










Identification of novel circulating microRNAs in advanced heart failure by next-generation sequencing

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Abstract

Aims Risk stratification in patients with advanced chronic heart failure (HF) is an unmet need. Circulating microRNA (miRNA) levels have been proposed as diagnostic and prognostic biomarkers in several diseases including HF. The aims of the present study were to characterize HF-specific miRNA expression profiles and to identify miRNAs with prognostic value in HF patients.

Methods and results We performed a global miRNome analysis using next-generation sequencing in the plasma of 30 advanced chronic HF patients and of matched healthy controls. A small subset of miRNAs was validated by real-time PCR ($P < 0.0008$). Pearson's correlation analysis was computed between miRNA expression levels and common HF markers. Multivariate prediction models were exploited to evaluate miRNA profiles' prognostic role. Thirty-two miRNAs were found to be dysregulated between the two groups. Six miRNAs (miR-210-3p, miR-22-5p, miR-22-3p, miR-21-3p, miR-339-3p, and miR-125a-5p) significantly correlated with HF biomarkers, among which N-terminal prohormone of brain natriuretic peptide. Inside the cohort of advanced HF population, we identified three miRNAs (miR-125a-5p, miR-10b-5p, and miR-9-5p) altered in HF patients experiencing the primary endpoint of cardiac death, heart transplantation, or mechanical circulatory support implantation when compared with those without clinical events. The three miRNAs added substantial prognostic power to Barcelona Bio-HF score, a multiparametric and validated risk stratification tool for HF (from area under the curve = 0.72 to area under the curve = 0.82).

Conclusions This discovery study has characterized, for the first time, the advanced chronic HF-specific miRNA expression pattern. We identified a few miRNAs able to improve the prognostic stratification of HF patients based on common clinical and laboratory values. Further studies are needed to validate our results in larger populations.

Keywords Biomarkers; Circulating microRNAs; Heart failure; Risk stratification

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Introduction

Heart failure (HF) prevalence is approximately 1–2% in the adult population of developed countries and is expected to rise, reflecting the chronic course of the disease as well as population growth and ageing.¹ Accurate prognostication

strategies to plan treatment and follow-up are still lacking.² In particular, the optimal timing for a non-medical intervention represents a conundrum in advanced stages when pharmacological treatment is no longer effective. So far, neither singular parameter nor risk score provides adequate guidance on whether the individual HF patient may benefit

from left ventricular assist devices (LVADs).³ The main challenge for the management of advanced HF patients should be to exploit new predictors, which, together with laboratory and clinical parameters, may allow to better stratify the risk of adverse cardiovascular events and, thus, correctly identify the patients for an invasive therapeutic strategy. This approach could reduce hospitalization rates and costs, improving patients' outcomes and quality of life.

MicroRNAs (miRNAs) are small non-coding RNAs involved in the regulation of protein translation at the post-transcriptional level. They can inhibit messenger RNA translation and/or induce messenger RNA degradation, playing a pivotal role as regulators of human physiology and development. MiRNAs are expressed in tissues, but they can also be isolated from blood plasma, where they are stable and present as cell-free circulating molecules or packed in extracellular vesicles.^{4–6} The potential role of miRNAs as diagnostic and prognostic biomarkers has been advocated in several diseases such as cancer, but there are also several studies showing promising results for some miRNAs in HF.^{7,8} However, the majority investigated the expression of candidate miRNAs.^{9–11}

The aims of the present study are (i) to produce expression profiles of cell-free circulating miRNAs in advanced HF patients, using next-generation sequencing (NGS); (ii) to identify differentially expressed miRNAs between patients and matched controls; (iii) to correlate miRNA expression levels with common HF markers; and (iv) to evaluate their prognostic role in this complex pathological setting, eventually including miRNA profiles in a predictive cardiovascular risk model.

Methods

This prospective, single-centre, case–control study was realized through the synergic work of three operational units: the *clinical unit*, namely, the local LVAD Hub Centre in the *Città della Salute e della Scienza University Hospital* of Turin, Northern Italy, organized the patients' screening and recruitment; the *biotechnology unit*, represented by the University of Turin, controlled the processing, handling, and storage of serum and plasma; and the *genomic unit*, represented by the Italian Institute for Genomic Medicine, Candiolo (TO), Italy, performed the NGS analyses and shared their expertise in miRNA detection in body fluids.

Study population

Consecutive ambulatory patients with stable advanced HF¹² admitted to our clinical unit from September 2016 to August 2019 were prospectively enrolled. Inclusion criteria were age ≥ 18 years old, all criteria for advanced HF as defined by

Metra *et al.*,¹² and INTERMACS profile between 4 and 7. To reduce the effect of the acute event on miRNA expression profile, a minimum time of 3 weeks of clinical stability after hospital admission for acute decompensated HF was required for the enrolment. Exclusion criteria were extracardiac pathologies with life expectancy lower than 2 years and hospitalization for acute coronary syndrome, percutaneous revascularization, or recent cardiac surgery (within 40 days). Patients with cancer disease including haematological neoplasm were also excluded because of possible interference with miRNA expression.

The control arm of the study included healthy volunteers matched by age and sex. All subjects provided written informed consent for study participation. The investigation conforms to the principles outlined in the *Declaration of Helsinki* and was approved by the local ethics committee.

Clinical and hemodynamic assessment

At the time of enrolment, clinical history (age, gender, cardiovascular risk factors, medications intake at home, and HF aetiology) was recorded, and physical examination and electrocardiogram (ECG) were performed in all patients. A cardiopulmonary exercise test was performed according to the clinician's decision. All control participants underwent a complete physical examination, risk factors assessment, and ECG.

Two-dimensional transthoracic echocardiography was performed in all patients, and haemodynamic data of right heart catheterization were reported when available. Further details are described in Supporting Information, Methods section.

Blood samples for clinical blood tests [creatinine, Na⁺, K⁺, urea, high-sensitivity cardiac troponin T (Hs-cTnT), total bilirubin, aspartate aminotransferase/alanine transaminase, N-terminal prohormone of brain natriuretic peptide (NT-proBNP), soluble ST2 (sST2), and copeptin] and for the analysis of miRNA expression profiling were obtained from each patient and control at the time of enrolment. The glomerular filtration rate was estimated by Modification of Diet in Renal Disease equation.

