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RESEARCH ARTICLE

The transcardiac gradient of cardio-microRNAs in the failing heart

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Aims Differential microRNA expression in peripheral blood has been observed in patients with heart failure, suggesting their value as potential biomarkers and likely contributors to disease mechanisms. In the present study, we aimed to evaluate the transcardiac gradient of 84 cardio-microRNAs in healthy and failing hearts to determine which microRNAs are released or absorbed by the myocardium in heart failure.

Methods and results Eight healthy volunteers and nine patients with congestive heart failure were included. Arterial and coronary sinus blood samples were collected, and microRNAs were extracted. The expression of microRNAs was analysed using real-time PCR by the miScript miRNA PCR Array Human Cardiovascular Disease. In coronary sinus samples, the microRNAs miR-16-5p, miR-27a-3p, miR-27b-3p, miR-29b-3p, miR-29c-3p, miR-30e-5p, miR-92a-3p, miR-125b-5p, miR-140-5p, miR-195-5p, miR-424-5p, and miR-451a were significantly down-regulated, and let-7a-5p, let-7c-5p, let-7e-5p, miR-23b-3p, miR-107, miR-155-5p, miR-181a-5p, miR-181b-5p and miR-320a were up-regulated in heart failure. Left ventricular filling pressure was negatively correlated with miR-195, miR-16, miR-29b-3p, miR-29c-3p, miR-451a, and miR-92a-3p. The failing heart released let-7b-5p, let-7c-5p, let-7e-5p, miR-122-5p, and miR-21-5p, and absorbed miR-16-5p, miR-17-5p, miR-27a-3p, miR-30a-5p, miR-30d-5p, miR-30e-5p, miR-130a-3p, miR-140-5p, miR-199a-5p, and miR-451a. *In silico* analyses suggest that the transcardiac gradient of microRNAs in heart failure may target pathways related to heart disease.

Conclusion We determined the transcardiac gradient of cardio-microRNAs in failing hearts, which supports the use of these microRNAs as potential biomarkers. The microRNAs described here may have a role in the pathophysiology of heart failure as they might be involved in pathways related to disease progression, including fibrosis.

Keywords miRNAs • Transcardiac gradient • Heart failure • Collagen

Introduction

MicroRNAs (miRNAs), small non-coding RNA molecules which regulate gene expression at the post-transcriptional level, have emerged as fundamental for heart development and disease.[1] Circulating miRNAs, such as those in plasma and serum samples, are relatively stable and are a new class of potential disease biomarkers.[2] Several circulating miRNAs have been associated with heart failure, and could be potentially used as biomarkers for diagnosis.[3] Whether these miRNAs accurately reflect cardiac turnover rather than systemic disturbances is unclear.

In the present study, we aimed to determine the coronary sinus and arterial levels of 84 miRNAs previously associated with cardiovascular development and disease (referred to here as 'cardio-miRNAs') between healthy controls and heart failure patients, and establish the transcardiac gradient in health and disease states. Although the transcardiac gradients of a few miRNAs (miR-29b-5p, miR-133a-5p, and miR-423-5p) have been analysed previously in heart failure,[4] this is the first study to analyse a large number of miRNAs across the myocardium and also to apply a new normalization method independent of single endogenous or exogenous miRNAs. After establishing the transcardiac gradient of

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miRNAs, we also performed a predictive evaluation of pathways that may be involved in the development of heart failure.

Methods

Human plasma samples

This study included eight healthy volunteers and nine patients with advanced heart failure. Healthy volunteers were recruited from the general community and were included in the absence of a history of cardiovascular disease and cardiovascular medications, and a normal cardiovascular examination. Heart failure patients included individuals undergoing assessment for heart transplantation or with advanced heart failure undergoing haemodynamic evaluation for optimization of therapy. All studies were performed in the morning, and antifailure medications were continued to avoid haemodynamic instability. In patients with heart failure, a balloon-tipped thermodilution catheter (7-Fr Arrow, Arrow International, Reading, PA, USA) was inserted via an introducer sheath placed in the right internal jugular vein or antecubital vein, including the determination of pulmonary arterial pressures, wedge pressure, and cardiac output in the patients with heart failure. A right radial or brachial arterial line was placed for arterial blood pressure (BP) measurement and blood sampling. After the haemodynamic assessment, a sampling catheter was positioned in the coronary sinus under fluoroscopic control. The tip of the catheter was positioned at least 2 cm proximal to the orifice of the coronary sinus, as confirmed by injection of radiographic contrast material. Arterial and coronary sinus blood samples were collected simultaneously in EDTA tubes to allow calculation of the transcardiac miRNA concentration gradient. After collection, blood samples were placed on ice. After the completion of the study, blood samples were centrifuged at 4 °C, and plasma was stored at −80 °C until subsequent assay. This study complied with the principles outlined in the Declaration of Helsinki, and was performed with the approval of the Alfred Hospital Ethics Review Committee. All subjects gave written informed consent.

