Serially Measured Circulating MicroRNAs and Adverse Clinical Outcome in Patients with Acute Heart Failure.

Nick van Boven[1,2,3*], Isabella Kardys[2,3*], Laura C. van Vark[2,3], K. Martijn Akkerhuis[2,3], Maurice W.J. de Ronde[4,5,6], Mohsin A.F. Khan[4], Daphne Merkus[3], Zhen Liu[7], Adriaan A. Voors[8], Folkert W. Asselbergs[9,10,11], Ewout-Jan van den Bos[12], Eric Boersma[2,3], Hans Hillege[8,13], Dirk J. Duncker[3#], Yigal M. Pinto[4#], Douwe Postmus[13#]

* These authors contributed equally to this manuscript

# These authors share senior authorship

[1]Department of Cardiology, Noordwest Ziekenhuisgroep, Alkmaar, The Netherlands; [2]Department of Cardiology, Thoraxcenter, Erasmus MC, Rotterdam, The Netherlands; [3]Cardiovascular Research School Erasmus MC (COEUR); [4]Department of Cardiology, Academic Medical Center, Amsterdam, The Netherlands; [5]Department of Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands; [6]Department of Clinical Epidemiology, Biostatistics and Bioinformatics, Academic Medical Center, Amsterdam, The Netherlands; [7]ACS Biomarker BV, Amsterdam, the Netherlands; [8]Department of Cardiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands; [9]Department of Cardiology, Division of Heart and Lungs, UMC Utrecht, Utrecht, The Netherlands; [10]Durrer Center for Cardiogenetic Research, ICIN-Netherlands Heart Institute, Utrecht, the Netherlands; [11]Institute of Cardiovascular Science, Faculty of Population Health Sciences, University College London, London, United Kingdom; [12]Department of Cardiology, Albert Schweitzer Hospital, Dordrecht, The Netherlands; [13]Department of Epidemiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Address correspondence to: Isabella Kardys, MD, PhD, Department of Cardiology, Erasmus Medical Centre, room Ba-561, 's Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands,

Telephone number +31 (0)10 703 50 48,

Email address: i.kardys@erasmusmc.nl

ABSTRACT

Aims: Previous studies have identified candidate circulating microRNAs (circmiRs) as biomarkers for heart failure (HF) by relatively insensitive arrays, validated in small cohorts. We used RNA sequencing to identify novel candidate circmiRs and compared this to previously identified circmiRs in a large, prospective cohort of acute HF (AHF) patients.

Methods and results: RNA sequencing of plasma from instrumented pigs was used to identify circmiRS produced by myocardium, and found production of known myomirs and microRNA(miR)-1306-5p. We next tested the prognostic value of this and 11 other circmiRs in a prospective cohort of 496 AHF patients, from whom blood samples were collected at several time points (max 7) during the study's 1-year follow-up. The primary endpoint (PE) was the composite of all-cause mortality and HF rehospitalization. In the prospective AHF cohort, 188 patients reached the PE, and higher values of repeatedly measured miR-1306-5p were positively associated with the risk of the PE at that same time-point (HR(95%CI):4.69(2.18–10.06)), independent of clinical characteristics and NT-proBNP. Baseline miR-1306-5p did not improve model discrimination/reclassification significantly compared to NT-proBNP. For miR-320a, miR-378a-5p, miR-423-5p and miR-1254 associations with the PE were present after adjustment for age and sex (HRs(95%CI):1.38(1.12–1.70), 1.35(1.04–1.74), 1.45(1.10–1.92),1.22(1.00–1.50), respectively). Detection rate of myomiRs miR208a-3p and miR499a-5p was very low.

Conclusion: Repeatedly-measured miR-1306-5p was positively associated with adverse clinical outcome in AHF, even after multivariable adjustment including NT-proBNP. Yet, baseline miR-1306-5p did not add significant discriminatory value to NT-proBNP. Low-abundant, heart-enriched myomiRs are often undetectable which mandates more sensitive assays.

Key words: MicroRNA, Biomarkers, Heart Failure, Prognosis, Serial Measurements.

INTRODUCTION

To date, natriuretic peptides are the only circulating biomarkers which are routinely used for diagnosis and prognostication of heart failure (HF).[1] Improved HF prognostication may identify patients that could benefit from closer follow-up and from more aggressive treatment. Therefore, exploration of novel prognostic markers of HF can improve clinical management.

Circulating microRNAs (circmiRs) have been proposed as an attractive new class of biomarkers because of their stability in the circulation, and their ensuing reliable assessment in easily accessible samples.[2] However, most published studies to date involve relatively small numbers of HF patients with most often discrepant findings between separate studies.[3-7] Larger studies are scarce and have not investigated the temporal patterns of microRNAs (miRs) in patients with HF.[8] Importantly, longitudinal circmiR measurements in HF patients may provide further insight into individual, temporal patterns and the patient's ensuing risk of disease progression and adverse outcome.

In the present study, we used an RNA sequencing discovery experiment in pigs to identify circmiRs produced by the myocardium. Subsequently, we tested the potential for prognostication of the most promising novel circmiR (miR-1306-5p) in a set of 475 patients who were prospectively included for serial sampling after an AHF admission and compared it to multiple miRs known to be cardiac-enriched or already previously linked to HF (miR-1254, miR-22-3p, miR-345-5p, miR-378a-3p, miR-423-5p, miR-320a, miR-133a-3p, miR-133b, miR-499a-5p, miR-622, and miR-208a-3p).

METHODS

Part I: Preclinical study design

Aortic Banding and plasma and tissue harvesting

Experiments were performed in Aortic Banding (AoB)-treated *(*n=29) and sham-operated *(*n=21) Yorkshire x Landrace swine (see Supplemental Material for details, including surgical procedures and sacrifice of the animals). Briefly, following thoracotomy, the proximal ascending aorta was dissected free and, in AoB animals a band was placed.[9] Up to eight weeks later, swine were instrumented for simultaneous arterial and coronary venous blood sampling, followed by excision of the heart and harvesting of myocardial tissue samples from the left ventricular anterior wall.

RNA Sequencing

RNA was isolated from myocardial tissue and from arterial and coronary venous plasma samples of AoB-treated *(*n=4) and sham-operated *(*n=4) swine at 8 weeks follow-up after sham and AoB. For subsequent sequencing, RNA was pooled from myocardial tissue samples and from plasma obtained from arterial and coronary venous samples from AoB-treated and sham-operated samples, respectively. Pooled RNA from each sample was then divided into two, to have 2 technical replicates per sample. This resulted in a total of 16 samples, which were sent to BGI Shenzhen (China) for sequencing of small RNAs. At the BGI, libraries were prepared using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® kit. Samples were sequenced on an Illumina NextSeq 500 platform and base-calling was performed using the bcl2fastq 2.0 Conversion Software from Illumina. Quality control of fastq files was performed using FASTQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Trimmomatic version 0.32 was used to carry out 3´ adapter clipping of reads, using a phred score cut-off of 30 in order to trim low quality bases whilst ensuring that reads with a length below 18 bases were discarded.[10]

Differential miR expression analysis

We analyzed differential expression in the RNA sequencing data using the R Bioconductor package, DESeq2.[11] MiRs were selected based on next-generation sequencing results. Only miRs that were differentially expressed or had a high abundance in heart tissue were analyzed. We used quantitative polymerase chain reaction (PCR) to analyze expression levels of selected miRs in coronary venous and arterial plasma samples from 21 sham pigs and 29 AoB pigs. Plasma samples were analyzed to obtain a trans-coronary gradient in a comparable fashion; sham arterial plasma vs. coronary venous plasma, and AoB arterial plasma vs. coronary venous plasma. Owing to the availability of replicates, the dispersion method “pooled” from DESeq2 was used to accurately estimate dispersion between each comparison. DESeq2's negative binomial model was used to estimate differentially expressed miRs for each analysis. At the end, only those miRs passing a fold-change (log2) cut-off of 1.0 together with a False Discovery Rate cut-off of 0.05 were deemed significantly differentially expressed.

Part II: Clinical study design

TRIUMPH was an observational, prospective study enrolling patients admitted with acute HF in 14 hospitals in The Netherlands, between September 2009 and December 2013. The study was designed to allow analysis of novel potential biomarkers for prognostication of HF patients, with a particular interest directed towards changes in blood-biomarker patterns over time and their value for prognostication in HF patients. The study was approved by the medical ethics committee at all participating centers. All patients provided written informed consent.

Patients

Patients were eligible if ≥18 years old and hospitalized for acute HF, resulting from decompensation of known, chronic HF or newly diagnosed HF, and all three of the following criteria were met: (1) natriuretic peptide levels elevated to ≥3 times the upper limit of normal (determined in each individual hospital); (2) evidence of sustained left ventricular dysfunction, defined as moderate to poor systolic function or grade II (pseudonormal) to grade IV (fixed restrictive) diastolic dysfunction on echocardiography during hospitalization; and (3) treatment with intravenous diuretics. Patients were excluded in case they suffered from HF precipitated by a non-cardiac condition, by an acute ST-segment elevation myocardial infarction or by severe valvular dysfunction without sustained left ventricular dysfunction. Furthermore, patients were excluded if they were scheduled for coronary revascularization, listed for heart transplantation, suffered from severe renal failure for which dialyses was needed, or had a coexistent condition with a life expectancy <1 year.

Patient management

Patient management was at the discretion of the treating clinician, in accordance with the guidelines of the European Society of Cardiology.[12] Of note, biomarker data obtained in the context of this study were unknown to the treating physicians and thus were not used for clinical decisions.

Study procedures

Blood samples were obtained from all patients during hospitalization at admission (day 1), once during days 2 to 4 and subsequently at discharge; thus, 3 samples per patient were drawn during hospitalization. Additionally, blood samples were obtained at outpatient clinic follow-up visits, planned 2 to 4 weeks, 3 months, 6 months, and 9 to 12 months after discharge; thus, 4 samples were drawn during follow-up. As such, a total of 7 samples were obtained for each patient, unless a patient was censored or died before all samples could be taken. A short medical evaluation was performed and blood samples were collected at every

follow-up visit. Adverse cardiovascular events and changes in medication were recorded in electronic case report forms.

MiR- and NT-proBNP measurements

MiRNAs were measured in all separate plasma samples as described in detail in the Supplemental Material. MiR-1254, miR-22-3p, 423-5p, miR-320a and miR-622 were selected because they were associated with HF in previous studies,[5,7,13] miR-378a-3p and miR-345-5p because of their enrichment in cardiomyocytes,[14] and miR133a-3p, miR133b, miR208a-3p and miR499a-5p are muscle specific miRs (so-called 'myomiRs'), of which the latter two are heart specific and are released during myocardial injury.[15,16] MiR486-5p was used for normalization of the other miRs, because endogenous miRs have been shown to carry advantages for normalization compared to spike-in (e.g. Cel39) or small RNAs.[17] In the RNA-sequencing experiment we noticed that miR486-5p is exceptionally abundant (representing the vast majority of all detected miRs in the circulation, see Results below) and stable compared to other miRs, making it a suitable candidate to use as a normalizer (details of normalization are described in the Supplementary Material NT-proBNP measurements are also described in the Supplemental Material.