Finally, for each patient, the risk of cardiovascular death and rehospitalization was estimated by the Barcelona (BCN) Bio-HF web calculator, a composite HF risk stratification tool suggested by the European Society of Cardiology. BCN Bio-HF score outperformed other multiparametric scores.^{13,14}

Blood sampling and RNA preparation

Blood samples were processed in the biotechnology unit laboratory within 4 h after collection. Blood samples

(5–8 mL per subject) were centrifuged for 10 min at 1000 *g*, and 200 μ L aliquots of plasma and serum were stored in long-term storage cryogenic vials at -80°C until use. Serum

samples were used to measure laboratory values (Tables 1 and 2). Total RNA from plasma was extracted in the genomic unit laboratory with the miRNeasy plasma/serum mini kit

Table 1 Clinical characteristics of chronic advanced heart failure

	HF group (n = 30)	Control group (n = 36)
Clinical history and risk profile		
Male gender	26 (87)	32 (89)
Age (years)	65 [58–68]	61.5 [57–65]
Body mass index	23.9 [21.6–26.0]	
Hypertension	18 (60)	
Diabetes mellitus	7 (23)	
Familiarity for coronary artery disease	7 (23)	
Current smoker	18 (60)	
Dyslipidaemia	15 (50)	
Ischaemic aetiology	18 (60)	
INTERMACS class	5 [5–5.7]	
VO ₂ max (mL/kg/min) ^a	12.1 [10.4–14.2]	
Treatments		
Furosemide (mg)	75 [50–100]	
Beta-blocker	28 (93)	
ACE-I	15 (50)	
MRA	25 (83)	
ICD	29 (97)	
CRT	11 (37)	
MitraClip	7 (23)	
Echocardiographic data		
Left ventricular EF (%)	21 [19–25]	
LVEDD (mm)	66.5 [61–70.7]	
RVEDD (mm)	42 [40–48.7]	
Mean E/e' (n)	17 [14–28]	
Moderate-to-severe mitral regurgitation	20 (67)	
Estimated systolic PAP (mmHg)	50 [42–69]	
TAPSE (mm)	16 [14–18]	
Haemodynamic variables^b		
CI (L/min/m ²)	1.8 [1.7–1.9]	
Mean WP (mmHg)	23 [15–29]	
Mean PAP (mmHg)	33 [22–39.5]	
TPG (mmHg)	9 [6–13]	
PVR (WU)	2.6 [1.6–3.7]	
Mean RAP (mmHg)	7 [4–10]	
PAPi	3.2 [2.6–4.5]	
Laboratory values		
Na ⁺ (mmol/L) ^{***}	135 [132–138]	141 [140–142]
K ⁺ (mmol/L) [*]	4.2 [3.9–4.5]	4.4 [4–4.6]
Creatinine (mg/dL) ^{***}	1.5 [1.2–1.8]	0.88 [0.8–0.94]
MDRD	50 [40–64]	
Urea (mg/dL) ^{***}	72 [52–97]	34.5 [30–38.2]
Copeptin (pmol/L) ^{***}	29.9 [26.1–58.6]	10.3 [6.5–15.1]
High-sensitivity cardiac troponin T (ng/L) ^{***}	33.6 [23.8–65]	6.1 [4.8–8.1]
Total bilirubin (mg/dL) ^{***}	0.8 [0.5–1.2]	0.6 [0.5–0.8]
AST (IU/L) ^{**}	19.5 [16–30.5]	16 [14–19]
ALT (IU/L) ^{***}	17.5 [9–25.7]	10.5 [8–14]
NT-proBNP (pg/mL) ^{***}	4406 [2360–9385]	32.9 [19.3–52.6]
sST2 (ng/mL) ^{***}	30.5 [20.8–44.6]	22.9 [18.1–25.8]

ACE-I, angiotensin-converting enzyme inhibitor; ALT, alanine transaminase; AST, aspartate aminotransferase; CI, cardiac index; CRT, cardiac resynchronization therapy; EF, ejection fraction; HF, heart failure; ICD, implantable cardiac defibrillator; LVEDD, left ventricular end-diastolic diameter; MDRD, Modification of Diet in Renal Disease; MRA, mineralocorticoid receptor antagonist; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; PAP, pulmonary artery pressure; PAPi, pulmonary artery pulsatility index; PVR, pulmonary vascular resistance; RAP, right atrial pressure; RVEDD, right ventricular end-diastolic diameter; sST2, soluble ST2; TAPSE, tricuspid annular plane systolic excursion; TPG, trans-pulmonary gradient; WP, wedge pressure; WU, Wood units.

Data are presented as median [inter-quartile range] or number (%).

^aVO₂ max was available in 21 patients.

^bRight heart catheterization data were available in 23 patients.

^{*}Significant differences between HF patients and controls ($P < 0.05$).

^{**}Significant differences between HF patients and controls ($P < 0.01$).

^{***}Significant differences between HF patients and controls ($P < 0.005$).

Table 2 Clinical characteristics of advanced HF patients with or without primary endpoint onset

