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Exercise responsive micro ribonucleic acids identify patients with coronary artery disease

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Abstract

Aims: Exercise is a trigger for acute coronary events especially in the untrained. Identifying subjects at risk remains a challenge. We set out to assess whether a distinct pattern of micro ribonucleic acids (miRNAs) expressed in response to an acute bout of all-out exercise might exist that would allow discrimination between health and disease.

Methods: Twenty healthy subjects and 20 patients with coronary artery disease (CAD) performed an all-out cycle ergometry. Total RNA was extracted from blood drawn before and after exercise. Each blood sample was analysed for 187 target miRNAs by quantitative reverse transcription polymerase chain reaction.

Results: At baseline, 18 miRNAs allowed discrimination between healthy subjects and CAD patients. In response to an acute all-out exercise in healthy subjects 51 miRNAs and in CAD patients 60 miRNAs were significantly modulated (all $p < 0.05$). Using logistic regression analysis, a unique pattern of pre-exercise miR-150-5p, post-exercise miR-101-3p, miR-141-3p and miR-200b-3p together with maximal oxygen uptake and maximal power corrected for bodyweight allowed discrimination between healthy subjects and CAD patients with an accuracy of 92.5%.

Conclusion: In this most comprehensive analysis of exercise effects on circulating miRNAs to date we demonstrate for the first time that a distinct combination of miRNAs together with variables of exercise capacity allow robust discrimination between healthy subjects and CAD patients. We postulate that miRNAs may eventually serve as biomarkers to identify patients with CAD and possibly even those at risk of exercise-induced cardiac events.

Keywords

Biomarker, risk prediction, circulating microRNA, cardiovascular disease, spiroergometry

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Introduction

Coronary artery disease (CAD) is the leading cause of death worldwide.[1] Whereas regular exercise training improves, among others, endothelial function,[2,3] clock-associated genes,[4] morbidity and mortality,[5,6] bouts of intensive exercise are known triggers for acute cardiac events especially in the untrained.[7] In clinical practice, it is often difficult to identify those at increased risk for CAD. Small non-coding micro ribonucleic acids (miRNAs) that regulate gene expression by translational inhibition might serve as candidate biomarkers, as they are easy to obtain, remarkably stable and known to be specific for certain physiological and pathophysiological conditions.[8] A large number of miRNAs has already been identified to be associated with endothelial function/dysfunction,[9] atherosclerosis[10] or myocardial infarction.[11] In addition, levels of expression have been shown to differ

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between various states of diseases.[12] Effects of exercise training on miRNA expression, however, have been studied only in healthy young men, and it remains unknown how miRNAs respond to acute exercise bouts in healthy elderly as well as in CAD patients.

Therefore, it was the aim of the present study to assess whether differences in miRNA expression exist between healthy subjects and CAD patients at rest as well as in response to an acute bout of all-out exercise.

Methods

Study participants

The effects of an acute all-out exercise bout were studied in 10 male and 10 female healthy subjects as well as 20 age- and sex-matched patients with CAD (Ethics accession no. 415-EP/73/342-2014 and 415-E/1734/10-2015, ClinicalTrials.gov: NTC02082106 and NCT02303379). The characteristics of the participants are shown in Table 1. Our study complied with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to beginning the study. All participants performed medical examination, routine blood testing and maximal ergospirometry.

Ergospirometry

Maximal all-out exercise tests were performed on cycle ergometers (Ergoline, Bitz, Germany). Depending on gender, self-reported fitness level and training status, starting loads were chosen and every minute increments were increased by 10–50 W so that maximal exhaustion was reached after 12–15 min. For measurements of gas exchange and ventilation during the test, a breath-by-breath spirometer (Carl Reiner, Wien, Austria) was used.

Sample collection and preparation

Blood samples were drawn in a fasting state before and directly after finishing the all-out ergospirometry. Here, 5 ml of EDTA blood was collected in vacuum tubes and put immediately on ice. Subsequently, within 1 h after blood collection, collection tubes were centrifuged for 30 min with 2580 *g* at 4°C. Blood plasma aliquots were immediately shock frozen in liquid nitrogen prior and stored at −80°C until analysis.