Quality control of human miR measurements

PCR of circulating miRs is sensitive to false or inaccurate signals, which may result in missing values.[18] Missing values may result from technical errors, but are most often due to template levels that are too low to measure reliably with qPCR. Therefore, we used a quality assessment algorithm to ensure the validity of each measurement. This algorithm is described more extensively elsewhere.[19] In brief, we distinguished three groups of measurements: 'detectable', 'non-detectable' (signal too low) and 'invalid'. If the measurement passed all the quality checks, it was considered valid and was marked 'detectable'. In case of a 'non-detectable' signal, the measurement was set to a low value, which was based on the PCR experiment parameters. If the measurement did not pass the

quality controls of the algorithm, it was defined as 'invalid'. Such measurements were not used in further analyses.

Endpoints

The primary endpoint comprised the composite of all-cause mortality and readmission for HF. The latter was defined as an unplanned rehospitalization due to acute HF, with at least two of the following three criteria: (1) elevated natriuretic peptide levels ≥3 times the upper limit of normal, (2) symptoms of cardiac decompensation (e.g. rales, edema or elevated central venous pressure), and (3) administration of intravenous diuretics. Secondary endpoints included the individual components of the primary endpoint and additionally cardiovascular mortality.

During follow-up, information on vital status and hospital readmissions was obtained until at least 9 months with a maximum of 400 days after the index hospital admission. We approached the civil registry, screened all medical records, and asked patients for information during their follow-up visits. A clinical event committee blinded to the biomarker results subsequently reviewed all collected information and adjudicated primary and secondary endpoints.

Statistical analysis

The associations between the baseline miR measurements and the risk of a study endpoint were assessed using Cox proportional hazards models. Abundant miRs were examined as continuous variables, while low-abundance miRs were entered into the models as dichotomous variables (detectable versus non-detectable, as defined by the algorithm described above), For repeated miR measurements, associations between the current level of each separate miR at a particular time point and the risk of an endpoint at that same time point were assessed using a joint modeling approach, which combines a linear mixed-effects model for the repeated miR measurements with a Cox proportional hazards model for the

risk of experiencing the event of interest.[20] A detailed description of the statistical analysis is provided in the Supplemental Material.

RESULTS

RNA sequencing in pigs samples

Post-quality control, the total number of reads per sample successfully aligned to pig-specific hairpin sequences ranged from 83.7 to 97.3 %. Combining all reads together, followed by discarding sequences longer than 25 nucleotides and those with low abundance (< 4 reads per sample) resulted in 373 x 10^6 reads that were successfully mapped to pig hairpin sequences. Aligning unmapped reads to hairpin sequences of other species increased the alignment rate by a negligible fraction (0.46%), suggesting that known hairpin sequences of Sus Scrofa were close to complete. We therefore, only used those sequences that were mapped to Sus scrofa hairpins.

Whilst calculating the number of reads aligned to each hairpin and mature miR sequence, a high abundance of miR-486-5p was observed in plasma samples (constituting 92.5-97% of all reads). There were a number of circmirs with a positive and significant trans-coronary gradient (figure 1). Among these were also known myomirs like miR-133a. In addition, less known circmirs like miR-1306 also showed a positive gradient. A comparison of next-generation sequencing based miR expression across tissue samples revealed a total of 16 miRs differentially expressed in sham-operated tissue compared to AoB-treated tissue (Table 1) among which miR-1306-5p was also significantly upregulated.

Given the positive trans-coronary gradient of miR-1306-5p and its significant upregulation in myocardial tissue of AoB compared to Sham pigs, we further evaluated the potential role of miR-1306-5p as a circulating biomarker. We compared the values obtained for miR-1306 in the control samples that are routinely taken along on the qPCR plates with the measurement of the HF samples, which showed that levels of circulating miR-1306-5p were significantly higher in the HF patients OR [95%CI] = 1.43 (1.033 – 1.98) in arbitrary

unit)/ln(pg/ml), p<0.05), further increasing the probability that circulating miR-1306-5p could serve as a novel biomarker for HF.

Prospective Clinical study: Baseline characteristics

A total of 496 patients were enrolled in the TRIUMPH clinical cohort and provided written informed consent. Three patients withdrew their informed consent. Eighteen patients were withdrawn from statistical analyses due to inclusion violation. These patients had no evidence of sustained systolic or diastolic left ventricular dysfunction on echocardiography. Accordingly, 475 patients compose the analysis set. Median age was 74 years (interquartile range (IQR) 65-80), 63% were men and median left ventricular ejection fraction was 30% (IQR 21-42) (Table 2). Median baseline NT-proBNP level was 4135 pg/mL (IQR 2123–9328).

Clinical endpoints

The composite primary endpoint was reached by 188 patients (40%) during a median follow-up of 325 (IQR 85–401) days. A total of 113 patients died, of which 77 were confirmed to die from a cardiovascular cause, and 123 patients were re-hospitalized for decompensated HF.

Circulating miR measurements

A total of 2214 blood samples were available for the current investigation. Median (IQR) number of miR measurements per patient was 3 (IQR 2–5). Supplemental table 1 displays the number of measurements that were detectable per miR. MiRs that were detectable in less than 700 out of 2214 samples were not used as continuous variables in further analyses but were dichotomized (detectable vs. non-detectable) as described above. MiRs that were examined as continuous variables were: miR-320a, miR-1254, miR-22-3p, miR-378a-3p, miR-423-5p, miR-345-5p and miR-1306-5p. MiRs that were dichotomized were: miR-133a-3p, miR-133b, and miR-499a-5p. MiR-486-5p was used for normalization of these miR levels. MiR-622 and miR-208a-3p were only detectable in 56 and 6 out of 2214 samples,

respectively. This low expression did not allow for meaningful statistical analysis of these miRs. Additionally, supplemental table 2 shows the baseline characteristics stratified by invalid versus valid measurement of baseline miR-1306-5p.

Finally, miR expression levels in patients with HF with reduced ejection fraction (HFrEF) vs. HF with preserved ejection fraction (HFpEF) are presented in supplemental table 3.

Associations between baseline miR levels and clinical endpoints

Figure 2 shows the difference in the risk of experiencing the primary endpoint for patients in different quartiles of baseline miR1306-5p levels ($p< 0.001$). This was confirmed in the subsequently fitted Cox models, where baseline miR1306-5p levels were significantly and independently associated with the primary endpoint (hazard ratios (HRs)(95%CI)): 1.13(1.03-1.23) (Table 3). From the other known miRs, only the baseline levels of miR-320a were significantly and independently associated with the primary endpoint (HRs(95%CI): 1.10(1.00-1.21)). Associations with secondary endpoints are shown in Supplemental Table 4. A sensitivity analysis on the subgroup of HFrEF patients, rendered a HR for baseline miR1306-5p in relation to the primary endpoint that was similar to the HR in the total group, but with a wider CI ((HR(95%CI): 1.09(0.95–1.25) (supplemental table 5). This was most likely caused by a decrease in statistical power in this subgroup.

Associations between temporal miR patterns and clinical endpoints

Repeatedly measured miR1306-5p level was positively and independently associated with the primary endpoint (HR(95%CI): (4.69(2.18–10.06)), $p< 0.001$ (Table 4). The temporal patterns of miR-320a, miR-378a-3p and miR-423-5p were positively associated with the primary endpoint after adjustment for age and sex. However, these associations disappeared after multivariable adjustment. The temporal pattern of miR-1254 displayed a borderline significant association with the primary endpoint after adjustment for age and sex

(HR(95%CI): 1.22(1.00-1.50). Associations of temporal patterns with secondary endpoints are shown in Supplemental Table 6.

Incremental prognostic value of miR-1306-5p

Adding miR-1306-5p to a model containing NT-proBNP age, sex, systolic blood pressure, diabetes mellitus, atrial fibrillation, BMI, previous hospitalization for HF during the last 6 months, ischemic HF, and baseline eGFR, we found a change in C-statistic of 0.012 (95%CI: -0.006–0.029), a continuous net reclassification (cNRI) improvement of 0.125(-0.016–0.267), and an integrated discrimination index (IDI) improvement of 0.020(-0.013–0.053), as shown in supplemental table 7. Thus, the incremental prognostic value of miR1306-5p on top of NT-proBNP did not reach statistical significance.

DISCUSSION

Direct RNA sequencing of plasma from instrumented pigs revealed a number of circmiRs to be produced by the pig myocardium, including miR-1306-5p which had not yet been identified as a miR related to the heart. Subsequently, we found in a prospective AHF cohort that repeatedly-assessed circulating miR-1306-5p is positively and independently associated with all-cause mortality and HF hospitalization. This association was independent of NT-proBNP. However, a model containing baseline miR-1306-5p measurements did not significantly improve model discrimination or reclassification when compared to NT-proBNP. Repeatedly-assessed circulating miR-320a, miR-378a-3p, miR-423-5p and miR-1254 were associated with the primary endpoint after adjustment for age and sex (albeit borderline for miR-1254), but not after further multivariable adjustment for clinical characteristics. Furthermore, an independent association was found between baseline values of miR-1306-5p and miR-320a and the primary endpoint.

Importantly, our findings are in line with those described in a manuscript where two large cohorts have been studied (Bayes-Genis et al, submitted back-to-back). In those two independent cohorts, miR-1306-5p was also positively and significantly associated with the risk of all-cause mortality or HF hospitalization. This further strengthens our findings and for the first time we see reproducible results on circulating miRs across three large cohorts. This contrasts with previous studies where usually one, mostly smaller cohort was analyzed,[21] and results have most often been discrepant between separate studies. To the best of our knowledge, the association between miR-1306-5p and cardiovascular disease has not been previously investigated in other studies, and further research is warranted on its expected targets.

RNA sequencing using plasma-derived RNA led to the discovery of miR-1306-5p produced by the heart. Akat et al also used RNA sequencing to analyze miRs potentially produced by the human heart.[22] However, their study was not designed to assess the clinical value of circmiRs as biomarkers. A word of caution concerns the large proportion of invalid and undetectable miR-1306-5p measurements which reduces power and illustrates the need

for more sensitive methods of miR assessment to enable optimal use of this marker for clinical prognostication. Nevertheless, the current study carried sufficient statistical power to demonstrate a significant association between repeatedly measured miR-1306-5p and the primary and secondary endpoints in spite of the proportion of invalid and undetectable measurements.

In line with our results, the study by Bayes-Genis et al. also found an association between miR-1254 and clinical outcome. Other existing data on miR-1254 are limited; of note is that Tijsen et al demonstrated upregulation of miR-1254 in HF cases compared to healthy controls.[5] An association between higher baseline miR423-5p levels and signs of progressive HF has been demonstrated in animal models,[6] and human studies with limited sample size.[3,5] Rising miR423-5p has also been related to worsening left ventricular function and has been shown to be upregulated in non-ST elevation myocardial infarction patients.[23] Our results agree with the findings of the aforementioned studies. Conversely, in recent a study in 236 acute HF patients, an inverse association was observed between miR423-5p and hospital readmission.[8] However, this finding could not be reproduced in the validation cohort which was examined.[8] Smaller studies have previously demonstrated higher circulating levels of miR-320a in HF patients compared to healthy individuals.[7,24] In addition, rat models have proven that overexpression of miR-320a leads to a greater loss of cardiomyocytes during infarction and that inhibition of miR-320a leads to reduced infarction size.[25] Furthermore, miR-320a showed a protective effect on left ventricular remodeling after myocardial ischemia-reperfusion injury in a rat model.[26] The results of the current study are in line with these previous studies, and further expand the evidence concerning miR-320a by showing that baseline measurements are independently associated with adverse prognosis in patients with HF, and that repetitively-measured miR-320a is independently associated with heart failure hospitalization in particular. The temporal pattern of miR-378a-3p was also associated with the primary endpoint. Naga Prasad et al showed downregulation of miR-378a-3p in left ventricular free wall tissue of HF patients with dilated cardiomyopathy.[4] In contrast, in the current study we examined circulating levels of miR-378a-3p. In addition,

Weber et al found higher levels of circulating miR-378a-3p in 5 patients with coronary artery disease, compared to 5 healthy controls.[27] However, studies other than ours on the prognostic value of miR-378a-3p in patients with HF are lacking.