	HF-noPE group (n = 16)	HF-PE group (n = 14)
Clinical history and risk profile		
Male gender	14 (88)	12 (86)
Age (years)	66 [63–70]	59 [55–65]
Body mass index	24 [23–26]	23 [21–26]
Hypertension	8 (50)	10 (71)
Diabetes mellitus	5 (31)	2 (14)
Familiarity for coronary artery disease	5 (31)	2 (14)
Current smoker	9 (56)	9 (64)
Dyslipidaemia	9 (56)	6 (43)
Ischaemic aetiology	7 (44)	4 (29)
INTERMACS class	5 [5–5]	5 [4–6]
VO ₂ max (mL/kg/min) ^{a,***}	14 [11–18]	11.4 [10.3–14]
BCN Bio-HF score*	42 [13.9–78.6]	25 [11.2–77.9]
Treatments		
Furosemide (mg)	75 [50–100]	75 [50–120]
Beta-blocker	15 (94)	13 (93)
ACE-I	7 (44)	8 (57)
MRA	13 (81)	12 (86)
ICD	15 (94)	14 (100)
CRT	6 (38)	5 (36)
MitraClip	4 (25)	3 (21)
Echocardiographic data		
Left ventricular EF (%)	23.5 [19.5–25.7]	20 [19.2–24.5]
LVEDD (mm)	63 [60.7–70.2]	67 [61.7–72.2]
RVEDD (mm)	42 [39.2–49]	43 [40.2–47.7]
Mean E/e' (n)	18.5 [15.2–25]	16.3 [14.1–28.5]
Moderate-to-severe mitral regurgitation	12 (75)	8 (57)
Estimated systolic PAP (mmHg)	49 [45–69.5]	52.5 [40.5–61.5]
TAPSE (mm)	15.5 [13.5–18]	16.5 [14.2–19]
Haemodynamic variables ^b		
CI (L/min/m ²)	1.9 [1.8–1.9]	1.8 [1.7–1.9]
Mean WP (mmHg)	22 [14–26]	24.5 [16.5–29]
Mean PAP (mmHg)	31 [19–36]	34.5 [25–45]
TPG (mmHg)*	7 [5–9]	11.5 [8.2–14.7]
PVR (WU)	2 [1.4–2.6]	3 [2.22–5.32]
Mean RAP (mmHg)	5 [4–6]	8.5 [5.5–10]
PAPi	4 [3.1–4.5]	4 [2.5–5.2]
Laboratory values		
Na ⁺ (mmol/L)	136 [133–138]	134 [132–138]
K ⁺ (mmol/L)	4.2 [4–4.4]	4.3 [3.8–4.6]
Creatinine (mg/dL)	1.7 [1.3–2.3]	1.5 [1.2–1.6]
MDRD*	42 [29–54]	56 [49–65]
Urea (mg/dL)	75 [59–128]	68 [53–86]
Copeptin (pmol/L)	40.5 [27.3–66.8]	29 [23–46]
High-sensitivity cardiac troponin T (ng/L)	39 [27.2–60.9]	30 [15–61.2]
Total bilirubin (mg/dL)	0.7 [0.5–1.4]	0.9 [0.6–1.1]
AST (IU/L)	19 [16–32]	20 [16–26]
ALT (IU/L)	18 [9–25]	12 [9–24]
NT-proBNP (pg/mL)	5137 [2027–11334]	3734 [2376–7293]
sST2 (ng/mL)	32 [19–45]	30 [27–45]

ACE-I, angiotensin-converting enzyme inhibitor; ALT, alanine transaminase; AST, aspartate aminotransferase; BCN, Barcelona; CI, cardiac index; CRT, cardiac resynchronization therapy; EF, ejection fraction; HF, heart failure; ICD, implantable cardiac defibrillator; LVEDD, left ventricular end-diastolic diameter; MDRD, Modification of Diet in Renal Disease; MRA, mineralocorticoid receptor antagonist; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; PAP, pulmonary artery pressure; PAPi, pulmonary artery pulsatility index; PVR, pulmonary vascular resistance; RAP, right atrial pressure; RVEDD, right ventricular end-diastolic diameter; sST2, soluble ST2; TAPSE, tricuspid annular plane systolic excursion; TPG, trans-pulmonary gradient; WP, wedge pressure; WU, Wood units.

Data are presented as median [inter-quartile range] or number (%).

^aVO₂ max was available in 21 patients.

^bRight heart catheterization data were available in 23 patients.

*Significant differences between HF patients with or without primary endpoint ($P < 0.05$).

***Significant differences between HF patients with or without primary endpoint ($P < 0.005$).

(Cat No. 217184, Qiagen, Hilden, Germany) using the QiaCube extractor (Qiagen) and following the manufacturer's instructions. For all samples, RNA concentration

was quantified by Qubit 2.0 Fluorometer with Qubit miRNA Assay Kit (Cat No. Q32880, Invitrogen, Thermo Fisher, Waltham, MA, USA).

MicroRNA profiling by next-generation sequencing and real-time PCR analysis validation

Small RNA transcripts were converted into barcoded cDNA libraries following the protocols developed in the genomic unit laboratory, as previously described.^{15–17} To validate NGS results, we planned to measure by real-time PCR the expression of a small subset of miRNAs identified to be differentially expressed between HF and control samples. This analysis was necessary to consolidate data obtained by NGS in a subgroup of patients and controls.

A more detailed description of the library preparation, Illumina sequencing protocols, and real-time PCR analysis is given in Supporting Information, Methods section.

Computational and statistical analyses

The data from small RNA sequencing were computationally processed and analysed as previously described¹⁸ and as specified in Supporting Information, Methods section.

Briefly, differential expression of miRNAs was tested with the DESeq2 package. Potential confounders were included as covariates: batch (library), sex, and age. *P*-values were adjusted for multiple testing by the Benjamini–Hochberg procedure. Pearson's correlation was computed between the base 2 logarithms of normalized miRNA expression and clinical variables. Significance of differences in the median of clinical variables between endpoints was tested by the Wilcoxon–Mann–Whitney test.

Primary endpoint and follow-up

Patients were prospectively followed with clinical examination, ECG, and transthoracic echocardiography for at least 12 months after the enrolment. The primary endpoint (PE) was a composite of cardiac death, elective or urgent heart transplantation, or LVAD implantation.

Prediction model for the diagnosis and prognosis of heart failure

Multivariate prediction models were based on logistic regression with ElasticNet penalty, and receiver operating characteristic curve and area under the curve (AUC) estimation was performed in cross-validation. More in detail, prediction models were built in Python with the scikit-learn module using the LogisticRegressionCV classifier. This classifier implements a logistic regression with ElasticNet penalty with an automatic selection of the best hyperparameters. We used AUC as a metric to assess the performance of the models that we built. To control for

overfitting, we performed 10-fold cross-validation repeated with different random seeds 50 times. A non-linear classifier such as the RandomForestClassifier did not provide better performance than results derived from the logistic regression.

Results

Baseline characteristics

Among 76 advanced HF patients screened, 46 did not meet the study criteria and were excluded as shown in Supporting Information, *Figure S1*. Finally, 30 patients were enrolled in the study and matched by age and sex with healthy controls (*Table 1*). All patients (mean age 65 years, 87% male, and 60% with ischaemic aetiology of the cardiomyopathy) presented advanced chronic HF: 100% New York Heart Association III, median NT-proBNP 4406 pg/mL, ejection fraction (EF) 21% (all patients showed a reduced EF), tricuspid annular plane systolic excursion 16 mm, VO₂ max 12.1 mL/kg/min, and optimal guideline-directed medical therapy including implantable cardioverter defibrillator in 97% and cardiac resynchronization therapy in 37% of patients. At right heart catheterization, performed in 23 patients, median cardiac index was 1.8 L/min/m², mean pulmonary artery pressure was 33 mmHg [22–39.5], and pulmonary artery pulsatility index was 3.2 [2.6–4.5]. Other baseline characteristics are shown in *Table 1*.