Selection of miRNA panel

Based on a prior conducted whole genome screen (data not shown) as well as a literature search 187 miRNAs were selected for analysis. A list of those miRNAs is shown in the Supplementary Material Table 1 online.

RNA extraction

Stored plasma samples were thawed on ice and centrifuged at 4°C for 10 min (2000 *g*). Total RNA, including

Table 1. Subject characteristics and performance parameters.

	Healthy $n = 20$	CAD $n = 20$	*p*-value
Subject characteristics			
Gender [male/female]	10/10	10/10	
Age [years]	53.8 ± 12.1	58.0 ± 7.7	0.199
Height [cm]	171.4 ± 11.5	170.1 ± 11.3	0.704
Weight [kg]	71.4 (56.8–106.0)	84.7 (55.5–129.6)	0.265†
BMI [kg/m^2]	26.4 ± 4.3	29.4 ± 5.1	0.056
Systolic blood pressure [mmHg]	121 ± 11	122 ± 16	0.910
Diastolic blood pressure [mmHg]	80 ± 9	79 ± 11	0.877
Performance parameter			
VO_{2max} [l/min per kg]	31.7 (20.7–54.5)	23.6 (11.0–43.4)	0.008*†
$P_{max\text{-}rel}$ [W/kg]	2.5 (1.5–4.4)	1.6 (0.8–3.8)	0.023*†
$P_{max\text{-}abs}$ [W]	188 (100–374)	146 (61–325)	0.108†
HR_{max} [beats/min]	169 ± 18	149 ± 21	0.002*
Max lactate [mmol/ml]	8.5 ± 2.4	8.1 ± 2.7	0.606

Values are mean ± standard deviation or if not normal distributed median and range; *p*-values are calculated with Student's *t*-test or normal if not distributed Mann–Whitney *U* test (marked with †).

*$p < 0.05$ = significance level healthy *vs.* CAD.

CAD: coronary artery disease; BMI: body mass index; VO_{2max}: maximum volume of oxygen; $P_{max\text{-}rel}$: maximum power relative to bodyweight; $P_{max\text{-}abs}$: absolute values of maximum power; HR_{max}: maximum heart rate

miRNA, was isolated from 200 µl plasma using QIAzol®Lysis Reagent (Qiagen, Maryland, USA) in combination with the column isolation kit NucleoSpin miRNA Plasma (Macherey-Nagel, Düren, Germany). In detail 200 µl plasma was mixed with 1000 µl lysis reagent. In addition, we added 1.25 µl of MS2 Phage-RNA (Roche, Basel, Switzerland; REF# 10165948001) to improve RNA yield and 1 µl of spike-in mix (UniSp2, UniSp4, UniSp5 RNA) (Exiqon, Vedbaek, Denmark) to control the isolation efficiency. All following steps were done as provided by the manufacturer in the user instructions. RNA was eluted in 30 µl of RNase free water and stored at −80°C until further analysis.

miRNA quantification by quantitative polymerase chain reaction

For quantitative polymerase chain reaction (PCR) 7µl of each RNA sample was reversely transcribed using the miRCURY LNA™ Universal RT microRNA PCR, polyadenylation and complementary-DNA (cDNA) synthesis kit. One hundred and eighty-seven selected miRNAs were analysed using a custom-made pick-and-mix panel (Exiqon, Vedbaek, Denmark) with ExiLENT SYBR Green master mix. cDNA samples were diluted 1:50 and assayed in 10 µl PCR reactions. PCR amplification was performed using a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland) and 384 well plates. Roche LC software was used for analysing the amplification curves and determination of the cycle threshold as well as for melting curve analysis. Quantitative PCR experiments were conducted at Exiqon Services, Denmark. Concentrations for each miRNA were calculated via standard curves and normalized with the global mean of the corresponding assays detected in all samples (relative miRNA expression).[13] The expression level at rest is shown in all figures as logarithm of the relative miRNA expression for better display. For the evaluation of exercise effects, relative expression values after exercise were compared with baseline levels. Regulations are calculated as percent change versus resting levels.