Plasma microRNA extraction

RNA was extracted from 100 μL of plasma from arterial and coronary sinus matching samples using the miRNeasy Serum/Plasma kit (Qiagen) according to the supplier. Briefly, 100 μL of plasma was mixed with 500 μL of QIAzol Lysis reagent, and incubated at room temperature for 5 min. We then mixed it with 3.5 μL of miRNeasy Serum/Plasma Spike-In Control (1.6×10^8 copies/μL), followed by 100 μL of chloroform. After vortexing and incubating it at room temperature for 3 min, we centrifuged it at 12 000 RCF at 4 °C for 15 min, to separate it into three phases. We transferred the aqueous phase to a new tube, and added 1.5 vols of 100% ethanol. The RNeasy MiElute spin column was used to wash and finally elute the RNA in 14 μL. No pooling of samples was performed.

MicroRNA expression by quantitative real-time PCR

The first-strand complementary synthesis reaction (cDNA) was performed using the miScript II Rt kit (Qiagen) according to the supplier. Briefly, 9 μL of isolated RNA was added to the cDNA master mix, composed of 5× miScript HiSpec Buffer, 10× miScript Nucleics Mix, miScript Reverse Transcriptase Mix, and water, to a total volume of 20 μL. The cDNA was incubated at 37 °C for 60 min, followed by 5 min incubation at 95 °C, and then diluted 11 times. Pre-amplification was not performed. Amplification reactions used the miScript miRNA PCR Array Human Cardiovascular disease (MIHS-113Z, Qiagen; Supplementary material online, *Table S1*) with a miScript SYBR Green PCR kit (Qiagen) in a 7300 qPCR system (Applied Biosystems), following the cycling conditions recommended by the supplier (15 min at 95 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 55 °C, and 30 s at 70 °C).

Statistical analyses

miScript miRNA PCR Array Data Analysis (Qiagen) was used for statistical analyses. A global normalization was performed, which included the average cycle threshold of the 84 cardio-miRNAs in the array plate, plus spike-in cel-miR-39 and endogenous small RNAs (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and RNU6). This is a more robust and preferred method for this type of study.[5] Significance was assessed by the $2^{-\Delta\Delta CT}$ method.[6] The transcardiac gradient was calculated by subtracting miRNA levels in arterial blood from miRNA levels in coronary sinus samples. An independent sample *t*-test (two-tail) was used to compare the data between groups, and between arterial and coronary sinus samples within groups. Volcano plots show only significant miRNAs with fold change >2. Hierarchical clustering was built using Euclidean distances in the miScript miRNA PCR Array Data Analysis tool. Data sets were tested for normal distribution using the D'Agostino and Pearson normality test. Pearson's or Spearman's correlations were used to correlate differentially expressed miRNAs and clinical data using GraphPad Prism (version 6). An SPSS (version 21) package was used for receiver operating characteristic (ROC) curve analysis. Significance was set at $P < 0.05$. The R package was used to calculate significance adjusted by the Benjamin–Hochberg false discovery rate (FDR).

Gene set enrichment analysis

The list of the transcardiac gradient of miRNAs in heart failure was uploaded into miRWalk.[7] Only mRNAs predicted in at least four out of five tools (miRanda, miRDB, miRWalk, RNA22, and TargetScan) were considered as possible miRNA targets. Gene set enrichment analysis (GSEA) was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID)[8,9] to ask which Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were enriched within the genes that contained binding sites for miRNAs being absorbed by or released from the failing heart. Only pathways with >10 genes, fold enrichment >1.5, and FDR <0.05 were considered.

Results

Patient population

The characteristics of healthy controls (mean ± SD 59.8 ± 8.8 years old) and heart failure patients (50.4 ± 10.1 years old, $P = 0.062$) are described in *Table 1*. Heart failure was due to non-ischaemic dilated cardiomyopathy in seven patients and ischaemic heart disease in two patients. The LV filling pressure (measured by the PCWP) was 8 ± 2 in healthy subjects and 20 ± 10 in heart failure patients ($P = 0.005$). Patients also had significantly lower systolic BP and greater LV mass, LV end-systolic diameter and LV end-diastolic diameter (*Table 1*). Concomitant medications are listed in *Table 1*.

Table 1 Characteristics of the healthy controls and heart failure patients

Demographics and clinical characteristics	Healthy (mean ± SD)	Heart failure (mean ± SD)	*P*-value
n	8	9	
Age, years	59.8 ± 8.8	50.4 ± 10.1	0.062
Male (%)	6 (75%)	6 (60%)	0.728
BMI, kg/m^2	25.1 ± 4.4	29.4 ± 5.6	0.118
ACE inhibitor (%)	0 (0%)	4 (44%)	
ARB (%)	0 (0%)	2 (22.2%)	
Beta-blocker (%)	0 (0%)	7 (77.8%)	
Spironolactone	0 (0%)	2 (22.2%)	
Right atrial pressure, mmHg	5 ± 1	9 ± 7	0.104
Pulmonary capillary wedge pressure, mmHg	8 ± 2	20 ± 10	**0.005**
Cardiac index	2.6 ± 0.5	2.1 ± 0.5	0.073
Systolic BP, mmHg	135 ± 17	106 ± 9	**<0.0001**
Diastolic BP, mmHg	68 ± 9	60 ± 13	0.169
Left ventricular mass	147 ± 42	301 ± 77	**0.0008**
Left ventricular end-systolic diameter, mm	32 ± 2	61 ± 10	**<0.0001**
Left ventricular end-diastolic diameter, mm	51 ± 3	70 ± 9	**0.001**

BMI, body mass index; BP, blood pressure.
P-values <0.05 (bold) were considered significant.

