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ORIGINAL ARTICLE

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Exercise dose affects the circulating microRNA profile in response to acute endurance exercise in male amateur runners

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The systemic response to exercise is dose-dependent and involves a complex gene expression regulation and cross-talk between tissues. This context ARISES the need for analyzing the influence of exercise dose on the profile of circulating microRNAs (c-miRNAs), as emerging posttranscriptional regulators and intercellular communicators. Thus, we hypothesized that different exercise doses will determine specific c-miRNA signatures that will highlight its potential as exercise dose biomarker. Nine active middle-aged males completed a 10-km race (10K), a half-marathon (HM), and a marathon (M). Blood samples were collected immediately before and after races. Plasma RNA was extracted, and a global screening of 752 microRNAs was analyzed using RT-qPCR. Three different c-miRNA profiles were defined according to the three doses. In 10K, 14 c-miRNAs were found to be differentially expressed between pre- and post-exercise, 13 upregulated and 1 downregulated. Regarding

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HM, 13 c-miRNAs were found to be differentially modulated, in all the cases up-regulated. A total of 28 c-miRNAs were found to be differentially expressed in M, 21 overexpressed and 7 repressed after this race. We had also found 3 common c-miRNAs between 10K and M and 2 common c-miRNAs between 10K and HM. In silico analysis supported a close association between exercise dose c-miRNA profiles and cellular pathways linked to energy metabolism and cell cycle. In conclusion, we have observed that different exercise doses induced specific c-miRNA profiles. So, our results point to c-miRNAs as emerging exercise dose biomarkers and as one of regulatory mechanisms modulating the response to endurance exercise.

KEYWORDS

circulating microRNAs, endurance exercise, exercise biomarkers, exercise dose

1 | INTRODUCTION

The systemic response to acute exercise and training has profound effects on gene expression and involves a complex cross-talk between tissues.[1,2] Although these adaptations are dose-dependent and exert systemic beneficial effects for health,[3] adverse consequences of acute prolonged exercise and chronic excessive endurance exercise have also been reported, suggesting that a safe upper-dose limit potentially exists.[4] However, the exact mechanisms by which gene expression is regulated to orchestrate this response remain partially unknown.[5]

During the last decade, several authors have suggested a prominent role of the non-coding transcriptome in the regulation of the physiological responses to exercise.[6] In this context, there emerges the need to assess the role of circulating microRNAs (c-miRNAs), as new players for intercellular communication and gene expression regulation,[7] as well as potential biomarkers of exercise dose.[8]

MicroRNAs (miRNAs) are short (≈22 nucleotides) non-coding RNA molecules that regulate posttranscriptional gene expression by promoting mRNA degradation or by repressing protein translation,[9] and they are predicted to target >60% of protein-coding genes.[10] Although miRNAs are intracellular regulators of gene expression, they have been detected in different body fluids in a stable form, including bloodstream,[11] mainly transported in extracellular vesicles,[12] associated to proteins[13] or lipoproteins.[14] It has been proposed that c-miRNAs can be secreted in a regulated manner as a response to stress, constituting a true intercellular communication system, or be passively released from injured, necrotic, or apoptotic cells.[15]

To date, an increasing number of studies have been published about the effect of acute exercise on the c-miRNA profile (reviewed in[8]). However, the response to different doses of acute exercise has been barely explored.[16-18] Surprisingly, despite the systemic nature of the response to exercise, none of them have addressed a global c-miRNA screening in this situation. The analysis of limited miRNA panels provides an incomplete perspective of their holistic regulatory role.[8] Therefore, analyzing the influence of exercise dose on the global response of c-miRNAs will provide a better understanding of their potential regulatory role on the systemic effect of acute exercise, as well as analyzing their value as biomarkers of exercise dose, which may also help in exploring the maximal limit for a safe and healthy exercise.

Thus, we hypothesized that different exercise doses will determine specific c-miRNA signatures that will highlight its potential as exercise dose biomarker.

To test this hypothesis, we performed a global screening of plasma c-miRNAs in response to different doses of acute endurance exercise in a group of active middle-aged males.

2 | SUBJECTS AND METHODS

2.1 | Ethics statement

All experimental procedures were approved by the Research Ethics Committee of the Principality of Asturias, Spain (reference 124/17). All participants gave written informed consent.

2.2 | Experimental design

In an observational repeated measures design, all subjects completed a 10 km race (10K), a half-marathon (HM), and a marathon (M), separated by 1 month.

Although all races involved the same type of exercise (endurance running), they differed in terms of duration, intensity, and energy demands, and thus represent distinct exercise doses.[18-20]

2.3 | Subjects

Volunteers were recruited among the members of MAPOMA Sports Association, which includes professional

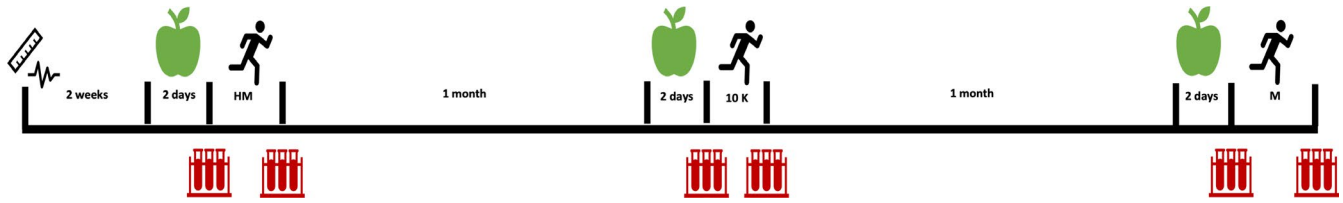

FIGURE 1 Study timeline

and amateur runners. A team of sports and health professionals design the training plans, promoting proper care and preparation.

