European Journal of Heart Failure (2015) **17**, 393–404
doi:10.1002/ejhf.223

Circulating microRNAs in heart failure with reduced and preserved left ventricular ejection fraction

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Received 31 July 2014; revised 11 November 2014; accepted 14 November 2014; online publish-ahead-of-print 23 January 2015

Aim The potential diagnostic utility of circulating microRNAs in heart failure (HF) or in distinguishing HF with reduced vs. preserved left ventricular ejection fraction (HFREF and HFPEF, respectively) is unclear. We sought to identify microRNAs suitable for diagnosis of HF and for distinguishing both HFREF and HFPEF from non-HF controls and HFREF from HFPEF.

Methods and results MicroRNA profiling performed on whole blood and corresponding plasma samples of 28 controls, 39 HFREF and 19 HFPEF identified 344 microRNAs to be dysregulated among the three groups. Further analysis using an independent cohort of 30 controls, 30 HFREF and 30 HFPEF, presented 12 microRNAs with diagnostic potential for one or both HF phenotypes. Of these, miR-1233, -183-3p, -190a, -193b-3p, -193b-5p, -211-5p, -494, and -671-5p distinguished HF from controls. Altered levels of miR-125a-5p, -183-3p, -193b-3p, -211-5p, -494, -638, and -671-5p were found in HFREF while levels of miR-1233, -183-3p, -190a, -193b-3p, -193b-5p, and -545-5p distinguished HFPEF from controls. Four microRNAs (miR-125a-5p, -190a, -550a-5p, and -638) distinguished HFREF from HFPEF. Selective microRNA panels showed stronger discriminative power than *N*-terminal pro-brain natriuretic peptide (NT-proBNP). In addition, individual or multiple microRNAs used in combination with NT-proBNP increased NT-proBNP's discriminative performance, achieving perfect intergroup distinction. Pathway analysis revealed that the altered microRNAs expression was associated with several mechanisms of potential significance in HF.

Conclusions We report specific microRNAs as potential biomarkers in distinguishing HF from non-HF controls and in differentiating between HFREF and HFPEF.

Keywords MicroRNAs • Biomarkers • Heart failure • Diagnosis

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Introduction

Circulating biomarkers are of prime importance in the clinical management of heart failure (HF)[1] and may provide insight into the underlying pathophysiology of HF with reduced vs. preserved ejection fraction (HFREF vs. HFPEF).[2] The cardiac natriuretic peptides, B-type natriuretic peptide (BNP) and its co-secreted congener, amino-terminal pro-B type natriuretic peptide (NT-proBNP) have proven diagnostic utility in acute HF and are independently related to prognosis at all stages of HF, leading to their use in HF diagnosis and management.[1,3] However, confounders including obesity and atrial fibrillation impair their diagnostic performance.[4,5] In asymptomatic left ventricular dysfunction, early symptomatic HF and treated HF, the discriminating power of B peptides is markedly diminished with half of all stable HFREF cases exhibiting BNP below 100 pg/mL and 20% have NT-proBNP below values employed to rule out HF in the acutely symptomatic state.[6] This loss of test performance is even more pronounced in HFPEF.[7] Therefore, there is an unmet need for markers that complement or replace B-type peptides in screening for HF in its early or partly treated state and in monitoring status in the chronic phase. This is particularly true for HFPEF.[8] In addition, as part of the effort to improve our understanding of the pathology of HFPEF compared with HFREF there is an ongoing search for biomarkers that may point to differing underlying mechanisms and subsequently to specific therapeutic targets in HFPEF.[9]

MicroRNAs (miRNAs), a class of small non-coding RNAs, are increasingly recognized as playing key roles in cardiac differentiation, development and in the cardiac response to stress and injury.[10] Several miRNAs arrays in human heart tissue have been reported[11,12] and a few have addressed plasma miRNAs profiles in HF. Tijsen *et al.*[13] suggested miR-423-5p as a diagnostic marker for HF. Others have identified miRNAs (miR-1, -133, -499, and -208) as markedly elevated in acute myocardial infarction (MI).[14,15] To date, only one report has considered the possibility that miRNAs may distinguish HFPEF from HFREF.[16] Reports of miRNAs profiles in whole blood in HF are even more sparse. Vogel *et al.*[17] reported several whole-blood miRNAs that correlate with disease severity in HFREF.

In the present report, we compare the circulating miRNAs profiles between controls without history of HF and patients with HFREF and HFPEF; all prospectively recruited in a nationwide HF study and characterized by comprehensive clinical examination, Doppler echocardiography and measurement of NT-proBNP.[18] We hypothesize that circulating miRNAs profiles could distinguish HF from non-HF controls, and HFREF from HFPEF.

Methods

HF and healthy control cohorts

Patients with HF were recruited from the Singapore Heart Failure Outcomes and Phenotypes (SHOP) study.[18] Patients were included if they presented with a primary diagnosis of HF or attended clinics for management of HF within 6 months of an episode of HF decompensation. Controls without overt coronary artery disease or history of HF were recruited through the ongoing epidemiological Singapore Longitudinal Ageing Study (SLAS).[2] All patients and controls underwent detailed clinical examination to establish the presence (or absence) of clinical HF, as well as comprehensive Doppler echocardiography. Left ventricular ejection fraction (LVEF) was assessed using the biplane method of disks on two-dimensional (2D) echo, as recommended by the American Society of Echocardiography (ASE) guidelines. Patients with validated HF and LVEF $\geq$50% were categorized as HFPEF, whereas those with LVEF $\leq$40% were classified as HFREF. Patients with EF between 40% and 50% were excluded. Assessments including blood samples were deliberately undertaken when patients had received treatment (typically for 3–5 days), were symptomatically improved with resolution of bedside physical signs of HF, and were considered fit for discharge. This ensured assessment of marker performance in the treated or 'chronic' phase of HF when B-peptides' test performance is challenged as described in the Introduction. This study complied with the Declaration of Helsinki. All individuals provided written informed consent and the cohort study protocol was approved by the National Health Group Domain Specific Review Board (NHG DSRB Reference Code: 2010/00114)) and the Institutional Review Board of National University of Singapore (NUS-IRB Reference Code: 04–140).

