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Circulating microRNA as predictors for exercise response in heart failure with reduced ejection fraction

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31

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Abstract

Aims.

Exercise training is a powerful adjunctive therapy in patients with heart failure with reduced ejection fraction (HFrEF), but one third of patients fail to improve VO_2peak. We hypothesize that circulating microRNAs (miRNAs), as epigenetic determinants of VO_2peak, can distinguish exercise responders (ER) from non-responders (ENR).

Methods.

We analyzed 377 miRNAs in 18 male HFrEF patients (9 ER and 9 ENR) prior to 15 weeks of exercise training using a miRNA array. ER and ENR were defined as change in VO_2peak of >20% or <6% respectively. First, unsupervised clustering analysis of the miRNA pattern was performed. Second, differential expression of miRNA in ER and ENR was analyzed and related to percent change in VO_2peak. Third, a gene set enrichment analysis was conducted to detect targeted genes and pathways.

Results.

Baseline characteristics and training volume were similar between ER and ENR. Unsupervised clustering analysis of miRNAs distinguished ER from ENR with 83% accuracy. A total of 57 miRNAs were differentially expressed in ENR vs ER. A panel of 7 miRNAs upregulated in ENR (Let-7b, miR-23a, miR-140, miR-146a, miR-191, miR-210, and miR-339-5p) correlated with %changeVO_2peak (all $p<0.05$) and predicted ENR with AUCs ≥0.77. Multiple pathways involved in exercise adaptation processes were identified.

Conclusion.

A fingerprint of 7 miRNAs involved in exercise adaptation processes is highly correlated with VO_2peak trainability in HFrEF, which holds promise for the prediction of training response and patient-targeted exercise prescription.

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Keywords: heart failure – microRNA – exercise training – VO_2peak – response - pathways

71 **Introduction**

72

73 Heart failure (HF) is an increasingly prevalent syndrome with considerable impact on quality of life.[1]
74 Exercise training is a powerful adjunctive therapy that improves morbidity and mortality and is therefore
75 recommended to all stable HF patients (Class I A indication).[2] Unfortunately, not all HF patients benefit
76 from this approach and ca. 55% of HF patients fail to demonstrate a clinically relevant increase in
77 aerobic capacity.[3] Peak oxygen consumption (VO_2peak) is one of the strongest prognostic factors in HF
78 and a failure to adequately increase VO_2peak adds to an adverse prognosis, independent of other risk
79 factors.[4]
80 Even with similar training volumes, the variability in VO_2peak response is high and underlying
81 mechanisms are not fully explained. Heritability accounts for at least 40-50% of the anticipated effect
82 of exercise training.[5] However, at present, it is not possible to predict which patients will show an
83 increase in VO_2peak following exercise training.

84

85 MicroRNAs (miRNAs) are non-coding RNAs that regulate gene expression at the post-transcriptional
86 level. miRNAs interrupt translation of messenger RNA (mRNA) through base pairing, provoking
87 translational repression or degradation of the mRNA. One miRNA may exert inhibitory effects on
88 several mRNAs.[6] miRNAs can be detected in plasma, either packed in exosomes or microparticles, or
89 bound to Ago proteins and HDL cholesterol, making them attractive as biomarkers.[7]
90 Plasma levels of miRNAs have been linked to aerobic capacity and they change dynamically following
91 exercise.[8] For example, miR-21, miR-210, miR-222 are elevated in healthy individuals with lower
92 VO_2peak[9], and miR-1, miR-20a, miR-146a and miR-486 are higher in endurance athletes with a higher
93 VO_2peak.[10,11] Exercise training increased plasma levels of miR-21, miR-146a, miR-221, and miR-222
94 in athletes.[10] In HF patients, an acute maximal exercise bout significantly upregulated circulating miR-
95 21, miR-378 and miR-940 levels immediately after the exercise test[12], and in patients with chronic
96 kidney disease, plasma levels of miR-146a showed a rapid downregulation after an acute exhaustive
97 exercise test.[13]