Quality control miRNA expression

Each sample was tested for extraction efficiency, cDNA synthesis performance and a general sample quality control was performed using five miRNAs and three synthetic spike-in miRNAs as recommended by Exiqon. Each test showed comparable results for all samples and no signs of inhibition were seen (data not shown). Further, quality control measurements have been carried out as described in Mayr et al.[14] In brief, optical density of plasma samples was evaluated as well as miRNAs for the assessment of haemolysis within the samples (data not shown).

Cluster building

For easier understanding of exercise-induced changes, we built miRNA clusters according to their known functions: pro-angiogenic, anti-angiogenic, pro-atherogenic, anti-atherogenic, hypoxic, pro-inflammatory, anti-inflammatory and lipid metabolism as well as one cluster for cardiomyocytes/vascular smooth muscle cells (VSMCs)/myocardial infarction and one for hypertension/obesity/exercise.

Statistical analysis

All data was tested for normal distribution using the Shapiro–Wilk test. In general, data is presented as mean ± standard deviation. According to data distribution, paired samples were compared using paired *t*-test or Wilcoxon-signed-rank-test, while group comparisons were analysed using Student's *t*-test or Mann–Whitney *U* test as well as multi-variance analyses. Correlation analyses were performed using Spearman's rho or Pearson's correlation according to normal distribution. In order to detect whether a putative miRNA pattern, meaning a couple of responsive miRNAs, might be used for discrimination between healthy subjects and CAD patients, a logistic regression analysis was used. This method calculated the likelihood ratio (LR) as well as sensitivity and specificity of parameters, and was used for the evaluation of the clinical impact of such a miRNA pattern. Areas under the receiver operating characteristic curves (AUCs) were calculated for the evaluation of the accuracy of correct classifications. A $p < 0.05$ was considered statistically significant. All statistical tests were conducted using IBM SPSS Statistics Version 21.0.0.1 software.

Results

Demographic and clinical variables

Table 1 shows performance-related data and clinical characteristics of the participants. Aerobic exercise capacity (VO_{2max}), maximal power corrected for bodyweight ($P_{max\text{-}rel}$) and maximal heart rate were significantly higher in healthy subjects than in CAD patients.

Baseline levels of miRNAs

Using quantitative real-time PCR, the comparison of expression levels at rest revealed 18 miRNAs with

significant differences between healthy subjects and patients with CAD (Figure 1). Significant differences in expression levels in healthy subjects as compared with CAD patients were found for individual miRNAs of the pro-angiogenic, pro-inflammatory, anti-inflammatory, hypertension/obesity/exercise, cardiomyocyte/VSMC/myocardial infarction or lipid metabolism clusters. Correlation analyses of all analysed miRNAs revealed a significantly negative correlation of a moderate strength for 11 miRNAs with participant's age (R −0.323 to −0.542; p all <0.05) and nine miRNAs with a weak positive correlation with the age (R 0.316 to 0.443; p all < 0.05). Also significant correlations with HbA1c, total cholesterol, high-density lipoprotein, low-density lipoprotein and triglycerides as well as high sensitive C-reactive protein or creatine kinase were found (Supplementary Material Table 2 online).

Effects of acute exercise

Following a single all-out exercise bout, the expression level of 77 out of 187 miRNAs was significantly modulated. Table 2 shows the direction of expression change (increased or decreased expression after exercise) of the significantly altered miRNAs according to their functional cluster. A detailed list of the miRNA changes in percent compared with baseline levels is shown in Supplementary Table 3.

