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RESEARCH ARTICLE

Circulating microRNA levels after exercise-induced muscle damage and the repeated bout effect

 Emeric Chalchat,^{1,2}  Vincent Martin,^{2,3} Keyne Charlot,^{1,4} Cyprien Bourrilhon,^{1,4} Stéphane Baugé,^{1,4} Stéphanie Bourdon,^{1,4} Arnaud Gruel,^{1,4} Benoit Lepetit,^{1,4} Sébastien Banzet,^{5,6} Sebastian Garcia-Vicencio,^{1,4,7*} and Julien Siracusa^{1,4*}

¹Département Environnements Opérationnels, Unité de Physiologie des Exercices et Activités en Conditions Extrêmes, Institut de Recherche Biomédicale des Armées, Bretigny-Sur-Orge, France; ²AME2P, Université Clermont Auvergne, Clermont-Ferrand, France; ³Institut Universitaire de France, Paris, France; ⁴Le Laboratoire de Biologie de l'Exercice pour la Performance et la Santé, Université de Evry, Institut de Recherches Biomédicales des Armées, Université Paris Saclay, Evry, France; ⁵Institut de Recherche Biomédicale des Armées, Clamart, France; ⁶INSERM UMRS-MD 1197, Université de Paris-Saclay, Clamart, France; and ⁷Human Motion Analysis, Humanfab, Aix-en-Provence, France

Abstract

The neuromuscular system can quickly adapt to exercise-induced muscle damage (EIMD), such that it is less affected by subsequent damaging exercise, a phenomenon known as the repeated bout effect (RBE). Circulating muscle-specific microRNAs (myomiRs) may be able to potentially predict the long-lasting maximal voluntary contraction (MVC) torque deficit (>24 h), an indicator of EIMD. We aimed to investigate: 1) how plasma myomiR levels are modified by the RBE and 2) whether plasma myomiRs can predict the long-lasting MVC torque deficit. Nineteen participants performed two identical bouts of loaded downhill walking separated by 2 wk. MVC torque, creatine kinase (CK) activity, myoglobin (Mb) concentration, and myomiR levels were measured before and up to 48 h after exercise. Correlation and multiple regression analyses were performed to assess the ability of these markers to predict the largest MVC torque loss beyond 24 h postexercise. Similar to MVC torque, CK activity, and the Mb concentration, the relative abundance of certain myomiRs (hsa-miR-1-3p, and hsa-miR-133a-3p) was less affected after the second bout of exercise relative to the first bout. The CK activity, Mb concentration, and level of several myomiRs (hsa-miR-1-3p, hsa-miR-133a-3p, and hsa-miR-206) correlated with long-lasting MVC torque loss. Multiple regression showed that the best combination of markers to predict the long-lasting deficit of MVC torque included several myomiRs, Mb, and CK. Certain myomiR levels increased less after *exercise bout 2* than after *exercise bout 1*, indicating the presence of the RBE. The measurement of myomiR levels in combination with Mb concentrations and CK activity could improve the prediction of the long-lasting MVC torque deficit.

biomarkers; creatine kinase; eccentric exercise; myoglobin; myomiRs

INTRODUCTION

Performing unaccustomed exercise may result in muscle damage, especially when muscles are activated while lengthening (eccentric contractions). Exercise-induced muscle damage (EIMD) results in the disruption of structural proteins in muscle fibers and/or connective tissues, characterized by a long-lasting deficit (>24 h) of force-generating capacity (1). In the clinical and/or sports context, it is important to monitor and accurately quantify the magnitude of EIMD to apply a recovery strategy as early as possible and/or modify training/rehabilitation programs. Long-lasting maximal voluntary contraction (MVC) torque deficit (i.e., >24 h) is generally considered to be a reliable and valid marker for the magnitude of EIMD

(1, 2). However, short-term (e.g., <2 h) MVC torque loss does not provide an accurate estimation of EIMD due to the confounding influence of muscle fatigue (2). Therefore, increases in plasma levels of intramyocellular constituents (e.g., creatine kinase, myoglobin) are often used to evaluate the magnitude of EIMD. However, the ability of these markers to predict the magnitude of the long-lasting MVC torque deficit (i.e., magnitude of EIMD) is limited (3). It is therefore necessary to search for alternative markers that increase rapidly after the end of exercise (i.e., within the first hours) and accurately predict the magnitude of MVC torque loss at 24 h postexercise to adapt the recovery strategy and training loads as soon as possible.