All runners at the amateur training group (n = 35) were invited to an informative briefing. A member of the research team presented the aims and methodology of the study, and answered the questions of the potential participants. Finally, 18 (51%) agreed to participate. The rest showed interest in the study, but various reasons (family and professional commitments, travel, and injuries) did not allow them to participate. Prior to participation, each volunteer underwent a thorough medical screening to determine eligibility. A number of inclusion and exclusion criteria were also established. Inclusion criteria were as follows: (a) men over 18 years of age; only men were selected as subjects due to the stability of their hormonal status, (b) regular trained, at least 50 km/wk, (c) have previously participated in at least two marathons, (d) be officially registered for the Madrid Marathon, (e) signing written informed consent. Exclusion criteria were as follows: (a) smokers and frequent passive smokers, (b) suffering from any chronic disease, (c) body mass index (BMI) over 30 kg/m^2, (d) be under dietary or pharmacological treatment during the time of the study. Although 14 volunteers met these criteria and participated, finally 9 subjects completed the whole study. The remaining 5 subjects were unable to finish at least one of the races, and their samples were excluded from the final analyses.

2.4 | Aerobic capacity and body composition assessment

VO_2max was determined by indirect calorimetry (Oxycon Pro, Jaeger) using an incremental protocol till exhaustion on a treadmill (LE- 600 C, Jaeger -HP Cosmos). Maximal heart rate (HRmax) and Maximal Aerobic Speed (MAS) were also recorded.

Before the test, the participants had their body composition assessed. The same, ISAK Level III certified anthropometrist measured height and body mass using a combined medical scale (model 778, Seca Ltd; precision 0.1 cm for height and 0.1 kg for weight). Body mass index (BMI) was then calculated from these measurements. The equation of Kyle et al[21] was used to estimate percent body fat (%BF) based on the information obtained using a multifrequency bioimpedance system device (Total Body Scan, Bio-Logic®). This equation was considered the most appropriate according to the position stand of the Spanish Group of Kinanthropometry.[22]

2.5 | Training schedule, athletic background, and in-race measurements

Volunteers were interviewed in order to determine their training history (years of training, number of 10K, HM and M races previously finished, and personal bests) and the volume of training in, at least, the last 3 months (days per week, hours per day, and km per week). Subjects were asked not to alter their usual training schedule, and all of them performed low intensity training the day before each race.

Mean speed and mean heart rate during each race were measured. From these data, the ratios Mean HR/HR max and Mean Speed/MAS were calculated.

2.6 | Dietary control

Participants were asked to keep a food diary 2 days before the first exercise bout and also on the test day (Figure 1). All foods and beverages were recorded using standard culinary measures[23]; for information about packed-foods and snacks, food labels were collected. Volunteers received specific oral guidelines and detailed written instructions about how to put this method into practice. A telephone number was available for them to answer any queries about the recording of their diets. In order to minimize the impact of food intake on the results, volunteers were asked to repeat the same intake pattern on the following races. They were also asked to keep a food diary, which were compared in terms of energy and macronutrients in order to certify that the same food and nutrient intake pattern was followed. Subjects were clearly asked not to alter their usual dietary pattern during the recording periods. No limitations for the type or the amount of food or beverages consumed were established at any time during the food-recording period. None of the volunteers reported the use of nutritional supplements. Food records were carefully reviewed immediately after completion, and subjects were contacted to clarify ambiguous information. Dietary records were analyzed using a software program for nutrient intake analysis (DIAL®, Alce Ingeniería).

2.7 | Blood sampling

Two blood samples were drawn, before and after the end of each exercise bout (Figure 1). In all cases, experienced technical staff, using standardized techniques and materials, obtained the samples. Subjects had their first blood sample taken about 1 hour and a half before the race, in fasting state, and before starting warming-up (Pre). Subjects then consumed their breakfast and performed warm-up exercises, after which point the race started. Another blood sample was drawn within 15 minutes after the cessation of exercise (Post). Blood draws and the immediate processing of the samples were carried out in a field laboratory installed near the starting and finishing lines, with the permission and cooperation of the organization staff.

The total volume of blood taken per exercise bout was <20 mL per individual. Blood samples were collected in vacutainers (No Additive (Z), Becton Dickinson), stored at room temperature, at least 15 minutes to allow clot formation, and immediately centrifuged at 1600 *g* for 15 minutes at 10°C. Serum samples were then aliquoted, immediately preserved in dry ice, and finally stored at −80°C for later analysis.