To show predominant differences in miRNA expression between the healthy subjects and CAD patients in relation to exercise, we used a multi-variance analysis. By this analysis we have evaluated three significant alterations in miRNA expression. These alterations in healthy subjects versus CAD patients were accounted for by anti-atherogenic miR-101-3p (−13.1%, $p = 0.035$); pro-inflammatory miR-141-3p (−76.7%, $p = 0.041$) and the hypoxia responsive miR-200b-3p (−58.8%, $p = 0.025$). Their changes in response to exercise are shown in Figure 2(a) to (c).

miRNA pattern for discrimination of healthy subjects and CAD patients

To discriminate between healthy subjects and CAD patients, we used a logistic regression analysis. Therefore, based on the results of the multi-variance analysis we generated receiver operating characteristic curves to assess whether or not patterns of miRNA expression at rest and/or in response to all-out exercise might be particular for healthy subjects or CAD patients. Using only performance variables VO_{2max} and $P_{max\text{-}rel}$ we received an AUC for the logistic regression model of 0.780 (95% confidence interval (CI): 0.741, 0.819; LR+: 2.00; LR–: 0.33) with an accuracy of 70% for correct classification, that is, correctly identifying a patient as such. The combination of baseline values of miR-150-5p and post-exercise values of miR-101-3p, miR-141-3p and miR-200b-3p had the same accuracy (70%) and an AUC of 0.840 (95% CI: 0.833, 0.847; LR+: 2.33; LR–: 0.43). To improve the accuracy, we combined both models, which improved correct classification of cases to 92.5%, AUC to 0.973 (95% CI: 0.965, 0.980; LR+: 9.50; LR–: 0.06), with a specificity of 95% and sensitivity of 90% (Figure 2(g)). All predictive variables (miR-101-3p,-141-3p,-200b-3p,-150-5p VO_{2max} and $P_{max\text{-}rel}$) were statistically significant.

Discussion

Circulating miRNAs are small, endogenous, non-coding RNA molecules that post-transcriptionally regulate eukaryotic gene expression of approximately two-thirds of all genes.[15] miRNAs are involved in the pathogenesis of various diseases, including CAD, by their paracrine-like cell to cell signalling, short range communication and gene regulation.[15] We hypothesized that expression levels would be different in healthy subjects and CAD patients and that a postulated pattern of distinct miRNAs might allow discrimination between the two. We therefore performed the most comprehensive analysis of miRNAs in current literature and found that patients with CAD indeed exhibit a miRNA expression pattern different from healthy subjects, not only at rest but also in response to exercise.

miRNA expression at rest

We found that at rest several miRNAs were differently expressed in healthy subjects as compared with CAD patients. For example, miR-150-5p, which plays a crucial role in resolving vascular injuries by restoring barrier functions by suppressing specific proteins,[16] was expressed in a higher concentration in CAD patients. This result may suggest a greater demand of vascular remodelling in CAD patients than in healthy subjects. On the other hand, 14 miRNAs had lower expression levels in patients than in healthy subjects. Five of these (let-7a-5p, -7c-5p, -7d-5p, -7f-5p, -7g-5p) are part of the let-7 family, which is known to be associated with cardiac hypertrophy, remodelling and fibrosis.[17] Interestingly, we found significant differences between groups at rest only in six of 10 functional clusters: no significant differences were seen in pro- or anti-atherogenic, anti-angiogenic or hypoxic clusters, suggesting similar demand of these miRNAs at rest independent of disease.