Blood and plasma sample collection

Peripheral venous blood drawn into ethylenediaminetetraacetic acid (EDTA) tubes were spun at 3488 g for 10 min and the plasma separated and stored at −80 °C before assay in batches. A separate 0.5 mL aliquot of blood was pipetted into a 2.0 mL microfuge tubes containing 1.3 mL RNALater (Ambion, Austin, TX, USA) and stored at −80 °C until further processing.

Measurement of NT-proBNP

Plasma NT-proBNP was measured by electrochemiluminescence immunoassay (Elecsys proBNP II assay) on an automated Cobas e411 analyser according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Measurements were made as single tests and the quality controls (high and low) measured in each batch run were within 2 standard deviation (SD) values, meeting the acceptance criteria for a valid assay as specified by the manufacturer. The range for detection for NT-proBNP was 5.22–35 000 pg/mL.

Total RNA extraction

Total RNA + miRNAs were isolated from whole blood using the Ribopure™ Blood RNA isolation Kit (Ambion) according to the manufacturers' protocols. Cel-miR-39 was used as a spike-in control for plasma miRNAs. Extraction of total RNA from plasma samples were performed using the Exiqon Biofluids Kit (Exiqon, Vedbaek, Denmark). Cel-miR-39 mimics were commercially purchased from Ambion and 10 pmoles of Cel-miR-39 was spiked into 60 μL of the lysis buffer. This was then added to 200 μL of plasma samples and processed according to manufacturer's recommendations. RNA concentration was determined using ND-1000 Spectrophotometer (Nanodrop™; Rockland, Wilmington, DE, USA). The integrity of whole blood RNA samples was verified using denaturing gel electrophoresis (15% polyacrylamide) and Bioanalyzer 2100 (Agilent Technology, Santa Clara, CA, USA). Whole-blood RNA samples displaying a RNA integrity number of $\geq$7.5 were subsequently selected for microarray and quantitative real-time polymerase chain reaction (PCR) (qPCR) analyses. All RNA samples were stored at −80 °C until further processing.

MiRNA microarray and statistical analysis

MiRNA profiling was performed using LNA™ modified oligonucleotide (Exiqon) probes (Sanger miRBase ver. 12.0, 16.0, and 18.0).[19] Total RNA (1 μg) was 3′-end-labelled with Hy3 dye and hybridized on miRCURY LNA™ arrays according to the manufacturer's protocol (Exiqon). The microarray chips were then washed and scanned using InnoScan700, (Innopsys, Carbonne, France) and analysed on Mapix® Ver4.5 software (Innopsys). Analysis was performed using Partek® 6.6 Genomics Suite software (Partek Inc., St Louis, MO, USA). Background-subtracted median signal intensities were normalized using two independent methods: endogenous and quantile normalization. The normalized values were log2 transformed and miRNAs that exhibited an absolute fold change ≥ 1.2 and ≤ -1.2 and $P < 0.05$ after Benjamini–Hochberg false discovery rate (FDR) correction following multiple comparisons were considered for further analysis. K-Means hierarchical clustering was performed using Partek 6.6 Genomics Suite software (Partek Inc.) with *z*-scores transformed from the original normalized values. By default, the expression of each gene was standardized to mean 0 and standard deviation of 1. Upregulated genes have positive values and are displayed red. Downregulated genes have negative values and are displayed green. All statistical analyses were performed using the statistical tools provided by the software. Results of the microarray data have been deposited into GEO database under the accession number GSE53437. Further selection of miRNAs was based on receiver-operator curve (ROC) and Pearson correlation analyses, which were performed using the online PSPP (http://www.gnu.org/software/pspp/) and MedCalc (http://www.medcalc.org/) statistical tools.

Quantitative PCR and statistical analysis

Verification of miRNAs in blood and plasma was carried out using TaqMan qPCR.[20] All reactions were carried out, using the Applied Biosystems 7900 HT sequence detection system, according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). Reactions were performed in triplicate using individual patient samples. RNU44 was used as the housekeeping gene for blood miRNAs. A 10 ng sample of total RNA was reverse transcribed using the respective miRNA/cel-miR-39 stem-loop primers. The cDNA was then used for qPCR amplification using the respective miRNA/cel-miR-39 specific Taqman primer-probes. The Ct (Threshold cycle) values obtained for each hsa-miRNA were then normalized to the respective RNU44/cel-miR-39 Ct values to obtain the ΔCt values and eventually used to plot relative expression values. The statistical significance of qPCR data was calculated using two-tailed *t*-tests. Receiver operator curves characterizing the diagnostic performance of candidate miRNAs and NT-proBNP were calculated using MedCalc software.

Results

Clinical characteristics

One hundred and seventy-six individuals participants were enrolled. Their clinical characteristics and demographics are summarized in *Table 1*. Cohort 1 with 86 individuals comprising 28 controls and 58 HF (39 HFREF and 19 HFPEF) patients was used for screening. Cohort 2 comprising 30 controls and 60 HF (30 HFREF and 30 HFPEF) patients was used for the validation study. Controls in both cohorts were free from chronic obstructive pulmonary disease (COPD), coronary artery disease (CAD), atrial fibrillation, and a history of stroke. Overall, HF patients exhibited significantly higher body mass index, NT-proBNP levels, and heart rate compared with controls.

MiRNA profiling in whole blood

MiRNA profiles were generated from cohort 1 blood samples where 806 miRNAs were commonly detected across all 86 arrays (miRBase versions 12–18). Using two independent methods of normalization, along with two-way ANOVA, Bonferroni correction (FDR $P < 0.05$) and a fold difference cut-off of >1.2 or < -1.2, 344 of 806 (~43%) showed a higher level of variation across the three categories; i.e. differences in miRNA levels were observed in the following comparisons: (i) HF vs. controls, (ii) HFREF vs. controls, (iii) HFPEF vs. controls, and (iv) HFREF vs. HFPEF. Hierarchical clustering of the miRNA profiles also showed segregation according to HF subtypes (see the Supplementary material online, *Figure S1*). Verification of profiling data was performed using qPCR on 10 randomly selected miRNAs and the data were seen to be consistent (see the Supplementary material online, *Figure S2*).

Identification of miRNAs in plasma

All 806 miRNAs detected in whole blood were also found in plasma, albeit at lower levels. Expression patterns of the 344 miRNAs were subjected to statistical scrutiny involving ROC and Pearson correlation analyses. This filtering identified 90 miRNAs satisfying either one of the conditions: area under the curve (AUC) >0.7 or Pearson correlation with respect to LVEF% (*r*-values >0.5 or < -0.5), were selected for further study (*Figure 1*; Supplementary material online, *Table S1*) The raw intensities obtained for the individual arrays for all 90 miRNAs are listed in the Supplementary material online, *Table S2*.