MicroRNA levels in aortic and coronary sinus samples in heart failure

The only miRNA differentially expressed in arterial samples in heart failure patients was miR-222-3p (controls 0.85 vs. patients 1.93, fold change 2.27, $P = 0.033$; Supplementary material online, *Figure S1A*). Out of the 84 miRNAs investigated, 12 miRNAs were significantly down-regulated in coronary sinus blood of heart failure patients (miR-16, miR-27a-3p, miR-27b-3p, miR-29b-3p, miR-29c-3p, miR-30e, miR-92a-3p, miR-125b, miR-140, miR-195, miR-424, and miR-451a), while 10 were up-regulated (let-7a, let-7c, let-7e, miR-23b-3p, miR-107, miR-155, miR-181a, miR-181b, and miR-320a) (*Table 2*; Supplementary material online, *Figure S1B*). miR-451a was the most down-regulated miRNA (fold change −6.89, $P = 0.044$), while miR-155 was the most up-regulated miRNA (fold change 6.73, $P = 0.002$). Hierarchical clustering showed a distinctive segregation between the majority of controls and heart failure patients (Supplementary material online, *Figure S2A*).

Correlations between clinical variables and microRNA expression

To determine whether differentially expressed miRNAs were associated with the clinical phenotype of heart failure, we determined correlations between miRNA expression and PCWP and LV mass. Amongst the differentially expressed miRNAs, PCWP was negatively correlated with miR-16 ($r = -0.49$, $P = 0.048$, *Figure 1A*), miR-195 ($r = -0.57$, $P = 0.017$, *Figure 1B*), miR-29b-3p ($r = -0.63$, $P = 0.001$, *Figure 1C*), miR-29c-3p ($r = -0.53$, $P = 0.030$, *Figure 1D*), miR-451a ($r = -0.64$, $P = 0.006$, *Figure 1E*), and miR-92a-3p ($r = -0.59$, $P = 0.014$, *Figure 1F*). LV mass was negatively correlated with miR-451a ($r = -0.55$, $P = 0.042$, *Figure 1G*). After removing outliers, the correlation between miR-451a and PCWP was still significant ($r = -0.60$, $P = 0.026$), while it was borderline with LV mass ($r = -0.54$ and $P = 0.071$). Based on these results, the sample size used here had a power of 66.2–89.7% to detect a significant association.

To determine the specificity and sensitivity of the miRNAs correlated with PCWP, we performed a ROC curve analysis. Briefly, those miRNAs that were correlated with PCWP ($P < 0.05$) were added as determinants of heart failure. The area under the curve (AUC) values varied from 0.806 to 0.875 for individual miRNAs (*Table 3*). The miRNAs with the highest AUC were miR-29b-3p, miR-29c-3p, and miR-451a (all 0.875). When we combined all six miRNAs, there was no improvement in the maximum AUC value (0.875). When we combined those with the highest AUC values (miR-29b-3p, miR-29c-3p, and miR-451a), there was a minor improvement to 0.906 (*Table 3*).

Transcardiac gradient of microRNAs

The transcardiac gradient of cardio-miRNAs was different in failing compared with normal hearts. The only miRNA released from the healthy heart was miR-140 (0.31 vs. 0.15, gradient = 0.16, $P = 0.021$, albeit not significant after FDR $q = 0.315$, *Table 4*; Supplementary material online, *Figure S1C*). Out of the 84 miRNAs studied, five were released from the heart of patients (let-7b, let-7c, let-7e, miR-122, and miR-21) and 10 were extracted (miR-16, miR-17, miR-27a-3p, miR-30a, miR-30d, miR-30e, miR-130a-3p, miR-140, miR-199a,and miR-451a) (*Table 4*; Supplementary material online, *Figure S1D*). The miRNA miR-21 had the highest gradient of release (71.6), while miR-451a had the highest gradient of extraction from the failing heart (−59.0). Hierarchical clustering showed segregation between the majority of coronary sinus and arterial blood samples (Supplementary material online, *Figure S2B*).

Pathways associated with transcardiac gradient of microRNAs in heart failure

We hypothesized that absorbed or released miRNAs from the failing heart could have the potential to contribute to the pathophysiology of the disease. The miRNAs being absorbed or released from the heart were predicted to target hundreds of mRNAs that collectively were predicted to regulate several pathways related to heart disease, amongst other pathways (*Figure 2*). This included the over-representation of pathways related to apoptosis (hsa04210), mitogen-activated protein kinase (MAPK) signalling (hsa04010), the transforming growth factor beta (TGF-β) signalling pathway (hsa04350), and cytokine–cytokine receptor interaction (hsa04060).

