

Circulating microRNAs for predicting and monitoring response to mechanical circulatory support from a left ventricular assist device

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Aims

There are few non-invasive techniques to predict and monitor patients' responses to left ventricular assist device (LVAD) therapy. MicroRNAs (miRs) are small non-coding RNAs with intricate roles in cardiovascular disease. They are stable in the circulation, readily quantified, and may be useful as new biomarkers. This study sought to identify candidate miR biomarkers for further investigation.

Methods and results

We studied 53 plasma and 20 myocardial samples from 19 patients who underwent HeartMate II LVAD implantation, and used a screening microarray to analyse the change in expression of 1113 miRs after 6 months LVAD support. Twelve miRs showed significant variation and underwent validation, yielding miR-1202 and miR-483-3p as candidate biomarkers. In the test cohort, circulating miR-483-3p showed early and sustained up-regulation with LVAD support, with median (interquartile range) fold changes from baseline of 2.17 (1.43–2.62; $P=0.011$), 2.27 (1.12–2.42; $P=0.036$), 1.87 (1.64–4.36; $P=0.028$), and 2.82 (0.70–10.62; $P=0.249$) at 3, 6, 9, and 12 months, respectively, whilst baseline plasma miR-1202 identified good vs. poor LVAD responders [absolute expression 1.296 (1.293–1.306) vs. 1.311 (1.310–1.318) arbitrary units; $P=0.004$]. Both miRs are enriched in ventricular myocardium, suggesting the heart as the possible source of the plasma fraction.

Conclusions

This is the first report of circulating miR biomarkers in LVAD patients. We demonstrate the feasibility of this approach, report the potential for miR-483-3p and miR-1202, respectively, to monitor and predict response to LVAD therapy, and propose further work to study these hypotheses and elucidate roles for miR-483-3p and miR-1202 in clinical practice and in underlying biological processes.

Keywords

MicroRNA • Ventricular assist device • Biomarker

Introduction

Left ventricular assist devices (LVADs) are implantable mechanical blood pumps that remain essential tools in patients with advanced

heart failure.¹ However, their use is limited by high rates of adverse events and high cost.^{2,3} Appropriate patient selection remains the key to optimizing patient outcomes, with clinical management relying on biomarkers to assist risk stratification and

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serial monitoring. Various parameters have been characterized for this purpose, particularly haemodynamic information from invasive right heart catheterization, but none provides adequate sensitivity and specificity for use as independent biomarkers.⁴

MicroRNAs (miRs) are tiny fragments of non-coding RNA typically 20–22 nucleotides in length, and there is accumulating evidence of their intricate role in cardiac physiology and disease.^{5,6} Their primary biological function is post-transcriptional regulation of gene expression by sequence-specific binding with mRNAs causing translational repression or mRNA degradation, and they might have further roles in cell–cell interactions controlling systemic alterations to the proteome.⁷ Analysis of miRs in the ventricular myocardium of heart failure patients undergoing LVAD implantation has shown plasticity of the miR-ome⁸ and specific expression patterns related to subsequent myocardial recovery and LVAD explant vs. persistent LVAD dependency.⁹ In addition to expression in the tissue of origin, miRs can be isolated from circulating blood, where they remain extremely stable due to their transport in association with microvesicles and exosomes, or in tight association with RNA-binding proteins that protect them from ribonuclease degradation.¹⁰ These characteristics and their ready quantification make them excellent biomarker candidates, and there are preliminary results identifying individual miRs or miR signatures for diagnosis of cardiovascular diseases including heart failure and acute myocardial infarction.^{11–13}

Circulating miRs have not been characterized in LVAD patients. Here we hypothesize that (i) circulating miRs are candidate biomarkers for predicting and monitoring clinical response to LVAD implantation; and that (ii) the circulating miR pattern is related to expression in ventricular myocardium.

Methods

Collection of human samples and clinical parameters

Human ventricular myocardial and plasma samples were obtained from patients who underwent implantation of a Thoratec HeartMate II LVAD at University Hospital Leuven, Belgium between November 2009 and September 2011. All samples were collected with informed consent and ethical approval as part of a previous research project (study number S-52-659; ethics approval number ML-6832). Plasma samples were obtained prior to LVAD implantation and at 3-month intervals after LVAD support until time of cardiac transplantation. Myocardial samples were collected at the time of LVAD implantation and subsequent cardiac transplantation. All samples were immediately frozen with liquid nitrogen and stored at -80°C until required for laboratory analysis. Clinical data were obtained contemporaneously with sample collection. Assays of NT-proBNP and estimation of LVEF were performed, respectively, by the clinical laboratories and core echocardiography laboratory at University Hospitals Leuven, Belgium.