Clinical endpoint

Fourteen patients (46.7%) met the primary composite endpoint at 12 month follow-up (HF-PE group, *Table 2*). Of them, three patients died for cardiovascular causes, five patients underwent heart transplantation, and six patients received LVAD implantation. Twenty-two patients were hospitalized for acute HF during the 12 month follow-up. Notably, among echocardiographic parameters of right and left ventricular function, no differences were noted between HF-PE group and patients' group without events (HF-noPE group, *Table 2*). Moreover, commonly used HF biomarkers (copeptin, NT-proBNP, sST2, and Hs-cTnT) did not show any significant difference between the two HF cohorts (*Table 2*). The most significantly different parameters were VO₂ max (although available in 21/30 patients) and BCN Bio-HF score values, with an inverse correlation with prognosis, and trans-pulmonary gradient (TPG, although available in 23/30 patients) with a direct correlation (*P*-value ranging from 0.002 to 0.05, *Table 2*).

Receiver operating characteristic curves were then built for the main clinical and laboratory variables, as shown in Supporting Information, *Figure S2*, to test their prediction capacity: VO₂ max, TPG (though with many missing values: 9/30 for VO₂ max and 7/30 for TPG), and the BCN Bio-HF score

were the most effective in our dataset (AUC = 0.88, 0.79, and 0.72, respectively).

MicroRNA profiling by next-generation sequencing

MicroRNA expression profiles were obtained by NGS performed in 30 enrolled HF patients and 36 healthy controls. From Illumina deep sequencing, a median of 12.7 million reads per sample was generated, ranging from 4.04 to 49.48 million reads. After adapter trimming, reads were aligned to a reference of human small non-coding RNAs as previously described.¹⁸ Alignments correctly mapping to miRNA sequences annotated in miRbase release 22 were in median 1.79 million per sample, ranging from 0.21 to 9.66 million reads. After removing miRNAs with expression lower than 20 reads in more than 50% of samples, a total of 329 unique miRNA sequences were detected in plasma and inserted in a count matrix with 66 samples and 329 miRNAs (Supporting Information, *Table S1*).

Case–control comparison

Thirty-two miRNAs out of the 329 detected were significantly differentially expressed in advanced HF patients when compared with healthy controls (adjusted *P*-value <0.05; *Table 3* and Supporting Information, *Figure S3A*). Of them, 20 miRNAs were significantly up-regulated and 12 down-regulated in HF patients compared with controls (*Table 3*). The most differentially expressed miRNA was miR-210-3p that resulted up-regulated in HF patients (\log_2 fold change = 0.8; adjusted *P*-value = 5.4×10^{-7}). The other significantly up-regulated miRNAs were miR-1287-5p, miR-148a-3p and miR-148a-5p, miR-629-5p, miR-22-5p, miR-339-3p, miR-1285-3p, miR-548o-3p, miR-22-3p, miR-550a-5p, miR-6515-5p, miR-152-3p, miR-330-5p, miR-3120-5p, miR-503-5p, miR-361-3p, miR-21-3p, miR-769-5p, and miR-576-3p. Among the down-regulated miRNAs, the most relevant were miR-628-3p and miR-125a-5p (\log_2 fold change = -0.5 and -0.7 ; adjusted *P*-value = 0.0008 and 0.001, respectively). The other significantly down-regulated miRNAs are reported in *Table 3*.

We validated by real-time PCR the expression levels of four selected miRNAs, characterized by high significance (*P*-value <0.0008) and large differential expression between case–control groups in the NGS analysis (\log_2 fold change >0.6). Validation was performed on 15 HF patients and their paired controls randomly chosen among all samples in the cohort. As shown in *Figure 1*, miR-210-3p, miR-1287-5p, miR-148a-5p, and miR-629-5p were differentially expressed in advanced HF patients in comparison with healthy controls. These results confirmed miRNA profiles observed by NGS.

Table 3 Differentially expressed microRNAs between HF patients and healthy controls

MiRNA name	Log ₂ fold change	<i>P</i> -values	Mean reads
miR-210-3p	0.85	0.00000054	129.46
miR-1287-5p	0.73	0.00030	29.25
miR-148a-3p	0.73	0.00033	74 769.39
miR-148a-5p	0.64	0.00077	160.43
miR-629-5p	0.68	0.00079	1464.03
miR-22-5p	0.38	0.0046	181.57
miR-339-3p	0.51	0.0071	169.04
miR-1285-3p	0.69	0.028	31.10
miR-548o-3p	0.44	0.028	99.51
miR-22-3p	0.37	0.028	6785.33
miR-550a-5p	0.58	0.034	52.85
miR-6515-5p	0.42	0.034	35.09
miR-152-3p	0.41	0.034	1656.89
miR-330-5p	0.34	0.040	55.25
miR-3120-5p	0.43	0.044	41.03
miR-503-5p	0.53	0.046	42.74
miR-361-3p	0.20	0.047	2601.47
miR-21-3p	0.61	0.048	36.70
miR-769-5p	0.24	0.048	197.93
miR-576-3p	0.41	0.049	126.85
miR-628-3p	-0.49	0.0008	205.15
miR-125a-5p	-0.69	0.0014	1245.26
miR-423-3p	-0.39	0.0019	17 516.91
miR-485-3p	-1.10	0.0052	135.97
miR-340-3p	-0.44	0.0081	375.54
miR-6721-5p	-0.51	0.012	45.93
miR-664a-3p	-0.50	0.018	23.79
miR-505-5p	-0.42	0.023	150.21
miR-181d-5p	-0.35	0.028	176.91
miR-484	-0.35	0.028	2545.13
miR-328-3p	-0.42	0.028	2135.29
miR-30d-5p	-0.16	0.034	75 352.14