Discussion

In the present study, we sought to analyse the transcardiac gradient of cardio-miRNAs in healthy and failing human hearts by the use of arterial and coronary sinus plasma samples. We also investigated which miRNAs were differentially expressed in arterial

Table 2 MicroRNAs differentially expressed between healthy controls and heart failure patients in coronary sinus blood samples

microRNA ID	Mean expression in controls	Mean expression in heart failure	95% confidence interval	*P*-value	FDR	Fold change
hsa-miR-451a	97.64	14.17	0.00001–0.34	0.044	0.044	−6.89
hsa-miR-424-5p	0.78	0.15	0.03–0.37	0.010	0.030	−5.07
hsa-miR-125b-5p	0.78	0.21	0.08–0.44	0.023	0.030	−3.78
hsa-miR-29b-3p	0.84	0.24	0.06–0.52	0.020	0.030	−3.48
hsa-miR-195–5p	27.14	8.33	0.05–0.57	0.019	0.030	−3.26
hsa-miR-92a-3p	99.51	31.84	0.07–0.57	0.041	0.043	−3.12
hsa-miR-16-5p	30.01	10.11	0.08–0.60	0.023	0.030	−2.97
hsa-miR-29c-3p	1.67	0.58	0.18–0.52	0.001	0.013	−2.88
hsa-miR-27a-3p	2.35	0.86	0.03–0.71	0.021	0.030	−2.72
hsa-miR-140-5p	0.31	0.11	0.21–0.53	0.0006	0.013	−2.71
hsa-miR-27b-3p	1.47	0.58	0.18–0.60	0.007	0.030	−2.54
hsa-miR-30e-5p	2.69	1.29	0.16–0.80	0.034	0.038	−2.08
hsa-miR-21-5p	60.93	119.29	0.27–3.65	0.032	0.037	1.96
hsa-let-7c-5p	7.11	14.13	0.78–3.20	0.023	0.030	1.99
hsa-miR-23b-3p	2.09	4.85	0.00001–4.91	0.029	0.036	2.32
hsa-miR-320a	30.89	74.25	0.87–3.93	0.008	0.030	2.40
hsa-let-7a-5p	24.94	69.87	0.32–5.28	0.019	0.030	2.80
hsa-miR-181b-5p	0.64	2.13	0.72–5.98	0.022	0.030	3.35
hsa-miR-181a-5p	0.06	0.22	0.92–6.20	0.011	0.030	3.56
hsa-let-7e-5p	4.94	18.12	0.45–6.89	0.018	0.030	3.67
hsa-miR-107	0.04	0.14	1.10–6.82	0.017	0.030	3.96
hsa-miR-155-5p	0.33	2.22	0.91–12.55	0.002	0.019	6.73

Fold change is expressed as heart failure over the control group.
Values shown are abundance relative to whole-plate normalization.
FDR, false discovery rate.

and coronary sinus blood. In coronary sinus samples, the miRNAs miR-16, miR-27a-3p, miR-27b-3p, miR-29b-3p, miR-29c-3p, miR-30e, miR-92a-3p, miR-125b, miR-140, miR-195, miR-424, and miR-451a were significantly down-regulated, and let-7a, let-7c, let-7e, miR-23b-3p, miR-107, miR-155, miR-181a, miR-181b, and miR-320a were up-regulated in heart failure patients. LV filling pressure, measured as PCWP, was negatively correlated with miR-16, miR-195, miR-29b-3p, miR-29c-3p, miR-451a, and miR-92a-3p, and these miRNAs had high specificity and sensitivity with heart failure. The failing heart released let-7b, let-7c, let-7e, miR-122, and miR-21, and absorbed miR-16, miR-17, miR-27a-3p, miR-30a, miR-30d, miR-30e, miR-130a-3p, miR-140, miR-199a, and miR-451a. These miRNAs have a net gradient turnover in the myocardium, suggesting that they may contribute to an active process in disease pathogenesis. Further studies, however, would be required to demonstrate their sensitivity and specificity as biomarkers for heart failure or cardiac remodelling. Finally, the miRNAs being absorbed or released in heart failure reported here could be involved in the pathophysiology of the disease, as they are predicted to target several pathways related to heart disease including apoptosis, MAPK signalling, TGF-β signalling, and cytokine–cytokine receptor interaction.