MicroRNAs (miRNAs), which are small noncoding RNAs involved in the posttranscriptional regulation of gene

*S. Garcia-Vicencio and J. Siracusa contributed equally to this work and share last authorship.

Correspondence: E. Chalchat (emchalchat@gmail.com).

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expression, could potentially serve as alternative markers of EIMD (4–6). Indeed, muscle-specific or muscle-enriched miRNAs (also called myomiRs), such as miR-1, -133a, -133b, -208a, -208b, and -499a (7), can be actively secreted by or passively leak out of injured myocytes and measured in the blood (5, 6). A large increase in plasma myomiRs has been reported in response to various muscle damage models, such as toxic muscle injury in rats (6) or after prolonged exercise, such as marathons (8–10) and ultramarathon runs (5). It has been found that the ability of myomiRs to distinguish damaged from nondamaged muscle is better than that of creatine kinase (CK) (4, 6), suggesting that myomiRs could more correctly diagnose muscle damage. However, the ability to predict the magnitude of EIMD, as evaluated by the long-lasting MVC torque deficit, was not evaluated in these studies.

Recently, we reported that myomiRs could better represent the magnitude of height jump loss at 24 h postexercise (i.e., magnitude of EIMD) than CK or myoglobin (Mb) after a 24-h ultramarathon in 11 elite runners (5). However, whether these observations from an extreme ultra-endurance exercise can be transposed to the monitoring of milder muscle damage in physical active people is unknown. An interesting paradigm to gain insight into the accuracy of myomiRs to predict the long-lasting (>24 h) MVC torque deficit is the repeated bout effect (RBE) (11). The RBE refers to adaptations in which a single “damaging exercise bout” (e.g., eccentrically biased exercise) confers protection against EIMD from subsequent bouts. Consequently, a repeat bout within the first weeks after the initial bout results in reduced symptoms of EIMD (e.g., torque loss, CK activity, Mb concentration) and enhanced recovery (11). Although it is known that a second eccentrically biased exercise bout can result in a reduced level of EIMD, it is not currently known if circulating myomiR levels may be lower than after the first bout, thus potentially providing an alternative marker for the RBE.

The aim of the present study was to examine the response of circulating myomiRs after two identical bouts of eccentrically biased exercise to investigate 1) how plasma myomiR levels are modified by the RBE, and 2) whether plasma myomiR levels can predict the long-lasting MVC torque deficit (>24 h). We hypothesized that circulating myomiR levels would 1) increase less after the second bout than after the first of eccentric exercise, and 2) display a stronger association with MVC torque loss than conventional markers (i.e., CK activity and Mb concentration).

MATERIALS AND METHODS

Participants

Nineteen young males (age: 31.3 ± 5.6 yr, body mass: 78.0 ± 10.7 kg, height: 1.80 ± 0.06 m, body mass index: 24.0 ± 2.3 kg·m⁻², and fat mass: $16.0 \pm 5.1\%$) were fully informed of the experimental procedures, gave their written informed consent, and agreed to participate in this study. Participants performed regular activity (between 3 and 8 h·w⁻¹); had no recent history of muscular, joint, or bone disorders; and did not take any medication that

could affect neuromuscular responses. None of them had experienced strenuous mountain trekking and/or downhill walking, and none had been involved in a resistance-training program in the previous 6 months. Participants were instructed not to perform unaccustomed activities or any interventions that could affect recovery, such as massage, icing, nutritional supplementation, or nonsteroidal anti-inflammatory drugs, during the experimental period. Breakfast was standardized throughout the experiment. Each participant participated in an inclusion session, consisting of a complete medical examination, including the collection of anthropometric data and complete familiarization with the experimental procedures. The study was conducted in accordance with the Declaration of Helsinki (12) and was approved by the Regional Ethics Committee (CPP Ile-de-France 8, France, Registration No. 2019-A01210-57, SMILE).

Protocol

Participants performed two bouts of forward downhill walking on a treadmill (pulsar 3p 4.0, HP cosmos, Warwickshire, UK) for 45 min while carrying a load equivalent to 30% of their body mass (carrying: a weighted vest of 10% of the body mass and a backpack of 20% of the body mass) separated by 2 wk (Fig. 1). The treadmill gradient was set to -25% and the velocity to 4.5 km·h⁻¹ and the participants were instructed to walk at their own preferred stride length and frequency. This downhill walking exercise was performed by the participants to induce EIMD, especially on the quadriceps muscles, due to the high proportion of active lengthening contractions. Five participants were unable to finish the walking bout and performed the first exercise to exhaustion (mean \pm SD: 30.2 ± 8.6 min). These participants performed the same exercise for the same duration during the second exercise. MVC torque was assessed before (PRE) and 24 and 48 h after the two bouts. Blood samples were obtained at PRE, POST, and 2, 6, 24, and 48 h after the two exercise bouts.