Expression of these 90 miRNAs was quantified in cohort 1 plasma by qPCR and highly detectable miRNAs, (i.e. with a cut-off Ct value <35 out of a total cycle of 50 cycles), were further selected. Thirty-two miRNAs (miR-1225-3p, -1233, -125a-5p, -1299, -130a-5p, -1322, -145-3p, -17-3p, -1825, -183-3p, -186-5p, -190a, -193b-3p, -193b-5p, -204-5p, -211-5p, -301a-3p, -320d, -326, -361-3p, -423-3p, -431-5p, -485-3p, -494, -509-3p, -545-5p, -550a-5p, -625−5p, -629-3p, -638, -671-5p, and -92b-3p) were shortlisted as their detection levels in HF plasma differed from controls. The expression of these 32 miRNAs was found to be comparable with those of the whole blood-derived array (see the Supplementary material online, *Table S3*).

Validation study in an independent cohort

Validation of the 32 selected miRNAs was undertaken using qPCR on plasma RNA obtained from the second cohort of participants (cohort 2; *Table 1*). Among these, a total of 12 miRNAs (miR-1233, -125a-5p, -183-3p, -190a, -193b-3p, -193b-5p, -211−5p, -494, -545−5p, -550a-5p, -638 and -671-5p) were found

Table 1 Clinical characteristics of the individuals involved in the study

	Cohort 1 (screening in blood and plasma)				Cohort 2 (validation in plasma)			
	Controls ($n = 28$)	All HF ($n = 58$)	HFREF ($n = 39$)	HFPEF ($n = 19$)	Controls ($n = 30$)	All HF ($n = 60$)	HFREF ($n = 30$)	HFPEF ($n = 30$)
Age, years	58.46 ± 1.62	60.21 ± 1.44	56.97 ± 1.35†	66.84 ± 2.91*†	65.93 ± 6.72	64.73 ± 8.18	65.38 ± 7.27	64.101 ± 9.05
BMI, kg/m^2	23.23 ± 0.68	26.93 ± 0.72*	26.84 ± 0.86*	27.12 ± 1.34*	26.66 ± 3.33	26.26 ± 5.68	24.15 ± 5.68*†	28.43 ± 4.88†
NT-proBNP, pg/mL	57.72 ± 8.90	3084.57 ± 421.27*	3598.45 ± 520.62*	2029.75 ± 670.13*	86.41 ± 83.19	4177.22 ± 5382.62*	6727.18 ± 6289.86*†	1712.26 ± 2638.03*
LVEF, %	63.44 ± 0.62	38.67 ± 2.32*	28.10 ± 1.66*†	60.38 ± 1.08*†	64.17 ± 4.05	41.25 ± 18.45*	25.12 ± 7.72*†	59.32 ± 5.17*†
NYHA classification	All class I	I: 6; II: 41; III: 10; IV:1	I: 5; II: 27; III: 7	I: 1; II: 14; III: 3; IV:1	All class I	I: 16; II: 30; III: 14; IV: 0	I: 8; II: 13; III: 9; IV: 0	I: 8; II: 17; III: 5; IV:
Heart rate, beats/min	65.14 ± 1.67	74.42 ± 1.95*	72.18 ± 1.82	78.89 ± 4.51*	66.70 ± 10.60	75.44 ± 13.51*	73.79 ± 13.69*	77.03 ± 13.36*
Systolic BP, mmHg	129.29 ± 3.32	120.02 ± 2.85	118.47 ± 3.6 7	123.11 ± 4.43	141.73 ± 20.80	121.86 ± 19.72*	118.55 ± 19.23*	125.07 ± 19.98*
Diastolic BP, mmHg	74.75 ± 1.64	69.91 ± 1.68	71.76 ± 2.31	66.21 ± 1.79*	78.63 ± 11.12	67.31 ± 9.25*	67.34 ± 9.69*	67.27 ± 8.96*
Clinical covariates								
Diabetes, %	3.6	62.1*	61.5*	63.2*	10.0	69.3*	73.3*	63.3*
Hypertension, %	7.1	81.0*	82.1*	78.9*	63.3	76.7	73.3*	80.0
CAD, %	0	69.0*	84.6*	36.8*†	0	48.3*	73.3*	23.3*†
Previous stroke, %	0	6.9	10.3	0	0	10.0	10.0	10.0
COPD, %	0	6.9	10.3	0	0	5.0	6.7	3.3
Atrial fibrillation, %	0	27.6*	12.8	57.9*†	0	25.0*	20.0*	30.0*
Medications (%)								
ACE-I or ARB	0	89.7*	92.3*	84.2*	20	75.0*	83.3*	66.7*
Diuretic	0	87.9*	92.3*	78.9*	10	88.3*	93.3*	83.3*
Beta blocker	7.1	91.4*	92.3*	89.5*	26.7	91.7*	93.3*	90*
Spironolactone	0	39.7*	56.4*	5.3†	0	38.3*	73.3*	3.3†
Anti-platelets	0	75.9*	84.6*	57.9*†	3.3	80.0*	93.3*	66.7*†
Nitrates	0	50.0*	53.8*	42.1*	0	48.3*	46.7*	50*
Calcium channel blockers	3.6	24.1*	15.4	42.1*†	20	33.3	20	46.7*†
Digoxin	0	22.4*	20.5*	26.3*	0	21.7*	23.3*	20*
Statins	7.1	86.2*	89.7*	78.9*	36.7	86.7*	93.3*	80*
Anti-diabetic agents	3.6	63.8*	64.1*	63.2*	6.7	60.0*	66.7*	53.3*

ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease; BMI, body mass index; BP, blood pressure; HFPEF, heart failure with preserved ejection fraction; HFREF, heart failure with reduced ejection fraction; NT-proBNP, *N*-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association. Values are mean ± SEM.

*$P < 0.05$ vs. control,

†$P < 0.05$ between HFREF and HFPEF by one-way analysis with Bonferroni correction or χ^2 test.