2.8 | Estimations of exercise intensity

All participants used heart rate (HR) monitors during the races. The ratio (%) between the mean HR during each race and the maximal HR (HRmax) during the incremental protocol on a treadmill was used to calculate the percent of HRmax (%HRmax), in order to determine the individual exercise intensity during all exercise bouts.[24]

Besides taking into account the VO_2max test and the mean speed of each race, the exercise intensity was also estimated as the percentage of the Maximal Aerobic Speed (MAS) for each race.

Time spent in completing each race was also recorded and compared with their personal best race time to calculate the percentage of personal best (%PB) as follows: race time 100/ personal best.

2.9 | RNA isolation and qRT-PCR

Total circulating RNA from 200 μL of serum was isolated using the miRCURY RNA isolation kit (Exiqon) following the manufacturer's instructions. For ulterior normalization, synthetic *Caenorhabditis elegans* miR-39-3p (cel-miR-39-3p), lacking sequence homology to human miRNAs, was added as an external reference. The mixture was supplemented with 1 μg of MS2 carrier RNA (Roche) to improve extracellular miRNA yield. The RNA Spike-in kit with synthetic RNA spike-in templates (UniSp2, UniSp4, UniSp5) (Exiqon) was also used in all extractions to monitor RNA isolation efficiency. RNA was eluted in 30 μL RNase-free H_2O and stored in a −80°C freezer.

For miRNA quantification, cDNA was synthesized using the universal cDNA synthesis kit II (Exiqon). The miRCURY LNA Universal RT microRNA PCR System offers a high sensitivity, specificity, and reproducibility.[25] Briefly, 10 μL RNA samples were reverse transcribed in 50 μL reactions. Additional spike-in (UniSp6) (Exiqon) was added to the cDNA synthesis reaction to check for RT and PCR inhibitors. RT reaction was performed with the following conditions: incubation for 60 minutes at 42°C, heat-inactivation for 5 minutes at 95°C, immediately cool to 4°C. Then, cDNA was stored at −80°C. For qPCR, cDNA was diluted 80x and 4 μL used in 10 μL qPCR reactions with ExiLENT SYBR Green master mix (Exiqon) on a 7900HT fast Real-Time PCR System (Applied Biosystems) with the following cycling conditions: 10 minutes at 95°C, 40 cycles of 10 seconds at 95°C and 1 minutes at 60°C, followed by a melting curve analysis. To discard the presence of nucleases, inhibitors, or hemolysis, the miRCURY miRNA Quality Control PCR Panel (Exiqon) was used before miRNA analysis. For whole genome screening, miRNAs were quantified using the human miRNome panels (752 human mature miRNAs) Version 3 (Exiqon). miRNA relative expression analysis was performed using the GenEx software (MultiD Analyses AB).

To ensure good quality data, synthetic spike-in RNA templates were analyzed to monitor the uniformity of the RNA extraction procedure and efficiency of RT and PCR reactions. The synthetic spike-in UniSp3 was also analyzed as an interplate calibrator. The SDS v2.3 software was used for both the determination of the quantification cycle (Cq) and for the melting curve analysis. The Cq was defined as the fractional cycle number at which the fluorescence exceeded a given threshold. The specificity of the PCR reaction was corroborated by melting curve analysis. The dCq (miR-23a-3p – miR-451a) method was used to confirm that none of the samples were affected by hemolysis (all samples had dCq value below 6). miRNAs were considered to be expressed when Cq values < 37 or were detected with at least 5 Cq below the negative control, as recommended by the manufacturer. For normalization purposes, the dCq method was used, where dCq = Cq[miRNA] − Cq[cel-miR-39]. Relative quantification to basal samples was performed using the 2^{-ddCq} method, where ddCq = dCq[miRNA] − dCq[mean miRNA prerace].[26]

2.10 | Pathway analysis and prediction

Pathway analysis of target genes of modulated c-miRNAs was performed to determine their possible implication in the biological response to exercise.

The R Bioconductor package mdgsa (version 1.8.0) was used for pathway analysis of differentially expressed miRNAs

in an integrative manner. This method allows for an accurate modeling of multiple miRNA's regulatory processes at a time, accounting for additive/cancelation effects of several miRNAs targeting the same gene based on the direction of their expression profile. For each miRNA, experimentally validated targets were retrieved from miRTarBase and miRWalk databases. Pathway annotations for each gene were retrieved from KEGG pathways. Thus, we obtained gene sets from both annotation databases, linked to the miRNAs targeting those genes. The results output a log odds ratio for each interrogated gene set, along with raw and false discovery adjusted *P*-values.

2.11 | Statistical analysis

Normality of variables was tested using Shapiro-Wilk's test. In light of the results obtained, descriptive values for anthropometric, dietetic, and performance variables are presented as means and standard deviations, and parametric methods were used for analytical statistics. General additive linear models, in which subject was considered as a random-effects covariate, were used for studying the effect of race length (10K, HM, M) on dietetic and performance variables. A multiple paired samples *t* test was performed and the *P*-values adjusted by the false discovery rate (FDR) criterion in order to compare pre- and post-exercise miRNA expression levels. Differences between pre- and post-samples were considered relevant if satisfied one of the following criteria: (a) *P*-value below (0.05) (computed by using the paired Student's *t*-test) and (b) difference between means larger than 1.5. A one-way repeated measures ANOVA was used to assess differences in c-miRNA expression between races. A customized R (www.r-project.org) function was used for all process.