98

99 As such, circulating miRNAs are promising as epigenetic markers of physical fitness and exercise-
100 induced cardiovascular adaptation, and they could even play a role in personalized exercise prescription.
101 In this study, we hypothesize that a specific miRNA signature could distinguish exercise non-responders
102 (ENR) from exercise responders (ER), hence allowing to predict which patients will show an increase
103 in VO_2peak following exercise training. To this end, we performed an unbiased miRNA screening in
104 HFrEF patients and related this to the VO_2peak response to exercise training.
105
106 **METHODS**
107
108 **Study design, exercise training and testing**
109 For this retrospective cohort study, male HFrEF patients (left ventricular ejection fraction (LVEF)
110 <40%) who followed an in-hospital training programme in the setting of a standardized longitudinal
111 study, were screened. To minimize the impact of sex-specific patterns on the miRNA profile, this study
112 was performed in male patients only.[14] All patients had to be clinically stable and optimally medically
113 treated for ≥6 weeks. Eighteen patients were included. The programme consisted of 15 weeks moderate
114 aerobic exercise training, three 50-minute sessions/week, with a continuous training intensity at 90% of
115 heart rate (HR) at the respiratory compensation point (RCP, n=17) or four 4-minute intervals at 90% of
116 HR at the RCP (n=1). In addition, all patients performed 10 minutes of moderate intensity resistance
117 exercise per session. At start and after 15 weeks of training, cardiopulmonary exercise test (CPET) was
118 performed on a bicycle or treadmill ergometer (the same modality at both visits) and VO_2peak was
119 determined as the mean VO_2peak during the final 30 seconds of exercise. RCP was identified using the
120 VE/VCO_2 curve and the CO_2-equivalent. Whole blood was collected at baseline and after 15 weeks after
121 an overnight fast in EDTA tubes, centrifuged within 30 minutes after collection and stored at -80°C.
122 miRNAs were quantified from baseline blood samples.
123 This study complied with the Declaration of Helsinki and was approved by the ethical committee of the
124 Antwerp University Hospital. Written informed consent was obtained from each participant.
125
126 **Definition of exercise responders (ER) and exercise non-responders (ENR)**

127 ER were defined as subjects with an increase of >20% in VO_2peak, and ENR as increase of <6% in
128 VO_2peak. An improvement of at least 6% in VO_2peak has been associated with reduced all-cause
129 mortality and all-cause hospitalization.[15] To increase the discriminatory capacity of the miRNA panel,
130 the cut-off for ER was set at >20% VO_2peak increase.
131

132 **miRNA array**

133 miRNAs were profiled and analyzed from plasma samples using TaqMan Low Density MicroRNA
134 Array (TLDA) Human Cards A (ThermoFisher), analyzing 377 human miRNAs, as previously
135 published.[16,17] Briefly, plasma samples were thawed on ice and centrifuged for 10 minutes (4°C,
136 16,000g). Total RNA was isolated using the mirVana Paris kit (ThermoFisher). Reverse transcription
137 and preamplification were performed with MegaPlex primer pools (ThermoFisher) following the
138 manufacturer's protocol.[16] The preamplification product was mixed with TaqMan Universal PCR
139 Master Mix No AmpErase UNG (ThermoFisher) and nuclease-free water before loading to the TLDA
140 card. The arrays were run in a 7900HT Fast Real-Time polymerase chain reaction (PCR) system
141 (ThermoFisher). Raw cycle quantification (Cq) values were calculated in SDS software v.2.4 using
142 automatic baseline and threshold settings. A miRNA was considered non-informative if Cq values were
143 >35 in >80% of samples. As suggested before, geNorm algorithm (*NormqPCR package*) was used for
144 normalization and relative miRNA levels were expressed as $2^{-\Delta Cq}$.[17]
145

146 **Technical validation by miRNA RT-qPCR**

147 Expression of the selected miRNA was repeated by conventional RT-qPCR. A new plasma aliquot of
148 the same individuals was thawed on ice and centrifuged for 10 minutes (4°C, 16,000g). RNA enriched
149 for small RNAs was isolated using the mirVana Paris Kit (ThermoFisher). As spike-in control, 20fmol
150 synthetic Ath-miR-159a (ThermoFisher) was added. Reverse transcription and preamplification were
151 performed using TaqMan miRNA primers (ThermoFisher) and multiplex qPCR was done in a CFX96
152 thermal cycler (BioRad).[13] Raw Cq values were calculated in BioRad CFX manager software v.3.1 using
153 automatic baseline and threshold settings. Data were normalized using Ath-miR-159a and relative
154 miRNA levels were expressed as $2^{-\Delta Cq}$.

155

156 **Statistical analysis**

157 Data were analyzed using R version 3.4.3, SPSS 26.0 and GraphPad Prism 8.3.0. Normality of
158 continuous variables was evaluated using Shapiro-Wilk test. Normally distributed data are expressed as
159 mean ± standard deviation (SD), skewed variables as median and range (1st – 3rd quartile). Categorical
160 variables were analyzed with Fisher-exact test, continuous variables with independent samples T-test or
161 Mann-Whitney U test where appropriate.