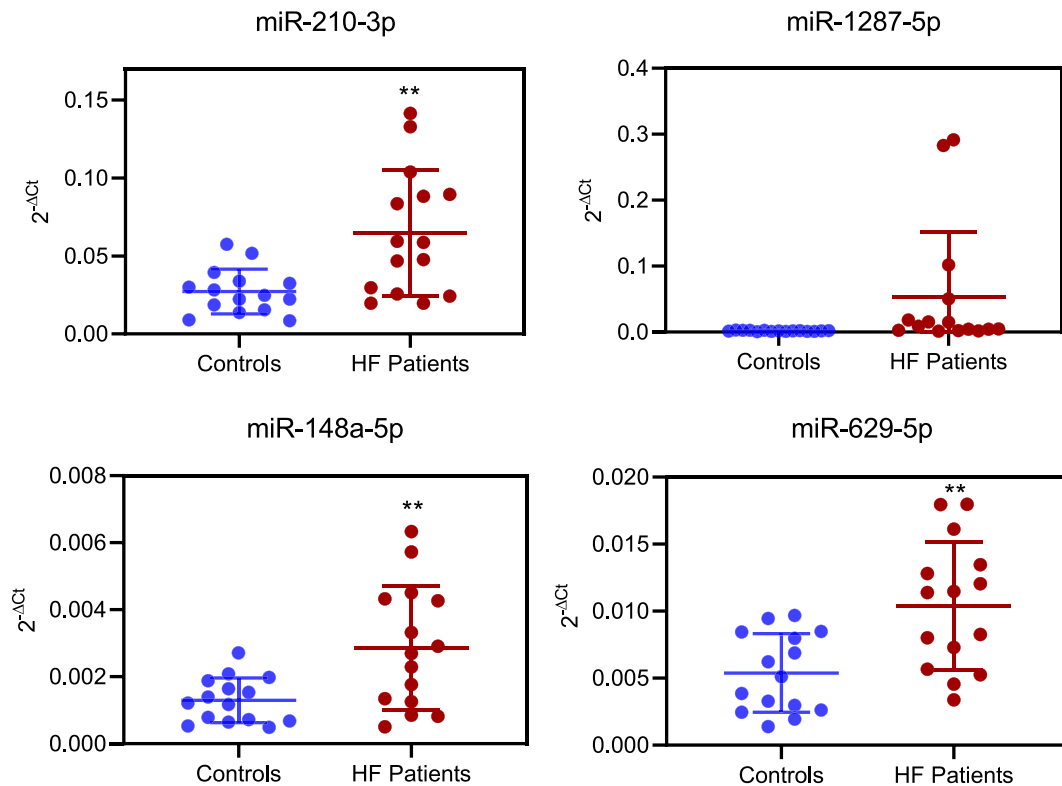
HF, heart failure; miRNA, microRNA.

MicroRNA profiles and heart failure markers

The 32 differentially expressed miRNAs between cases and controls were compared with established biomarkers of HF severity. Among the 32 differentially expressed miRNAs between cases and controls, six resulted associated by regression analysis with NT-proBNP serological levels (*Figure 2* and Supporting Information, *Figure S3*), which confirmed as the best common HF biomarker (AUC = 1, Supporting Information, *Figure S4*). The up-regulated miR-210-3p, miR-22-5p, miR-22-3p, miR-21-3p, and miR-339-3p positively correlated with NT-proBNP ($R \geq 0.30$, *Figure 2*). Also, these miRNAs showed a positive correlation with at least another HF biomarker, among which copeptin, sST2, and hs-cTnT ($R \geq 0.30$, *Figure 2*). The down-regulated miR-125a-5p negatively correlated with NT-proBNP and sST2 ($R < -0.30$, *Figure 2*). Moreover, miR-210-3p, miR-21-3p, and miR-339-3p positively correlated with the BCN Bio-HF score ($R < 0.30$), while miR-125a-5p showed a negative correlation ($R = -0.36$, *Figure 2*).

However, the performance of these miRNAs alone as HF biomarkers was worse than that of common laboratory

Figure 1 Validation by real-time PCR of a subset of four microRNAs differentially expressed between heart failure (HF) cases and controls in the next-generation sequencing analyses (P -value <0.0008 and \log_2 fold change >0.6). Blue: controls. Red: HF patients. ** P -value ≤ 0.01 .



values when tested by AUC analysis, even in a prediction model including all 32 miRNAs (Supporting Information, Figure S4).

Circulating microRNAs related to clinical outcomes

Out of 329 miRNAs detected during the discovery phase, three miRNAs were significantly differentially expressed among HF patients experiencing primary endpoint (HF-PE) and those without clinical events (HF-noPE, Figure 3A). In particular, miR-125a-5p and miR-10b-5p were significantly down-regulated (\log_2 fold change = -0.9 and -1.8 , respectively; adjusted P -value = 0.04 for both), while miR-9-5p was significantly up-regulated (\log_2 fold change = 1; adjusted P -value = 0.04; Figure 3A).

Therefore, we compared the levels of these three miRNAs with clinical variable significant predictive markers. As reported in Figure 3B, in the group of HF-noPE patients, miR-9-5p showed the best negative correlation with VO_2 max ($R = -0.62$, Figure 3B). Interestingly, in the HF-PE group, miR-125a-5p positively correlated with the TPG