3 | RESULTS

3.1 | Physical characteristics, training habits, and dietary control

The subject characteristics and training habits were previously described elsewhere.[16] No differences were observed in the daily energy and macronutrient intake of volunteers during five consecutive days before, during, and after the exercise bouts (Table 1).

3.2 | Performance parameters

As expected, performance parameters defined three doses of maximal endurance exercise determined by significant differences in relative intensity for the different volumes (Table 2).

TABLE 1 Nutritional intake of volunteers (n = 9) before and after the race days

	10K	Half-marathon	Marathon
Energy (kcal)	2533 ± 525	2620 ± 528	2581 ± 530
Carbohydrates			
g	321 ± 75	332 ± 74	335 ± 81
%energy	51 ± 4	51 ± 4	52 ± 5
Proteins			
g	90 ± 19	93 ± 21	90 ± 20
%energy	14 ± 1	14 ± 1	14 ± 1
Lipids			
g	98 ± 22	102 ± 23	98 ± 31
%energy	35 ± 4	35 ± 5	35 ± 5

Note: Data are presented as mean ± standard deviation of 3 d food records. %energy: Percentage of the total daily energy intake provided by the different macronutrients.

TABLE 2 Performance parameters of participants in a 10K race, a half-marathon and a marathon

	10 km	Half-marathon	Marathon
Race time (h:min)	0:42 ± 0:03	1:37 ± 0:10	3:54 ± 0:36
Personal best (h:min)	0:42 ± 0:04	1:35 ± 0:08	3:35 ± 0:32
Mean HR (bpm)	169 ± 3[a]	162 ± 4	157 ± 7[b]
Mean HR/HR max (%)	92.3 ± 2.1[a]	90.2 ± 0.3	86.7 ± 2.8
Mean speed (km/h)	14.3 ± 1.0[a]	13.1 ± 1.3	10.9 ± 1.6[b]
Mean speed/MAS (%)	83.9 ± 4.1[a]	76.1 ± 4.6	64.1 ± 5.9[b]

Note: Data are presented as mean ± standard deviation.

Abbreviations: bpm, beats per minute; HR max, Maximal Heart Rate; HR, Heart rate; MAS, Maximum Aerobic Speed.

[a]Significantly different ($P < 0.05$) from half-marathon and marathon races;

[b]Significantly different ($P < 0.05$) from 10-km and half-marathon.

Participants performed close to their personal best in 10K (%PB: 100.2 ± 2.5%) and HM (%PB: 102.7 ± 3.0%), while performed worse in M (%PB: 109.1 ± 8.8%), maybe due to adverse weather conditions, although the difference to their personal bests was not significant.

3.3 | Circulating miRNAs

To explore the effect of acute endurance exercise on the non-coding miRNA transcriptome, the expression levels of 752

human circulating miRNAs, under three races representing three differing doses of maximal endurance exercise, were screened by qRT-PCR (Table 3). A total of 378 individual miRNAs were detected in at least one of the races. If we differentiate by race, we find that 321 miRNAs were detected in 10K race samples (Table S1). From these, 14 c-miRNAs were found to be differentially expressed between pre- and post-exercise, 13 upregulated and 1 downregulated. Regarding HM, 266 c-miRNAs were detected (Table S1), from which 13 c-miRNAs were found to be differentially modulated, in all the cases upregulated. Finally, 339 c-miRNAs were detected in M (Table S1), from which a total of 28 c-miRNAs were found to be differentially expressed, 21 overexpressed and 7 repressed after race. A total of 239 microRNAs were detected in all the races (Table S1), which represents 74% of those detected in the basal samples of 10K, 90% in HM, and 70% in M.

The expression of 168 of the common baseline c-microRNAs detected was significantly different between races (Table S1).

We have used a Venn diagram to represent the profile of c-miRNAs differentially expressed between pre- and post-exercise samples in each race, as well as the overlapping between races (Figure 2). No common c-miRNAs were found for all races. However, 2 common miRNAs were observed between 10K and HM: miR-33a-5p and miR-505-3p, both upregulated. Comparing 10K and M races, 3 common miRNAs were observed: miR-1260a, miR-345-5p, and miR-424-3p, all of them were upregulated in both races. Figure 3 shows the acute response (pre-post)

TABLE 3 Significant circulating miRNAs after a 10 km race, a half-marathon, and a marathon