162 Unsupervised agglomerative hierarchical clustering was performed using Manhattan distances and
163 Ward linkage, and a heatmap was constructed (*cluster* and *Heatplus* packages). The accuracy of this
164 unsupervised model was calculated by superposing unblinded information (responder and non-
165 responder status of the patients) to the clusters.

166 To identify a miRNA panel that discriminates ENR from ER, miRNA expression between ER and ENR
167 was compared using multiple T-tests and resulting p-values were adjusted for multiple testing using the
168 Benjamini and Hochberg procedure controlling the false discovery rate at 5%. miRNAs were considered
169 significantly differentially expressed when fold change was <0.66 or >1.5, and the adjusted p-value was
170 <0.05, between ER and ENR.

171 Next, a supervised shrunken centroid model was fitted to predict responder status from baseline miRNA
172 expression (*pamr* package, threshold 1.0).[18] We predefined criteria to further refine the miRNA
173 selection: since we are interested in a biomarker of ENR, miRNAs of interest had to be 1) expressed in
174 all ENR, 2) upregulated in ENR, 3) p <0.005 for the difference in fold change expression between ENR
175 vs ER, and 4) similarly expressed in arrays and RT-qPCR.

176 Correlation between miRNAs and percent change in VO_2peak (%changeVO_2peak) was assessed using
177 Spearman correlation analysis. A univariate logistic regression model to predict ENR using relevant
178 baseline characteristics (age, baseline LVEF and NHYA class)[19] was fitted. Area under the receiver
179 operating characteristic curve (AUC) was calculated to predict ENR using miRNAs of interest. Linear
180 mixed models were fitted using time (baseline vs. 15 weeks) and group (ER vs. ENR) as fixed effects
181 and patient ID as random effect, to asses change in CPET characteristics, BMI, LVEF, strength and
182 NYHA class. A two-sided p-value <0.05 was considered significant.

183

184 **Interaction network**

185 To perform a pathway analysis, a three-layer interaction network was constructed:

186 First, a protein-protein interaction matrix was constructed using STRING database v10.5, specific for
187 heart, blood vessel, skeletal muscle and kidney - tissues relevant for heart failure pathophysiology - and
188 annotated with their corresponding genes using GTex database v7. Interactions were relevant when read
189 counts for both genes were >10 in at least one of the relevant tissues.

190 Second, a miRNA-miRNA interaction matrix was constructed based on a mutual information network
191 after applying the Aracne algorithm, using default settings, on the miRNA expression data.

192 Third, TargetScan database v7.0 was used to predict human miRNA-gene interactions.

193 Then, a Markov random walk algorithm was used to smooth expression fold changes for miRNAs with
194 a significantly altered expression over the constructed three-layer network. Three different restart
195 probabilities (0.8, 0.5 and 0.2, respectively limited, average and extensive smoothing) were executed,
196 thereby simulating the amount of information flow through the network and generating a probability
197 distribution reflecting the likelihood that a gene in the network is perturbed by a miRNA of interest.
198 Next, a gene set enrichment analysis was performed, using the genes ranked in decreasing order
199 according to the perturbation probabilities. Here 56 gene sets associated with biological processes and
200 signal transduction pathways retrieved from the Kyoto Encyclopaedia of Genes and Genomes (KEGG,
201 retrieved on 20/11/2017) were used. The enrichment score normalized for gene set length and the
202 associated significance level, estimated using 10.000 permutations, were recorded for each of the
203 evaluated gene sets. Resulting p-values were adjusted for multiple testing using the Benjamini and
204 Hochberg procedure controlling the false discovery rate at 5%. Consolidated pathways were those that
205 remained after removal of gene overlap and only positive enriched pathways were selected.

206 This analysis was performed using data obtained with the different restart probability levels. For network
207 visualization, a minimal spanning tree algorithm was first applied onto the network, to extract the most
208 representative interactions from these dense networks.

209

210 **RESULTS**

211

212 **Patient characteristics and association with response**

213 From a total of 41 HFrEF patients, 9 ER (>20% change in VO_2peak) and 9 age-matched ENR (<6%

214 change in VO_2peak) that underwent an identical training program, were withheld for further analysis.