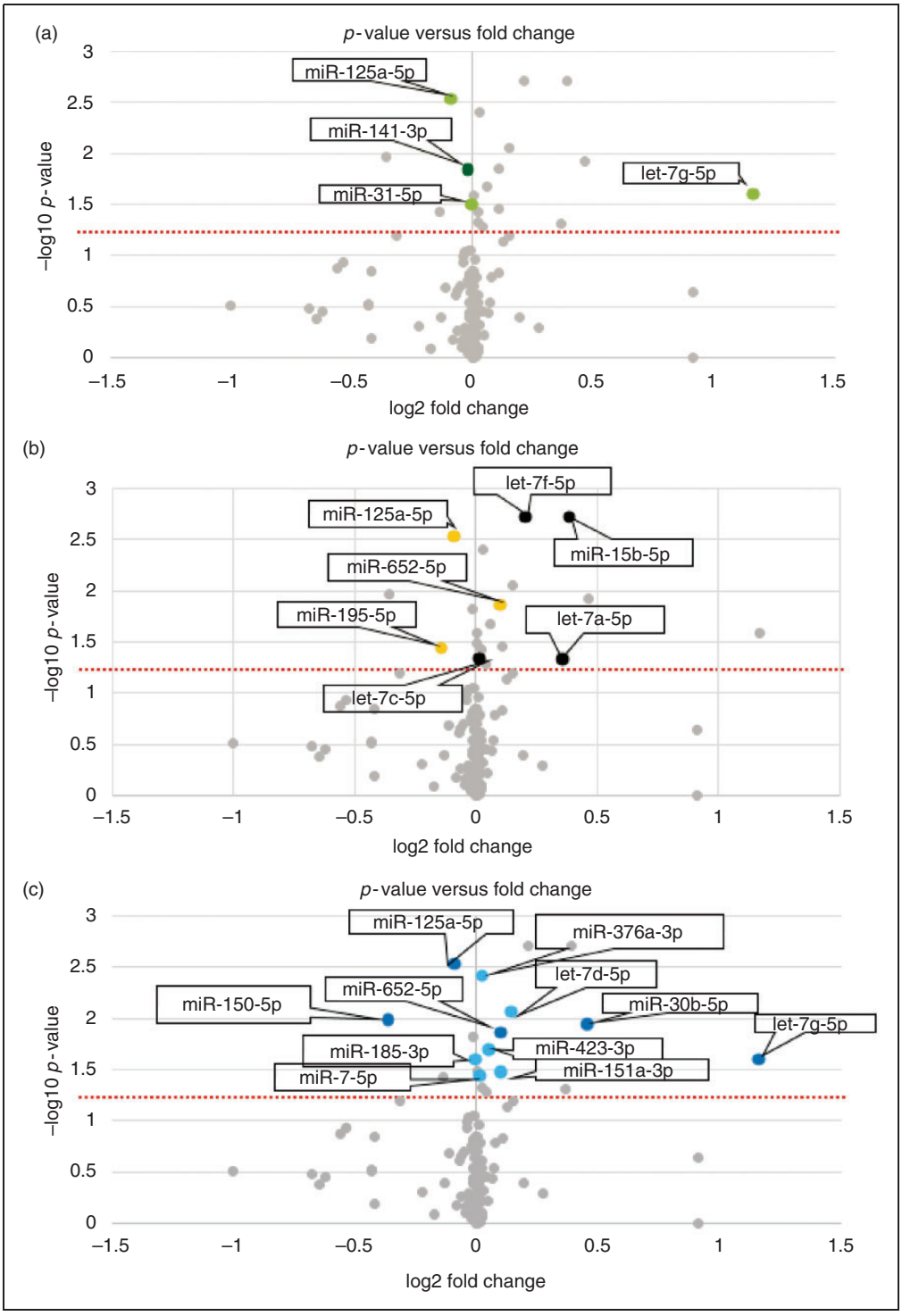
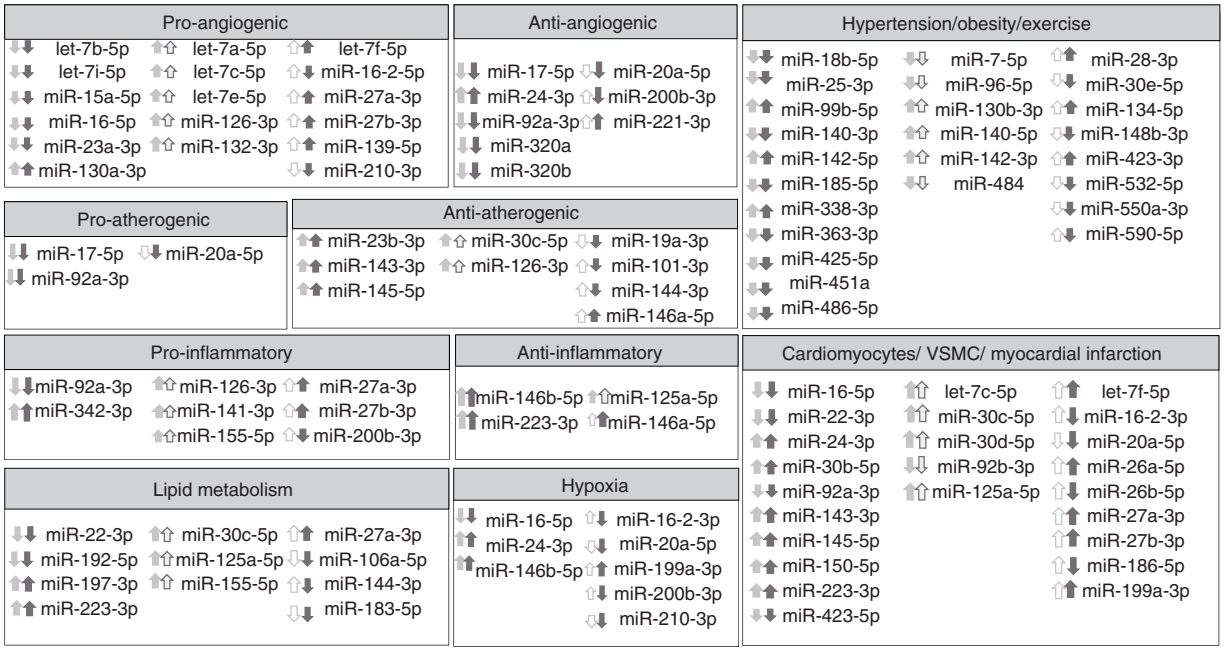