Total RNA isolation

All RNA studies were performed in the Institute for Molecular and Translational Therapeutic Strategies in Hannover Medical School, Hannover, Germany. Total RNA isolation was performed using the Qiagen miReasy Mini Kit and 96 Kit (Qiagen, Venlo, The Netherlands)

with QIAzol Lysis Reagent used for cell lysis. For plasma samples, a fixed volume (200 μL) was used and the homogenate spiked with RNA from *Caenorhabditis elegans* gene 39 (5 μL of 1 fmol/ μL RNA) as external control. For myocardial tissue samples, the concentration of total RNA in the final eluate was determined using ultraviolet spectroscopy, and samples prepared each containing a fixed quantity of total RNA. RNU48 was used as endogenous control for tissue samples.

Polymerase chain reaction microarray for microRNA expression profiling

RNA was pooled into four pairs of samples for miR expression profiling. cDNA was prepared for real-time quantitative polymerase chain reaction (RT-qPCR) from these pooled samples using QuantiMir (BioCat GmbH, Heidelberg, Germany), and miR expression profiling was performed with the BioCat Human Genome Wide microRNA 384-well RT-qPCR Array (BioCat GmbH) using the BioRad CFX384 Touch RT qPCR Detection System (BioRad, Hemel Hempstead, UK). This array uses SYBR Green reagents and detects 1113 known miRs identified in the Sanger miRBase Version 15.

Validation and quantification with TaqMan real-time quantitative polymerase chain reaction

The miRs identified in the screening microarray were validated in individual samples using the more specific TaqMan RT-qPCR technique. The validated miRs were quantified in all samples using fresh preparations of total RNA isolated from all available myocardial and plasma samples. For all these reactions, cDNA transcripts were prepared using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), and real-time qPCR was done using the ViiA 7 Real-Time PCR System (Applied Biosystems) using Absolute Blue reagents. All TaqMan reactions were performed in duplicate.

Analysis of real-time quantitative polymerase chain reaction data

For the microarray, data were normalized by two separate methods each generating comparable results. The first method used *C. elegans* spike-in reference, to control for differing efficiencies of RNA isolation and individual qPCR runs, and the second method used global normalization, whereby the mean expression data across the plate are used to normalize individual values. After normalization, candidate miRs were identified if they were expressed in all test samples and showed a fold change in expression >3 times or <0.33 times between baseline and 6 months. miRs with threshold cycle (Ct) values >35 were highlighted as unreliable. For candidate identification, fold change in expression between baseline and 6 months was calculated for each miR, and paired *t*-tests were used to identify statistically significant differences. For TaqMan reactions, normalization was performed using *C. elegans* spike-in reference only, due to the small number of RT-qPCRs per plate. In all cases, the selection of thresholds for detection was performed by personnel blinded to the study groups. miR quantification is expressed as 'log₂[40 – Ct value]'.¹⁴

Study design and statistics

The primary outcome was change in miR expression at 6 months. For assessment of predictive biomarkers, we classified the clinical

Table 1 Patient characteristics at the time of left ventricular assist device implantation

	Whole cohort (n = 19)	Good responders (n = 7)	Poor responders (n = 6)	P-value
Gender, n (%)				
Female	4 (21)	2 (29)	1 (17)	0.612
Male	15 (79)	5 (71)	5 (83)	
Age in years, median (IQR)	52 (30–61)	40 (14–62)	55 (36–60)	0.731
Aetiology, n (%)				
Ischaemic	10 (53)	2 (29)	3 (50)	0.429
Non-ischaemic	9 (47)	5 (71)	3 (50)	
Body mass index (kg/m ²), median (IQR)	23.4 (22.5–26.6)	22.1 (20.9–25.4)	23.3 (22.8–27.8)	0.181
Current or previous smoker, n (%)	7 (37)	1 (14)	4 (67)	0.053
Diabetes mellitus, n (%)	1 (5)	1 (14)	0 (0)	0.335
Chronic renal failure, n (%)	0 (0)	0 (0)	0 (0)	–
Medications, n (%)				
Intravenous inotrope	10 (53)	4 (57)	3 (50)	0.797
ACE inhibitor	12 (63)	5 (71)	4 (67)	0.853
ARB	1 (5)	0 (0)	1 (17)	0.261
Beta-blocker	11 (58)	6 (86)	3 (50)	0.164
MRA	6 (32)	0 (0)	4 (67)	0.009
Digoxin	3 (16)	3 (43)	0 (0)	0.067
Diuretic	14 (74)	5 (71)	5 (83)	0.612
Platelet inhibitor	6 (32)	2 (29)	1 (17)	0.612
Anticoagulant	0 (0)	0 (0)	0 (0)	–
INTERMACS profiles 1–3, n (%)	15 (80)	6 (86)	4 (67)	0.416
NT-proBNP (ng/L), median (IQR)	4724 (1526–20 890)	17499 (4724–30 212)	1443 (1306–3858)	0.008
Change in NT-proBNP after 3 months LVAD support	0.23 (0.07–0.62)	0.08 (0.04–0.13)	0.62 (0.54–1.14)	0.001
LVEF (%), median (IQR)	15 (15–25)	20 (10–30)	15 (13–23)	0.662

Data are shown for the whole cohort and for the good and poor responder subgroups. The P-value refers to comparison of the subgroups.