215 The evolution of VO_2peak is shown in **Figure 1**. At baseline, ER and ENR were similar with regard to

216 patient demographics, clinical, pharmacological and CPET characteristics (all p>0.05, **Table 1)**.

217 Adherence to training was excellent in both groups with 37 completed sessions out of 45 total sessions.

218 ENR trained at 106% and ER at 95% of their target HR during the first 4 weeks (p=0.040), and ENR at

219 111% and ER at 96% of their target HR from week 5-15 (p=0.036). Age, baseline LVEF and NYHA

220 class did not predict exercise response in univariate logistic regression analysis (p> 0.05). There was no

221 difference in change in medical therapy between the groups (p>0.05, data not shown). Whereas not

222 significantly different between groups (p-value for interaction >0.05), there was a trend towards

223 improved strength, LVEF and NYHA class in the ER compared to ENR group (**Table S1**).

224

225 **Differential miRNA expression between ENR and ER**

226 Unsupervised analysis of the miRNA array expression data in all patients revealed 2 separate clusters

227 of patients (**Figure 2**). These clusters represent ENR and ER with an accuracy of 83%, and a

228 classification error in ER = 22%, in ENR = 11%, which results in an overall classification error of 17%

229 (1 ENR misclassified in the ER group, 2 ER in the ENR group). Clear differences in miRNA expression

230 between the two clusters are illustrated in a heatmap (**Figure 2**).

231 Supervised analysis revealed differential expression of 57 miRNAs in the ENR versus ER: 26

232 miRNAs were upregulated and 31 were downregulated in ENR (**Figure 3**, p<0.05). A supervised

233 shrunken centroid model correctly classified 89% of patients as ER or ENR (two ER were

234 misclassified as ENR) using only baseline miRNA expression and responder status as input.

235

236 **Discriminative miRNA signature for ENR**

237 To develop a discriminative miRNA signature for ENR, predefined selection criteria were applied.

238 miRNAs of interest had to be 1) expressed in all ENR, 2) upregulated in ENR, 3) p<0.005 for the

239 difference in fold change expression between ENR and ER, and 4) similarly expressed in arrays and
240 RT-qPCR. This resulted in ENR miRNA signature of 7 miRNAs: miR-23a; miR-339-5p, miR-140, miR-
241 191, miR-210, miR-146a and Let-7b (**Figure 3**). All 7 miRNAs were significantly correlated with
242 %changeVO_2peak ($p<0.05$), and all miRNAs of interest had an AUC ≥0.77 for the identification of ENR
243 (**Table 2, Figure S1**).
244
245 **Targets of differentially regulated miRNAs**
246 In order to detect the genes and pathways targeted by the miRNA profile of ENR and ER, a pathway
247 analysis was performed. First, a miRNA-gene interaction network was constructed and a Markov
248 random walk diffusion algorithm was used to rank network genes based on their probability of being
249 perturbed by individual or joint miRNA expression changes. The most important interactions in the
250 miRNA-gene network are shown in **Figure S2 A and B**, with key roles for PIK3C2A, DNM2, RAB5C
251 and HSPA8 genes (targeted by upregulated miRNAs in ENR), and CRK, EIF4B and PRKG1 genes
252 (targeted by upregulated miRNAs in ER). Next, a gene set enrichment analysis was performed,
253 translating the network-based gene perturbation probabilities into pathways. KEGG pathways related to
254 nucleotide-binding oligomerization domain-like receptors (NOD-like receptors), transforming growth
255 factor β (TGF-β), toll-like receptor, adherens junction, apelin signaling, neurotrophin signaling and
256 miRNAs in cancer were consolidated in the analysis of upregulated miRNAs in ENR, whereas Notch,
257 mitogen-associated protein kinase (MAPK) and vascular endothelial growth factor (VEGF), epidermal
258 growth factor receptor (EGFR), tyrosin kinase inhibitor resistance, hippo signaling, adherens junction,
259 apelin signaling, neurotrophin signaling pathways and miRNAs in cancer were consolidated in the
260 analysis of upregulated miRNAs in ER (**Figure 4 A and B**).
261
262 **DISCUSSION**
263
264 The present study describes for the first time a plasma miRNA profile that distinguishes HFrEF patients
265 with a favourable response to exercise training from exercise non-responders, despite excellent training
266 adherence and similar patient characteristics in both groups. The panel of 7 miRNAs (Let-7b, miR-23a,