10K			HM			M		
c-miRNA	**FC**	***P***	**c-miRNA**	**FC**	***P***	**c-miRNA**	**FC**	***P***
miR-199b-5p	22.81	.02	miR-425-3p	22.08	.02	miR-1972	71.65	.03
miR-424-3p	9.74	.03	miR-33a-5p	16.52	.05	miR-940	13.72	.02
miR-33a-5p	8.26	.03	miR-338-3p	11.65	.05	miR-424-3p	12.75	.04
miR-551a	7.75	.04	miR-339-5p	11.10	.02	miR-130b-5p	11.06	.04
miR-1537	4.52	.02	miR-106b-3p	10.02	.00	miR-223-5p	8.67	.04
miR-223-5p	3.94	.02	miR-502-3p	8.57	.04	miR-145-3p	8.43	.02
miR-1260a	3.29	.04	miR-27a-3p	6.37	.05	miR-181c-3p	8.42	.05
let-7b-3p	3.04	.04	miR-660-5p	5.85	.05	miR-501-3p	7.40	.04
miR-150-5p	2.55	.04	miR-505-3p	5.60	.03	miR-1260a	7.40	.01
miR-423-5p	2.17	.04	miR-100-5p	5.25	.05	miR-675-3p	7.25	.04
miR-223-3p	2.13	.03	miR-22-3p	4.40	.05	miR-345-5p	5.96	.04
miR-345-5p	2.04	.02	miR-30e-5p	4.38	.05	miR-424-5p	5.38	.04
miR-505-3p	1.94	.04	miR-497-5p	2.76	.05	miR-1-3p	4.62	.03
miR-346	−4.69	.02				miR-34a-5p	3.74	.03
						miR-629-5p	3.17	.04
						miR-30a-5p	3.01	.02
						miR-148a-3p	2.68	.02
						miR-596	2.46	.00
						miR-10b-5p	2.37	.01
						miR-30d-5p	1.93	.05
						miR-320d	1.60	.04
						miR-192-5p	−1.52	.04
						miR-20b-5p	−1.72	.04
						miR-103a-3p	−1.74	.04
						miR-106b-5p	−1.89	.01
						miR-144-3p	−2.69	.01
						miR-665	−3.26	.04
						miR-486-3p	−3.85	.04

Abbreviations: 10K, 10 km race; FC, Fold change; HM, Half- marathon;M, Marathon; *P*-V, *P*-Value.

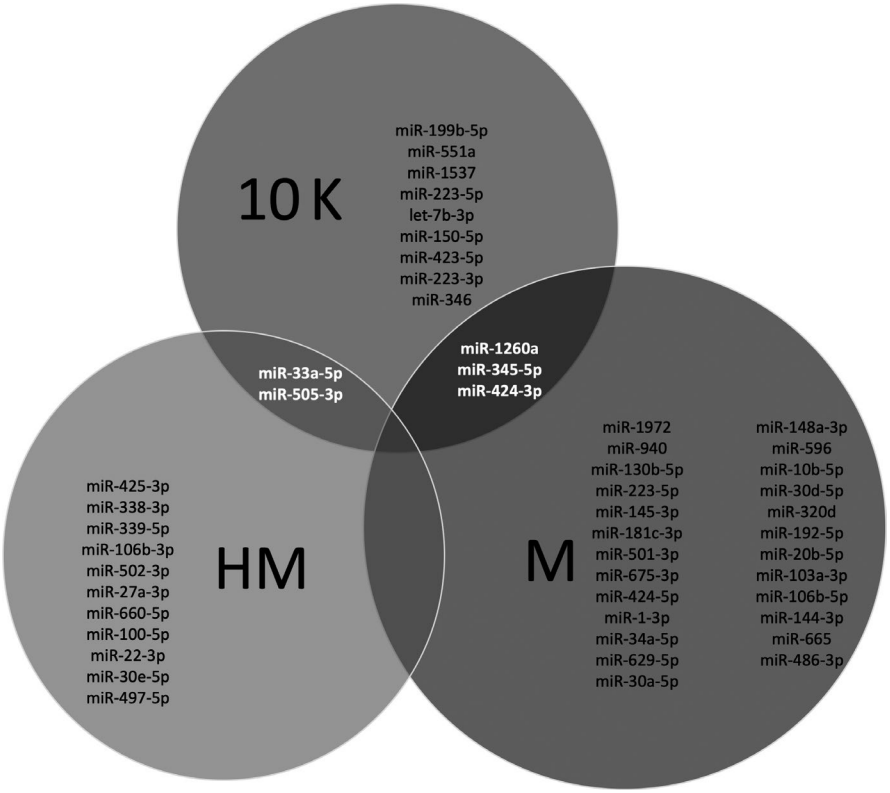

FIGURE 2 Venn diagram analysis of modulated (overexpressed and repressed) circulating miRNAs in response to different doses of acute exercise. In black bold font, repressed miRNAs. In white bold font, common miRNAs, all of them overexpressed

of significantly changed common circulating miRNAs, as well as the differences between exercise doses. No common c-miRNAs were found to change in response to HM and M. Furthermore, the magnitude of change for miR-33a-5p, miR-505-3p, miR-1260a, and miR-345-5p was significantly different between races, being highest for M in most cases, except for miR-33a-5p.

3.4 | Target pathways analysis

Pathway analyses of target genes of modulated c-miRNAs were performed to determine their possible implication in the biological response to exercise. Using experimentally validated miRNA-target interaction databases (TarBase v7),[27] we performed pathway analysis using KEGG database.