IQR, interquartile range; INTERMACS, Interagency Registry for Mechanically Assisted Circulatory Support; LVAD, left ventricular assist device; MRA, mineralocorticoid receptor antagonist.

response to LVAD therapy based on their change in NT-proBNP at 3 months, and divided the cohort at the 50th centile for a binary outcome. Patients whose change in NT-proBNP was above the 50th percentile (i.e. smaller change) were identified as poor responders, and patients whose change in NT-proBNP was below the 50th centile (i.e. larger change) were identified as good responders. Descriptive statistics are shown as the median (interquartile range). Final data showed non-normal distribution, and non-parametric tests were used for outcome analysis. All analysis was done using IBM SPSS Statistics Version 21 with log-transformed data. Results with $P < 0.05$ were considered statistically significant.

Results

Patient characteristics and clinical response to left ventricular assist device therapy

We retrospectively obtained 53 serial plasma and 20 ventricular myocardial samples from 19 patients with severe advanced heart failure who underwent LVAD implantation. Patients' baseline characteristics are summarized in Table 1 and reflect the advanced nature of their disease, with 80% in INTERMACS profiles 1–3, NT-proBNP concentration 4724 (1526–20890) ng/L,

and LVEF 15 (15–25) %. These values are similar to parameters reported in previous clinical trials, confirming that this sample is a representative LVAD population.^{14,15} All patients were implanted with a HeartMate II LVAD at baseline. NT-proBNP levels show inverse correlation with duration of LVAD support ($r = -0.508$, $P = 0.0001$), with NT-proBNP concentration falling to 924 (653–2792) ng/L and 674 (524–1421) ng/L by 3 and 6 months, respectively. During the study period, 15 patients (79%) underwent cardiac transplantation after 200 (133–299) days, and at the time of transplantation NT-proBNP was 1116 (828–2130) ng/L.

Identification of candidate biomarkers

To identify candidate miR biomarkers (Figure 1), we studied a screening cohort for whom we had paired plasma samples from baseline and after 6 months LVAD support ($n = 8$). After total RNA isolation and cDNA synthesis, we used an RT-qPCR screening microarray to compare differential expression of 1113 plasma miRs in four pairs of pooled samples (see the Methods). In one of the sample pools ($n = 2$), there was failure of the *C. elegans* spike-in to amplify, alongside a high rate of amplification failure for other target miRs, probably related to the presence of heparin or PCR inhibitors. These data were excluded from the analysis, making a final $n = 6$ in the screening cohort.