Figure 1. Volcano plots of micro ribonucleic acid (miRNA) *p*-values versus fold change between healthy subjects and coronary artery disease patients at rest.

Dotted red lines represent the level of significance ($p < 0.05$) of miRNA expression level differences at rest between both groups. Cluster miRNAs are marked as follows: (a) dark green dots, pro-inflammatory; light green dots, anti-inflammatory; (b) black dots, pro-angiogenic; yellow dots, lipid metabolism; (c) light blue dots, hypertension/obesity/exercise cluster; dark blue dots, cardiomyocytes/vascular smooth muscle cells/myocardial infarction cluster.

Table 2. Exercise-induced responses in micro ribonucleic acid grouped by physiological/pathophysiological function.



Direction of arrows, upward, expression increase; downward, expression decrease; light grey arrows, healthy subjects; dark grey arrows, CAD patients; filled arrows, $p < 0.05$; unfilled arrows, non-significant regulation.
VSMC: vascular smooth muscle cell.

miRNA expression in response to all-out exercise

Focusing on the effects of exercise, 77 of the 187 miRNAs studied changed their expression levels in response to all-out exercise. Furthermore, many of them have not been described to be responsive to exercise before. Only two previous studies analysed effects of all-out exercise-induced changes in miRNA in healthy volunteers. In keeping with our results, Baggish et al.[12] observed an increase of miR-21-5p after all-out exercise in young male elite rowers, and Uhlemann et al.[18] reported an upregulation of miR-126-3p during exercise testing in young, healthy volunteers. Since both studies reported resting expression levels higher than ours, it may be speculated that expression of these two miRNAs might be age-dependent, as participants in our study were considerably older (19.1 ± 0.6 *vs.* 30.4 ± 2.0 *vs.* 53.8 ± 12.1 years, respectively). Indeed, findings reported by Ameling et al., who identified a significant positive correlation between miR-126-3p, miR-21-5p and age,[19] support this assumption.