Repeatedly measured, highly-abundant miRs only showed age-and sex-adjusted significant associations with the primary endpoint, and associations disappeared after multivariable adjustment. Possibly, prognostic information of these circmiRs, which are probably not produced by the heart, can be easily diluted. Conversely, myomiRs, i.e. miRs which are skeletal- and cardiac-muscle specific, carry potential to provide prognostic information that is incremental to clinical characteristics. Such myomiRs play a central role in myogenesis regulation and muscle remodeling.[28,29] Although the main sources of circulating myomiRs, and in particular the relationship between myomiRs in tissue and plasma have yet to be fully elucidated, an association between cardiac damage (caused by myocardial infarction or myocarditis) and upregulation of circulating myomiRs has been previously demonstrated.[15] Moreover, circulating myomiR levels have been associated with skeletal muscle wasting.[30] We examined several myomiRs in the current investigation (miR133a-3p, miR133b, miR208a-3p and miR499a-5p). However, myomiRs are lowly expressed in the circulation, as illustrated by the fact that they were non-detectable in a large proportion of the samples available in our study. Thus, we were forced to perform a simplified analysis and examined the association between presence of detectable myomiR levels at baseline and occurrence adverse events. The loss of information inherent to such an analysis may have obscured potential associations with the outcome. Therefore, more sensitive assays are needed to properly examine the roles of myomiRs in HF.

To remove noise by less robust QPCR results we designed and implemented a strict and conservative algorithm to remove unreliable QPCR data, and at the same time retain reliable assessment of 'too low to detect' signals. Furthermore, we used miR486-5p to normalize our data, as using such endogenous miRs for this purpose has been shown to carry advantages.[17] We have separately described our quality control algorithm we used here (provided for review purposes) and given the strong concordance between three large

cohorts we have thus measured strengthens the point of view that such algorithms help to remove noise and improve reproducibility.

Some aspects of this study warrant consideration. First, aortic banding has been used to model heart failure. This is a model that shows strong similarity to the TAC model in mice and has previously been used in multiple studies as a model for pressure-overload hypertrophy.[31-34] This model may not be fully representative of human left ventricular dysfunction. However, our observation that miR 1306-5p, identified in our swine model, does provide prognostic potential in the clinic, underscores the validity of our approach. Second, we did not adjust our analyses for multiple comparisons, because the miRs we examined were not selected in a hypothesis-free manner but had resulted from previous fundamental and clinical studies. Nevertheless, if we applied Bonferroni correction, the results would remain statistically significant. The association between repeated miR1306-5p and the primary endpoint rendered a HR(95%CI) of 4.69(2.18–10.06) and a p-value < 0.0001; since we examined 7 repeatedly measured miRs, the Bonferroni threshold for the p-value would be 0.05/7=0.007. Furthermore, we focused on patients with known heart failure. Studies using a healthy control group may provide insights into temporal miR patterns in healthy persons.

In conclusion, in patients hospitalized for AHF, baseline and repeatedly-assessed miR-1306-5p was independently associated with adverse clinical outcome. Associations of temporal patterns of miR-320a, miR-378a-5p, miR-423-5p and miR-1254 with adverse clinical outcome were not independent of clinical characteristics. Myocyte-specific miRs were non-detectable in a large proportion of the samples. More sensitive myomiR assays are needed in order to precisely estimate the risk associated with elevated levels of miRs such as miR1306-5p, and to investigate whether cardiac specific myomiRs on their part are capable of providing additional information to established, clinical risk predictors.

ACKNOWLEDGEMENTS

None.

FUNDING

This work was supported by the framework of CTMM (Center for Translational Molecular Medicine), projects TRIUMPH (grant 01C-103) and ENGINE (grant 01C-401). Folkert W. Asselbergs is supported by a Netherlands Heart Foundation Dekker scholarship - Junior Staff Member 2014T001 and UCL Hospitals NIHR Biomedical Research Centre.

CONFLICT OF INTEREST

Zhen Liu is employed by ACS Biomarker BV, Amsterdam, The Netherlands. Yigal Pinto has a commercial interest in ACS Biomarker BV (<5%) and is named as an inventor on a submitted patent application regarding miR-1306. Adriaan Voors is a patent holder of circulating miRs described in ref 21. All other authors have no conflict to declare.

REFERENCES

1. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JG, Coats AJ, Falk V, Gonzalez-Juanatey JR, Harjola VP, Jankowska EA, Jessup M, Linde C, Nihoyannopoulos P, Parissis JT, Pieske B, Riley JP, Rosano GM, Ruilope LM, Ruschitzka F, Rutten FH, van der MP. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur J Heart Fail* 2016;**18**:891-975.

2. Vegter EL, van der MP, De Windt LJ, Pinto YM, Voors AA. MicroRNAs in heart failure: from biomarker to target for therapy. *Eur J Heart Fail* 2016;**18**:457-468.

3. Goldraich LA, Martinelli NC, Matte U, Cohen C, Andrades M, Pimentel M, Biolo A, Clausell N, Rohde LE. Transcoronary gradient of plasma microRNA 423-5p in heart failure: evidence of altered myocardial expression. *Biomarkers* 2014;**19**:135-141.

4. Naga Prasad SV, Duan ZH, Gupta MK, Surampudi VS, Volinia S, Calin GA, Liu CG, Kotwal A, Moravec CS, Starling RC, Perez DM, Sen S, Wu Q, Plow EF, Croce CM, Karnik S. Unique microRNA profile in end-stage heart failure indicates alterations in specific cardiovascular signaling networks. *J Biol Chem* 2009;**284**:27487-27499.

5. Tijsen AJ, Creemers EE, Moerland PD, De Windt LJ, van der Wal AC, Kok WE, Pinto YM. MiR423-5p as a circulating biomarker for heart failure. *Circ Res* 2010;**106**:1035-1039.

6. Dickinson BA, Semus HM, Montgomery RL, Stack C, Latimer PA, Lewton SM, Lynch JM, Hullinger TG, Seto AG, van Rooij E. Plasma microRNAs serve as biomarkers of therapeutic efficacy and disease progression in hypertension-induced heart failure. *Eur J Heart Fail* 2013;**15**:650-659.

7. Goren Y, Kushnir M, Zafrir B, Tabak S, Lewis BS, Amir O. Serum levels of microRNAs in patients with heart failure. *Eur J Heart Fail* 2012;**14**:147-154.

8. Seronde MF, Vausort M, Gayat E, Goretti E, Ng LL, Squire IB, Vodovar N, Sadoune M, Samuel JL, Thum T, Solal AC, Laribi S, Plaisance P, Wagner DR, Mebazaa A, Devaux Y. Circulating microRNAs and Outcome in Patients with Acute Heart Failure. *PLoS One* 2015;**10**:e0142237.

9. Duncker DJ, Zhang J, Bache RJ. Coronary pressure-flow relation in left ventricular hypertrophy. Importance of changes in back pressure versus changes in minimum resistance. *Circ Res* 1993;**72**:579-587.

10. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;**30**:2114-2120.

11. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;**15**:550.

12. McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Bohm M, Dickstein K, Falk V, Filippatos G, Fonseca C, Gomez-Sanchez MA, Jaarsma T, Kober L, Lip GY, Maggioni AP, Parkhomenko A, Pieske BM, Popescu BA, Ronnevik PK, Rutten FH, Schwitter J, Seferovic P, Stepinska J, Trindade PT, Voors AA, Zannad F, Zeiher A. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. *Eur Heart J* 2012;**33**:1787-1847.

13. Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, Benjamin H, Kushnir M, Cholakh H, Melamed N, Bentwich Z, Hod M, Goren Y, Chajut A. Serum microRNAs are promising novel biomarkers. *PLoS One* 2008;**3**:e3148.

14. Rao PK, Toyama Y, Chiang HR, Gupta S, Bauer M, Medvid R, Reinhardt F, Liao R, Krieger M, Jaenisch R, Lodish HF, Blelloch R. Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. *Circ Res* 2009;**105**:585-594.

15. Corsten MF, Dennert R, Jochems S, Kuznetsova T, Devaux Y, Hofstra L, Wagner DR, Staessen JA, Heymans S, Schroen B. Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. *Circ Cardiovasc Genet* 2010;**3**:499-506.

16. Bostjancic E, Zidar N, Stajer D, Glavac D. MicroRNAs miR-1, miR-133a, miR-133b and miR-208 are dysregulated in human myocardial infarction. *Cardiology* 2010;**115**:163-169.

17. Kok MG, Halliani A, Moerland PD, Meijers JC, Creemers EE, Pinto-Sietsma SJ. Normalization panels for the reliable quantification of circulating microRNAs by RT-qPCR. *FASEB J* 2015;**29**:3853-3862.

18. Zampetaki A, Willeit P, Tilling L, Drozdov I, Prokopi M, Renard JM, Mayr A, Weger S, Schett G, Shah A, Boulanger CM, Willeit J, Chowienczyk PJ, Kiechl S, Mayr M. Prospective study on circulating MicroRNAs and risk of myocardial infarction. *J Am Coll Cardiol* 2012;**60**:290-299.

19. de Ronde MW, Ruijter JM, Lanfear D, Bayes-Genis A, Kok M, Creemers E, Pinto YM, Pinto-Sietsma SJ. Practical data handling pipeline improves performance of qPCR-based circulating miRNA measurements. *RNA* 2017.

20. Rizopoulos D. JM: An R Package for the Joint Modelling of Longitudinal and Time-to-Event Data. *Journal of Statistical Software* 2010;**35**:1-33.

21. Ovchinnikova ES, Schmitter D, Vegter EL, Ter Maaten JM, Valente MA, Liu LC, van der HP, Pinto YM, de Boer RA, Meyer S, Teerlink JR, O'Connor CM, Metra M, Davison BA, Bloomfield DM, Cotter G, Cleland JG, Mebazaa A, Laribi S, Givertz MM, Ponikowski P, van der MP, van Veldhuisen DJ, Voors AA, Berezikov E. Signature of circulating microRNAs in patients with acute heart failure. *Eur J Heart Fail* 2016;**18**:414-423.

22. Akat KM, Moore-McGriff D, Morozov P, Brown M, Gogakos T, Correa DR, Mihailovic A, Sauer M, Ji R, Ramarathnam A, Totary-Jain H, Williams Z, Tuschl T, Schulze PC. Comparative RNA-sequencing analysis of myocardial and circulating small RNAs in human heart failure and their utility as biomarkers. *Proc Natl Acad Sci U S A* 2014;**111**:11151-11156.