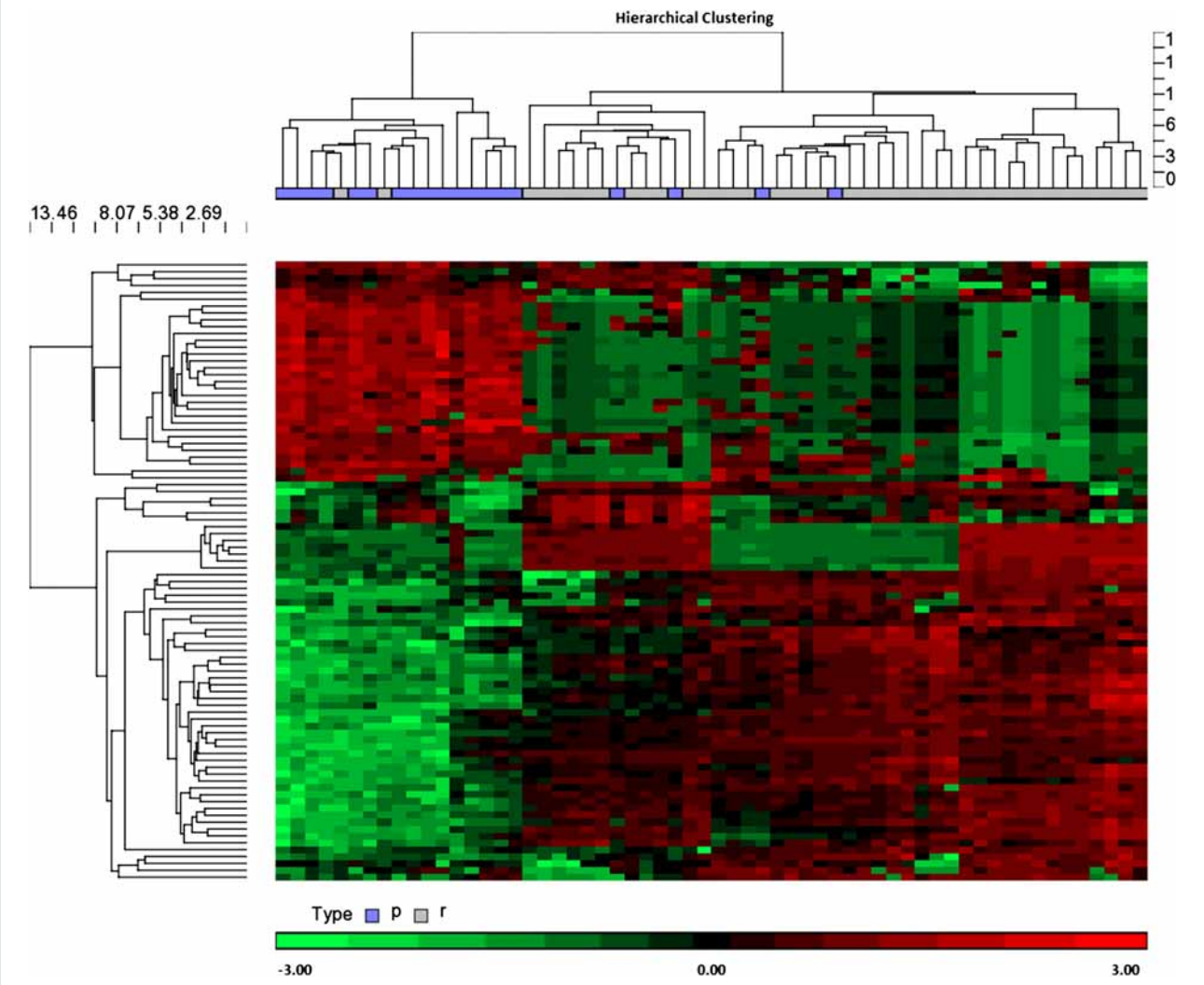

Figure 1 Differentially detected microRNAs (miRNAs) in heart failure relative to controls. Ninety miRNAs were found to be differentially detected (based on receiver operating characteristic (ROC) analysis and Pearson correlation analysis in relation to left ventricular ejection fraction (LVEF) data) in blood profiles of heart failure (HF) patients compared with non-HF controls. Grey denotes patients clinically classified as heart failure with reduced ejection fraction (HFrEF) and purple denotes heart failure with preserved ejection fraction (HFpEF).

to be dysregulated with several of them being significant in more than one category (see the Supplementary material online, *Table S3*). These 12 miRNAs could segregate HF, HFREF, and HFPEF from non-HF controls as well as HFREF from HFPEF.

HF vs. controls

Expression levels of miR-1233 and miR-671-5p were found to be upregulated in overall HF patients while miR-183−3p, -190a, -193b-3p, 193b-5p, -211-5p, and -494 were found to be downregulated in overall HF patients when compared with controls (*Figure 2*).

HFREF vs. controls

Expression levels of miR-125a-5p, -183-3p, -193b-3p, -211-5p, -494, -638 and -671-5p were observed to differ significantly between HFREF and control samples. MiR-125a-5p and -671-5p were upregulated whereas the rest were downregulated in HFREF. MiR-671-5p showed the highest expression (3.47 ± 0.57; $p < 0.01$) in HFREF patients (*Figure 2*; Supplementary material online, *Table S3*).

HFPEF vs. controls

Six miRNAs, miR-1233, -183-3p, -190a, -193b-3p, -193b-5p and -545-5p, showed significant differences in expression between HFPEF and controls. Expressions of miR-1233 and -545-5p were increased whereas the rest were decreased in HFPEF patients compared with controls (*Figure 2*). The most significant increase in expression for HFPEF was seen in miR-545−5p (3.02 ± 1.13; $P < 0.05$; *Figure 2*; Supplementary material online, *Table S3*).