In this study, we also reported for the first time a direct comparison of the effects of acute exercise in CAD patients versus healthy subjects in the same experimental setting. This comparison enables us to compare directly the miRNA expression after all-out exercise in healthy subjects and CAD patients. This led to the result that some of the significantly responsive miRNAs showed considerably higher regulations in healthy subjects. Those miRNAs included the anti-atherogenic miR-145-5p and miR-143-3p [20] as well as the pro-inflammatory miR-141-3p. Additionally, we found miRNAs which were responsive in opposite directions, as was the case with the pro-angiogenic miR-16-2-5p, the hypoxia responsive miR-200b-3p and the anti-atherogenic miR-101-3p showing a slight upregulation in healthy subjects but pronounced downregulation in CAD patients. These results may indicate that several miRNAs are dysregulated in CAD patients, which promotes a pathophysiological state, generating cardiac complications, or were caused in direct relation to the cardiac event.

miRNA pattern for discrimination between healthy subjects and CAD patients

In order to assess the differences in the pattern of miRNAs between healthy subjects and CAD patients, we performed a logistic regression analysis. Indeed, we found that a miRNA pattern that included miR-150-5p measured at rest and miR-101-3p, miR-141-3p and miR-200b-3p measured in response to exercise was able to discriminate between healthy subjects and CAD patients. When VO_{2max} and $P_{max\text{-}rel}$, both relative to the bodyweight, were added, sensitivity and