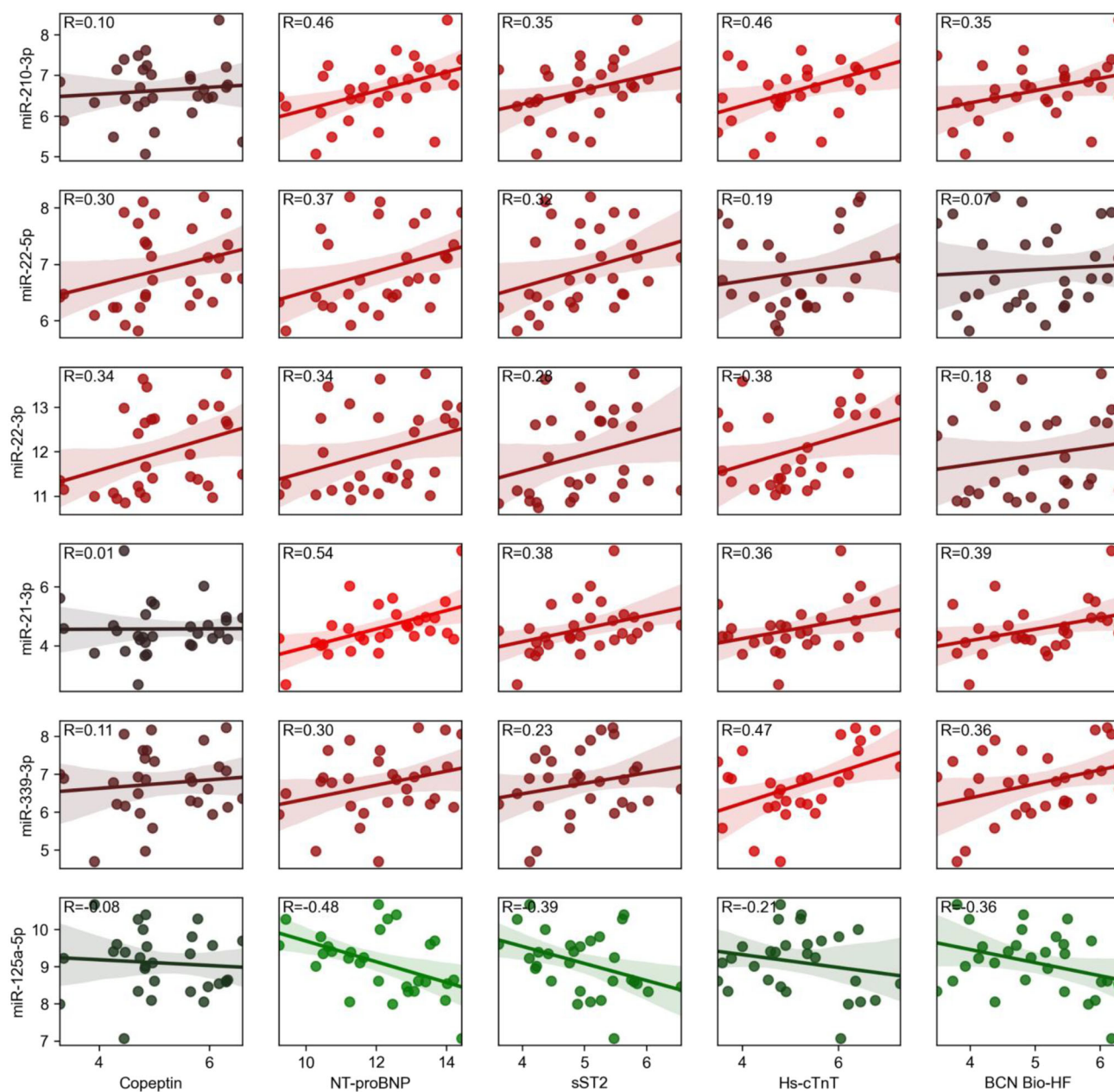
haemodynamic variable ($R = 0.35$, Figure 3B). In the HF-noPE group, all the three miRNAs also correlated with BCN Bio-HF score (Figure 3B).

The final objective of our work was to evaluate if some miRNAs could eventually improve a prognostic model solely based on clinical and laboratory values in advanced HF patients: we therefore tested the additive power of these three selected miRNAs to the variables showing the best AUC for endpoint prediction. Interestingly, while a moderate prognostic power characterized the model including all three miRNAs (AUC = 0.69, Figure 3C), their addition to the model including BCN Bio-HF (AUC = 0.82; Figure 3C), VO_2 max (AUC = 0.90; Supporting Information, Figure S5A), or TPG (AUC = 0.83; Supporting Information, Figure S5B) remarkably increased the AUC: miRNAs eventually improved the prognostic stratification of advanced HF.

Discussion

To the best of our knowledge, this is the first study to characterize the complete miRNA expression profile through the NGS technology in a highly selected population of

Figure 2 Differentially expressed microRNAs significantly correlated with heart failure (HF) biomarkers [copeptin, N-terminal prohormone of brain natriuretic peptide (NT-proBNP), soluble ST2 (sST2), and high-sensitivity cardiac troponin T (Hs-cTnT)] and Barcelona (BCN) Bio-HF score. Bright red ($R > 0.40$) and bright green ($R < -0.40$) colours are associated with stronger positive and negative correlations, respectively. Dark red and dark green colours are weak correlation values. MicroRNA expression and clinical variable values are reported as base 2 logarithms. The correlation coefficients are shown (R).