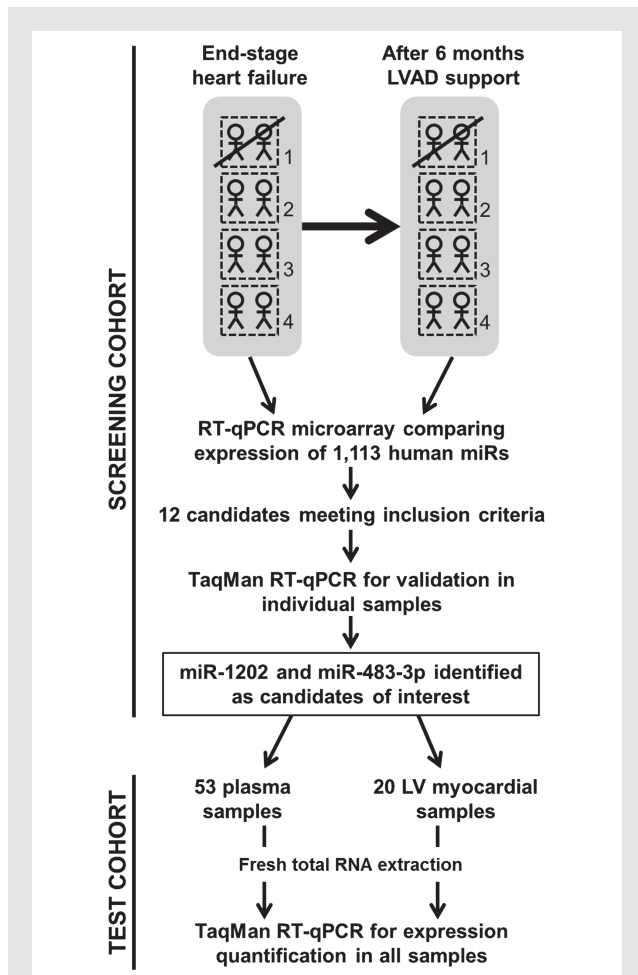


Figure 1 Schematic diagram illustrating the strategy for identification and validation of candidate microRNAs (miRs). For identification of candidate miRs we used a screening cohort of eight patients with paired plasma samples from before and after 6 months of left ventricular assist device therapy. Total RNA was extracted, and this was pooled into four test samples for each time point. We used a real-time quantitative polymerase chain reaction (RT-qPCR) microarray for unbiased expression profiling. Amplification failed for one sample pair and therefore this was excluded from analysis, giving a final $n = 6$ for the screening cohort. The microarray yielded 12 candidate miRs. These underwent validation using TaqMan RT-qPCR for greater specificity to minimize false-positive results, yielding miR-1202 and miR-483-3p as candidate miRs of interest. After fresh RNA extraction, these two miRs were quantified in the test cohort of all 73 samples (53 plasma and 20 LV myocardial tissue). All TaqMan reactions in the screening and test cohorts were performed in duplicate. See the Methods for more details.

One hundred and thirteen miRs met the criteria for consideration, of which 12 miRs showed statistically significant variation. These were (fold change, P -value): miR-1254 (5.75, $P < 0.01$), miR-33a (118.25, $P < 0.01$), miR-219-1-3p (4.15, $P < 0.01$), miR-548l (0.16, $P = 0.01$), miR-1250 (11.12, $P = 0.02$), miR-938 (121.43, $P = 0.03$), miR-483-3p (5.78, $P = 0.03$), miR-4266 (30.57,

$P = 0.03$), miR-4325 (0.13, $P = 0.03$), miR-1202 (59.50, $P = 0.03$), miR-557 (20.00, $P = 0.05$), and miR-1275 (5.24, $P = 0.05$).

For greater specificity, these findings were validated using TaqMan RT-qPCR in the individual samples used during the screening microarray ($n = 14$). miR-1202 and miR-483-3p were detected robustly (mean Ct values across all samples were 19.365 ± 1.090 and 31.497 ± 1.342 cycles, respectively) and showed change in expression consistent with the microarray but with a lesser amplitude (fold change 1.91, $P = 0.10$; and fold change 1.90, $P = 0.11$, respectively). These lesser degrees of statistical confidence were accepted for candidate selection at this stage, given the limitations of the small sample size. The remaining miRs were false positives, showing either robust expression but no significant difference, or unreliable expression data (e.g. Ct > 35 cycles, high standard deviation between duplicates).

Thus the screening yielded miR-1202 and miR-483-3p as candidate miR biomarkers that show significant change during 6 months of LVAD support, and these were taken forward for further assessment.

After left ventricular assist device implantation, miR-483-3p is up-regulated in plasma and in ventricular myocardium

Having identified two candidate biomarkers, we characterized how their expression varied after LVAD implantation in plasma and ventricular myocardium. We used a test cohort including all available myocardial and plasma samples from all 19 patients. Fresh total RNA isolation was performed, followed by quantification of miR-1202 and miR-483-3p in all plasma and myocardial samples using TaqMan RT-qPCR ($n = 73$). Of the 53 plasma samples, there was poor amplification in two, and these were excluded. In the remaining plasma samples ($n = 51$), both miRs were expressed robustly [Ct values 19.64 (19.25–19.87) and 31.85 (31.25–32.44) cycles for miR-1202 and miR-483-3p, respectively]. Both miRs were strongly expressed in the ventricular myocardium [$n = 20$; Ct values 21.19 (21.05–21.32) and 25.28 (24.83–25.92) cycles for miR-1202 and miR-483-3p, respectively].

For temporal assessment of circulating miR expression, duration of LVAD support was categorized as 0 months ($n = 14$; before LVAD implantation), 3 months [$n = 9$; 91 (83–91) days after implant], 6 months [$n = 8$; 187 (181–193) days], 9 months [$n = 7$; 271 (265–272) days], and 12 months [$n = 6$; 371 (361–392) days]. For comparison of plasma and myocardial expression, we studied paired samples of ventricular myocardium at baseline and after LVAD support at the time of transplantation, and compared this with corresponding plasma expression ($n = 10$ pairs).

Circulating miR-483-3p showed early and sustained up-regulation with LVAD support, with fold changes from baseline of 2.17 (1.43–2.62; $P = 0.011$), 2.27 (1.12–2.42; $P = 0.036$), 1.87 (1.64–4.36; $P = 0.028$), and 2.82 (0.70–10.62; $P = 0.249$) at 3, 6, 9, and 12 months, respectively (see Table 2 and Figure 2). These results mirrored the reduction in NT-proBNP levels [e.g. at 6 months, fold change 0.30 (0.08–0.43; $P = 0.004$)]. Myocardial expression of miR-483-3p also showed up-regulation, with absolute expression

Table 2 Temporal changes in plasma levels of microRNA-483-3p, microRNA-1202 and plasma concentration of N-terminal pro brain natriuretic peptide after implantation of a left ventricular assist device