MVC Torque

MVC torque was measured using an isokinetic dynamometer (Cybex Norm, Lumex, Ronkonkoma, NY). Participants were comfortably positioned on an adjustable chair with the hip joint flexed at 70° (0° = neutral supine position). The axis of rotation of the dynamometer was aligned with the femoral condyles of the femur, and the lever arm was attached 1 to 2 cm above the malleolus with a Velcro strap. Measurements were taken from the participant's right leg, which was fixed at 90° (0° = knee fully extended). Torque data were corrected for gravity, digitized, and exported at a rate of 2 kHz to an external analog-to-digital converter (Powerlab 16/35; ADInstruments, New South Wales, Australia) driven by Labchart pro 8.1 software (ADInstruments, New South Wales, Australia). The participants performed two isometric MVCs of the knee extensors with 2 min of rest and were instructed to grip the lateral handles of the seat to stabilize the pelvis and strongly encouraged by the investigator. The MVC torque was determined as the peak torque reached during the maximal effort. This was continuously monitored

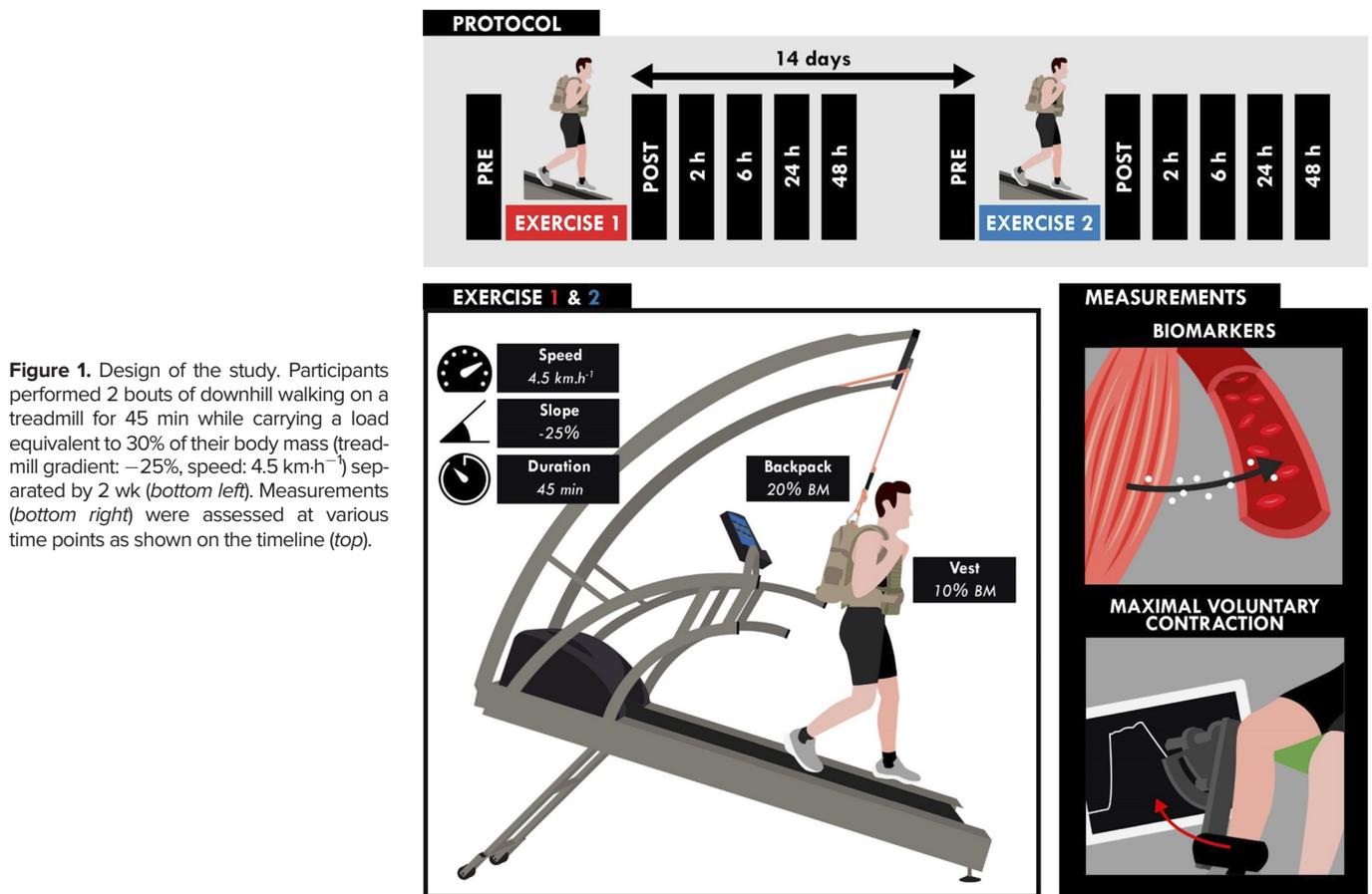


Figure 1. Design of the study. Participants performed 2 bouts of downhill walking on a treadmill for 45 min while carrying a load equivalent to 30% of their body mass (treadmill gradient: -25% , speed: $4.5 \text{ km}\cdot\text{h}^{-1}$) separated by 2 wk (*bottom left*). Measurements (*bottom right*) were assessed at various time points as shown on the timeline (*top*).

during the experiments. When the level of variability was higher than 5%, a third MVC was performed. The best of the two/three values was used for further analysis. Before the MVCs, the participants performed a warm-up consisting of eight contractions at submaximal and progressive intensities (2 contractions at 20%, 40%, 60%, and 80% of their perceived maximal voluntary contraction).

Muscle Soreness

The magnitude of muscle soreness of the whole quadriceps was assessed using a visual analog scale, consisting of a 100-mm line representing “no pain” at one end (0 mm) and “very, very painful” at the other (100 mm), while performing a squat over a 90° -knee range of motion.

Biological Measurements