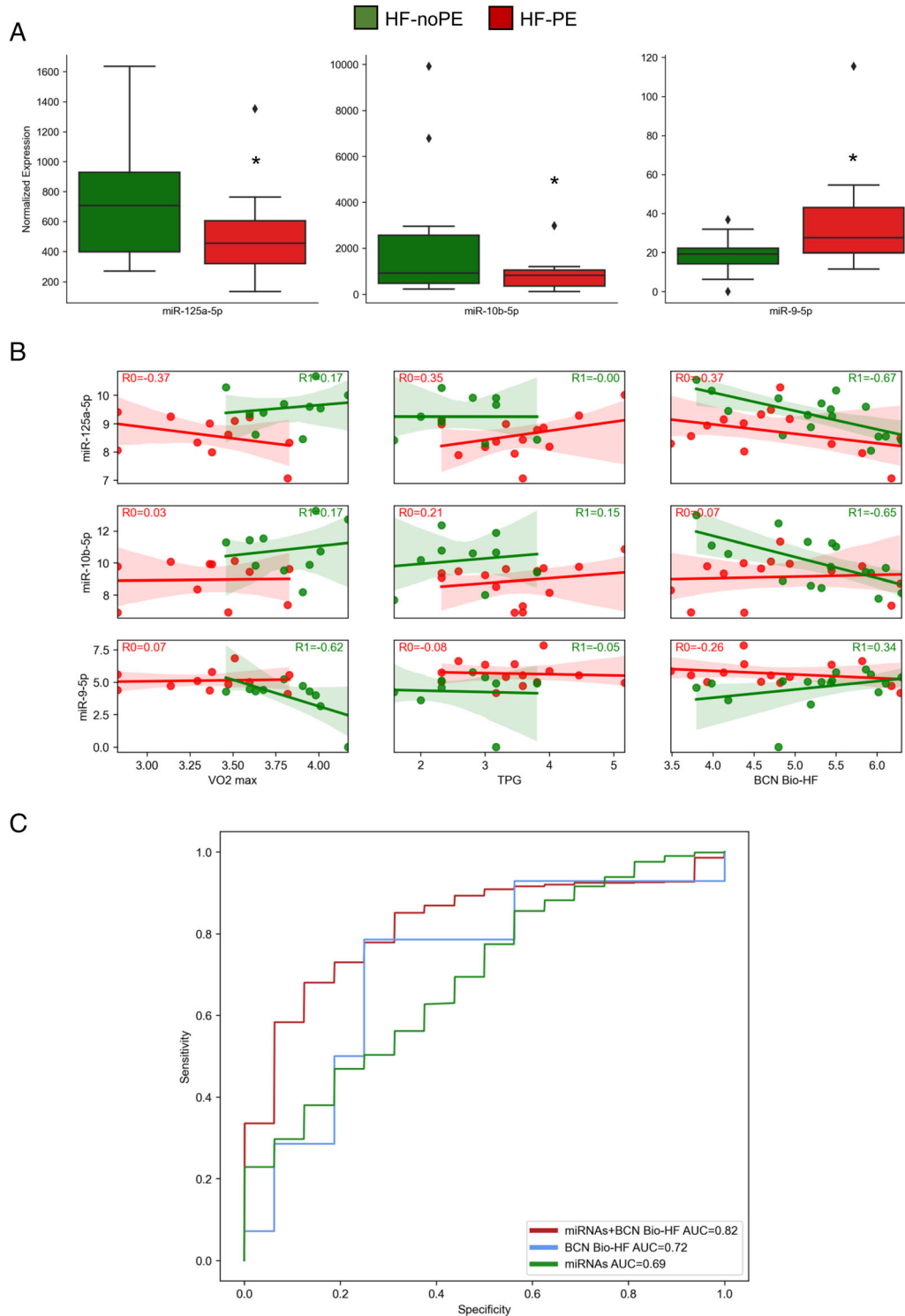


patients with advanced HF. Our main findings are the following: (i) on the global miRNome analysis, a unique profile of 32 miRNAs differentially expressed between advanced HF patients and controls; (ii) six miRNAs significantly correlated with HF markers; and (iii) among the advanced HF population, three miRNAs were significantly differentially

expressed and correlated with the composite endpoint of cardiac death, heart transplantation or mechanical circulatory support implantation and implemented the prognostic prediction model solely based on clinical values.

In the puzzling setting of advanced HF, miRNAs still represent a mostly undiscovered chapter as compared

Figure 3 Circulating microRNAs (miRNAs) related to clinical outcomes. (A) miRNAs differentially expressed among heart failure (HF) patients experiencing primary endpoint (HF-PE, red) and those without clinical events (HF-noPE, green). *Adjusted *P*-value = 0.04. *Outlier sample values. (B) Correlation levels of the three differentially expressed miRNAs in HF-PE (red) vs. HF-noPE (green) with clinical variable significant predictive markers (Table 2). miRNA expression and clinical variable values are reported as base 2 logarithms. The correlation coefficients are shown (*R*). (C) Receiver operating characteristic curves built for the three differentially expressed miRNAs among HF-PE and HF-noPE groups (green), Barcelona (BCN) Bio-HF score (blue), and three miRNAs + BCN Bio-HF score (red). The value of area under the curve (AUC) was reported. TPG, trans-pulmonary gradient.



with other diseases in which genomic medicine is confirming its promising perspectives. In particular, the idea of a serological parameter with a role in the gene regulation at a post-transcriptional level and that could implement prognostic prediction in HF makes these non-coding molecules exceptionally appealing. This is especially important when considering the relatively low prognostic power of the available markers and scores and the low availability, high costs, and complication burden of non-pharmacological interventions, as mentioned earlier.

Our research relied on NGS application, a powerful forefront technique able to evaluate the entire miRNome with a hypothesis-free approach. The majority of previous studies, performed in acute and chronic HF patients, used a candidate approach considering a limited and predefined number of miRNAs.^{11,19–28} In contrast, only few papers exploited the NGS approach to determine the abundance of myocardial and circulating miRNAs in HF patients before and after LVAD implantation.^{29–31}

In the present study, we carefully selected an advanced HF population, as proven by clinical, echocardiographic, and laboratory parameters and by the high adverse event rate at 12 month follow-up. In this population, we obtained an HF-specific panel of 32 differentially expressed miRNAs in comparison with matched controls. Some (14) of these dysregulated miRNAs have already been described in HF or in other cardiovascular diseases. Five miRNAs (miR-210-3p, miR-22-5p, miR-22-3p, miR-21-3p, and miR-339-3p) were positively correlated to NT-proBNP levels, and miR-125a-5p was negatively correlated. These miRNAs also correlated with at least one HF biomarker among copeptin, sST2, and Hs-cTnT. As expected, though, they did not show a higher diagnostic force than those of biomarkers themselves, probably because the end-stage HF disease involves multiple organs and is characterized by high inflammatory state, and miRNAs are expected to reflect more than the original cardiac pathology. While the aim of our work is not to improve diagnostics in HF, we intend to describe a correlation of the miRNA expression profile with the commonly available markers.

The major hypoxia-inducible miR-210³² is dysregulated in several cardiovascular diseases, among which HF and myocardial infarction,^{33,34} while up-regulated levels of this miRNA have been found in patients with stable and end-stage HF.²⁹ Intramyocardial injection of miR-210 carrying vector during myocardial infarction improved cardiac function, reducing the infarct size and promoting angiogenesis,³⁵ therefore suggesting its association with a repair response to organ stress.

MiR-21 was found dysregulated in different cardiovascular diseases, among which HF, cardiac hypertrophy, and myocardial ischaemia.^{36–38} This important miRNA may attenuate HF damage by promoting angiogenesis and reducing fibrosis and apoptosis after myocardial infarction.³⁹ However, its role in

the cardiac fibrotic process is controversial, because the majority of scientific results describe a pro-fibrotic action in the infarcted heart tissue³⁸ and high levels have been found in the fibroblasts⁴⁰ and macrophages⁴¹ of failing hearts.