Duration of LVAD support (months)	MicroRNAs		miR-1202		NT-proBNP						
	n	Exp., AU	FC from 0 months	P-value ^a	Exp., A.U.	FC from 0 months	P-value ^a	n	Concentration, ng/mL	RC from 0 months	P-value ^a
0	14	0.884 (0.857–0.957)	—	—	1.308 (1.296–1.313)	—	—	17	4724 (1526–20 890)	—	—
3	9	0.915 (0.906–0.941)	2.17 (1.43–2.62)	0.011	1.308 (1.306–1.319)	1.04 (0.85–1.57)	0.515	13	924 (653–2792)	0.23 (0.07–0.62)	0.006
6	8	0.907 (0.891–0.923)	2.27 (1.12–2.42)	0.036	1.307 (1.304–1.326)	0.94 (0.79–1.51)	1.000	11	674 (524–1421)	0.30 (0.08–0.43)	0.004
9	7	0.922 (0.881–0.942)	1.87 (1.64–4.36)	0.028	1.314 (1.304–1.323)	1.07 (0.71–1.71)	0.612	9	873 (420–1300)	0.14 (0.05–0.42)	0.008
12	6	0.897 (0.851–1.011)	2.82 (0.70–10.62)	0.249	1.313 (1.290–1.320)	1.14 (0.54–1.50)	0.917	7	1190 (534–1367)	0.26 (0.10–0.64)	0.028

LVAD, left ventricular assist device; Exp, plasma expression in arbitrary units (AU); FC, fold change in miR expression from 0 months; miR, microRNA; RC, relative change in NT-proBNP as a proportion of NT-proBNP concentration at 0 months.

^aP-value for comparison vs. 0 months.

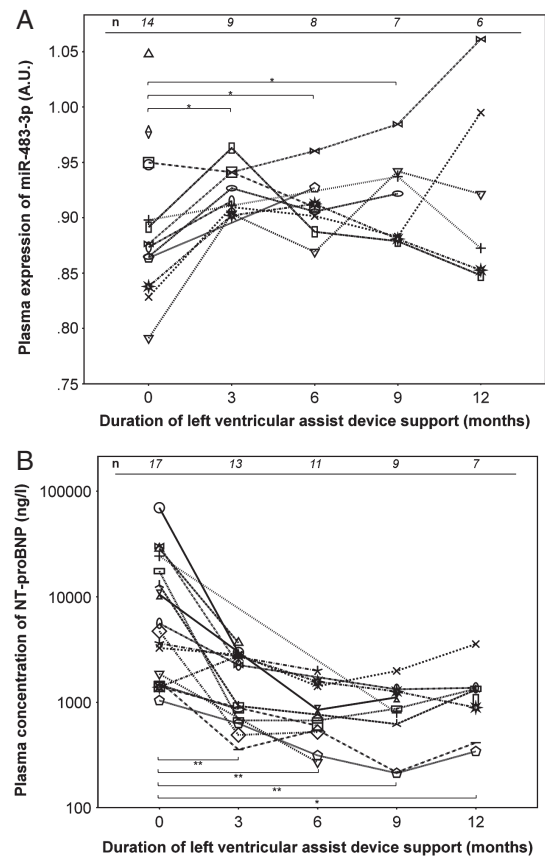


Figure 2 Temporal changes in plasma levels of microRNA-483-3p (miR-483-3p) and plasma concentration of NT-proBNP after implantation of a left ventricular assist device (LVAD). There is significant early and sustained up-regulation of plasma miR-483-3p after LVAD implantation (A), which mirrors the reduction in levels of NT-proBNP (B). Each line represents one patient. For miR-483-3p, data are shown for $n = 14$, $n = 9$, $n = 8$, $n = 7$, and $n = 6$ for 0, 3, 6, 9, and 12 months, respectively. For NT-proBNP, data are shown for $n = 14$, $n = 13$, $n = 11$, $n = 9$, and $n = 7$ for 0, 3, 6, 9, and 12 months respectively. * $P < 0.05$; ** $P < 0.01$.

increased from 1.153 (1.093–1.171) arbitrary units (AU) at the time of LVAD implantation to 1.180 (1.167–1.191) AU at the time of transplantation, equating to a fold change of 1.80 (0.72–4.72; $P = 0.169$; Figure 3).

In contrast, detection of circulating miR-1202 showed only small changes from baseline at all time points in the test cohort, and the change in miR-1202 expression between baseline and 6 months that was observed in the screening cohort was not replicated [fold change 0.94 (0.79–1.51; $P = 1.000$)]. Tissue expression of miR-1202 also showed no change with LVAD support [fold change 0.99 (0.81–1.07); $P = 0.575$; Figure 3].