Blood was drawn from the antecubital vein into two separate tubes (5 mL, Becton Dickinson vacutainer, Franklin Lakes, NJ), one containing EDTA and the other being a serum-separating tube (SST). Blood in the SST was allowed to clot at ambient temperature for 30 min and centrifuged ($2,000 \text{ g}$, 4°C , 10 min). Serum from the STT and EDTA plasma was aliquoted and frozen at -80°C for further analysis. Serum CK activity and Mb concentration were analyzed using an autoanalyzer (Advia 1800; Siemens Healthineers, Erlangen, Germany), and plasma

miRNAs were analyzed by real-time quantitative PCR (RT-qPCR).

Plasma miRNA Extraction

Total RNA was extracted from plasma as previously described (4–6). Briefly, total RNA was extracted from 200 μL plasma using the mirVana PARIS Kit (Ambion, Austin, TX) and low-binding tubes (1.5 mL; DNA LoBind Tubes, Eppendorf, Hamburg, Germany) according to the manufacturer's protocol. An additional precipitation step was performed as follows. Column elution was performed with 180 μL sterile water. Then, 18 μL 3 M sodium acetate (Sigma-Aldrich, Saint-Quentin-Fallavier, France), 396 μL 100% ethanol, and 1 μL GlycoBlue (Ambion) were added. Tubes were vortexed, and the RNA was allowed to precipitate for 20 min at -20°C . After centrifugation ($12,000 \text{ g}$, 4°C , 10 min), the pellet was washed with 400 μL 70% ethanol and allowed to dry for 30 min. The RNA was finally suspended in 12 μL RNase-free water, incubated for 10 min at 50°C , and frozen at -80°C until cDNA synthesis.

Reverse Transcription

cDNA was synthesized from 5 μL total RNA diluted 1:6 in a 10- μL reaction volume, including the UniSp6 RNA spike-in, using the miRCURY LNA RT Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol.

Real-Time Quantitative qPCR

RT-qPCR was performed on a 96-well LightCycler 480 instrument (Roche Applied Science, Mannheim, Germany) with 4 μ L cDNA (diluted 1:40 in sterile water), 1 μ L PCR primer mix, and 5 μ L 2x miRCURY SYBR Green Master Mix (Qiagen) in a 10 μ L reaction volume. We measured hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, hsa-miR-206, hsa-miR-208a-3p, hsa-miR-208b-3p, hsa-miR-378a-3p, and hsa-miR-499a-5p. We chose these miRNAs due to their muscle-specific expression (13) and their documented responses to toxic (6) and exercise-related muscle damage (4, 5). The PCR protocol consisted of one activation cycle (95°C, 2 min), 52 amplification cycles (denaturation 95°C, 10 s, and annealing/amplification 56°C, 1 min), and a melting curve analysis step, as recommended by the manufacturer. Undetectable values were handled as described by De Ronde et al. (14) by adding one quantification cycle (C_q) to the highest C_q observed for a miRNA.