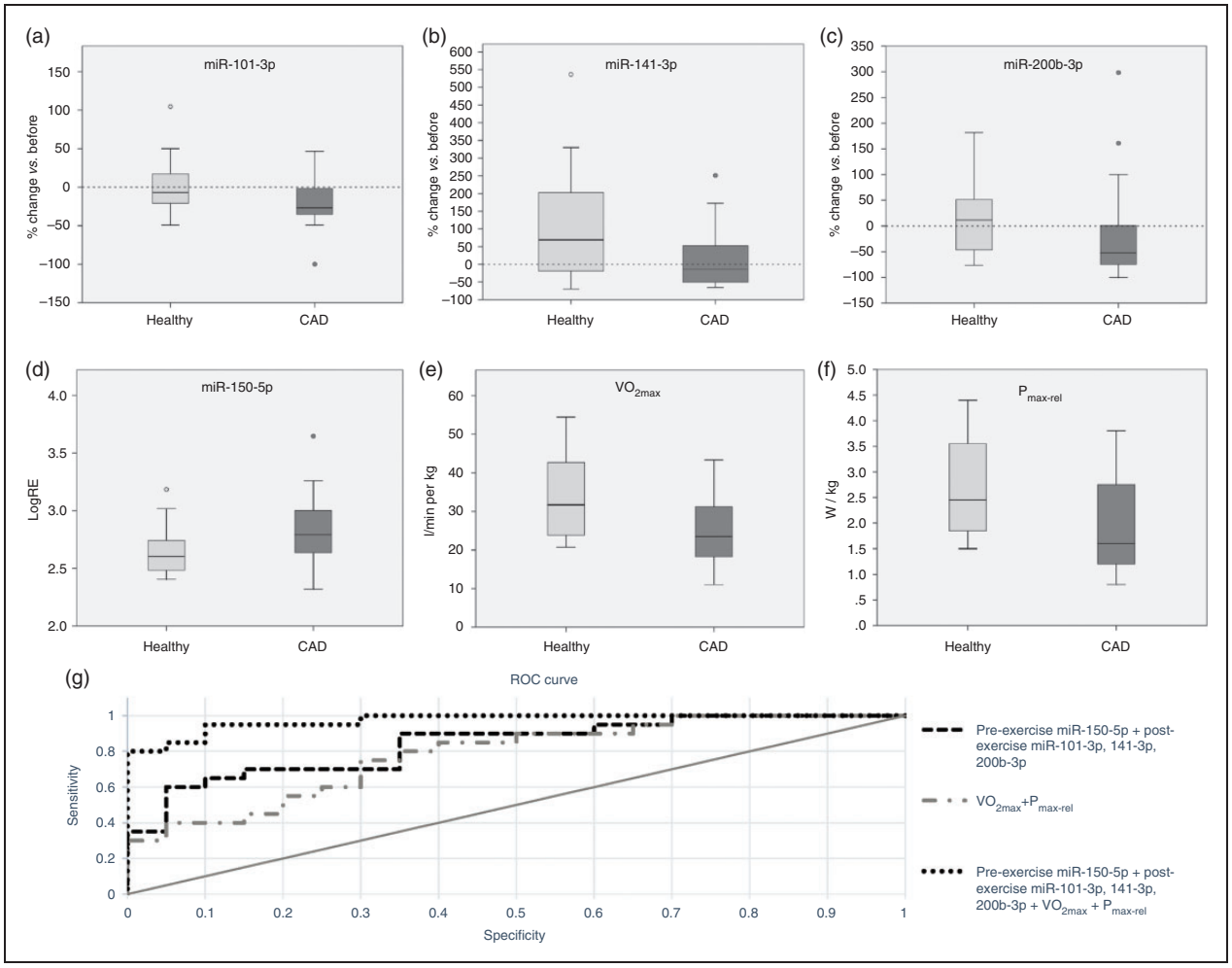


Figure 2. Micro ribonucleic acid (miRNA) patterns that allow discrimination between healthy subjects and coronary artery disease patients.
(a)–(c): values are depicted as percent change versus beginning. Below 0% (below broken line) describes a down regulation. Above 0% (above broken line) describes an up regulation versus beginning; (d) mean logarithm of the relative expression of the baseline values of miR-150-5p ($p = 0.011$). (e) maximum oxygen uptake relative to the bodyweight ($p = 0.008$). (f) maximum power relative to bodyweight ($p = 0.023$). (g) ROC-curve analysis (broken line curve) representing area under the curve for miRNAs only, chain line curve representing only the performance parameters and dotted line curve representing miRNA pattern plus performance parameters VO_{2max} and $P_{max\text{-}rel}$, which resulted in superior sensitivity and specificity.
CAD: coronary artery disease; VO_{2max}: maximum oxygen uptake; $P_{max\text{-}rel}$: maximum power relative to bodyweight; ROC: receiver operating characteristic.

specificity improved from 70% for both to 90% and 95%, respectively.

In this study, we also found that the exercise-induced expression of miRNA was less pronounced in CAD patients as compared with healthy individuals. This, however, was expected as miRNAs can interact with over 2000 target genes, which in turn exert effects on a broad spectrum of cellular processes. Not all target genes are needed during adaptation to exercise; however, several proteins are targets of miRNA expression and explain varying effects on the regulation of glucose and lipid metabolism as well as myogenesis, which are all processes involved in the response to exercise.[21]

Limitations

This is an explorative study performed in a rather small sample size. Therefore, translation of our findings must be done with caution. Further studies should be conducted not only in larger cohorts but also in different age groups and, where possible, in patients of different ethnicity.

Conclusion

In summary, we report in this most comprehensive analysis of exercise effects on miRNA expression to date

the novel finding that differences in healthy subjects as compared with CAD patients not only exist at rest, but are even more pronounced in response to exercise. Most importantly, we demonstrate for the first time that a distinct pattern of circulating miRNAs in combination with variables of exercise capacity allows discrimination between healthy subjects and CAD patients. We therefore speculate that this combination of miRNAs (miR-150-5p, miR-101-3p, miR-141-3p, miR-200b-3p) with parameters of the exercise capacity (VO_{2max}, $P_{max\text{-}rel}$) may eventually serve as biomarkers for the identification of subjects at risk for the development of CAD and/or exercise-induced cardiac events.

Author contribution

BM, EEM, MS, HBK, JN contributed to the conception or design of the work. BM, EEM, CS, SD contributed to the acquisition, analysis, or interpretation of data for the work. BM drafted the manuscript. EEM, MS, HBK, CS, SD, JN critically revised the manuscript. All gave final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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