The transcardiac gradient of specific miRNAs has been previously reported in the literature in healthy hearts (miRNAs studied: miR-34a, miR-92a-3p, miR-155, miR-378-3p, miR-1-3p, miR-133a-3p, and miR-499),[10] CAD (miR-133a, miR-499, miR-208a, miR-126, miR-92a, miR-155, and miR-233),[11] coronary endothelial dysfunction (miR-17, miR-92a, miR-126, miR-34, miR-181b, miR-221, miR-222, miR-208, miR-133, miR-21, miR-145, and miR-155),[12] and heart failure (miR-29b, miR-133a, and miR-423).[4] Similarly to our study, Goldraich and colleagues[4] analysed samples collected from the coronary sinus of healthy and failing hearts. Our study, however, had several strengths compared with previous transcardiac miRNA gradient studies, regardless of the health or disease state analysed.[4,10–12] First, we used a global normalization method instead of exogenous spike-in (cel-miR-39) normalization. This method included both exogenous and endogenous miRNAs, and is a preferred method over exogenous normalization only.[13] Secondly, we analysed 84 miRNAs previously identified to have a role in cardiovascular development and disease ('cardio-miRNAs') instead of selecting a few miRNAs. Whilst this methodology ensured that the majority of the miRNAs analysed would be detected in cardiac samples, our study was not confined to miRNAs previously found as biomarkers of heart failure or associated with one or another pathway. Thirdly, besides identifying new differentially expressed miRNAs and determining the transcardiac gradient of miRNAs in heart failure, our study was the first to predict pathways associated with several miRNAs released from or absorbed by the failing heart.

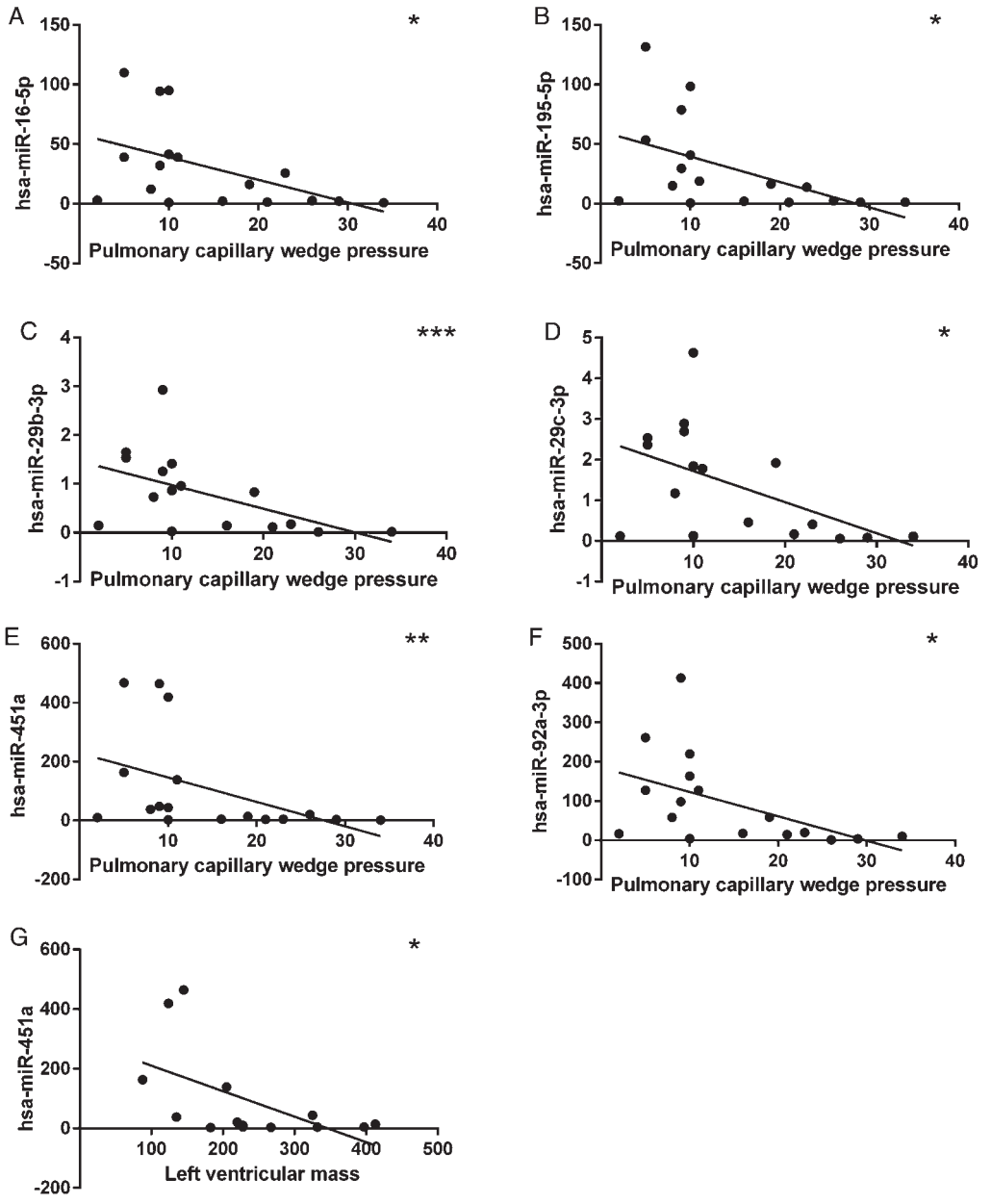

Figure 1 Correlations between microRNA expression, pulmonary capillary wedge pressure (PCWP), and LV mass. PCWP was negatively correlated with (**A**) miR-16 ($r = -0.49$, $P = 0.048$, $n = 17$), (**B**) miR-195 ($r = -0.57$, $P = 0.017$, $n = 17$), (**C**) miR-29b-3p ($r = -0.63$, $P = 0.001$, $n = 16$), (**D**) miR-29c-3p ($r = -0.53$, $P = 0.030$, $n = 17$), (**E**) miR-451a ($r = -0.64$, $P = 0.006$, $n = 17$), and (**F**) miR-92a-3p ($r = -0.59$, $P = 0.014$, $n = 17$). (**G**) LV mass was negatively correlated with miR-451a ($r = -0.55$, $P = 0.042$, $n = 14$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P \leq 0.001$.