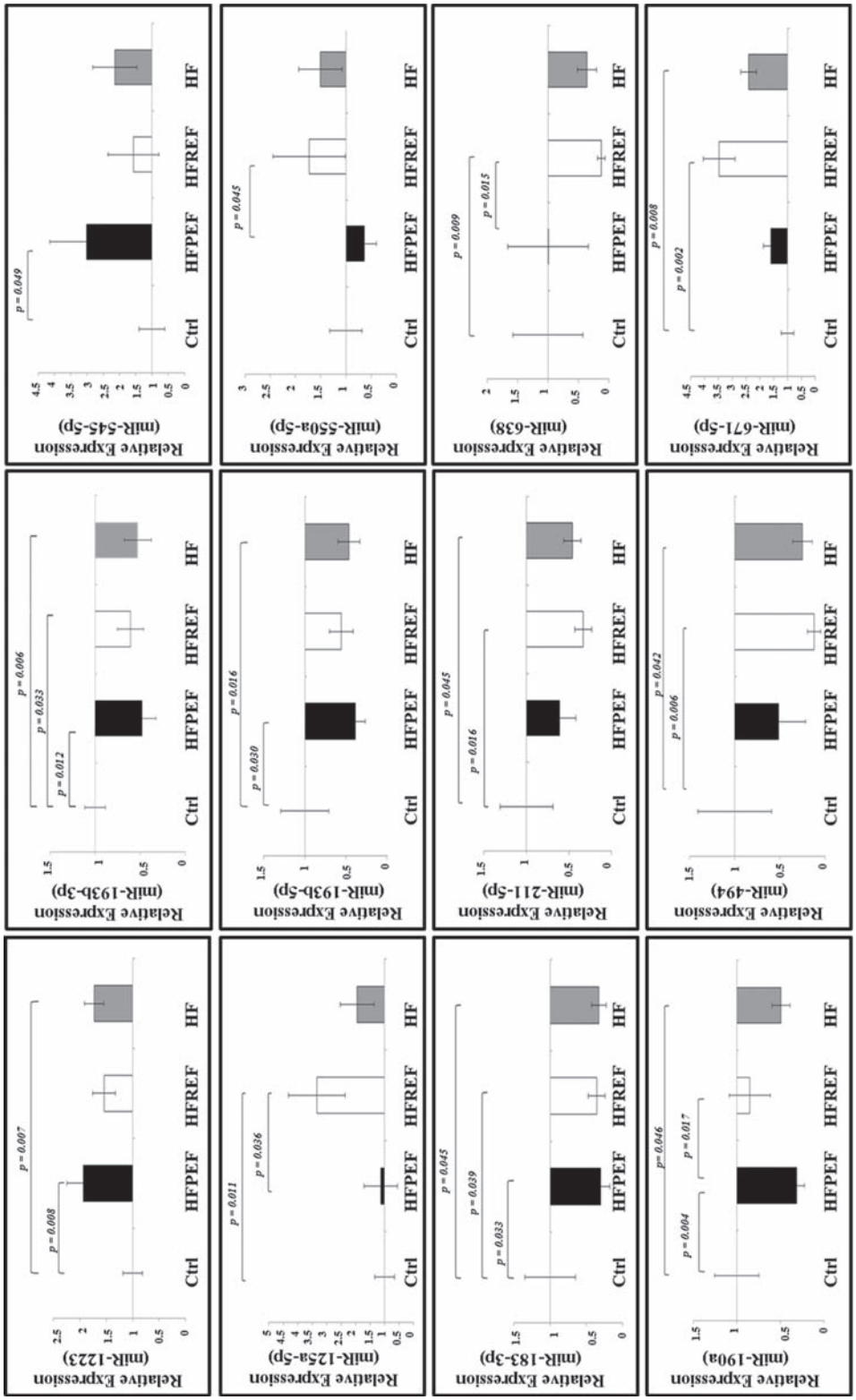

Figure 2 Significantly detected microRNAs (miRNAs) in the plasma of heart failure (HF) patients. Findings in blood were validated in plasma samples using quantitative stem-loop real-time polymerase chain reaction (PCR) in an independent cohort of 90 individuals. After selection, 12 miRNAs showed significant change in levels between HF and non-HF controls and/or between heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF). Eight miRNAs were identified as potential HF markers (miR-1233, -183-3p, -190a, -193b-3p, -193b-5p, 211-5p, -494, and -671-5p). Seven miRNAs were identified as potential HFREF markers (miR-125a-5p, -183-3p, -193b-3p, -211-5p, -494, -638, and -671-5p). Six miRNAs were identified as potential HFPEF markers (miR-1233, -183-3p, -190a, -193b-3p, -193b-5p, and -545-5p). Four miRNAs showed significant differences between HFREF from HFPEF (miR-125a-5p, -190a, -550a-5p, and -638). Data represented in bar charts are mean ± SD Statistical analysis was performed using Student's *t*-test.

HFREF vs. HFPEF

The expressions of miR-125a-5p, -190a, -550a-5p, and -638 were found to differ significantly between HFREF and HFPEF patients (*Figure 2*). Notably, miR-125a-5p (FC = 3.34, $P < 0.05$) was highly upregulated in HFREF but remained close to control levels in HFPEF (*Figure 2*; Supplementary material online, *Table S3*). Conversely, mir-638 (FC = −8.33, $P < 0.01$) was downregulated in HFREF but remained close to control levels in HFPEF (*Figure 2*; see the Supplementary material online, *Table S3*). MiR-190a was significantly downregulated in both HFPEF (FC = −3.18, $P < 0.01$) and HF (FC = −2.03, $P < 0.01$) compared with controls. However, expression of miR-190a in HFREF (FC = 1.18, $P < 0.05$) was similar to that in controls. Notably, among these four miRNAs, miR-550a-5p showed a directionally opposite expression pattern between HFREF (FC = 1.73, $P < 0.05$) and HFPEF (FC = −1.58, $P < 0.05$).

Determination of the diagnostic potential of selected miRNAs

To determine the diagnostic potential of the 12 miRNAs, ROC analyses were performed on the qPCR data from cohort 2. The diagnostic potential for each miRNA is listed for the respective categories (*Table 2*). The highest predictive potential was seen for miR-671-5p (AUC = 0.75), miR-671-5p (AUC = 0.89), miR-1233/miR-193b-3p (AUC = 0.69) and miR-190a (AUC = 0.66), respectively, for comparison of HF, HFREF, HFPEF and HFREF vs. HFPEF. Combined ROC analyses for miRNA panels strengthened the diagnostic potential by increasing the AUC values to 1.0, 1.0, 0.89, and 0.80 for the HF, HFREF, HFPEF, and HFREF vs. HFPEF categories, respectively. In comparison, NT-proBNP exhibited 0.94, 1.0, 0.89, and 0.83 AUC values for HF, HFREF, HFPEF, and HFREF vs. HFPEF categories, respectively. Thus the HF miRNA panel was a stronger discriminator than NT-proBNP for distinguishing all HF from control, whereas the HFREF and HFPEF miRNA panels were of equal power (*Table 2*). HFREF vs. HFPEF miRNA panel showed slightly lower discriminative power when compared with NT-proBNP.