267 miR-140, miR-146a, miR-191, miR-210, and miR-339-5p), upregulated in exercise non-responders, is
268 involved in exercise adaptation processes such as angiogenesis, skeletal muscle function, and
269 inflammation. *In silico* gene set enrichment analysis revealed several pathways involved in the
270 regulation of exercise response, which may serve as novel therapeutic targets.
271
272 ***Clinical variables to predict exercise response***
273 Early identification of ENR is of great importance, as 55% of HFrEF patients show impaired VO_2peak
274 response (<6% increase in VO_2peak) after standardized cardiac rehabilitation[3]. Indeed, patient-tailored
275 training modifications, either through changing the type or through increasing the duration, intensity or
276 frequency of the training programme, might result in a more favourable and cost-effective VO_2peak
277 response.[19–21] In the past decade, several studies have investigated different clinical and training-related
278 determinants of exercise response, but a clear discriminative marker is still lacking. In the SMARTEX-
279 HF study, investigating the effect of exercise intensity in 215 HFrEF patients, lower NYHA class,
280 younger age, higher LVEF and high intensity interval training (HIIT) or moderate continuous training
281 (MCT) significantly increased the odds for being a VO_2peak responder.[19] In a large study on VO_2peak
282 trainability, including 677 participants of both healthy, elderly and clinical populations (coronary artery
283 disease, type-2 diabetes and metabolic syndrome), age, sex, exercise volume, population group, and the
284 average between pre and post training VO_2peak explained only 17% of the variance in VO_2peak
285 trainability.[20] In patients with coronary artery disease, older age, history of elective percutaneous
286 coronary intervention and higher baseline VO_2peak significantly predicted ENR.[22]
287 In contrast with the SMARTEX-HF findings[19], a significant association between age, baseline LVEF,
288 NYHA class and exercise response was not found in the present study, possibly due to its smaller sample
289 size.
290
291 ***Epigenetics to predict exercise response***
292 In the past two decades, extensive research on (epi)genetic biomarkers of aerobic capacity has been
293 performed. The HERITAGE Family Study attributed 47% of the variability in VO_2peak response to
294 genetic factors, and Karvinen et al. confirmed that the genome plays a significant role in exercise

295 participation with a narrow-sense heritability of physical activity estimated at 53%.[23,24] Furthermore, the
296 latter also suggested that genetic pleiotropy might partly explain the association between high physical
297 activity, cardiorespiratory fitness and survival.[24] As epigenetic regulators of exercise response, several
298 miRNAs have been investigated in healthy individuals or well-trained athletes. To the best of our
299 knowledge, we are the first to describe a miRNA signature to identify non-responders to exercise
300 training prior to exercise prescription. Plasma-derived miRNAs are stable and relatively easy detectable
301 with conventional RT-qPCR techniques, and circulating miRNAs are withheld as promising diagnostic
302 or prognostic biomarkers in different pathologies such as cancer and heart failure. Moreover, their role
303 as therapeutic targets is currently being investigated in various clinical conditions.[25,26]
304 The proposed miRNA panel, consisting of Let-7b, miR-23a, miR-140, miR-146a, miR-191, miR-210,
305 and miR-339-5p, is upregulated in patients with an unfavourable VO_2peak response. All miRNAs
306 correlated with the change in VO_2peak and showed good performance for the prediction of non-response
307 (AUCs $\geq$77%). Circulating miR-210 has been inversely related to aerobic capacity in healthy subjects[9],
308 and plasma miR-146a has been positively correlated with VO_2peak in endurance athletes.[10] Both acute
309 and chronic exercise also upregulate plasma levels of miR-146a in patients with chronic kidney disease
310 and in healthy athletes.[10,13]
311
312 ***Epigenetics to identify underlying mechanisms of exercise response***
313 Since the expression profiles of the proposed miRNA panel were significantly different between ER and
314 ENR, miRNAs could also aid in unravelling the underlying mechanisms of the lack of response to
315 exercise training in HFrEF patients. miRNAs can be found in the circulation in resting conditions, but
316 also upon release by skeletal muscle or endothelial cells during exercise, thereby mediating exercise
317 adaptation pathways. Previous research demonstrated that an increase in plasma volume with endurance
318 training contributes to the increase in VO_2peak.[27] To unravel epigenetic mechanisms of exercise
319 response, we performed an *in silico* analysis that revealed several pathways involved in exercise
320 adaptation processes such as VEGF, Notch, apelin, MAPK, NOD-like and toll-like receptor pathways,
321 providing potential therapeutic targets which may contribute to achieving higher VO_2peak response
322 rates in the future.