The specific miRNA profiles detected for each exercise dose have a specific validated target signature (Table S2). These profiles have 44, 65, and 57 significant validated targets for upregulated miRNAs and 3, 0, and 64 significant validated targets for downregulated miRNAs, considering 10K, HM, and M, respectively. The large number of validated targets supports the hypothesis that c-miRNA has potentially enormous influence on gene responses to exercise. Pathways related to energy metabolism and cell cycle control are the most represented. Interestingly, AMPK and mTOR pathways are targeted by most of the up and downregulated miRNAs in the acute response to HM and M.

Considering only the common miRNAs between races, which are shown in Table S3, all of them target lysine degradation pathway and cell cycle or p53. All these common miRNAs were upregulated.

4 | DISCUSSION

We have found that, according to the aim proposed, different exercise doses define specific c-miRNA profiles. Three doses of exercise in-field conditions were assayed in the same subjects. All the races were maximal endurance efforts at different volumes, with an inverse relationship with intensity. This approach, together with the global c-miRNA screening performed (>750 miRNAs analyzed), provided a new perspective on this field, enhancing the value of c-miRNAs as biomarkers of endurance exercise dose.

Other authors have previously analyzed the acute effect of different doses of endurance exercise on the c-miRNA profile. de Gonzalo-Calvo et al[17] described a relationship between two doses of endurance exercise (10 km and Marathon) and the c-miRNA response, although using a selection of 106 specific inflammatory miRNAs instead of a global screen. Furthermore, Ramos et al[18] defined, under laboratory conditions, the isolated effect of intensity or duration of treadmill running on the c-miRNA profile. A very restricted selection of 7 miRNAs was analyzed in two separate groups of volunteers: variable intensity and variable duration cohorts. However, most of the intensities tested in the variable

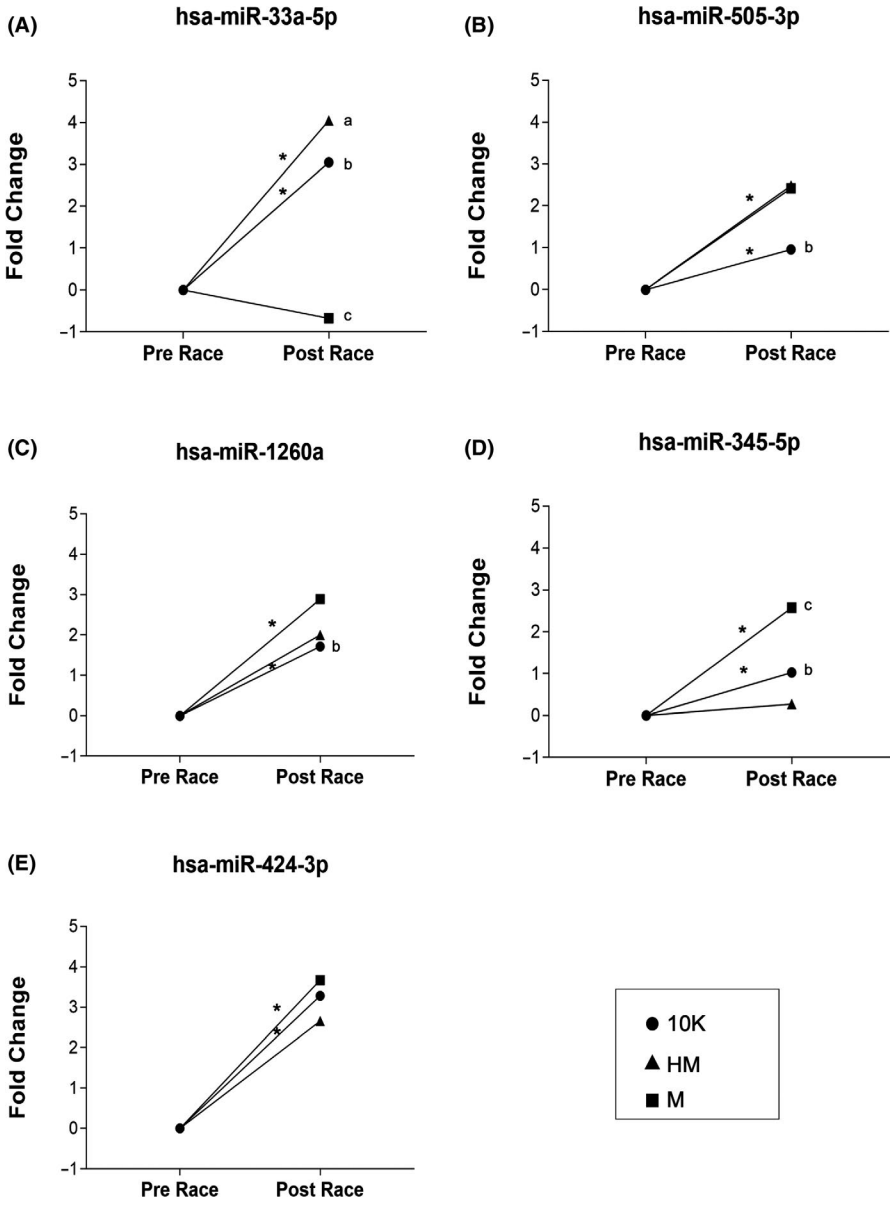

FIGURE 3 Acute response (pre-post) of significantly changed common circulating miRNAs (A-E) between exercise doses. Data are presented as means. *P-values adjusted by the false discovery rate (FDR) <0.05 and pre-post fold change >1.5. [a]Significant differences (P-vale < 0.05) between 10 km and Half-Marathon. [b]Significant differences (P-vale < 0.05) between 10 km and Marathon. [c]Significant differences (P-vale < 0.05) between Half-Marathon and Marathon