23. Olivieri F, Antonicelli R, Lorenzi M, D'Alessandra Y, Lazzarini R, Santini G, Spazzafumo L, Lisa R, La Sala L, Galeazzi R, Recchioni R, Testa R, Pompilio G, Capogrossi MC, Procopio AD. Diagnostic potential of circulating miR-499-5p in elderly patients with acute non ST-elevation myocardial infarction. *Int J Cardiol* 2013;**167**:531-536.

24. Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, Doevendans PA, Mummery CL, Borlak J, Haverich A, Gross C, Engelhardt S, Ertl G, Bauersachs J. MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. *Circulation* 2007;**116**:258-267.

25. Ren XP, Wu J, Wang X, Sartor MA, Qian J, Jones K, Nicolaou P, Pritchard TJ, Fan GC. MicroRNA-320 is involved in the regulation of cardiac ischemia/reperfusion injury by targeting heat-shock protein 20. *Circulation* 2009;**119**:2357-2366.

26. Song CL, Liu B, Diao HY, Shi YF, Li YX, Zhang JC, Lu Y, Wang G, Liu J, Yu YP, Guo ZY, Wang JP, Zhao Z, Liu JG, Liu YH, Liu ZX, Cai D, Li Q. The Protective Effect of MicroRNA-320 on Left Ventricular Remodeling after Myocardial Ischemia-Reperfusion Injury in the Rat Model. *Int J Mol Sci* 2014;**15**:17442-17456.

27. Weber M, Baker MB, Patel RS, Quyyumi AA, Bao G, Searles CD. MicroRNA Expression Profile in CAD Patients and the Impact of ACEI/ARB. *Cardiol Res Pract* 2011;**2011**:532915.

28. Townley-Tilson WH, Callis TE, Wang D. MicroRNAs 1, 133, and 206: critical factors of skeletal and cardiac muscle development, function, and disease. *Int J Biochem Cell Biol* 2010;**42**:1252-1255.

29. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet* 2006;**38**:228-233.

30. Donaldson A, Natanek SA, Lewis A, Man WD, Hopkinson NS, Polkey MI, Kemp PR. Increased skeletal muscle-specific microRNA in the blood of patients with COPD. *Thorax* 2013;**68**:1140-1149.

31. Kupper T, Pfitzer P, Schulte D, Arnold G. Pressure induced hypertrophy in the hearts of growing dwarf pigs. *Pathol Res Pract* 1991;**187**:315-323.

32. Modesti PA, Vanni S, Bertolozzi I, Cecioni I, Polidori G, Paniccia R, Bandinelli B, Perna A, Liguori P, Boddi M, Galanti G, Serneri GG. Early sequence of cardiac adaptations and growth factor formation in pressure- and volume-overload hypertrophy. *Am J Physiol Heart Circ Physiol* 2000;**279**:H976-H985.

33. Marshall KD, Muller BN, Krenz M, Hanft LM, McDonald KS, Dellsperger KC, Emter CA. Heart failure with preserved ejection fraction: chronic low-intensity interval exercise training preserves myocardial O2 balance and diastolic function. *J Appl Physiol (1985)* 2013;**114**:131-147.

34. Xiong Q, Zhang P, Guo J, Swingen C, Jang A, Zhang J. Myocardial ATP hydrolysis rates in vivo: a porcine model of pressure overload-induced hypertrophy. *Am J Physiol Heart Circ Physiol* 2015;**309**:H450-H458.

555 **Figure titles and legends**

556

557 Figure 1: Trans-coronary gradients in plasma microRNAs.

558 *The number indicates the number of pigs (out of a total of 44 pigs) with both a detectable*
559 *venous and arterial microRNA value. The gradient is calculated as arterial minus venous Ct*
560 *value of the microRNA, and shown as Mean±SEM. A negative value indicates release of the*
561 *microRNA by the myocardium, and a positive value indicates uptake. The p-value is*
562 *calculated using a paired samples T-test, and indicates the difference between arterial and*
563 *venous Ct value of the microRNA.*

564

565 Figure 2: Kaplan-Meier survival curves for the primary endpoint of death or readmission for
566 HF in the four quartiles of baseline miR-1306-5p levels.

567 *Q1 lowest quartile, Q4 highest quartile.*

Serially Measured Circulating MicroRNAs and Adverse Clinical Outcome in Patients with Acute Heart Failure.

Nick van Boven[1,2,3]*, Isabella Kardys[2,3]*, Laura C. van Vark[2,3], K. Martijn Akkerhuis[2,3], Maurice W.J. de Ronde[4,5,6], Mohsin A.F. Khan[4], Daphne Merkus[3], Zhen Liu[7], Adriaan A. Voors[8], Folkert W. Asselbergs[9,10,11], Ewout-Jan van den Bos[12], Eric Boersma[2,3], Hans Hillege[8,13], Dirk J. Duncker[3#], Yigal M. Pinto[4#], Douwe Postmus[13#]

* These authors contributed equally to this manuscript

# These authors share senior authorship

[1]Department of Cardiology, Noordwest Ziekenhuisgroep, Alkmaar, The Netherlands; [2]Department of Cardiology, Thoraxcenter, Erasmus MC, Rotterdam, The Netherlands; [3]Cardiovascular Research School Erasmus MC (COEUR); [4]Department of Cardiology, Academic Medical Center, Amsterdam, The Netherlands; [5]Department of Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands; [6]Department of Clinical Epidemiology, Biostatistics and Bioinformatics, Academic Medical Center, Amsterdam, The Netherlands; [7]ACS Biomarker BV, Amsterdam, the Netherlands; [8]Department of Cardiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands; [9]Department of Cardiology, Division of Heart and Lungs, UMC Utrecht, Utrecht, The Netherlands; [10]Durrer Center for Cardiogenetic Research, ICIN-Netherlands Heart Institute, Utrecht, the Netherlands; [11]Institute of Cardiovascular Science, Faculty of Population Health Sciences, University College London, London, United Kingdom; [12]Department of Cardiology, Albert Schweitzer Hospital, Dordrecht, The Netherlands; [13]Department of Epidemiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Address correspondence to: Isabella Kardys, MD, PhD, Department of Cardiology, Erasmus Medical Centre, room Ba-561, 's Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands,

Telephone number +31 (0)10 703 50 48,

Email address: i.kardys@erasmusmc.nl

ABSTRACT

Aims: Previous studies have identified candidate circulating microRNAs (circmiRs) as biomarkers for heart failure (HF) by relatively insensitive arrays, validated in small cohorts. We used RNA sequencing to identify novel candidate circmiRs and compared this to previously identified circmiRs in a large, prospective cohort of acute HF (AHF) patients.

Methods and results: RNA sequencing of plasma from instrumented pigs was used to identify circmiRS produced by myocardium, and found production of known myomirs and microRNA(miR)-1306-5p. We next tested the prognostic value of this and 11 other circmiRs in a prospective cohort of 496 AHF patients, from whom blood samples were collected at several time points (max 7) during the study's 1-year follow-up. The primary endpoint (PE) was the composite of all-cause mortality and HF rehospitalization. In the prospective AHF cohort, 188 patients reached the ~~primary endpoint~~PE, and higher values of repeatedly measured miR-1306-5p were positively associated with the risk of the ~~primary endpoint~~PE at that same time-point (HR(95%CI):4.69(2.18–10.06)), independent of clinical characteristics and NT-proBNP. Baseline miR-1306-5p did not improve model discrimination/reclassification significantly compared to NT-proBNP. For miR-320a, miR-378a-5p, miR-423-5p and miR-1254 associations with the PE were present after adjustment for age and sex (HRs(95%CI):1.38(1.12–1.70), 1.35(1.04–1.74), 1.45(1.10–1.92),1.22(1.00–1.50), respectively). Detection rate of myomiRs miR208a-3p and miR499a-5p was very low.

Conclusion: ~~MiR-1306-5p is produced by the myocardium and higher levels of r~~Repeatedly-measured miR-1306-5p was positively associated with adverse clinical outcome in AHF, even after multivariable adjustment including NT-proBNP. Yet, baseline miR-1306-5p did not add significant discriminatory value to NT-proBNP.~~provide prognostic information beyond NT-proBNP.~~ Low-abundant, heart-enriched myomiRs are often undetectable which mandates more sensitive assays.

Key words: MicroRNA, Biomarkers, Heart Failure, Prognosis, Serial Measurements.

INTRODUCTION

To date, natriuretic peptides are the only circulating biomarkers which are routinely used for diagnosis and prognostication of heart failure (HF).[1] Improved HF prognostication may identify patients that could benefit from closer follow-up and from more aggressive treatment. Therefore, exploration of novel prognostic markers of HF can improve clinical management.

Circulating microRNAs (circmiRs) have been proposed as an attractive new class of biomarkers because of their stability in the circulation, and their ensuing reliable assessment in easily accessible samples.[2] However, most published studies to date involve relatively small numbers of HF patients with most often discrepant findings between separate studies.[3-7] Larger studies are scarce and have not investigated the temporal patterns of microRNAs (miRs) in patients with HF.[8] Importantly, longitudinal circmiR measurements in HF patients may provide further insight into individual, temporal patterns and the patient's ensuing risk of disease progression and adverse outcome.

In the present study, we used an RNA sequencing discovery experiment in pigs to identify circmiRs produced by the myocardium. Subsequently, we tested the potential for prognostication of the most promising novel circmiR (miR-1306-5p) in a set of 475 patients who were prospectively included for serial sampling after an AHF admission and compared it to multiple miRs known to be cardiac-enriched or already previously linked to HF (miR-1254, miR-22-3p, miR-345-5p, miR-378a-3p, miR-423-5p, miR-320a, miR-133a-3p, miR-133b, miR-499a-5p, miR-622, and miR-208a-3p).

METHODS

Part I: Preclinical study design

Aortic Banding and plasma and tissue harvesting

Experiments were performed in Aortic Banding (AoB)-treated *(*n=29) and sham-operated *(*n=21) Yorkshire x Landrace swine (see Supplemental Material for details, including surgical procedures and sacrifice of the animals). Briefly, following thoracotomy, the proximal ascending aorta was dissected free and, in AoB animals a band was placed.[9] Up to eight weeks later, swine were instrumented for simultaneous arterial and coronary venous blood sampling, followed by excision of the heart and harvesting of myocardial tissue samples from the left ventricular anterior wall.