323

324 *Endothelial function and angiogenesis*

325 Both miR-146a and miR-210 stimulate VEGF expression by decreasing the expression of neurofibromin
326 2 and increasing Ras-related C3 botulinum toxin substrate 1 and p21-activated kinase-1, and by
327 decreasing runt-related transcription factor-3 respectively.[28,29] Furthermore, the proposed miRNA panel
328 is involved in angiogenesis, with clear roles for miR-23a (inhibits Semaphorin-6A and -6D, and sprouty
329 homolog 2)[30], miR-146a (increases fibroblast growth factor binding protein 1 expression[31]), and miR-
330 191 (targets nuclear factor kappa B (NF-kB) signaling and vascular endothelial zinc finger 1).[32] Since
331 these miRNA appeared to be upregulated in ENR, either the stimulation of endothelial function and
332 angiogenesis is not sufficient or downstream pathways are impaired, which could contribute to ENR.

333

334 *Skeletal muscle mass and function*

335 Some of the miRNAs have been related to skeletal muscle; Let-7b and miR-191-5p regulate myogenesis
336 through respectively paired box protein-7 inhibition[33] and MAPK, interleukin-6 signaling, and
337 serine/threonine-protein phosphatase PP1-β catalytic subunit, and signal transducer and activator of
338 transcription 3 pathway.[34] Furthermore, miR-140 and miR-23 protect against skeletal muscle atrophy
339 through inhibiting Wnt family member 11 expression[35] and the ubiquitin-proteasome pathway
340 respectively,[36] and miR-146a-5p has been related to TGF-β, which is one of the key pathways in cardiac
341 remodeling and fibrosis and in skeletal muscle repair after exercise.[37] Additionally, miR-23a and miR-
342 140-3p mediate cardiac hypertrophy, through targeting the ubiquitin-proteasome pathway and GATA
343 binding protein 4 respectively.[38,39]

344

345 *Inflammation*

346 Several of the proposed miRNAs have been related to inflammatory processes; overexpression of miR-
347 23a could downregulate heat shock protein 90 and NF-kB protein in inflammatory macrophages and
348 foam cells.[40] miR-146a suppresses inflammation through increasing erb-b2 receptor tyrosine kinase 4
349 expression and decreasing TNF receptor associated factor 6, interleukin-1 receptor-associated kinase 1,

350 NF-kB, early growth response factor 1 expression and toll-like receptor 4 activation[41], and miR-339-
351 targets fibroblast growth factor receptor substrate 2.[42]
352
353 *Future perspectives*
354 The proposed miRNA signature for identification of ENR is promising. Whether the high expression of
355 the 7-miRNA panel in ENR reflects an increased release in the circulation or a compensatory rise to the
356 lack of downstream response, remains to be elucidated before miRNA can assist in patient-tailored
357 exercise prescription or can lead toward new therapeutic targets. Therefore, these findings deserve
358 validation in a larger independent cohort .
359
360 **Limitations**
361 The findings of this retrospective study should be validated in a large prospective trial. A second
362 limitation relates to the analysis of the miRNA expression data in relation to the constructed interaction
363 networks in order to define biological themes, as the number of genes and interactions were limited to
364 only those relevant in tissues of interest. Hence, we emphasize that the presented computational analysis
365 is merely hypothesis-generating.
366
367 **Conclusion**
368 In HFrEF patients participating in exercise training, we investigated whether circulating miRNAs are
369 able to predict VO_2peak response. In this discovery cohort, we found a significantly different expression
370 pattern of baseline plasma miRNA levels between ENR and ER, which could distinguish ENR from ER
371 with 83% accuracy in an unsupervised analysis and 89% in a supervised analysis. A fingerprint of 7
372 miRNAs was strongly correlated with %changeVO_2peak and showed AUCs of $\geq$0.77 for predicting
373 ENR. Pathway analysis revealed several targets involved in exercise adaptation processes such as
374 angiogenesis, skeletal muscle adaptation and inflammation. Therefore, the proposed miRNA panel can
375 be an asset in optimizing personalized exercise prescription and could even open new therapeutic
376 avenues in heart failure. Therefore, these findings deserve prospective validation in a large cohort of
377 HFrEF patients.