The only miRNA that was associated with a transcardiac gradient in heart failure patients in the study by Goldraich and colleagues was miR-423-5p.[4] Only miR-423-3p, however, was investigated in the present study (Supplementary material online, *Table S1*), and therefore no comparison can be made. Moreover, in our study, miR-155 and miR-181b were up-regulated in coronary sinus samples from heart failure patients, and miR-17 was absorbed by the failing heart. Albeit previously investigated, these miRNAs were not found to be differentially expressed in the previous studies.[11,12] This is likely to be due to the differences in the diseases studied, where biological material was sampled, how samples were processed, and the normalization methods used.

Here we also reported that coronary sinus plasma levels of the miRNAs miR-16, miR-195, miR-29b-3p, miR-29c-3p, miR-451a, and miR-92a-3p were negatively correlated with LV filling pressure. These miRNAs, especially miR-29b-3p, miR-29c-3p, and miR-451a, had high specificity and sensitivity in the clinical diagnosis of heart failure. The diagnostic value of these miRNAs, however, could not

Table 3 Area under the curve values for microRNAs differentially expressed in coronary sinus samples from heart failure patients that were correlated with pulmonary capillary wedge pressure

microRNAs	AUC values	Standard error	95% CI	*P*-value
miR-16	0.806	0.117	0.58–1.00	0.034
miR-195	0.819	0.121	0.58–1.00	0.027
miR-29b-3p	0.875	0.097	0.69–1.00	0.012
miR-29c-3p	0.875	0.086	0.71–1.00	0.009
miR-451	0.875	0.106	0.67–1.00	0.009
miR-92a-3p	0.833	0.107	0.623–1.00	0.021
miR-16, miR-195, miR-29b-3p, miR-29c-3p, miR-451, and miR-92a-3p	0.875	0.097	0.69–1.00	0.012
miR-29b-3p, miR-29c-3p, and miR-451	0.906	0.092	0.73–1.00	0.006

These microRNAs were subjected to receiver operating characteristic (ROC) analysis individually and in combination.
AUC, area under the curve; CI, confidence interval.

Table 4 Transcardiac gradient of microRNAs in healthy and failing hearts

microRNA ID	Controls						Heart failure					
	CS	Arterial	Gradient CS–arterial	Absorbed or released by the heart	*P*-value	FDR	CS	Arterial	Gradient CS–arterial	Absorbed or released by the heart	*P*-value	FDR
hsa-let-7c-5p	7.11	6.99	0.12		0.598	0.815	14.13	2.79	11.34	Released	0.002	0.030
hsa-miR-122-5p	7.21	5.55	1.66		0.782	0.902	8.69	2.11	6.57	Released	0.010	0.047
hsa-let-7e-5p	4.94	4.52	0.42		0.734	0.902	18.12	4.47	13.65	Released	0.042	0.047
hsa-let-7b-5p	53.95	47.38	6.58		0.55	0.815	83.04	21.03	62.01	Released	0.029	0.047
hsa-miR-21-5p	60.93	34.05	26.89		0.359	0.814	119.29	47.69	71.59	Released	0.033	0.047
hsa-miR-140-5p	0.31	0.15	0.16	Released	0.021	0.315	0.11	0.19	−0.08	Absorbed	0.044	0.047
hsa-miR-30d-5p	2.61	1.77	0.84		0.437	0.815	1.33	3.04	−1.70	Absorbed	0.047	0.047
hsa-miR-30a-5p	3.80	2.77	1.03		0.995	0.995	1.72	4.07	−2.35	Absorbed	0.047	0.047
hsa-miR-17-5p	2.48	3.31	−0.83		0.33	0.814	1.47	3.98	−2.50	Absorbed	0.029	0.047
hsa-miR-30e-5p	2.69	1.96	0.73		0.955	0.995	1.29	3.69	−2.40	Absorbed	0.021	0.047
hsa-miR-16-5p	30.01	39.92	−9.91		0.38	0.814	10.11	30.34	−20.24	Absorbed	0.022	0.047
hsa-miR-199a-5p	0.07	0.13	−0.06		0.18	0.814	0.11	0.39	−0.28	Absorbed	0.026	0.047
hsa-miR-27a-3p	2.35	2.00	0.35		0.57	0.815	0.86	3.13	−2.27	Absorbed	0.017	0.047
hsa-miR-130a-3p	0.42	0.62	−0.20		0.22	0.814	0.30	1.12	−0.82	Absorbed	0.011	0.047
hsa-miR-451a	97.64	188.56	−90.92		0.243	0.814	14.17	73.17	−59.00	Absorbed	0.044	0.047