RNA Sequencing

RNA was isolated from myocardial tissue and from arterial and coronary venous plasma samples of AoB-treated *(*n=4) and sham-operated *(*n=4) swine at 8 weeks follow-up after sham and AoB. For subsequent sequencing, RNA was pooled from myocardial tissue samples and from plasma obtained from arterial and coronary venous samples from AoB-treated and sham-operated samples, respectively. Pooled RNA from each sample was then divided into two, to have 2 technical replicates per sample. This resulted in a total of 16 samples, which were sent to BGI Shenzhen (China) for sequencing of small RNAs. At the BGI, libraries were prepared using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® kit. Samples were sequenced on an Illumina NextSeq 500 platform and base-calling was performed using the bcl2fastq 2.0 Conversion Software from Illumina. Quality control of fastq files was performed using FASTQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Trimmomatic version 0.32 was used to carry out 3´ adapter clipping of reads, using a phred score cut-off of 30 in order to trim low quality bases whilst ensuring that reads with a length below 18 bases were discarded.[10]

Differential miR expression analysis

We analyzed differential expression in the RNA sequencing data using the R Bioconductor package, DESeq2.[11] MiRs were selected based on next-generation sequencing results. Only miRs that were differentially expressed or had a high abundance in heart tissue were analyzed. We used quantitative polymerase chain reaction (PCR) to analyze expression levels of selected miRs in coronary venous and arterial plasma samples from 21 sham pigs and 29 AoB pigs. Plasma samples were analyzed to obtain a trans-coronary gradient in a comparable fashion; sham arterial plasma vs. coronary venous plasma, and AoB arterial plasma vs. coronary venous plasma. Owing to the availability of replicates, the dispersion method “pooled” from DESeq2 was used to accurately estimate dispersion between each comparison. DESeq2's negative binomial model was used to estimate differentially expressed miRs for each analysis. At the end, only those miRs passing a fold-change (log2) cut-off of 1.0 together with a False Discovery Rate cut-off of 0.05 were deemed significantly differentially expressed.

Part II: Clinical study design

TRIUMPH was an observational, prospective study enrolling patients admitted with acute HF in 14 hospitals in The Netherlands, between September 2009 and December 2013. The study was designed to allow analysis of novel potential biomarkers for prognostication of HF patients, with a particular interest directed towards changes in blood-biomarker patterns over time and their value for prognostication in HF patients. The study was approved by the medical ethics committee at all participating centers. All patients provided written informed consent.

Patients

Patients were eligible if ≥18 years old and hospitalized for acute HF, resulting from decompensation of known, chronic HF or newly diagnosed HF, and all three of the following criteria were met: (1) natriuretic peptide levels elevated to ≥3 times the upper limit of normal (determined in each individual hospital); (2) evidence of sustained left ventricular dysfunction, defined as moderate to poor systolic function or grade II (pseudonormal) to grade IV (fixed restrictive) diastolic dysfunction on echocardiography during hospitalization; and (3) treatment with intravenous diuretics. Patients were excluded in case they suffered from HF precipitated by a non-cardiac condition, by an acute ST-segment elevation myocardial infarction or by severe valvular dysfunction without sustained left ventricular dysfunction. Furthermore, patients were excluded if they were scheduled for coronary revascularization, listed for heart transplantation, suffered from severe renal failure for which dialyses was needed, or had a coexistent condition with a life expectancy <1 year.

Patient management

Patient management was at the discretion of the treating clinician, in accordance with the guidelines of the European Society of Cardiology.[12] Of note, biomarker data obtained in the context of this study were unknown to the treating physicians and thus were not used for clinical decisions.

Study procedures

Blood samples were obtained from all patients during hospitalization at admission (day 1), once during days 2 to 4 and subsequently at discharge; thus, 3 samples per patient were drawn during hospitalization. Additionally, blood samples were obtained at outpatient clinic follow-up visits, planned 2 to 4 weeks, 3 months, 6 months, and 9 to 12 months after discharge; thus, 4 samples were drawn during follow-up. As such, a total of 7 samples were obtained for each patient, unless a patient was censored or died before all samples could be taken. A short medical evaluation was performed and blood samples were collected at every

follow-up visit. Adverse cardiovascular events and changes in medication were recorded in electronic case report forms.

MiR- and NT-proBNP measurements

MiRNAs were measured in all separate plasma samples as described in detail in the Supplemental Material. MiR-1254, miR-22-3p, 423-5p, miR-320a and miR-622 were selected because they were associated with HF in previous studies,[5,7,13] miR-378a-3p and miR-345-5p because of their enrichment in cardiomyocytes,[14] and miR133a-3p, miR133b, miR208a-3p and miR499a-5p are muscle specific miRs (so-called 'myomiRs'), of which the latter two are heart specific and are released during myocardial injury.[15,16] MiR486-5p was used for normalization of the other miRs, because endogenous miRs have been shown to carry advantages for normalization compared to spike-in (e.g. Cel39) or small RNAs.[17] In the RNA-sequencing experiment we noticed that miR486-5p is exceptionally abundant (representing the vast majority of all detected miRs in the circulation, see Results below) and stable compared to other miRs, making it a suitable candidate to use as a normalizer (details of normalization are described in the Supplementary Material NT-proBNP measurements are also described in the Supplemental Material.

Quality control of human miR measurements

PCR of circulating miRs is sensitive to false or inaccurate signals, which may result in missing values.[18] Missing values may result from technical errors, but are most often due to template levels that are too low to measure reliably with qPCR. Therefore, we used a quality assessment algorithm to ensure the validity of each measurement. This algorithm is described more extensively elsewhere.[19] In brief, we distinguished three groups of measurements: 'detectable', 'non-detectable' (signal too low) and 'invalid'. If the measurement passed all the quality checks, it was considered valid and was marked 'detectable'. In case of a 'non-detectable' signal, the measurement was set to a low value, which was based on the PCR experiment parameters. If the measurement did not pass the

quality controls of the algorithm, it was defined as 'invalid'. Such measurements were not used in further analyses.

Endpoints

The primary endpoint comprised the composite of all-cause mortality and readmission for HF. The latter was defined as an unplanned rehospitalization due to acute HF, with at least two of the following three criteria: (1) elevated natriuretic peptide levels ≥3 times the upper limit of normal, (2) symptoms of cardiac decompensation (e.g. rales, edema or elevated central venous pressure), and (3) administration of intravenous diuretics. Secondary endpoints included the individual components of the primary endpoint and additionally cardiovascular mortality.

During follow-up, information on vital status and hospital readmissions was obtained until at least 9 months with a maximum of 400 days after the index hospital admission. We approached the civil registry, screened all medical records, and asked patients for information during their follow-up visits. A clinical event committee blinded to the biomarker results subsequently reviewed all collected information and adjudicated primary and secondary endpoints.

Statistical analysis

The associations between the baseline miR measurements and the risk of a study endpoint were assessed using Cox proportional hazards models. Abundant miRs were examined as continuous variables, while low-abundance miRs were entered into the models as dichotomous variables (detectable versus non-detectable, as defined by the algorithm described above), For repeated miR measurements, associations between the current level of each separate miR at a particular time point and the risk of an endpoint at that same time point were assessed using a joint modeling approach, which combines a linear mixed-effects model for the repeated miR measurements with a Cox proportional hazards model for the

risk of experiencing the event of interest.[20] A detailed description of the statistical analysis is provided in the Supplemental Material.

RESULTS

RNA sequencing in pigs samples

Post-quality control, the total number of reads per sample successfully aligned to pig-specific hairpin sequences ranged from 83.7 to 97.3 %. Combining all reads together, followed by discarding sequences longer than 25 nucleotides and those with low abundance (< 4 reads per sample) resulted in 373 x 10^6 reads that were successfully mapped to pig hairpin sequences. Aligning unmapped reads to hairpin sequences of other species increased the alignment rate by a negligible fraction (0.46%), suggesting that known hairpin sequences of Sus Scrofa were close to complete. We therefore, only used those sequences that were mapped to Sus scrofa hairpins.

Whilst calculating the number of reads aligned to each hairpin and mature miR sequence, a high abundance of miR-486-5p was observed in plasma samples (constituting 92.5-97% of all reads). There were a number of circmirs with a positive and significant trans-coronary gradient (figure 1). Among these were also known myomirs like miR-133a. In addition, less known circmirs like miR-1306 also showed a positive gradient. A comparison of next-generation sequencing based miR expression across tissue samples revealed a total of 16 miRs differentially expressed in sham-operated tissue compared to AoB-treated tissue (Table 1) among which miR-1306-5p was also significantly upregulated.

Given the positive trans-coronary gradient of miR-1306-5p and its significant upregulation in myocardial tissue of AoB compared to Sham pigs, we further evaluated the potential role of miR-1306-5p as a circulating biomarker. We compared the values obtained for miR-1306 in the control samples that are routinely taken along on the qPCR plates with the measurement of the HF samples, which showed that levels of circulating miR-1306-5p were significantly higher in the HF patients OR [95%CI] = 1.43 (1.033 – 1.98) in arbitrary

unit)/ln(pg/ml), p<0.05), further increasing the probability that circulating miR-1306-5p could serve as a novel biomarker for HF.

Prospective Clinical study: Baseline characteristics

A total of 496 patients were enrolled in the TRIUMPH clinical cohort and provided written informed consent. Three patients withdrew their informed consent. Eighteen patients were withdrawn from statistical analyses due to inclusion violation. These patients had no evidence of sustained systolic or diastolic left ventricular dysfunction on echocardiography. Accordingly, 475 patients compose the analysis set. Median age was 74 years (interquartile range (IQR) 65-80), 63% were men and median left ventricular ejection fraction was 30% (IQR 21-42) (Table 2). Median baseline NT-proBNP level was 4135 pg/mL (IQR 2123–9328).

Clinical endpoints

The composite primary endpoint was reached by 188 patients (40%) during a median follow-up of 325 (IQR 85–401) days. A total of 113 patients died, of which 77 were confirmed to die from a cardiovascular cause, and 123 patients were re-hospitalized for decompensated HF.

Circulating miR measurements

A total of 2214 blood samples were available for the current investigation. Median (IQR) number of miR measurements per patient was 3 (IQR 2–5). Supplemental table 1 displays the number of measurements that were detectable per miR. MiRs that were detectable in less than 700 out of 2214 samples were not used as continuous variables in further analyses but were dichotomized (detectable vs. non-detectable) as described above. MiRs that were examined as continuous variables were: miR-320a, miR-1254, miR-22-3p, miR-378a-3p, miR-423-5p, miR-345-5p and miR-1306-5p. MiRs that were dichotomized were: miR-133a-3p, miR-133b, and miR-499a-5p. MiR-486-5p was used for normalization of these miR levels. MiR-622 and miR-208a-3p were only detectable in 56 and 6 out of 2214 samples,

respectively. This low expression did not allow for meaningful statistical analysis of these miRs. Additionally, supplemental table 2 shows the baseline characteristics stratified by invalid versus valid measurement of baseline miR-1306-5p.

Finally, miR expression levels in patients with HF with reduced ejection fraction (HFrEF) vs. HF with preserved ejection fraction (HFpEF) are presented in supplemental table 3.

Associations between baseline miR levels and clinical endpoints

Figure 2 shows the difference in the risk of experiencing the primary endpoint for patients in different quartiles of baseline miR1306-5p levels ($p< 0.001$). This was confirmed in the subsequently fitted Cox models, where baseline miR1306-5p levels were significantly and independently associated with the primary endpoint (hazard ratios (HRs)(95%CI)): 1.13(1.03-1.23) (Table 3). From the other known miRs, only the baseline levels of miR-320a were significantly and independently associated with the primary endpoint (HRs(95%CI): 1.10(1.00-1.21)). Associations with secondary endpoints are shown in Supplemental Table 4. A sensitivity analysis on the subgroup of HFrEF patients, rendered a HR for baseline miR1306-5p in relation to the primary endpoint that was similar to the HR in the total group, but with a wider CI ((HR(95%CI): 1.09(0.95–1.25) (supplemental table 5). This was most likely caused by a decrease in statistical power in this subgroup.