378

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387

388 **CONFLICT OF INTEREST**

389 The Authors declare that there is no conflict of interest.

390

391 **AUTHOR CONTRIBUTIONS**

392 IW, AG, HH, SVL AVC and EVC contributed to the conception and design of the work. IW, AG
393 and SVL contributed to the formal analysis. IW, NP, PB, AV contributed to data acquisition. IW,
394 AG, AVC and EVC drafted the manuscript. AG, NP, PB, AV, HH, SVL, AVC and EVC critically
395 revised the manuscript. All gave final approval and agree to be accountable for all aspects of
396 work ensuring integrity and accuracy.

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508 **FIGURE LEGENDS**

509 **Figure 1. Change in exercise capacity, measured by VO_2peak**. Linear mixed model using time and
510 group as fixed effects and patient ID as random effect. ER= exercise responder, ENR = exercise non-
511 responder V1 = visit 1, V2 = visit 2.

512 **Figure 2: Unsupervised cluster analysis and heatmap**. Each row represents a miRNA and each
513 column a HFrEF patient. Two ER patients and one ENR patient are misclassified, resulting in an overall
514 classification error of 17% and an accuracy of 83%. ER = exercise responder, ENR = exercise non-
515 responder. Red = downregulated miRNAs, green = upregulated miRNAs.

516 **Figure 3**: **Volcanoplot of fold changes**. Applying predefined criteria, miRNAs of interest had to be
517 expressed in all ENR, upregulated in ENR and $p<0.005$ for the difference in fold change expression
518 between ENR and ER (red dots). Green dots = highly significantly upregulated miRNAs in ER (p
519 <0.005). Blue dots = significantly upregulated miRNAs in ENR and ER ($p <0.05$). ER = exercise
520 responder, ENR = exercise non-responder.

521 **Figure 4 Positively enriched signaling pathways.** These result from a gene set enrichment analysis,
522 grouping genes in KEGG pathways. A. KEGG pathways targeted by upregulated miRNAs in ENR, B.
523 KEGG pathways targeted by upregulated miRNAs in ER. NES is plotted for each pathway and for 3
524 different restart probabilities (respectively 0.2 = extensive smoothing, 0.5 = average smoothing and 0.8
525 =limited smoothing) of the Markov random walk diffusion. Blue dot= consolidated pathways. KEGG =
526 Kyoto Encyclopaedia of Genes and Genomes, NES = normalized enrichment score.

527 **Figure S1: Receiver Operating Characteristic (ROC) curves** for A. Let-7b, B. miR-23a, C. miR-
528 140, D. miR-146a, E. miR-191, F. miR-210 and G. miR-339-5p for the identification of ENR. AUC =
529 area under the curve.

530 **Figure S2 Gene regulatory network.** *In silico* analysis of A. miRNAs upregulated in ENR, B. miRNAs
531 upregulated in ER. Gene regulatory network obtained from smoothing expression fold changes of
532 miRNAs with a significantly altered expression over an interaction network using a Markov random
533 walk diffusion algorithm with 0.8 restart probability. This pathway analysis revealed several key genes
534 involved in exercise-adaptation processes; PIK3C2A, DNM2, RAB5C, HSPA8 are involved in
535 angiogenesis, PIK3C2A, DNM2 and HSPA8 are also related to skeletal muscle, and HSPA8 and CRK

536 are involved inflammation. PRKG1 has cardioprotective, antihypertrophic effects. EIF4B has not been
537 related to exercise-adaptation processes and could represent a new research area. Nodes are bigger when
538 they have more connections.
539

540 **APPENDICES**

541 **Figure S1.**
542 **Figure S2.**
543 **Table S1.**

544 **TABLES**

545 **Table 1. Patient characteristics**

Clinical characteristics			
	ER (n=9)	**ENR (n=9)**	**p-value**
Age (years)	59.4 (50.7 – 65.4)	62.2 (59.8 – 65.6)	0.387
Male sex	100%	100%	
BMI (kg/m^2)	30.5 ±3.9	30.3 ±5.2	0.940
Diabetes (%)	22%	33%	1.0
Hypertension (%)	56%	33%	0.637
NYHA class	II=89%, III=11%	II=44%, III=56%	0.131
Etiology of heart failure			
Ischemic cardiomyopathy	6/9 (67%)	4/9 (44%)	0.793
Dilated cardiomyopathy	2/9 (22%)	4/9 (44%)	
Toxic cardiomyopathy (ethyl)	1/9 (11%)	1/9 (11%)	
LV ejection fraction (%)	26.9 ±7.7	24.6 ±9.9	0.584
CRT or ICD	CRT 11% ICD 22%	CRT 11% ICD 56%	0.361
Pharmacological therapy			
ACE inhibitor (%)	89%	78%	1.0
ARB (%)	0%	22%	0.471
Beta blocker (%)	100%	100%	
Aldosteron antagonist (%)	56%	56%	1.0
Diuretic (%)	89%	89%	1.0
Cardiopulmonary exercise test variables			
Resting heart rate (bpm)	68 ±16.6	76 ±15.0	0.280
Baseline VO_2peak (ml/kg/min)	17.5 ±3.4	17.2 ±3.0	0.868
% Predicted VO_2peak (%)	63.7 ±11.1	64.4 ±8.6	0.882