Considering all plasma samples ($n = 51$), there was a trend towards correlation between NT-proBNP levels and plasma expression of miR-1202 ($r = -0.270$, $P = 0.055$), but no association of NT-proBNP with miR-483-3p ($P = 0.406$) and no correlation

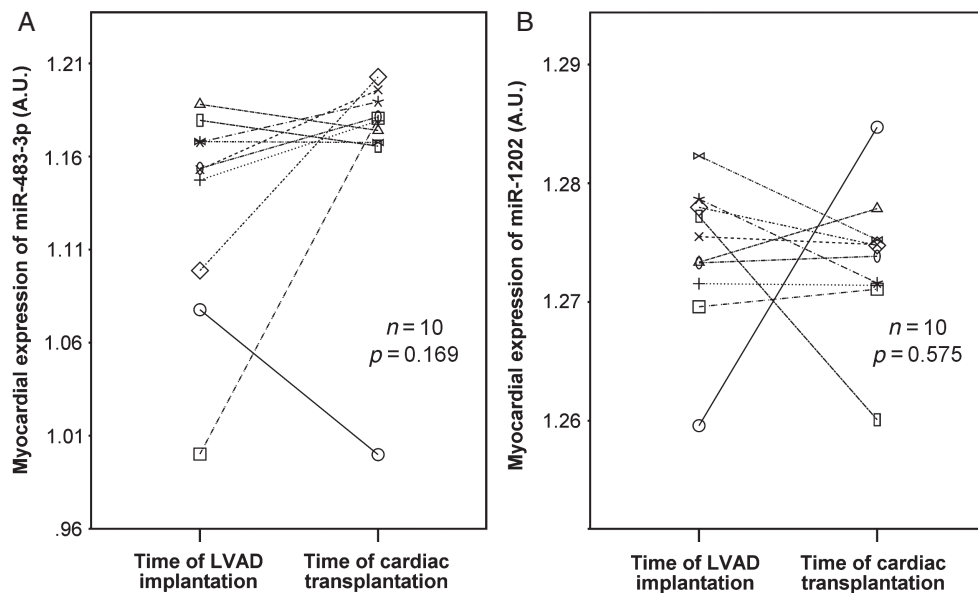


Figure 3 Changes in myocardial expression of the microRNAs (miRs) miR-483-3p and miR-1202 between the time of left ventricular assist device (LVAD) implantation and time of subsequent cardiac transplantation. There were paired samples from the time of LVAD implantation and time of cardiac transplantation from 10 patients. The median time to transplantation was 200 (133–299) days. After LVAD support, there was up-regulation of myocardial miR-483-3p expression [A, median fold change 1.799 (0.717–4.719; $P = 0.169$)], but no significant change in myocardial miR-1202 expression [B, median fold change 0.988 (0.808–1.070; $P = 0.575$)]. Each line represents one patient.

between duration of LVAD support in days and fold change in plasma expression of miR-483-3p ($P = 0.498$) or miR-1202 ($P = 0.759$). Despite the relationship between plasma and myocardial expression for each miR, we found no direct correlation between plasma and myocardial expression in the limited number of patients for whom we had paired plasma–myocardial samples ($n = 10$). Furthermore, there was no difference in plasma expression of either miR (miR-483-3p or miR-1202) by gender ($P = 0.733$, $P = 0.839$) or heart failure aetiology ($P = 0.282$, $P = 0.228$).

miR-1202 as a biomarker for predicting response to left ventricular assist device support

We hypothesized that baseline measurement of the candidate miRs might provide novel biomarkers that predict individual patients' response to LVAD support. We classified patients' clinical response to LVAD therapy based on their change in NT-proBNP at 3 months ($n = 13$). There was a strong correlation between baseline miR-1202 expression and change in NT-proBNP at 3 months ($r = 0.604$, $P = 0.029$). We divided the cohort at the 50th centile for change in NT-proBNP at 3 months to identify good responders ($n = 7$) and poor responders ($n = 6$; see the Methods and Figure 4). Expression of circulating miR-1202 varied significantly between these groups [1.296 (1.293–1.306) vs. 1.311 (1.310–1.318) AU; $P = 0.004$]. Finally, receiver operator characteristic (ROC) curve analysis to compare baseline circulating miR-1202, baseline NT-proBNP, and the pre-operative

INTERMACS profile identifies baseline miR-1202 as the best predictor of change in NT-proBNP at 3 months [($n = 13$; area under the curve (AUC) 0.976 (0.904–1.000), 0.714 (0.397–1.000), and 0.071 (0.000–0.210) for pre-implant miR-1202, INTERMACS profile, and NT-proBNP respectively, $P = 0.04$].

Discussion

This is the first time that circulating miRs have been characterized in heart failure patients treated with LVAD therapy. We have performed a large-scale unbiased microarray screen and identified novel miRs of relevance to this patient cohort. We have confirmed the feasibility of this approach, and demonstrated the plasticity of miR-483-3p expression in plasma and in ventricular myocardium in response to LVAD support. Furthermore, we have shown potential for circulating miRs to act as biomarkers for predicting (miR-1202) and monitoring (miR-483-3p) individual patients' response to LVAD therapy.