Associations between temporal miR patterns and clinical endpoints

Repeatedly measured miR1306-5p level was positively and independently associated with the primary endpoint (HR(95%CI): (4.69(2.18–10.06)), $p< 0.001$ (Table 4). The temporal patterns of miR-320a, miR-378a-3p and miR-423-5p were positively associated with the primary endpoint after adjustment for age and sex. However, these associations disappeared after multivariable adjustment. The temporal pattern of miR-1254 displayed a borderline significant association with the primary endpoint after adjustment for age and sex

(HR(95%CI): 1.22(1.00-1.50). Associations of temporal patterns with secondary endpoints are shown in Supplemental Table 6.

Incremental prognostic value of miR-1306-5p

Adding miR-1306-5p to a model containing NT-proBNP age, sex, systolic blood pressure, diabetes mellitus, atrial fibrillation, BMI, previous hospitalization for HF during the last 6 months, ischemic HF, and baseline eGFR, we found a change in C-statistic of 0.012 (95%CI: -0.006–0.029), a continuous net reclassification (cNRI) improvement of 0.125(-0.016–0.267), and an integrated discrimination index (IDI) improvement of 0.020(-0.013–0.053), as shown in supplemental table 7. Thus, the incremental prognostic value of miR1306-5p on top of NT-proBNP did not reach statistical significance.

DISCUSSION

Direct RNA sequencing of plasma from instrumented pigs revealed a number of circmiRs to be produced by the pig myocardium, including miR-1306-5p which had not yet been identified as a miR related to the heart. Subsequently, we found in a prospective AHF cohort that repeatedly-assessed circulating miR-1306-5p is positively and independently associated with all-cause mortality and HF hospitalization. This association was independent of NT-proBNP. However, a model containing baseline miR-1306-5p measurements did not significantly improve model discrimination or reclassification when compared to NT-proBNP. Repeatedly-assessed circulating miR-320a, miR-378a-3p, miR-423-5p and miR-1254 were associated with the primary endpoint after adjustment for age and sex (albeit borderline for miR-1254), but not after further multivariable adjustment for clinical characteristics. Furthermore, an independent association was found between baseline values of miR-1306-5p and miR-320a and the primary endpoint.

Importantly, our findings are in line with those described in a manuscript where two large cohorts have been studied (Bayes-Genis et al, submitted back-to-back). In those two independent cohorts, miR-1306-5p was also positively and significantly associated with the risk of all-cause mortality or HF hospitalization. This further strengthens our findings and for the first time we see reproducible results on circulating miRs across three large cohorts. This contrasts with previous studies where usually one, mostly smaller cohort was analyzed,[21] and results have most often been discrepant between separate studies. To the best of our knowledge, the association between miR-1306-5p and cardiovascular disease has not been previously investigated in other studies, and further research is warranted on its expected targets.

RNA sequencing using plasma-derived RNA led to the discovery of miR-1306-5p produced by the heart. Akat et al also used RNA sequencing to analyze miRs potentially produced by the human heart.[22] However, their study was not designed to assess the clinical value of circmiRs as biomarkers. A word of caution concerns the large proportion of invalid and undetectable miR-1306-5p measurements which reduces power and illustrates the need

for more sensitive methods of miR assessment to enable optimal use of this marker for clinical prognostication. Nevertheless, the current study carried sufficient statistical power to demonstrate a significant association between repeatedly measured miR-1306-5p and the primary and secondary endpoints in spite of the proportion of invalid and undetectable measurements.

In line with our results, the study by Bayes-Genis et al. also found an association between miR-1254 and clinical outcome. Other existing data on miR-1254 are limited; of note is that Tijsen et al demonstrated upregulation of miR-1254 in HF cases compared to healthy controls.[5] An association between higher baseline miR423-5p levels and signs of progressive HF has been demonstrated in animal models,[6] and human studies with limited sample size.[3,5] Rising miR423-5p has also been related to worsening left ventricular function and has been shown to be upregulated in non-ST elevation myocardial infarction patients.[23] Our results agree with the findings of the aforementioned studies. Conversely, in recent a study in 236 acute HF patients, an inverse association was observed between miR423-5p and hospital readmission.[8] However, this finding could not be reproduced in the validation cohort which was examined.[8] Smaller studies have previously demonstrated higher circulating levels of miR-320a in HF patients compared to healthy individuals.[7,24] In addition, rat models have proven that overexpression of miR-320a leads to a greater loss of cardiomyocytes during infarction and that inhibition of miR-320a leads to reduced infarction size.[25] Furthermore, miR-320a showed a protective effect on left ventricular remodeling after myocardial ischemia-reperfusion injury in a rat model.[26] The results of the current study are in line with these previous studies, and further expand the evidence concerning miR-320a by showing that baseline measurements are independently associated with adverse prognosis in patients with HF, and that repetitively-measured miR-320a is independently associated with heart failure hospitalization in particular. The temporal pattern of miR-378a-3p was also associated with the primary endpoint. Naga Prasad et al showed downregulation of miR-378a-3p in left ventricular free wall tissue of HF patients with dilated cardiomyopathy.[4] In contrast, in the current study we examined circulating levels of miR-378a-3p. In addition,

Weber et al found higher levels of circulating miR-378a-3p in 5 patients with coronary artery disease, compared to 5 healthy controls.[27] However, studies other than ours on the prognostic value of miR-378a-3p in patients with HF are lacking.

Repeatedly measured, highly-abundant miRs only showed age-and sex-adjusted significant associations with the primary endpoint, and associations disappeared after multivariable adjustment. Possibly, prognostic information of these circmiRs, which are probably not produced by the heart, can be easily diluted. Conversely, myomiRs, i.e. miRs which are skeletal- and cardiac-muscle specific, carry potential to provide prognostic information that is incremental to clinical characteristics. Such myomiRs play a central role in myogenesis regulation and muscle remodeling.[28,29] Although the main sources of circulating myomiRs, and in particular the relationship between myomiRs in tissue and plasma have yet to be fully elucidated, an association between cardiac damage (caused by myocardial infarction or myocarditis) and upregulation of circulating myomiRs has been previously demonstrated.[15] Moreover, circulating myomiR levels have been associated with skeletal muscle wasting.[30] We examined several myomiRs in the current investigation (miR133a-3p, miR133b, miR208a-3p and miR499a-5p). However, myomiRs are lowly expressed in the circulation, as illustrated by the fact that they were non-detectable in a large proportion of the samples available in our study. Thus, we were forced to perform a simplified analysis and examined the association between presence of detectable myomiR levels at baseline and occurrence adverse events. The loss of information inherent to such an analysis may have obscured potential associations with the outcome. Therefore, more sensitive assays are needed to properly examine the roles of myomiRs in HF.

To remove noise by less robust QPCR results we designed and implemented a strict and conservative algorithm to remove unreliable QPCR data, and at the same time retain reliable assessment of 'too low to detect' signals. Furthermore, we used miR486-5p to normalize our data, as using such endogenous miRs for this purpose has been shown to carry advantages.[17] We have separately described our quality control algorithm we used here (provided for review purposes) and given the strong concordance between three large

cohorts we have thus measured strengthens the point of view that such algorithms help to remove noise and improve reproducibility.

Some aspects of this study warrant consideration. First, aortic banding has been used to model heart failure. This is a model that shows strong similarity to the TAC model in mice and has previously been used in multiple studies as a model for pressure-overload hypertrophy.[31-34] This model may not be fully representative of human left ventricular dysfunction. However, our observation that miR 1306-5p, identified in our swine model, does provide prognostic potential in the clinic, underscores the validity of our approach. Second, we did not adjust our analyses for multiple comparisons, because the miRs we examined were not selected in a hypothesis-free manner but had resulted from previous fundamental and clinical studies. Nevertheless, if we applied Bonferroni correction, the results would remain statistically significant. The association between repeated miR1306-5p and the primary endpoint rendered a HR(95%CI) of 4.69(2.18–10.06) and a p-value < 0.0001; since we examined 7 repeatedly measured miRs, the Bonferroni threshold for the p-value would be 0.05/7=0.007. Furthermore, we focused on patients with known heart failure. Studies using a healthy control group may provide insights into temporal miR patterns in healthy persons.

In conclusion, in patients hospitalized for AHF, baseline and repeatedly-assessed miR-1306-5p was independently associated with adverse clinical outcome. Associations of temporal patterns of miR-320a, miR-378a-5p, miR-423-5p and miR-1254 with adverse clinical outcome were not independent of clinical characteristics. Myocyte-specific miRs were non-detectable in a large proportion of the samples. More sensitive myomiR assays are needed in order to precisely estimate the risk associated with elevated levels of miRs such as miR1306-5p, and to investigate whether cardiac specific myomiRs on their part are capable of providing additional information to established, clinical risk predictors.

ACKNOWLEDGEMENTS

None.

FUNDING

This work was supported by the framework of CTMM (Center for Translational Molecular Medicine), projects TRIUMPH (grant 01C-103) and ENGINE (grant 01C-401). Folkert W. Asselbergs is supported by a Netherlands Heart Foundation Dekker scholarship - Junior Staff Member 2014T001 and UCL Hospitals NIHR Biomedical Research Centre.

CONFLICT OF INTEREST

Zhen Liu is employed by ACS Biomarker BV, Amsterdam, The Netherlands. Yigal Pinto has a commercial interest in ACS Biomarker BV (<5%) and is named as an inventor on a submitted patent application regarding miR-1306. Adriaan Voors is a patent holder of circulating miRs described in ref 21. All other authors have no conflict to declare.

REFERENCES

1. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JG, Coats AJ, Falk V, Gonzalez-Juanatey JR, Harjola VP, Jankowska EA, Jessup M, Linde C, Nihoyannopoulos P, Parissis JT, Pieske B, Riley JP, Rosano GM, Ruilope LM, Ruschitzka F, Rutten FH, van der MP. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur J Heart Fail* 2016;**18**:891-975.

2. Vegter EL, van der MP, De Windt LJ, Pinto YM, Voors AA. MicroRNAs in heart failure: from biomarker to target for therapy. *Eur J Heart Fail* 2016;**18**:457-468.

3. Goldraich LA, Martinelli NC, Matte U, Cohen C, Andrades M, Pimentel M, Biolo A, Clausell N, Rohde LE. Transcoronary gradient of plasma microRNA 423-5p in heart failure: evidence of altered myocardial expression. *Biomarkers* 2014;**19**:135-141.

4. Naga Prasad SV, Duan ZH, Gupta MK, Surampudi VS, Volinia S, Calin GA, Liu CG, Kotwal A, Moravec CS, Starling RC, Perez DM, Sen S, Wu Q, Plow EF, Croce CM, Karnik S. Unique microRNA profile in end-stage heart failure indicates alterations in specific cardiovascular signaling networks. *J Biol Chem* 2009;**284**:27487-27499.

5. Tijsen AJ, Creemers EE, Moerland PD, De Windt LJ, van der Wal AC, Kok WE, Pinto YM. MiR423-5p as a circulating biomarker for heart failure. *Circ Res* 2010;**106**:1035-1039.

6. Dickinson BA, Semus HM, Montgomery RL, Stack C, Latimer PA, Lewton SM, Lynch JM, Hullinger TG, Seto AG, van Rooij E. Plasma microRNAs serve as biomarkers of therapeutic efficacy and disease progression in hypertension-induced heart failure. *Eur J Heart Fail* 2013;**15**:650-659.