Even if miR-22, differently from miR-210 and miR-21, has been studied mainly in the cancer field,⁴² it may be associated with regulation of cardiac remodelling⁴³ and has been recently reported as one of the most abundant miRNA in the heart, with higher levels in acute myocardial infarction and in stable chronic HF.^{28,44} For these characteristics, it has been proposed as candidate diagnostic marker.⁴⁵

Conversely, miR-339 has not been implicated in cardiac pathophysiology so far, only sparse data showed an up-regulation of this miRNA after cardiac surgery with cardiopulmonary bypass and cardioplegic arrest⁴⁶ and in cardiac amyloidosis.⁴⁷

The role of miR-125a-5p in the cardiovascular system is poorly known, it has been proposed to differentiate HF patients with reduced left ventricular EF from those with preserved EF.⁴⁸

It is worthy to note that the muscle-related myomiRs, such as miR-499 and miR-208, did not contribute to the HF signature of our patients. In the present study, these two specific miRNAs were not included in the count matrix because not expressed, whereas miR-1 and miR-133 did not show differences in the expression levels between HF patients and controls. These results are consistent with previous reports showing that myomiRs are biomarkers of the acute phase of the disease, returning to the baseline expression levels in the chronic and stable period.^{29,49} Overall, a better understanding of the role of these miRNAs in HF may highlight molecular and cellular mechanisms of the cardiovascular disease and allow to improve HF patients' management and therapies.

Concerning prognostication in our population, commonly available markers mostly did not help in the prognostic stratification; the parameters with the strongest predictive capacities were the VO₂ max and TPG (AUC = 0.88 and 0.79, respectively). Noteworthy, also the validated BCN Bio-HF score did not perform well (AUC = 0.72). The three differentially expressed miRNAs in the HF-PE group (miR-125a-5p, miR-10b-5p, and miR-9-5p) added substantial prognostic power to the BCN Bio-HF score. Little is known about the cardiovascular function of miR-10b-5p and miR-9-5p. On the other hand, miR-125a-5p has been found down-regulated in HF patients vs. controls showing a potential role as HF biomarker. Interestingly, the three miRNAs are involved in the cardiac apoptotic process: miR-125a-3p has been associated with cell survival stimulation and apoptosis inhibition in cardiac myocytes.⁵⁰ miR-10b-5p was found to improve cardiac function in a murine model of myocardial infarction and exerted anti-apoptotic function in cardiomyocytes *in vitro*.⁵¹ Finally, for miR-9, the role on apoptosis is

controversial: the knockdown of this miRNA led to inhibition of hypoxia-induced cardiomyocyte apoptosis,⁵² but a recent work suggested its anti-apoptotic function during myocardial infarction progression.⁵³ Overall, comparing our miRNA expression results with published data, we may speculate that they are involved in the worsening of HF progression through an effect on apoptosis and therefore play a prognostic role.

The main limitation of our paper is undoubtedly the small number of patients enrolled: this may lead to an overestimation of the role of some miRNAs and conversely to an underestimation of others, whose significance could have been blunted or enhanced in a larger sample size. For example, miR-423-5p, whose dysregulation has been previously associated with poor long-term outcome in acute HF patients,²⁵ was found to be also down-regulated in our HF-PE group in comparison with HF-noPE patients (fold change = -1, data not shown), but only with nominal significance (nominal *P*-value = 0.003; adjusted *P*-value = 0.06). Notwithstanding, this was conceived as a pilot 'discovery' study, it is the first of its kind, and other analogous works not investigating the whole miRNome enrolled a similar number of patients. Moreover, the authors faced the difficult task of standardizing the timing of sampling in phases of relative clinical stability of the complex setting of advanced HF.

Another limitation of the study is the lack of external validation of our predictive model. Even if the internal validation (10-fold cross-validation repeated with different random seeds 50 times) strengthens our results, further studies should investigate the role of our predictive model in an external cohort of selected advanced HF patients.

It could also be argued that the Seattle Heart Failure Model or Heart Failure Survival Score could have been used instead of the BCN Bio-HF and may have produced higher AUC for prognostic prediction. However, BCN Bio-HF is one of the scores recommended by the European Society of Cardiology and has been proven to outperform other risk models. Moreover, not all HF patients may be able to perform a cardiopulmonary exercise test, as was the case of 30% of our population, and this percentage may be higher in a real-world setting.

We also acknowledge the low percentage of female included in our study: though in line with other registries and clinical trials of HF patients,⁵⁴ this may limit the generalizability of our results.

In conclusion, in this discovery study on patients with advanced HF, we sequenced the plasma miRNome and identified differentially expressed miRNAs between cases and controls with some miRNAs also altered between patients with and without adverse events at follow-up. A prediction model including three of these miRNAs improved the prognostic stratification based on standard clinical and

laboratory values. Further studies are needed to validate and confirm our results in larger populations.

Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Workflow of the study.

Figure S2. Receiver operating characteristic (ROC) curves built for the main clinical and laboratory variables that resulted differentially distributed among patients encountering primary endpoint (HF-PE group) and those without events (HF-noPE group). TPG:trans-pulmonary gradient. The values of area under the curve (AUC) were reported.

Figure S3. (A) Volcano Plot reporting all miRNAs differentially expressed between cases and controls (red: up-regulated, green: down-regulated). The six miRNAs better correlated with NT-proBNP are reported. (B) Heatmap reporting the six miRNAs better correlated with NT-proBNP and significantly differentially expressed between cases (HF,red) and controls (CTL,blue).

Figure S4. Receiver operating characteristic (ROC) curves built for HF biomarkers (NT-proBNP, Hs-cTNT, Copeptin, and sST2) that resulted differentially distributed among cases and controls, for 32 differentially expressed miRNAs between cases and controls, and 6 miRNAs better correlated with NT-proBNP and significantly differentially expressed between cases and controls. The values of area under the curve (AUC) were reported.

Figure S5. Receiver operating characteristic (ROC) curves built for the differentially expressed miRNAs among patients encountering primary endpoint (HF-PE group) and those without events (HF-noPE group), the 3 differentially expressed miRNAs alone or together with (A) VO2 max or (B) TPG. TPG: trans-pulmonary gradient. The values of area under the curve (AUC) were reported.

Table S1. MiRNA raw counts metrics stratified for all samples included in the study.

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