intensity group were submaximal. Furthermore, in the variable duration cohort, a submaximal intensity was also fixed. Interestingly, Ramos et al[18] described that miR-21 and miR-210 did not respond to any of the intensities and durations tested, which is in accordance with our results. However, although they describe that miR-1, miR-24, miR-146a, miR-133a, and miR-222 increase at different volumes and/or intensities, we found no significant changes in these miRNAs, except for the overexpression of miR-1-3p in response to M. This result suggests that, under laboratory conditions, miR-1-3p is a good biomarker of both exercise volume and intensity, but in field situations, this miRNA only changes in high-volume conditions at maximal efforts. The upregulation of miR-1-3p in response to marathon had been previously described by Baggish et al,[28] Mooren et al,[29] and Clauss et al[30] although no other exercise doses were tested. Here, we provide data to confirm that miR-1-3p is a good biomarker of high-volume maximal endurance exercise, while in low-volume doses, there is an absence of response for this c-miRNA.

It was also clear that an adaptive response of c-microRNAs occurred during the season. The objective of the athletes who participated in this study was to run a marathon, for which they followed a structured training plan, complemented by participation in different races. In this case, the first race was HM, followed a month later by the 10K, which took place a month before M (Figure 1). The expression levels of 168 out of 239 c-miRNAs detected in the pre-exercise samples of all races were significantly different between races, which represents 70% of all the baseline c-miRNAs detected (Table S1), although more than 70% of the miRNAs detected were the same. Therefore, the baseline miRNA profiles were very similar, but not the expression levels of the miRNAs detected. These results suggest that the differences observed in the basal expression levels of certain c-miRNAs

in the different races may be related to an adaptive response to training. In this sense, it should be taken into account that the races were separated by 1 month. Furthermore, potential confounding factors, such as previous food intake or training, were strictly controlled. Very few authors have analyzed the response of c-miRNAs to a training intervention,[8] and heterogeneous results were observed. Thus, Aoi et al[31] described a decrease in miR-486-5p after 4 weeks of training in cycle ergometer, while Nielsen et al[32] observed no changes in this miRNA, and Baggish et al[33] did not analyze it. We have observed a significant decrease in the baseline levels of expression of this miRNA between HM and 10K, with no differences between HM and M (Table S1). On the other hand, Nielsen et al[32] described a decrease in miR-21 in response to 12 weeks of training in cycle ergometer, while Baggish et al[33] described an increase after 90 days of team-based rowing training in open water and indoor ergometer, and Aoi et al[31] did not analyze it. Our results show a significantly lower baseline level of expression of this miRNA in 10K and M compared to HM (Table S1). The heterogeneity in the catalogue of miRNAs analyzed by each author makes it difficult to establish further comparisons, but what seems clear is that the c-miRNA profile varies in response to training. In this sense, the changes that we have observed in the basal levels of expression of a significant number of c-miRNAs in the different races could also be indicating an adaptation to training. It should also be noted that, in order to compare pre-post changes in c-miRNA expression, we used the $2^{(-\Delta\Delta ct)}$ method defined by Livak and Schmittgen in 2001,[26] for which the relative expression of a gene of interest is calculated relative to some internal control gene and to a reference sample (in this case, basal sample). Therefore, the differences described in c-miRNA expression in basal samples from different races would be normalized by using this method, and therefore, comparisons between races can be carried out. Thus, we have observed that in response to M not only is higher the number of c-miRNAs that change significantly, but also the magnitude of that change (Figure 3), which could be indicating a greater regulatory role for c-miRNAs in response to this exercise dose.

Another interesting result of the present study was that, at the same exercise dose, up- and downregulated c-miRNAs were observed (Table 3). This response is evident at 10K (lowest volume, highest relative intensity) and at M (highest volume, lowest relative intensity). This suggests that there is an interaction between volume and intensity with the c-miRNA response and could be indicating an active secretion and absorption of c-miRNAs in the acute response to exercise. Since secretion and uptake of c-miRNAs are thought to be facilitated by extracellular vesicle (EV) carriers and EVs have been hypothesized to carry out inter-tissue cross-talk during exercise,[2] it is conceivable that assessments of c-miRNAs in serum reflect a snapshot of this complex process.