7. Goren Y, Kushnir M, Zafrir B, Tabak S, Lewis BS, Amir O. Serum levels of microRNAs in patients with heart failure. *Eur J Heart Fail* 2012;**14**:147-154.

8. Seronde MF, Vausort M, Gayat E, Goretti E, Ng LL, Squire IB, Vodovar N, Sadoune M, Samuel JL, Thum T, Solal AC, Laribi S, Plaisance P, Wagner DR, Mebazaa A, Devaux Y. Circulating microRNAs and Outcome in Patients with Acute Heart Failure. *PLoS One* 2015;**10**:e0142237.

9. Duncker DJ, Zhang J, Bache RJ. Coronary pressure-flow relation in left ventricular hypertrophy. Importance of changes in back pressure versus changes in minimum resistance. *Circ Res* 1993;**72**:579-587.

10. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;**30**:2114-2120.

11. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;**15**:550.

12. McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Bohm M, Dickstein K, Falk V, Filippatos G, Fonseca C, Gomez-Sanchez MA, Jaarsma T, Kober L, Lip GY, Maggioni AP, Parkhomenko A, Pieske BM, Popescu BA, Ronnevik PK, Rutten FH, Schwitter J, Seferovic P, Stepinska J, Trindade PT, Voors AA, Zannad F, Zeiher A. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. *Eur Heart J* 2012;**33**:1787-1847.

13. Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, Benjamin H, Kushnir M, Cholakh H, Melamed N, Bentwich Z, Hod M, Goren Y, Chajut A. Serum microRNAs are promising novel biomarkers. *PLoS One* 2008;**3**:e3148.

14. Rao PK, Toyama Y, Chiang HR, Gupta S, Bauer M, Medvid R, Reinhardt F, Liao R, Krieger M, Jaenisch R, Lodish HF, Blelloch R. Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. *Circ Res* 2009;**105**:585-594.

15. Corsten MF, Dennert R, Jochems S, Kuznetsova T, Devaux Y, Hofstra L, Wagner DR, Staessen JA, Heymans S, Schroen B. Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. *Circ Cardiovasc Genet* 2010;**3**:499-506.

16. Bostjancic E, Zidar N, Stajer D, Glavac D. MicroRNAs miR-1, miR-133a, miR-133b and miR-208 are dysregulated in human myocardial infarction. *Cardiology* 2010;**115**:163-169.

17. Kok MG, Halliani A, Moerland PD, Meijers JC, Creemers EE, Pinto-Sietsma SJ. Normalization panels for the reliable quantification of circulating microRNAs by RT-qPCR. *FASEB J* 2015;**29**:3853-3862.

18. Zampetaki A, Willeit P, Tilling L, Drozdov I, Prokopi M, Renard JM, Mayr A, Weger S, Schett G, Shah A, Boulanger CM, Willeit J, Chowienczyk PJ, Kiechl S, Mayr M. Prospective study on circulating MicroRNAs and risk of myocardial infarction. *J Am Coll Cardiol* 2012;**60**:290-299.

19. de Ronde MW, Ruijter JM, Lanfear D, Bayes-Genis A, Kok M, Creemers E, Pinto YM, Pinto-Sietsma SJ. Practical data handling pipeline improves performance of qPCR-based circulating miRNA measurements. *RNA* 2017.

20. Rizopoulos D. JM: An R Package for the Joint Modelling of Longitudinal and Time-to-Event Data. *Journal of Statistical Software* 2010;**35**:1-33.

21. Ovchinnikova ES, Schmitter D, Vegter EL, Ter Maaten JM, Valente MA, Liu LC, van der HP, Pinto YM, de Boer RA, Meyer S, Teerlink JR, O'Connor CM, Metra M, Davison BA, Bloomfield DM, Cotter G, Cleland JG, Mebazaa A, Laribi S, Givertz MM, Ponikowski P, van der MP, van Veldhuisen DJ, Voors AA, Berezikov E. Signature of circulating microRNAs in patients with acute heart failure. *Eur J Heart Fail* 2016;**18**:414-423.

22. Akat KM, Moore-McGriff D, Morozov P, Brown M, Gogakos T, Correa DR, Mihailovic A, Sauer M, Ji R, Ramarathnam A, Totary-Jain H, Williams Z, Tuschl T, Schulze PC. Comparative RNA-sequencing analysis of myocardial and circulating small RNAs in human heart failure and their utility as biomarkers. *Proc Natl Acad Sci U S A* 2014;**111**:11151-11156.

23. Olivieri F, Antonicelli R, Lorenzi M, D'Alessandra Y, Lazzarini R, Santini G, Spazzafumo L, Lisa R, La Sala L, Galeazzi R, Recchioni R, Testa R, Pompilio G, Capogrossi MC, Procopio AD. Diagnostic potential of circulating miR-499-5p in elderly patients with acute non ST-elevation myocardial infarction. *Int J Cardiol* 2013;**167**:531-536.

24. Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, Doevendans PA, Mummery CL, Borlak J, Haverich A, Gross C, Engelhardt S, Ertl G, Bauersachs J. MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. *Circulation* 2007;**116**:258-267.

25. Ren XP, Wu J, Wang X, Sartor MA, Qian J, Jones K, Nicolaou P, Pritchard TJ, Fan GC. MicroRNA-320 is involved in the regulation of cardiac ischemia/reperfusion injury by targeting heat-shock protein 20. *Circulation* 2009;**119**:2357-2366.

26. Song CL, Liu B, Diao HY, Shi YF, Li YX, Zhang JC, Lu Y, Wang G, Liu J, Yu YP, Guo ZY, Wang JP, Zhao Z, Liu JG, Liu YH, Liu ZX, Cai D, Li Q. The Protective Effect of MicroRNA-320 on Left Ventricular Remodeling after Myocardial Ischemia-Reperfusion Injury in the Rat Model. *Int J Mol Sci* 2014;**15**:17442-17456.

27. Weber M, Baker MB, Patel RS, Quyyumi AA, Bao G, Searles CD. MicroRNA Expression Profile in CAD Patients and the Impact of ACEI/ARB. *Cardiol Res Pract* 2011;**2011**:532915.

28. Townley-Tilson WH, Callis TE, Wang D. MicroRNAs 1, 133, and 206: critical factors of skeletal and cardiac muscle development, function, and disease. *Int J Biochem Cell Biol* 2010;**42**:1252-1255.

29. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet* 2006;**38**:228-233.

30. Donaldson A, Natanek SA, Lewis A, Man WD, Hopkinson NS, Polkey MI, Kemp PR. Increased skeletal muscle-specific microRNA in the blood of patients with COPD. *Thorax* 2013;**68**:1140-1149.

31. Kupper T, Pfitzer P, Schulte D, Arnold G. Pressure induced hypertrophy in the hearts of growing dwarf pigs. *Pathol Res Pract* 1991;**187**:315-323.

32. Modesti PA, Vanni S, Bertolozzi I, Cecioni I, Polidori G, Paniccia R, Bandinelli B, Perna A, Liguori P, Boddi M, Galanti G, Serneri GG. Early sequence of cardiac adaptations and growth factor formation in pressure- and volume-overload hypertrophy. *Am J Physiol Heart Circ Physiol* 2000;**279**:H976-H985.

33. Marshall KD, Muller BN, Krenz M, Hanft LM, McDonald KS, Dellsperger KC, Emter CA. Heart failure with preserved ejection fraction: chronic low-intensity interval exercise training preserves myocardial O2 balance and diastolic function. *J Appl Physiol (1985)* 2013;**114**:131-147.

34. Xiong Q, Zhang P, Guo J, Swingen C, Jang A, Zhang J. Myocardial ATP hydrolysis rates in vivo: a porcine model of pressure overload-induced hypertrophy. *Am J Physiol Heart Circ Physiol* 2015;**309**:H450-H458.

Figure titles and legends

Figure 1: Trans-coronary gradients in plasma microRNAs.

The number indicates the number of pigs (out of a total of 44 pigs) with both a detectable venous and arterial microRNA value. The gradient is calculated as arterial minus venous Ct value of the microRNA, and shown as Mean±SEM. A negative value indicates release of the microRNA by the myocardium, and a positive value indicates uptake. The p-value is calculated using a paired samples T-test, and indicates the difference between arterial and venous Ct value of the microRNA.

Figure 2: Kaplan-Meier survival curves for the primary endpoint of death or readmission for HF in the four quartiles of baseline miR-1306-5p levels.

Q1 lowest quartile, Q4 highest quartile.

A large section of the discussion is based on the consistency between the results of this study and the results recently found by Bayes-Genis's group (Bayes-Genis et al, Ms. No.: EURJHF-17-436-MDR), which is still unpublished. As stated in the cover letter, Dr. Pinto, last author of this manuscript, but also last author of the manuscript by Bayes-Genis et al, and Dr. Bayes-Genis, provide permission to use the data from Bayes-Genis et al, Ms. No.: EURJHF-17-436-MDR in the current manuscript. Please see also the added email sent by Dr. Bayes-Genis, which was added for review only, in which he provides written permission to use the data from Bayes-Genis et al, Ms. No.: EURJHF-17-436-MDR. We also added cover letter of the Bayes-Genis manuscript, Ms. No.: EURJHF-17-436-MDR, for review purposes only.

Furthermore, the manuscript by Bayes-Genis et al, Ms. No.: EURJHF-17-436-MDR, has been resubmitted at the same time as this manuscript, and both manuscripts are currently in the same stage of the review process.

We thank the Reviewers very much for their comments. Please find our response to the suggestions of the Editor and Reviewers below. We have incorporated all suggestions into the manuscript.

Editorial comments:

A large section of the discussion is based on the consistency between the results of this study and those recently found by Bayes-Genis's group, though still unpublished. Let me remind to the Authors that, as stated in our instructions for authors, “Authors should get permission from the source to cite unpublished data.” These are original data and therefore the issue is more sensitive. We recommend one of the following options: 1) delete any reference to Bayes-Genis data, 2) have a written permission by Bayes- Genis who should likely approve all the written text where his data are used. Obviously, this issue would not exist once the Bayes-Genis data are published.

Response: As stated in the cover letter, Dr. Pinto, last author of this manuscript, but also last author of the manuscript by Bayes-Genis et al, and Dr. Bayes-Genis, provide permission to use the data from Bayes-Genis et al, Ms. No.: EURJHF-17-436-MDR in the current manuscript. Please see also the added email sent by Dr. Bayes-Genis, which was added for review only, in which he provides written permission to use the data from Bayes-Genis et al, Ms. No.: EURJHF-17-436-MDR. We also added cover letter of the Bayes-Genis manuscript, Ms. No.: EURJHF-17-436-MDR, for review purposes only.

Furthermore, the manuscript by Bayes-Genis et al, Ms. No.: EURJHF-17-436-MDR, has been resubmitted at the same time as this manuscript, and both manuscripts are currently in the same stage of the review process.

Ref. 21 needs to be updated

Response: As requested, we have updated Ref. 21.

Reviewers' comments:

Reviewer #1: I would like to thank the authors for their very considered responses to the points I made at the first review. I agree that consideration of total or recurrent events is not necessary. Otherwise, I think that their responses are very appropriate.

Response: We thank the reviewer for the constructive comments which have improved the paper.