Values shown are abundance relative to whole-plate normalization.
CS, coronary sinus; FDR, false discovery rate.

be compared with BNP as the latter was not measured in these subjects. Additionally, the difficulty in acquiring coronary sinus blood samples would be a limitation for their use as biomarkers of heart failure. The miRNAs miR-29b-3p and miR-29c-3p are part of the miR-29 family, previously found to regulate collagen and cardiac fibrosis.[14] Moreover, the down-regulation of miR-451 was present in heart tissue from hypertrophic cardiomyopathy patients[15] and in a transverse aortic constriction model,[16] and *in vitro* it increased the cell surface area of neonatal cardiomyocytes.[15] These findings are consistent with our results showing a negative correlation between miR-451 and LV mass, which may be necessary for ischaemic pre-conditioning cardioprotection.[17] Thus, in line with our results, miR-29b-3p, miR-29c-3p, and miR-451a are likely to be involved in the pathophysiology of heart failure.

The miRNA families let-7 (let-7a, let-7c, and let-7e), miR-15 (miR-16 and miR-195), and miR-181 (miR-181a and miR-181b) were over-represented in the coronary sinus of heart failure patients. The detailed involvement of the let-7 family in cardiovascular disease is reviewed elsewhere.[18] Highlighted are higher levels of some let-7 miRNAs in endothelial to mesenchymal transition, possibly contributing to cardiac fibrosis,[19] and inhibition of let-7 *in vivo* preserving cardiac function post-infarction and reducing fibrosis.[20] The miR-15 family was previously found to govern cardiomyocyte proliferation and binucleation during development, by inducing cell cycle arrest.[21] Inhibition of the miR-15 family during the neonatal period resulted in higher proliferation of both cardiomyocytes and non-cardiomyocytes following infarction in the adult myocardium.[22] Transgenic mice overexpressing the miR-15 family in the heart had lower survival, development of congenital heart defects and cardiomyopathy with ageing, and premature death.[21] This is in line with our findings that miR-16 was absorbed by the myocardium, which is likely to result in higher levels of miR-16 and contribute to the development of the pathology. Moreover, we have previously found that miR-181a was differentially expressed in human[23] and mouse hypertensive kidneys.[24] Consistent with the present study, we have also shown

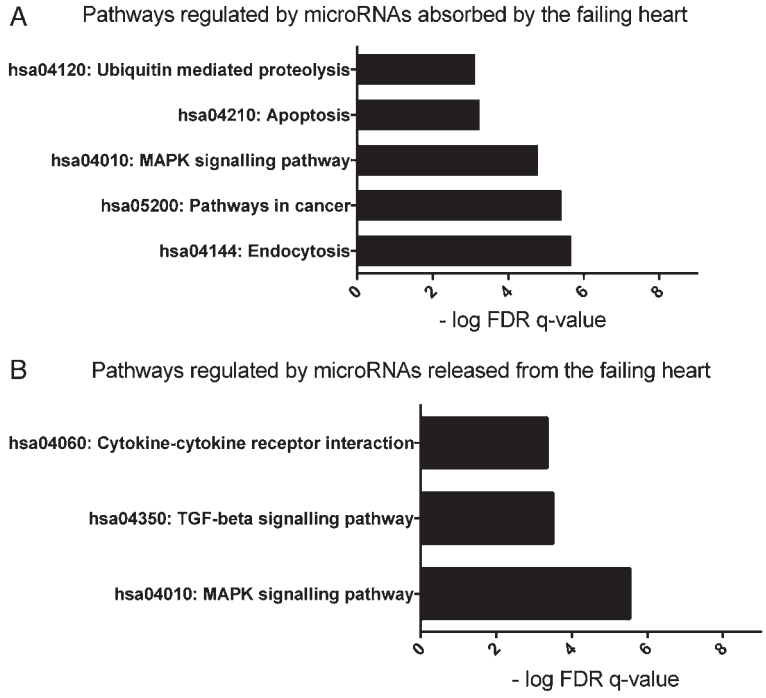

Figure 2 Gene set enrichment analyses of pathways targeted by transcardiac gradient microRNAs in the failing heart. (**A**) Pathways being regulated by microRNAs absorbed by or (**B**) released from the failing heart, showing −log false discovery rate (FDR) *q*-value (all $q < 0.05$). MAPK, mitogen-activated protein kinase; TGF, transforming growth factor.

recently that circulating miR-181a was associated with higher systolic and diastolic BP, through regulation of renin and pathways associated with the immune system and inflammation,[23,25] consistent with immune system activation in heart failure.[26] The circulating miRNA miR-30e-5p is down-regulated in patients with acute heart failure.[27] In our study, we also found miR-30e-5p to be down-regulated in coronary sinus samples from patients with congestive heart failure and it was absorbed by the failing heart.