Although NT-proBNP showed stronger discriminative power than any individual miRNA, the data obtained thus far showed that miRNA panels have comparable performance to NT-proBNP. Furthermore, miRNAs increase the discriminative performance of NT-proBNP. The combination of miR-125a-5p and NT-proBNP improved the AUC value to 0.91, which was better than using NT-proBNP alone with an AUC of 0.83 to differentiate HFREF from HFPEF.

Pathways putatively regulated by the dysregulated cluster of miRNAs

Pathway analyses were performed on miRNAs that were identified as dysregulated in HFREF and HFPEF specifically to identify possible pathophysiological mechanisms. Among the seven miRNAs in the HFREF marker cluster, miR-125a-5p, -193b-3p, and -671-5p that exhibited significant dysregulation as well as significant AUC values were selected for pathway analyses. MiR-1233, -193b-3p, -193b-5p and -545-5p were included in the HFPEF cluster analysis. According to DIANA-mirPath (http://diana.cslab.ece.ntua.gr/pathways/),[21] a total of 26 pathways were found to be potentially altered ($P < 0.05$), as reflected in changes in miR-125a-5p, -193b-3p and -671-5p (*Figure 3A*). Signalling pathways identified, including neurotrophin (highest enrichment score), Wnt, Toll-like receptor, insulin, ErbB, as well as p53 have been reported to be associated with HF pathogenesis. Analyses for the HFPEF miRNAs identified a total of 30 pathways potentially significantly dysregulated (*Figure 3B*). Unlike the HFREF cluster, metabolism-associated pathway including fatty acid biosynthesis and PI3K-AKT signaling were those implicated most. Common pathways between the HFPEF and HFREF clusters of miRNAs included neurotrophin and Wnt signalling as well as p53 signalling.

Discussion

As the roles of miRNAs in cardiovascular diseases are increasingly being recognized, we aimed to investigate the potential of circulating miRNAs as biomarkers in HF. We report here a comprehensive miRNA profiling study performed in both whole blood and plasma samples from patients of Asian ethnicity. To our knowledge, this is the first study, with the largest cohort ($n = 176$) reported so far, incorporating screening of circulating miRNAs in both HFREF (LVEF ≤40%) as well as HFPEF (LVEF ≥50%). In addition, the present study takes common comorbidities such as diabetes and hypertension exhibited by HF patients (common confounders) into consideration. Hence, our control participants consisted of non-HF individuals exhibiting similar comorbidities as HF patients. Vogel *et al.*[17] validated miRNAs expression from 92 individuals in both whole blood and serum, comparing HFREF (<50% LVEF) and controls. They showed that miRNAs were able to distinguish HFREF from controls and miR-558,-122*, -520-5p, -622, -519*, and -200b* correlated with disease severity.

Blood samples are most frequently used in making diagnosis as they are readily accessible. This makes blood the obvious source for biomarker discovery.[17,19,22] Blood samples may be stored in non-coagulated form or, more often, plasma is separated from anticoagulant samples. Hence, it is important that potential miRNA biomarkers can be detected in both fractions for versatility. In the present study, whole blood was used for miRNA identification because it represents the most complete pool of blood fractions. In this way, the complete library of miRNAs detected in blood is used for the initial screening platform. Furthermore, a study by Heneghan *et al.*[23] has shown that higher yields of miRNAs were obtained from whole blood compared with serum/plasma. The miRNA microarray from whole blood provided a global view of the circulating miRNA profile in HF. We identified 806 commonly detected miRNAs, of which 344 showed striking dysregulation in the blood of HF patients. All the miRNAs detected in blood could also be detected in plasma samples albeit at generally lower level. This is not surprising as plasma is a component of whole blood.[24] For the purpose of this study, it is important that the selected

Table 2 Area under the curve (AUC) values for selected miRNAs. MiRNAs found to be significantly altered in plasma samples and identified as possible markers for each category were subjected to receiver operating characteristic (ROC) analysis individually and in combinations as shown below. The discriminative power increased when certain miRNAs (underlined) are selected and combined to constitute the miRNA panels.

Category	AUC values			
	Individual marker	95% Confidence interval	Combined (miRNA + NT-proBNP)	95% Confidence interval
Markers for heart failure				
miR-1233	0.63	0.500–0.752	0.96	0.884–0.993
miR-183-3p	0.62	0.492–0.744	0.92	0.824–0.977
miR-190a	0.59	0.438–0.734	0.94	0.820–0.989
miR-193b-3p	0.67	0.553–0.782	0.95	0.866–0.988
miR-193b-5p	0.60	0.466–0.735	0.93	0.837–0.982
miR-211-5p	0.66	0.497–0.824	0.93	0.819–0.981
miR-494	0.58	0.422–0.734	0.93	0.824–0.979
miR-671-5p	0.75	0.591–0.901	0.97	0.836–1.000
miRNA panel	1.00	0.782–1.000	1.00	0.782–1.000
NT-proBNP	0.94	0.883–1.000		
Markers for reduced ejection fraction				
miR-125a-5p	0.70	0.601–0.894	1.00	0.872–1.000
miR-183-3p	0.72	0.486–0.786	1.00	0.905–1.000
miR-193b-3p	0.80	0.505–0.888	1.00	0.918–1.000
miR-211-5p	0.73	0.535–0.895	1.00	0.894–1.000
miR-494	0.66	0.482–0.836	1.00	0.907–1.000
miR-638	0.67	0.468–0.769	1.00	0.912–1.000
miR-671–5p	0.89	0.646–0.954	1.00	0.832–1.000
miRNA panel	1.00	0.692–1.000		0.841–1.000
NT-proBNP	1.00	1.000–1.000		
Markers for preserved ejection fraction				
miR-1233	0.69	0.514–0.789	0.93	0.812–0.983
miR-183-3p	0.60	0.456–0.751	0.87	0.737–0.953
miR-190a	0.62	0.513–0.835	0.88	0.715–0.972
miR-193b-3p	0.69	0.556–0.820	0.91	0.792–0.976
miR-193b-5p	0.68	0.483–0.782	0.88	0.742–0.961
miR-545-5p	0.62	0.433–0.744	0.90	0.768–0.971
miRNA panel	0.89	0.735–0.967	1.00	0.868–1.000
NT-proBNP	0.89	0.774–0.999		
Markers to distinguish between reduced and preserved ejection fraction				
miR-125a-5p	0.61	0.437–0.789	0.91	0.738–0.984
miR-190a	0.66	0.332–0.689	0.91	0.737–0.984
miR-550a-5p	0.58	0.429–0.731	0.79	0.630–0.898
miR-638	0.60	0.445–0.754	0.86	0.709–0.953
miRNA panel	0.80	0.608–0.923	1.00	0.794–1.000
NT-proBNP	0.83	0.701–0.951		