The stability of reference miRNAs (hsa-miR-16-5p, hsa-miR-20a-5p, hsa-miR-21-5p, hsa-miR-103a-3p, hsa-miR-185-5p, hsa-miR-192-5p, and hsa-miR-320a-3p) was assessed using geNorm, BestKeeper, NormFinder, the ΔC_T method, and RefFinder. GeNorm determined that all reference miRNAs were necessary for the optimal normalization of the target miRNAs. Final quantification is expressed as arbitrary units (AU) and consisted of the geometrical mean of the quantification performed with each reference miRNA. qPCR primer information is supplied for the target and reference miRNA in Supplemental Spreadsheet S1 (see <https://doi.org/10.5281/zenodo.6938518>).

Index of Protection

To assess the magnitude of the protective effect conferred by the first bout of downhill walking, an index of protection was calculated for MVC torque using an equation adapted from Chen et al. (15): [relative changes from PRE after *exercise bout 2* – relative changes from PRE after *exercise bout 1*], with PRE corresponding to 100% and relative changes corresponding to the lower MVC torque value between 24 h and 48 h (i.e., the largest decrease). For example, if the MVC torque represents 70% (i.e., –30%) of the PRE value after the first exercise and 90% after the second exercise, the calculation is: $90 - 70 = 20$. The result indicates that the decrease in MVC torque is attenuated by 20% after the second exercise relative to the first. The equation was adapted because the initial version from Chen et al. (15) was applicable to mean values but not individual values.

Statistical Analysis

Statistical analyses were performed to assess: 1) the changes between the PRE and, POST, 2, 6, 24, and 48 h post-exercise for both downhill walking bouts and 2) the effect of exercise (i.e., downhill walking bout). Because of the absence of a normal distribution according to the Shapiro-Wilk test, we performed a nonparametric Friedman analysis of variance to assess the main effect of time on muscle soreness, miRNA, CK activity, and Mb concentration levels. When a significant main effect was identified, Dunn's multiple comparisons were used to identify which time points were significantly different to baseline for these

variables. Statistical differences between matched time points (i.e., *exercise bout 1* vs. *exercise bout 2*) were assessed using Mann-Whitney *U* test. Each *P* value was adjusted to account for multiple comparisons. MVC torque results were analyzed by two-way ANOVA (time \times exercise) with repeated measures for paired values. When an interaction between time and exercise was found by the two-way ANOVA, Holm post hoc tests were applied to test for differences between the means. All time points (24 and 48 h) were compared with PRE and matched time points were compared between *exercise bout 1* and *exercise bout 2* (e.g., 24 h after the *exercise bout 1* vs. 24 h after the *exercise bout 2*).

Correlations were performed between changes in myomiR levels from PRE *exercise bout 1* to PRE *exercise bout 2* and the index of protection for MVC torque to investigate whether long-term adaptations in myomiR levels could be involved in the protection conferred by the initial bout. Similarly, correlations were performed between peak changes in myomiR levels from PRE *exercise bout 1* (hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, hsa-miR-206, hsa-miR-208a-3p, hsa-miR-208b-3p, hsa-miR-378a-3p, and hsa-miR-499a-5p) and the index of protection for MVC torque to investigate whether myomiR levels could be representative of the adaptations conferred by the initial exercise bout.

Correlations were performed between peak changes in myomiR levels and peak changes in CK activity and Mb concentration (i.e., conventional EIMD markers). Correlations were also performed between the long-lasting MVC torque deficit (>24 h) and miRNA levels, CK activities, and Mb concentrations measured at POST, 2 h, and 6 h to assess the ability of these markers to predict the largest MVC torque loss beyond 24 h postexercise. The level of association between parameters was assessed using Spearman's rank correlation coefficient (ρ). The strength of the correlation ρ was defined as follows (16): 0.00–0.39: weak correlation; 0.40–0.69: moderate correlation; 0.70–1.00: strong correlation. To determine whether a combination of circulating markers measured at POST, 2 h, or 6 h postexercise could improve the prediction of MVC torque loss at 24 h, we performed backward multiple linear regression analyses using IBM SPSS Statistics v26 software (IBM Corp.). The independent variables entered the analyses were hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, hsa-miR-206, hsa-miR-208a-3p, hsa-miR-208b-3p, hsa-miR-378a-3p, and hsa-miR-499a-5p levels, CK activity, and Mb concentration. The results are presented as the means \pm SD. Significance was defined as $P < 0.05$. Except for multiple linear regression, analyses were performed using GraphPad Prism (Version 8.4.3, GraphPad Software).