At this point, the following question arises: which tissues or cell types could have released the c-miRNAs detected? Although the cell origin of c-miRNAs in response to exercise is unknown, but potentially diverse,[32] most authors analyzing exercise-induced c-miRNAs in humans have focused on muscle-specific miRNAs, the so-called myomiRs: miR-1-3p, miR-1-5p, miR-133a-3p, miR-133a-5p, miR-133b, miR-206, miR-208a-3p, miR-208a-5p, miR-208b-3p, miR-208b-5p, miR-486-3p, miR-486-5p, miR-499a-3p, miR-499a-5p, and miR-499b-5p.[34] Although the results obtained for circulating myomiRs in humans in response to an acute bout of exercise are inconsistent or diverse in the different studies, mainly due to different methodological approaches and experimental designs,[8] there is unanimous agreement that their circulating levels are not influenced by passive release from damaged muscle tissue.[35] In the present study, all myomiRs were analyzed, except miR-208a and miR-499a, and detected at every sampling point. However, only miR-1-3p and miR-486-3p changed in the acute response to M. Interestingly, miR-1-3p was overexpressed, while miR-486-3p was repressed. It has been described that miR-1 is actively secreted to the bloodstream in EVs.[35] Furthermore, a large proportion of EVs secreted in response to exercise are taken by the liver.[2] Therefore, it is reasonable thinking that miR-1-3p is actively secreted by the skeletal muscle during exercise in a regulated manner, depending on the dose, with the liver being a potential target.

Regarding miR-486-3p, it has been barely analyzed in the different studies, but the acute repression observed is in accordance with what Aoi et al[31] described in response to a 1 hour of exercise on cycle ergometer at 70% VO_2max. This lower plasma level might be indicating a repressed expression or secretion of this specific miRNA in skeletal muscle, but also an intense uptake by some unknown tissue.

As mentioned before, little information is available about the target tissues of the circulating miRNAs that respond to exercise, which limits its understanding as inter-tissue communicators.[8] However, a pathway analysis of validated gene targets provides interesting information about their potential regulatory role (Tables S2 and S3). In this sense, the extensive number of validated targets on AMPK and mTOR, pathways, particularly of those miRNAs that respond to higher exercise doses, highlights the potential systemic regulatory action of these miRNAs on energy metabolism. AMPK and mTOR activities have been widely studied in the context of exercise metabolism.[1,36] Surprisingly, their acute response to marathon or half-marathon has not been explored in any tissue, although a significant response is plausible. Based on our data, in which both acutely overexpressed and suppressed circulating miRNAs target those pathways, an inter-tissue balanced regulation of energy metabolism mediated by these miRNAs could be proposed. This regulatory effect of circulating miRNAs may not be restricted to active muscle tissue. Previous studies on

obese mice have shown a regulatory role of certain miRNAs on hepatic energy metabolism both when associated with[37] and without EVs.[38] These findings, alongside data suggesting uptake of EVs to the liver with exercise,[2] may support a role of miRNAs on hepatic metabolism during exercise. Lysine degradation, a cross-cutting target of the miRNAs described, is a mainly liver process.[39] Lysine cannot be used in the muscle as a metabolic fuel, as happens with other amino acids, like branched-chain amino acids.[40] These facts contrast with a decrease of lysine plasma concentration in response to marathon[41] so our data suggested a cross-talk between tissues and regulatory role of miRNAs in response to exercise dose.

In summary, our results add novel evidence that acute endurance exercise induces specific c-miRNA profiles depending on exercise dose. Moreover, we provide evidence that certain c-miRNAs are overexpressed and repressed, which points out their possible balanced regulatory role during acute exercise and training adaptations.

5 | STRENGTHS AND LIMITATIONS

The strengths of our study are the strict control and characterization of the subjects, including dietary habits, the repeated measures experimental design, in which the same subjects performed the different exercise doses tested, and the global c-miRNA screening performed, which boosts the discovery component of this study. Considering the enormous heterogeneity that has been described in the c-miRNA response between different individuals, the repeated measures nature of this study helps in reducing the variability in the response and strengthening the ulterior statistical analysis. Furthermore, we recruited amateur athletes, who represent a great proportion of participants in endurance events and for which exploring the maximal limit of healthy exercise dose is relevant. Some limitations should also be noted. First, a larger number of volunteers would have been desirable, although, in the repeated measures design of the present study, the same 9 subjects participated in three different exercise trials and provided two samples, one before and one after each race, which led us to analyze more than 50 samples. Second, this study was performed in male subjects; whether sex difference may exert a different response is not known. Third, no samples were taken during exercise. We do not discard that exercise-induced changes in miRNA levels are not lineal, as it happens for other plasma biomarkers in response to exercise.[42] Finally, as our candidate c-miRNAs are highly expressed in a variety of cell types, their real source/s and target/s are not known, and it is out of the scope of this study to go deeper than an in silico analysis. Mechanistic in vitro and in vivo studies are necessary to experimentally validate these findings.

6 | PERSPECTIVE

There is indubitable evidence about the beneficial systemic effects of regular exercise to health throughout the lifespan. However, controversy persists on the effect of acute exercise, even for trained individuals. Thus, elucidating the molecular signaling pathways and effectors of acute exercise is important for the development of further healthy exercise recommendations that may include a maximal safe exercise dose.[43] In this sense, our data suggest that the modulation of certain c-miRNAs could be also achieved by physical exercise. Therefore, c-miRNAs emerge as exercise dose biomarkers.[8,16] Therapeutic modulation of miRNA function involves both, the inhibition or gain of function of a particular miRNA, and both features have been observed in the c-miRNA response to endurance exercise in active middle-aged individuals.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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