NT-proBNP, *N*-terminal pro-brain natriuretic peptide.

miRNA biomarkers exhibit similar expression pattern in blood and plasma so that accurate diagnosis can be derived from either miRNA source. Multiple statistical analyses were carried out to shortlist 90 miRNAs that specifically distinguish the HF categories and these were eventually validated in an independent cohort of plasma samples.

We found that levels of at least 12 miRNAs (miR-125a-5p, -183-3p, -190a, -193b-3p, -193-5p, -211-5p, -494, -545–5p, -550a-5p, -638, -671–5p and -1233) differed significantly between HF and non-HF controls and/or between HFREF and HFPEF in both blood and plasma. Thus far, only Ellis *et al.*[16] have examined the miRNA profiles between HFREF and HFPEF in plasma. They investigated a heterogeneous population presenting with breathlessness including many with dyspnoea of non-cardiac origin. However, their findings on initial screening were not corroborated in the validation phase. In contrast, we found that expression of miR-125a-5p, -190a, -550a-5p and -638 could significantly differentiate HFREF from the HFPEF group. Initial discovery (in blood and

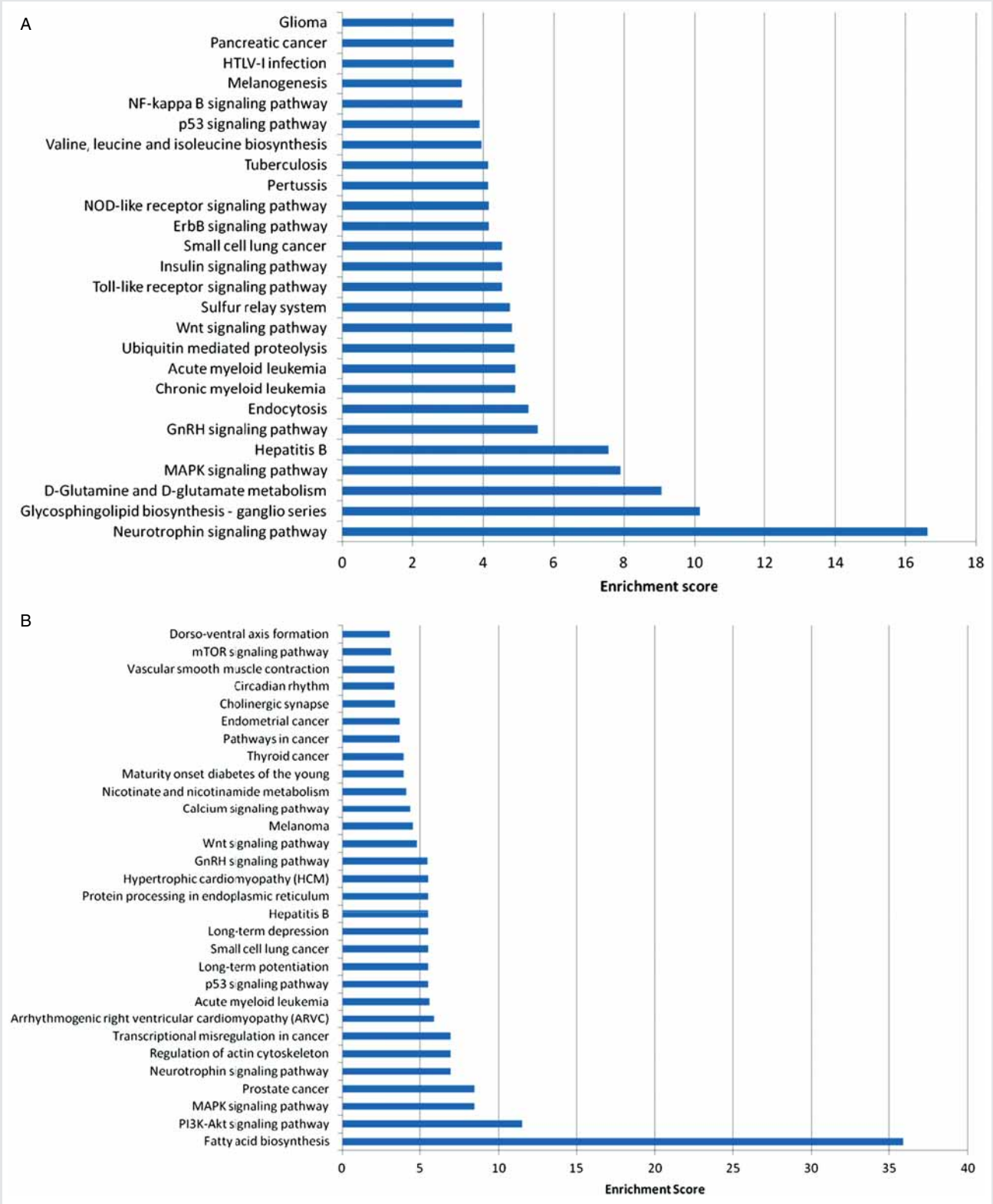

Figure 3 Pathways regulated by heart failure with reduced ejection fraction (HFrEF) or heart failure with preserved ejection fraction (HFpEF) specific cluster of microRNAs (miRNAs). (A) 125a-5p, -193b-3p, and -671-5p were selected from the HFREF cluster of miRNAs; (B) miR-1233, -193b-3p, -193b-5p, and -545-5p were shortlisted from the HFPEF cluster of miRNAs. Pathway analyses were performed using DIANA-mirPath (http://diana.cslab.ece.ntua.gr/pathways/).

plasma) confirmed in an independent cohort of plasma samples. We have also shown that miR-183-3p, -190a, -193-3p, -193-5p, and -545-5p distinguished HFPEF from non-HF controls. Furthermore, we found that miR-183-3p, -190a, -193b-3p, -193b-5p, -211-5p, -494, -671-5p, and -1233 could differentiate HF from non-HFs. Tijsen *et al.*[13] identified miR-423-5p as a biomarker of HF. However, we did not find miR-423-5p levels in HF to differ from those in controls. The stage of disease (acute, chronic, treated or untreated), ethnic differences and, more importantly, the selected controls may underlie some of the lack of concordance between these reports.