RESULTS

PRE Exercise Measurements

No significant differences were found between *exercise bout 1* and *exercise bout 2* for the absolute PRE values of any variables (Table 1).

MVC Torque

A significant interaction (time \times exercise, $P < 0.001$) was found for MVC torque (Fig. 2). MVC torque was below the baseline value up to 48 h after *exercise bout 1*, whereas no

Table 1. Absolute PRE values of exercise bout 1 and exercise bout 2 for all variables

Absolute PRE Values	Exercise Bout 1	Exercise Bout 2	P
MVC torque, N·m	312 ± 51	311 ± 67	0.98
hsa-miR-1-3p, AU	7.9 ± 6.2	10.0 ± 10.9	0.95
hsa-miR-133a-3p, AU	14.1 ± 13.0	11.3 ± 9.0	0.70
hsa-miR-133b, AU	10.0 ± 10.4	10.6 ± 14.0	0.79
hsa-miR-206, AU	1.55 ± 2.78	1.09 ± 1.53	0.98
hsa-miR-208a-3p, AU	0.02 ± 0.04	0.01 ± 0.01	0.43
hsa-miR-208b-3p, AU	0.26 ± 0.86	0.12 ± 0.41	0.60
hsa-miR-378a-3p, AU	24.1 ± 14.1	25.5 ± 18.6	0.91
hsa-miR-499a-5p, AU	0.4 ± 1.27	0.34 ± 1.03	0.79
CK activity, U/L	200 ± 135	154 ± 52	0.32
Mb concentration, ng/mL	72 ± 14	71 ± 20	0.72

Data are expressed as means ± SD. AU, arbitrary units; MVC, maximal voluntary torque; hsa, homo sapiens; CK, creatine kinase; Mb, myoglobin. Results with a P value < 0.05 were considered significant.

significant changes were observed the days after the second bout of exercise (Fig. 2).

Muscle Soreness

A significant effect of time was found for muscle soreness ($P < 0.001$). Indeed, muscle soreness was increased after the first exercise (baseline: 2 ± 6 mm) at 24 h (46 ± 22 mm; $P < 0.001$) and 48 h postexercise (53 ± 26 mm; $P < 0.001$). Similarly, muscle soreness was increased after the second exercise (baseline: 2 ± 9 mm) at 24 h (21 ± 16 mm; $P < 0.001$) and 48 h postexercise (18 ± 19 mm; $P < 0.001$). However, muscle soreness was significantly lower after the second bout of exercise than after the first at 24 h ($P < 0.001$) and 48 h ($P < 0.001$) postexercise.

Circulating miRNA Levels

A significant effect of time was found for hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, and hsa-miR-206 levels ($P < 0.001$ for all these myomiRs) (Fig. 3) but not for those of hsa-

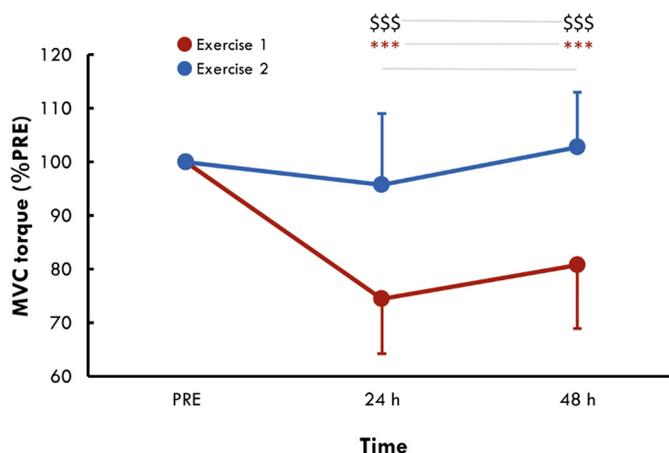


Figure 2. Time course of changes in maximal voluntary contraction (MVC) torque from PRE to 24 h and 48 h for both exercise bout 1 and exercise bout 2. AU, arbitrary units. Significant different from the PRE value: *** $P < 0.001$, significant difference between exercise bout 1 and exercise bout 2: \$\$\$ $P < 0.01$. These P values were extracted from the two-way ANOVA (time × exercise) with repeated measures and Holm post hoc test ($n = 19$). Data are expressed as means ± SD.