Our study showed that five miRNAs were released from the failing heart, while 10 were absorbed. This supports that the majority of circulating miRNAs previously associated with heart failure[3] were mostly unlikely to have been released by the myocardium and, therefore, not involved in the mechanisms resulting in the disease itself. The miRNAs miR-21 and miR-122, previously described as biomarkers of heart failure in circulating samples,[28−30] were also released from the failing heart in our study. Interestingly, miR-21 has previously been associated with the development of interstitial fibrosis and cardiac hypertrophy in a mouse model.[31] With the use of small RNA-sequencing of plasma RNA from advanced heart failure patients compared with healthy controls, let-7c-5p was found to be up-regulated, while miR-17−5p and miR-199-5p were down-regulated, consistent with our results showing that these miRNAs were released or absorbed, respectively, from the failing heart.[32] This supports the use of these miRNAs as biomarkers for heart failure in the circulation.

Several pathways were predicted to be regulated by the miRNAs being released or absorbed by the failing heart by *in silico* analyses, including those previously associated with heart disease. The role of these pathways is supported by extensive literature. TGF-β signalling plays an important role in cardiac fibrosis and hypertrophic remodelling,[33] while MAPK signalling is associated with cardiomyocyte growth and cardiac hypertrophy.[34] Apoptosis of cardiomyocytes is an essential process in the development and progression of heart failure.[35] Similarly, the role of proinflammatory cytokines in cardiac dysfunction is well established, and patients with heart failure have more circulating cytokines and cytokine receptors.[36,37] Whether these pathways were truly associated with the miRNAs being absorbed or released from the failing heart will need to be determined by laboratory experiments, but this was outside the scope of the present study.

Our study has some limitations. While the sample size analysed was relatively small, the study employed invasive cardiac catheterization methodology including a normal healthy control group. Although a larger cohort or validation in a second independent cohort would be preferential, the samples studied here were well characterized. The sample size, Iso, had enough statistical power to detect significance for all of the analyses. Another limitation was the lack of cardiac flow measurements, which would allow for calculations about the net rate of release or absorption of miRNAs from the heart. Changes in miRNAs between healthy controls and heart failure patients could be, in part, due to the borderline difference in age between the two groups. Previous findings regarding ageing and the miRNAs let-7a-5p[38] and miR-181a-5p[39] support that the associations described here are not due to age. As

stated above, we acknowledge that the diagnostic value of the miRNAs differentially expressed in coronary sinus samples of patients with heart failure is a limitation, especially because it could not be compared with BNP levels. Finally, miRNAs are responsive to therapeutic interventions,[40] and the higher prevalence of the use of ACE inhibitors and beta-blockers in our cohort (*Table 1*) could have influenced the levels of miRNAs observed here, including the down-regulation of miR-16 in heart failure. Although the use of medication can be a confounding factor, we would expect that some miRNAs were differentially expressed due to the severe phenotype of the patients.

In the present study we did not detect a net production of miR-499. Previous studies have shown that myocardial levels of miR-499 are increased in failing and hypertrophied hearts and that elevated levels of miR-499 may alter a range of relevant targets particularly in relation to stress response and hypertrophy.[41,42] The lack of detection of a transcardiac miR-499 gradient in the present study could be explained by the sensitivity of the present assay for miR-499 or the possibility that differential release kinetics exist for specific cardiomyocyte miRNAs.

Our study described for the first time the expression of cardio-miRNAs in the transcardiac gradient, showing that the failing heart releases let-7b, let-7c, let-7e, miR-122, and miR-21, and absorbs miR-16, miR-17, miR-27a-3p, miR-30a, miR-30d, miR-30e, miR-130a-3p, miR-140, miR-199a, and miR-451a. These miRNAs could have a role in the pathophysiology of cardiac failure as they have been predicted to be involved in pathways related to disease progression, such as fibrosis. Some of these miRNAs may have potential as therapeutic targets and biomarkers, especially miR-21, miR-122, let-7c, miR-17, and miR-199, as supported by the literature, since there is a net gradient turnover in the myocardium.

Supplementary Information

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

Figure S1. Volcano plots of transcardiac and differentially expressed microRNAs (miRNAs) in healthy and failing hearts. (A) Only one miRNA was differentially expressed between arterial samples of healthy and failing hearts. (B) Several miRNAs were differentially expressed between coronary sinus (CS) samples of healthy and failing hearts. (C) Transcardiac gradient of miRNAs in the healthy heart, showing that only one miRNA was released. (D) Transcardiac gradient of miRNAs in the failing heart. Blue line shows *P*-value cut off ($P < 0.05$). Green dots represent down-regulated or released miRNAs with a fold change >2, while red dots represent up-regulated or extracted miRNAs with a fold change >2.

Figure S2. Hierarchical clustering using Euclidean distance. (A) Comparison between differentially expressed microRNAs in coronary sinus samples in heart failure patients (mostly on the left columns) and controls (mostly on the right columns). (B) Comparison between coronary sinus ("cs", mostly on the right columns) and arterial ("a", mostly on the left columns) samples in heart failure patients. Red depicts microRNAs up-regulated and green those down-regulated.

Table S1. Cardio-microRNAs investigated in this study.

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