seven male and 13 female individuals and did not find any gender-specific deviations (P-values ranged from 0.21 to 0.95). Another limitation is the relatively small size of our replication cohort. This may have led to the fact that we could only achieve significance for a part of the studied miRNAs. Finally, we could benchmark miRNAs against NT-proBNP only in a subgroup of individuals and quantification of LVEF was routinely done by LV angiography, not by echocardiography.

Different options in heart failure treatment are applied in a stepwise manner according to disease severity. As mentioned earlier, this should not be solely based on the patients’ symptoms that may be subjective or inconsistent, but should be complemented by quantitative measures based on cardiac imaging or molecular biomarkers. Despite their tremendous clinical importance in disease detection and risk prediction, even well-established HF biomarkers such as natriuretic peptides may show relatively moderate AUC values of 0.71–0.83 depending on the cohorts studied. In our low risk cohort with only three cardiovascular deaths within a median follow-up period of 924 days, we find a better sensitivity for four miRNAs compared with NT-proBNP, making them attractive candidates for further translation into clinical application. Additionally, the miRNAs show a good correlation to disease severity as judged by LVEF and in case of...
Figure 8 Validation of whole-blood miRNAs in whole blood and in serum. (A) Selected miRNAs (miR-519e*, miR-520d-5p, miR-1231, miR-200b*, miR-622, miR-1228*, miR-551b*, miR-345, and miR-let-7g) were assessed by quantitative real-time PCR in an independent cohort. Relative fold quotients and AUC values are given for each miRNA. (B) Relative expression of miRNAs in CD3 (T cells), CD14 (Monocytes), CD15 (Granulocytes), CD19 (B cells), and CD56 (NK cells) positive cells as indicated. (C) In serum samples, miR-200b*, miR-622, and miR-1228* also show significant up-regulation in heart failure with reduced ejection fraction patients when compared with controls (red dashed line), while miR-1231 shows a trend towards up-regulation ($^{*}P < 0.05; ^{**}P < 0.01$). miR-520d-5p, miR-558, miR-122*, and miR-519e* could not be detected in serum. AUC, area under the curve.
miR-519e also to patients’ outcome. However, there are several studies showing extraordinary sensitivity and good specificity of natriuretic peptides in large HF cohorts and it will be interesting to assess the here identified miRNAs in equally sized cohorts. 32, 33

Many uncertainties remain, which could explain the fact that some miRNA biomarker candidates failed replication in independent studies. 34 Technical challenges such as phenotyping, biosampling, sample handling, RNA processing, and miRNA quantification could impact on this fact. For instance, it is well known that the concordance rate between micro-arrays (hybridization-based) and real-time PCR (PCR-based) is not complete. The here used MPEA assay provides a high correlation to PCR-based approaches and shows little cross-hybridization between similar miRNA sequences. 35 However, we do not see a complete concordance between our screening and replication cohort exemplified by the diverging behaviour of miR-let7g. This might have technical or biological reasons. Only recently, a genetic testing for the channelopathies and cardiomyopathies this document was developed as a partnership between the Heart Rhythm Society (HRS) and the European Heart Rhythm Association (EHRA). Heart Rhythm 2011;8:1308–1339.