miR-208a-3p (exercise bout 1: $P = 0.321$; exercise bout 2: $P = 0.908$), hsa-miR-208b-3p (exercise bout 1: $P = 0.475$; exercise bout 2: $P = 0.553$), hsa-miR-378-3p (exercise bout 1: $P = 0.333$; exercise bout 2: $P = 0.823$), or hsa-miR-499a-5p (exercise bout 1: $P = 0.062$; exercise bout 2: $P = 0.228$) (Supplemental Table S1; see <https://doi.org/10.5281/zenodo.6458050>). After the first bout of exercise, the levels were significantly higher for hsa-miR-1-3p (Fig. 3A), hsa-miR-133a-3p (Fig. 3B), hsa-miR-133b (Fig. 3C), and hsa-miR-206 (Fig. 3D) after 2 and 6 h. After the second bout of exercise, the levels were significantly higher for hsa-miR-206 (Fig. 3D) at POST, for hsa-miR-1-3p (Fig. 3A) and hsa-miR-133b (Fig. 3C) after 2 h, and for hsa-miR-133a-3p (Fig. 3B) and hsa-miR-133b (Fig. 3C) after 6 h. Moreover, the levels were significantly lower for hsa-miR-1-3p (Fig. 3A) and hsa-miR-133a-3p (Fig. 3B) 6 h after the second bout of exercise than after the first.

Creatine Kinase Activity

A significant effect of time was found for CK activity ($P < 0.001$). Indeed, CK activity was increased from 2 h to 48 h after both exercises. CK activity was significantly lower 6 h, 24 h, and 48 h after the second bout of exercise than after the first (Fig. 4A).

Myoglobin Concentration

A significant effect of time was found for Mb concentration ($P < 0.001$). Indeed, the Mb concentration was significantly higher up to 6 h after both exercises (Fig. 4B). The Mb concentration was significantly lower 2 and 6 h after the second bout of exercise than after the first (Fig. 4B).

Correlation between Changes in myomiR Levels from PRE Exercise Bout 1 and the Index of Protection for MVC Torque

There was no correlation between peak changes in myomiR levels from PRE exercise 1 and the index of protection for MVC torque (Supplementary Table S2a; see <https://doi.org/10.5281/zenodo.6458054>). Similarly, there was no correlation between changes in myomiR levels from PRE exercise bout 1 to PRE exercise bout 2 and the index of protection for MVC torque (Supplementary Table S2b; see <https://doi.org/10.5281/zenodo.6458054>).

Correlation between CK Activity, Mb Concentration, and Circulating myomiR Levels

Peak changes in CK activity and Mb concentration significantly correlated with peak changes in hsa-miR-1-3p (CK: $P < 0.001$, $\rho = 0.54$; Mb: $P = 0.005$, $\rho = 0.50$), hsa-miR-133a-3p (CK: $P < 0.001$, $\rho = 0.58$; Mb: $P = 0.048$, $\rho = 0.36$), hsa-miR-133b (CK: $P = 0.002$, $\rho = 0.50$; Mb: $P = 0.056$, $\rho = 0.35$), and hsa-miR-206 (CK: $P = 0.03$, $\rho = 0.35$; Mb: $P = 0.007$, $\rho = 0.48$).

Correlation between Markers and Long-Lasting (>24 h) MVC Torque Loss

There was no correlation between long-lasting (>24 h) MVC torque loss and CK activity (Fig. 5, A and C) or miRNA levels measured POST and 2 h postexercise, whereas there was a significant correlation with Mb concentration at 2 h postexercise (Fig. 5D). Moreover, there was a significant correlation between long-lasting MVC