The need for new biomarkers

There is a paucity of non-invasive biomarkers that assist patient selection and serial monitoring of LVAD patients. Contemporary risk stratification relies on an experienced clinician integrating multiple prognostic parameters including clinical situation, maximal oxygen uptake, levels of natriuretic peptides, results from invasive right heart catheterization, and risk scores such as the Seattle Heart Failure Model.^{1,4,16,17} After LVAD implantation there are

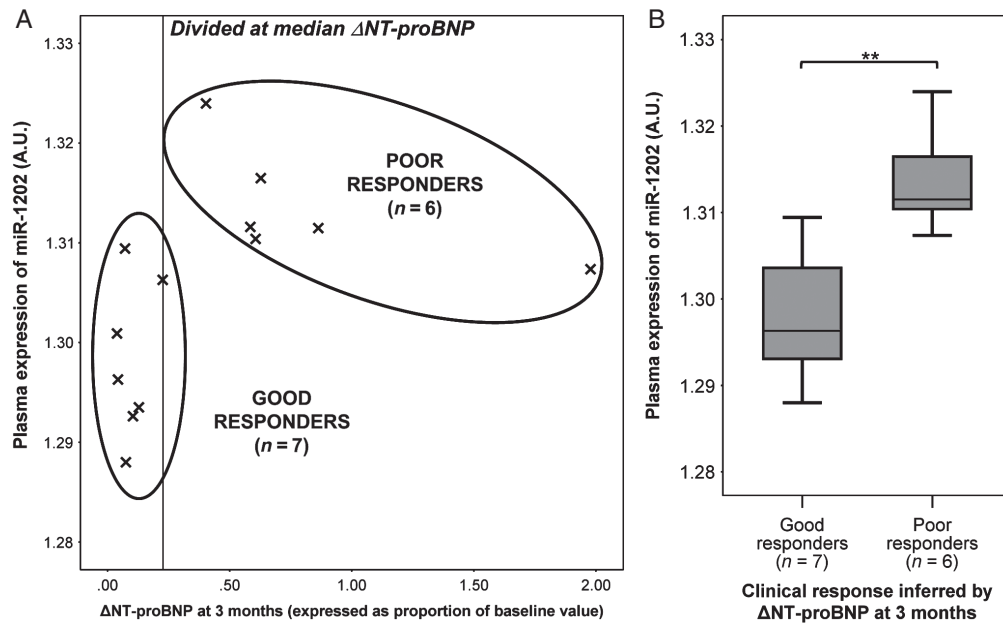


Figure 4 microRNA-1202 (miR-1202) identifies patients at high risk of inadequate response to left ventricular assist device (LVAD) support. We used change in change in NT-proBNP after 3 months of LVAD support as a marker of clinical response to LVAD support ($n = 13$). Dividing the cohort at the median identified good ($n = 7$) and poor ($n = 6$) responders. The scatterplot (A) and boxplot (B) show plasma miR-1202 expression before LVAD implantation, identifying good vs. poor responders. Expression of circulating miR-1202 varied significantly between these groups [good, 1.296 (1.293–1.306) vs. poor, 1.311 (1.310–1.318) AU; $P = 0.004$].

inadequate tools for assessing changes in ventricular function during ongoing support: cardiac catheterization is informative but invasive; echocardiography studies the unloaded LV and is limited by obstruction of apical views;¹⁸ and others such as exercise testing and natriuretic peptides give information about overall circulatory function yielded by the heart and pump together.¹⁹ Use of the ‘turn down’ study, where the LVAD speed is temporarily reduced to challenge the ventricle with volume loading, partially overcomes this to allow functional, haemodynamic, or echocardiographic assessments, but is time consuming and carries a theoretical risk of LVAD thrombosis due to transiently low pump speeds.^{20,21} Here, we hypothesized that circulating miRs could provide additional non-invasive tools to aid these assessments.

Monitoring response to left ventricular assist device therapy

This cohort of 19 patients had severely advanced heart failure at enrolment, with 80% in INTERMACS profiles 1–3, median NT-proBNP 4724 ng/L, and median LVEF 15%. All were implanted with a HeartMate II LVAD with varying degrees of response, with median NT-proBNP falling to 674 ng/L by 6 months. Circulating miR-483-3p shows a significant up-regulation with LVAD support that mirrors the suppression of NT-proBNP levels. miR-483-3p in the ventricular myocardium is also up-regulated with LVAD support, though to a lesser extent (and not reaching statistical significance in this small sample). These changes in

miR-483-3p expression could provide a more specific assessment of ventricular function that complements the changes in systemic neuroendocrine milieu recorded by serial measurement of natriuretic peptides. This hypothesis requires further assessment in a larger cohort to determine how changes in miR-483-3p expression correspond to changes in functional and haemodynamic indices, and long-term outcomes.