Work economy (watt/ml/kg/min)	6.5 ±1.6	5.8 ±0.9	0.293
Peak systolic blood pressure (mmHg)	124 ±36.0	135 ±35.6	0.532
Load (Watt)	116.7 ±46.1	101.1 ±25.7	0.390
VE/VCO_2 slope	27.7 ±5.0	33.2 ±7.7	0.094
Training adherence			
Sessions completed (max. 45 sessions)	37 ±4.2	37 ±3.4	0.719

546 **Table 1.** Baseline clinical, pharmacological and cardiopulmonary exercise test, and training
547 characteristics. ER, exercise responder, ENR, exercise non-responder, BMI, body mass index, NYHA
548 class, New York Heart Association functional class, LV, left ventricular, CRT, cardiac
549 resynchronization therapy, ICD, implantable cardioverter-defibrillator, ACE, angiotensin-converting
550 enzyme, ARB, angiotensin II receptor blockers, bpm, beats per minute.

551

552 **Table 2. Array miRNA panel**

miRNA	Amplification	ER vs ENR		Spearman correlation with %changeVO_2peak		AUC to predict ENR
		Fold change	p-value	rho	p-value	
Let-7b	18/18	0.321	0.004	-0.64	0.004	0.89
miR-23a	13/18	0.234	<0.001	-0.74	<0.001	0.98
miR-140	18/18	0.443	0.001	-0.86	<0.001	0.94
miR-146a	18/18	0.456	0.003	-0.70	0.001	0.88
miR-191	18/18	0.330	0.004	-0.72	0.001	0.91
miR-210	12/18	0.165	0.004	-0.64	0.004	0.91
miR-339-5p	9/18	0.076	<0.001	-0.52	0.027	0.77

553 **Table 2:** miRNA amplification and fold changes in miRNA arrays. Serial two-sample T-tests comparing
554 miRNA fold changes in ER vs. ENR. The association of miRNAs of interest with %changeVO_2peak
555 was assessed using Spearman correlation, the association with ENR using area under the curve. Fold
556 change p-values are false-discovery rate-adjusted. ER, exercise responder, ENR, exercise non-
557 responder, AUC, area under the curve.

558

559 **Table S1. Change in key variables with exercise training**

	ER (n=9)			ENR (n=9)			p-value for interaction
	Baseline	15 weeks	p-value	Baseline	15 weeks	p-value	
BMI	30.5 ±3.9	29.9 ±3.3	1.0	30.3 ±5.2	31.6 ±7.3	0.187	0.057
VO_2peak (ml/kg/min)	17.5 ±3.4	24.9 ±5.4	**<0.001**	17.2 ±3.0	15.3 ±2.0	**0.014**	**<0.001**
Peak load (Watt)	116.7 ±46.1	147.8 ±33.1	<0.001	101.1 ±25.7	108.9 ±19.6	0.625	0.068
VE/VCO_2 slope	27.7 ±5.0	26.1 ±3.7	0.123	33.2 ±7.7	35.1 ±8.4	0.078	**0.010**
LVEF (%)	26.9 ±7.7	32.6 ±11.0	0.154	24.6 ±9.9	23.1 ±9.9	1.0	0.099
Quadriceps (kg)	43.0 ±12.0 (n=5)	55.0 ±12.9 (n=4)	0.016	50.9 ±14.8 (n=8)	57.8 ±9.8 (n=8)	0.187	0.238
NYHA class	II= 89% III=11%	I=67% II=33%	<0.001	II=44% III=56%	II=89% III=11%	0.012	0.165

560 **Table S1**: Change in key variables with exercise training. Linear mixed models with time and group as
561 fixed effect, patient ID as random effect. ER, exercise responder, ENR, exercise non-responder, BMI,
562 body mass index, LVEF, left ventricular ejection fraction, NYHA class, New York Heart Association
563 functional class.