Further statistical evaluation showed that the miRNAs identified in this study hold potential diagnostic utility. The discriminative power of HF and HFREF miRNA panels identified in this study was shown to match or even exceed that of NT-proBNP (AUC = 1.0; *Table* 2). Although single miRNA markers for each category showed a lower AUC value when compared with NT-pro-BNP, they exhibited markedly improved discriminative power when used in panels or in combination with NT-proBNP. For example, NT-proBNP showed an AUC of 0.83 for discriminating HFREFs from HFPEFs and this was increased to 0.91 with the inclusion of miR-125a-5p. The predictive value was further improved to almost 1 when a panel of miRNAs for each category was used. This could result from the multitarget properties of miRNAs, which allows a single miRNA to be involved in multiple pathways, in other words limiting its specificity. Nonetheless, the identification of a unique cluster of miRNAs belonging to a certain key biological pathway/network will increase the specificity of their implication in a particular pathogenesis. Thus, combining individual miRNA with NT-proBNP or employing a unique panel of multiple miRNA biomarkers can improve the performance of diagnostic assays.

We have identified several miRNAs not previously associated with HF-related signalling. Conceivably, these circulating miRNAs may exert paracrine effects,[25,26] despite their low abundance, and be involved in putative signalling pathway associated with HF. The potential source and roles of these miRNAs in HF warrants further investigation. Williams *et al.*[27] suggested that the majority of circulating miRNAs are contributed by blood components but also confirmed the presence of tissue specific miRNAs, albeit at low abundance. Akat *et al.*[28] showed the possibility of an increase in circulating miRNA levels in individuals diagnosed with advanced heart failure suggesting complex regulation of these small RNAs. In our study, the highest enrichment score observed in the HFREF miRNA cluster was the neurotrophin signalling pathway which regulates many essential physiological systems. Its dysfunction is implicated in cardiovascular disease potentially by modulating the strength of cardiac sympathetic drive.[26] The fatty acid biosynthesis pathway exhibited the highest enrichment score for the HFPEF miRNA cluster. Although there is no report directly linking this pathway to HFPEF, the impact of comorbidities incorporating derangement of lipid and fatty acid metabolism, such as hypertension, diabetes and obesity, are more frequent and possibly more often reflective of underlying causative molecular pathology in HFPEF.[29] The analyses pointing towards the putative signalling pathways discussed above can be considered as of 'hypothesis-generating' value only. Critical genes involved in these signalling pathways that are predicted to be targets of these miRNA clusters need to be quantitated.[30]

Circulating miRNAs serve as biomarkers of myocardial injury but their utility as bona fide HF biomarkers must be carefully examined.[28] Our study cohort consisted of individuals who were carefully selected and characterized into non-HF and HF categories, and there is a need to extend our findings to the 'real life"' settings, as conducted for the cardiac peptides,[31] on unbiased consecutive series of patients presenting with uncertain diagnosis and non-specific symptoms such as breathlessness.[32] The effects of age, obesity, atrial fibrillation, and other potential confounders upon miRNA test performance also need to be defined. However, our current findings suggest an assay of selected miRNAs in addition to NT-proBNP will potentially provide substantial improvement in our ability to screen and monitor in early, partially compensated or partly treated HF, including in the challenging setting of HFPEF.

Conclusion

Our study identified clusters of circulating miRNAs that distinguished HF from non-HF controls and HFREF from HFPEF. These miRNAs may complement the diagnostic value of BNP/NT-proBNP. Further exploration of these miRNA clusters may offer diagnostic and prognostic applications and insights into pathology pointing to new phenotype-specific therapeutic targets.

Acknowledgements

We thank the study participants and coordinators from SHOP and SLAS studies. We also acknowledge the nurses as well as colleagues from Cardiovascular Research Institute especially Oi Wah Liew, Cui Xia Ang, Yu Pei Peh, and Abby S. Y. Wee who contributed to this paper.

Supplementary Information

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

Figure S1. Profile of 344 circulating miRNAs in HF patients with HFREF or HFPEF normalized against controls. Grey denotes patients clinically classified as HFREF and purple denotes HFPEF. Hierarchical clustering showed clear segregation between majority of the HFREF and HFPEF patients with some outliers.

FigureS2: Verification of blood miRNAs array. Ten randomly selected miRNAs was verified using quantitative stem-loop real-time PCR and compared to miRNAs profiling data. Level of miRNAs was determined in heart failure samples clustered according to their clinical diagnosis. Data is represented as a relative expression with respect to healthy controls ± SD (n = 8 per group).

Table S1: Significantly dysregulated miRNAs in HF patients. List of 90 miRNAs which were significantly dysregulated (FDR < 0.05) in total blood in HF patients are given. The 90 miRNAs were selected from blood profiling based on ROC (AUC > 0.7) and

Pearson correlation analysis (>0.5 or < −0.5). Expression data are represented as normalized median ± S.D (Controls, n = 28; HFPEF, n = 19; HFREF, n = 39). The corresponding expression levels of the 90 miRNAs in randomly selected plasma samples (n = 6 in each group) of HF patients is also given. Majority of these miRNAs was detected at lower intensities in the plasma samples.

Table S2: Raw intensity values of the selected 90 miRNAs.

Table S3: Validation of miRNAs in plasma in cohort 2. List of 32 selected miRNAs based on cohort 1 study and validated in an independent cohort 2 comprising 30 controls, 30 HFREF and 30HFPEF. MiRNAs were significantly dysregulated ($p < 0.05$) in comparison between HF and non-HF controls, HFREF and controls, HFPEF and controls as well as HFREF from HFPEF are highlighted in grey.

Funding

This research was supported by NUS-CVRI start-up grant (R-172-000-206-733), NMRC-IRG (R-183-000-290-213) and NMRC-TCR (NMRC/TCR/006-NUHS/213-Theme 2: NR13NMR108) grants.

Conflict of interest: none declared.

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