Identifying the likely non-responder

The heterogeneity of clinical response to LVAD support can be challenging, and better tools for identifying patients unlikely to benefit from this invasive and expensive therapy could significantly reduce morbidity, mortality, and treatment cost. Our data identify miR-1202 as a biomarker that predicts early response to LVAD support. Baseline plasma miR-1202 levels correlate with change in NT-proBNP at 3 months, and stratify the cohort into poor vs. good responders with greater accuracy than either baseline NT-proBNP or pre-operative INTERMACS profile, parameters which are crucial aids to decision-making around the time of implant. Clinically this would be valuable for judging the likelihood of good response to LVAD support, such that patients identified *a priori* as poor LVAD responders could be put forward for an alternative therapy such as urgent cardiac transplantation. However, this analysis is limited by the cohort size, and by the use of change in NT-proBNP as a surrogate endpoint for clinical response. In the long term, miR-1202 will need to be validated in a larger, prospective trial with long-term clinical outcome and survival data.

Strengths and limitations

The key advantage of circulating miR is that it is a non-invasive test and as such has the capacity to improve management for this patient cohort. This relies in turn on the stability of miRs in the circulation and in *ex vivo* storage, and their ready isolation and quantification. However, levels of circulating miRs are dependent on multiple factors, many independent of the cardiac disease itself. We noted two points of particular relevance here. First, the presence of heparin interferes with miR quantification,^{22,23} though this is mitigated in chronic LVAD patients because they are preferentially anticoagulated with warfarin. Secondly, antiplatelet medications can affect the circulating miR profile and in some patients are commenced after LVAD implantation, though our candidates show no overlap with the platelet-responsive targets identified recently.²⁴ Nonetheless these factors and their implications for RNA extraction efficiency may explain our observation that miR-1202 expression showed significant variation at 6 months in the microarray and validation steps, but not in the test cohort after re-isolation of RNA.

It is interesting that we have not identified miRs previously characterized in the heart failure or LVAD populations.^{8–12} Moreover having identified novel miR candidates pertaining to this precise clinical scenario, we chose not to include other miRs previously associated with heart failure. There are several reasons for this. First, our cohort represents the extreme end-stage of the heart failure spectrum, in comparison with others studies with largely NYHA II–III patients. Secondly, we have taken an unbiased microarray screening approach, and studied a larger panel of miRs than previous studies using rigorous candidate selection. Finally, our microarray was designed to compare differences between baseline and post-LVAD therapy where others studies have compared differences between healthy controls and heart failure, and miR-ome plasticity differs between these scenarios.

The limitations of our conclusions are reliance on a retrospective analysis of a small, heterogeneous clinical sample, and the necessity, in the absence of superior alternatives, to use change in NT-proBNP as a surrogate marker for treatment response. Whilst this is not a standard outcome measure in heart failure, there is well-established evidence of the prognostic role of natriuretic peptides,²⁵ and evidence that changes in BNP after LVAD implantation are accompanied by changes in cellular markers of heart failure.²⁶ These points underline the preliminary nature of our conclusions, and the need for further confirmatory studies in appraising these novel biomarkers for potential clinical use.

Further work

This study has forged a new avenue of enquiry in this fast moving field, but several questions remain unaddressed. First, we have generated hypotheses that miR-1202 and miR-483-3p could be useful biomarkers in LVAD patients, but these need prospective testing in larger cohorts. Secondly, based on the current data, we cannot draw firm conclusions about the source of the circulating miR-1202 and miR-483-3p, nor about their biological functions. We have shown that both miRs are enriched in ventricular myocardium

as well as in circulating plasma, but this does not establish the heart as the origin of the circulating fraction. Further studies in animal models of heart failure and in human patients could include tissue fractionation studies to identify the cellular origins of these miRs, and functional studies to consider both local functions at the cell or tissue level in the heart and paracrine-type signalling to other organs through plasma expression. Finally, the plasticity of the miR-ome may vary between different modes (e.g. pulsatile vs. continuous flow devices) and degrees (e.g. full vs. partial) of mechanical circulatory support which alter the quality of blood flow. For example, we know that the vascular endothelial cell miR-ome can be modulated by shear stress,²⁷ and other cells such as cardiomyocytes exhibit similar mechanosensitivity likely to involve miR pathways. These factors combined may yield a unique circulating miR-ome dependent on the quality of blood flow after LVAD implantation.

Conclusion

There is an unmet need for non-invasive techniques for use in serial assessment of LVAD patients, and this requirement is likely to grow. We have demonstrated the feasibility of circulating miRs as biomarkers in this patient group and proposed specific candidates that warrant further evaluation for potential clinical use.

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