Reviewer #2: The authors have replied correctly to my comments. The fact that miR-1306-5p does not add to NT-proBNP, the benchmark prognostic marker in HF, in terms of discrimination (reclassification), must be clearly indicated in the abstract and in the discussion.

Response: As requested by the reviewer, we have indicated the fact that miR-1306-5p does not add to NT-proBNP, in terms of discrimination, in the abstract and in the discussion:

Abstract, lines 46-47:- ***Baseline miR-1306-5p did not improve model discrimination/reclassification significantly compared to NT-proBNP.***

Abstract, lines 51-53: ***"Repeatedly-measured miR-1306-5p was positively associated with adverse clinical outcome in AHF, even after multivariable adjustment including NT-proBNP. Yet, baseline miR-1306-5p did not add significant discriminatory value to NT-proBNP."***

Discussion, lines 312-314: ***"This association was independent of NT-proBNP. However, a model containing baseline miR-1306-5p measurements did not significantly improve model discrimination or reclassification when compared to NT-proBNP. "***

Reviewer #3: I congratulate the authors. The answers are adequately answered.

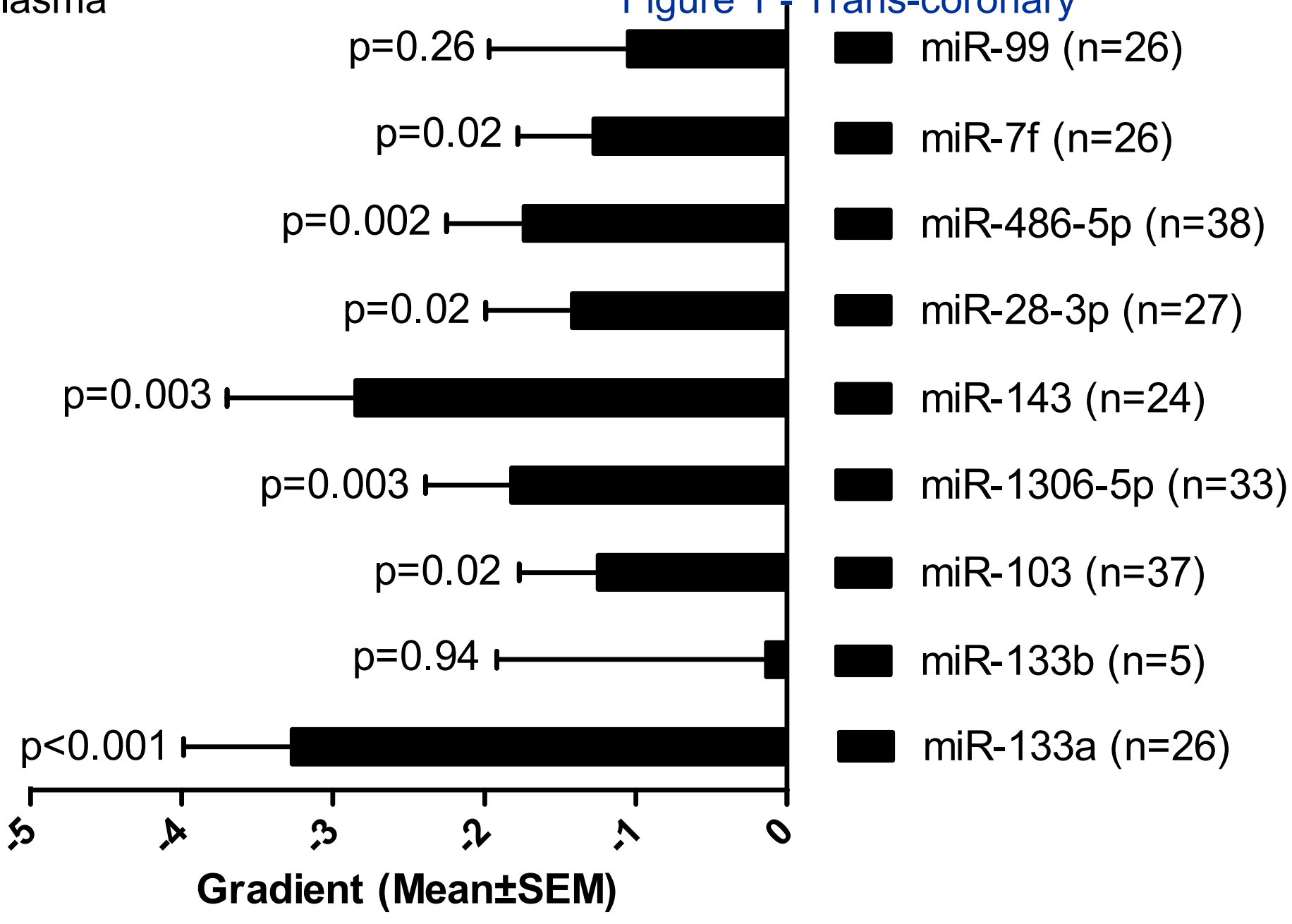
Response: We thank the reviewer for the suggestions that have indeed improved the paper.

Word count: 3429 words

Word count revision: 3895 words

Figure 1 - Trans-coronary gradients in plasma

Click here to download Figure
Figure 1 - Trans-coronary



igure 2 - Kaplan-Meier survival

Click here to download Figure Figure 2 - Kaplan-Meier survival curves.pdf

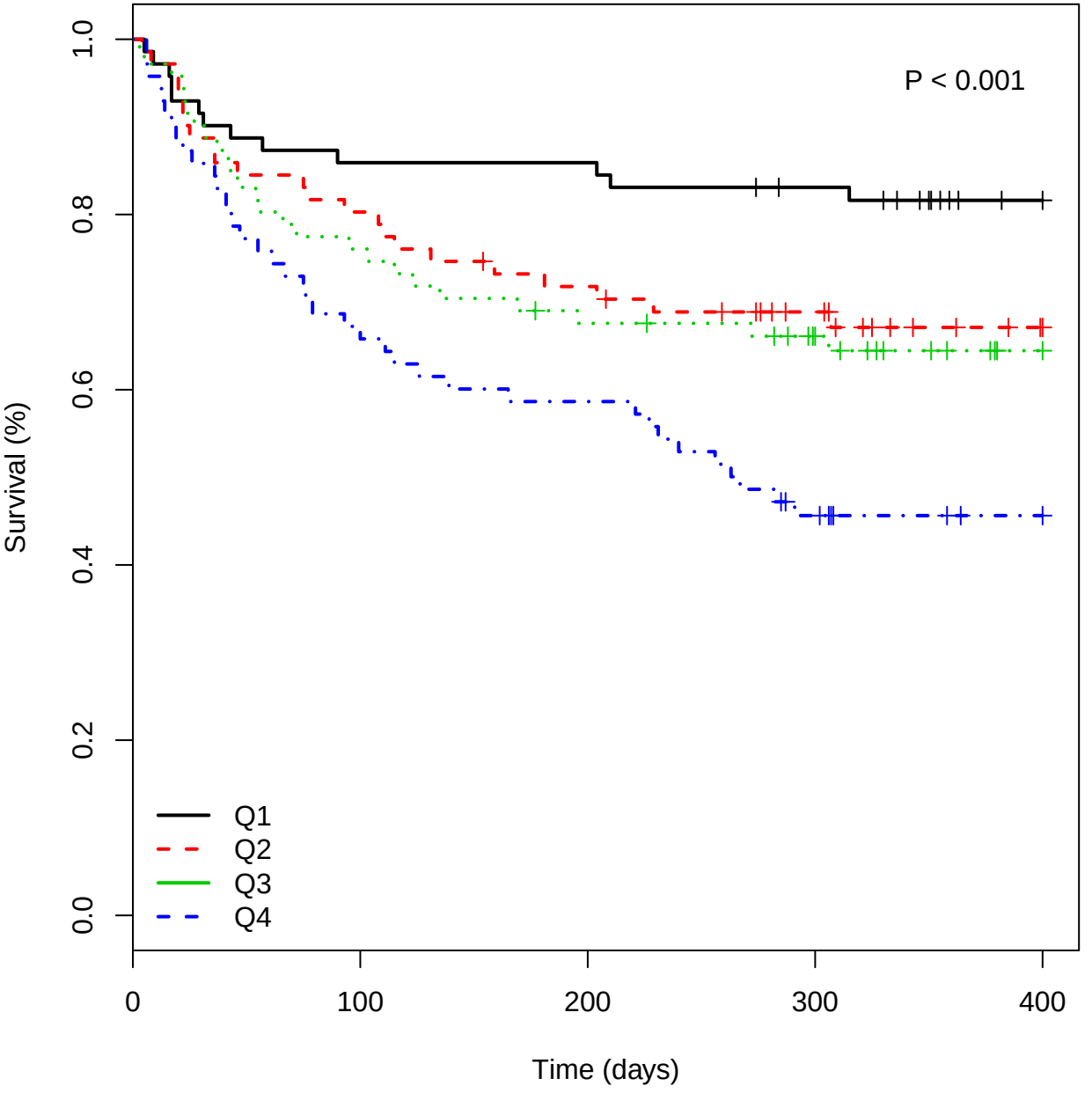


Table 1 – Differentially expressed microRNAs across tissue samples

MiR	Fold change*	Adjusted p-value
306-5p	1,354	0.002
132	1,554	0.013
133a-3p	1,107	0.004
142-5p	1,992	<0.001
144	1,457	0.004
144-5p	2,621	<0.001
150	1,767	0.006
15b	1,996	<0.001
15b-5p	1,922	<0.001
342	1,932	<0.001
365-3p	1,507	<0.001
451	3,015	<0.001
532-3p	1,956	0.001
7139-3p	1,889	<0.001
92b-3p	1,04	0.015
99b-3p	-1,225	0.023
133b	0,69	0,07
103	-0,198	0,72
143-3p	-0,251	0,75
143-5p	-0,297	0,755
28-3p	-0,347	0,53
486-5p	0,166	0,77
7f	0,472	0,51
99	-0,53	0,11

Myocardial samples were obtained from the left ventricular free wall and compared between sham-operated and TAC-treated swine. P-values were calculated using the negative binomial model from DESeq. MiR = microRNA.

* Log2 fold change

Table 2 – Baseline characteristics

Variables	Overall sample (n=475)
Demographic characteristics, median [IQR] or number (%)	
Age, years	73 [64 - 80]
Female, %	36.6 (167)
Caucasian, %	94.3 (430)
Measurements at baseline, median [IQR] or number (%)	
Body mass index, kg/m2	27.5 [24.7 - 31.1]
Systolic blood pressure, mmHg	125 [110 - 147]
Diastolic blood pressure, mmHg	75 [65 - 85]
Heart rate, bpm	85 [72 - 100]
eGFR	46 [34.4 - 61.7]
Left ventricular ejection fraction, %	30 [21 - 42]
Heart failure with reduced ejection fraction, %	79.8 (289)
NT-proBNP (pg/ml)	4143.7 [2097.5 - 9053.2]
Medical history, number (%)	
Previous heart failure admission within 6 months	19.8 (90)
Ischemic heart failure	48.1 (219)
Myocardial infarction	40.4 (184)
Hypertension	50 (228)
Atrial fibrillation	42.5 (194)
Diabetes Mellitus	36.5 (166)
Stroke	17.5 (80)

IQR = Inter-quartile range, eGFR = estimated glomerular filtration rate.