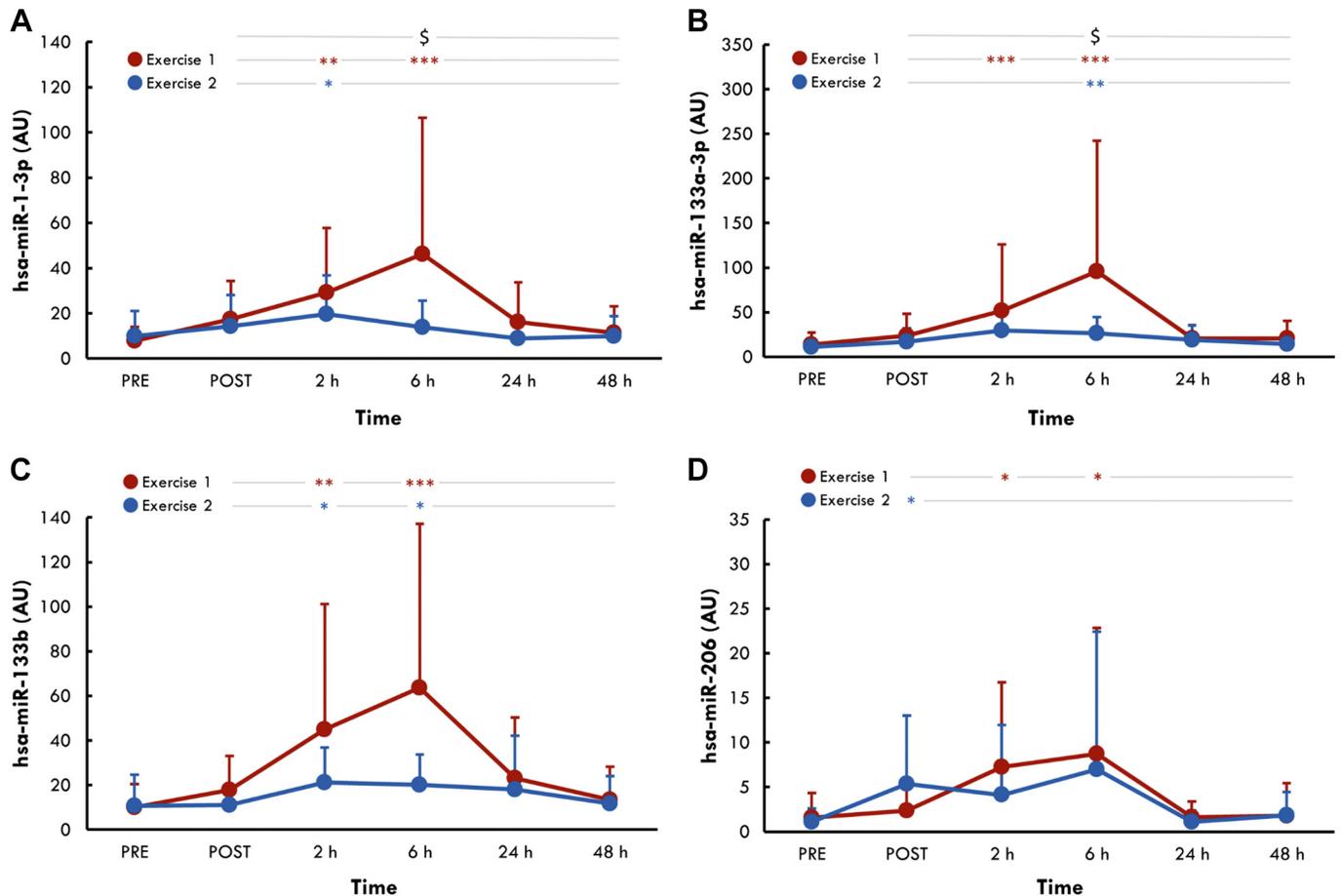


Figure 3. Time course of changes in homo sapiens (hsa)-miR-1-3p (A), hsa-miR-133a-3p (B), hsa-miR-133b (C), and hsa-miR-206 levels (D) from PRE to 48 h after *exercise bout 1* [PRE (1), POST (1), 2 h (1), 6 h (1), 24 h (1), and 48 h (1)] and *exercise bout 2* [PRE (2), POST (2), 2 h (2), 6 h (2), 24 h (2), and 48 h (2)]. Data are expressed as relative abundance arbitrary units (AU). Significant differences from the PRE value are indicated: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Significant difference between *exercise bout 1* and *exercise bout 2*: \$ $P < 0.05$. These P values were extracted from Friedman test and Dunn's multiple comparisons ($n = 19$). Data are expressed as means \pm SD.

torque loss and the measurements at 6 h postexercise for CK activity (Fig. 5E), Mb concentration (Fig. 5F), and hsa-miR-1-3p (Fig. 6A), hsa-miR-133a-3p (Fig. 6B; nearly significant, $P = 0.053$), and hsa-miR-206 (Fig. 6D) levels. However, there was no significant correlation between long-lasting MVC torque loss and hsa-miR-133b (Fig. 6C), miR-208a, hsa-miR-208b, hsa-miR-378, or hsa-miR-499a-5p levels (Table 2). At POST, multiple linear regression showed the best combination of markers included as follows: Mb, CK, hsa-miR-1-3p, hsa-miR-133b, and hsa-miR-206 ($r^2: 0.485$, $P < 0.001$). The best combination included Mb and CK at 2 h postexercise ($r^2: 0.440$, $P < 0.001$), whereas the combination of Mb and hsa-miR-1-3p ($r^2: 0.300$, $P = 0.002$) was better at 6 h postexercise.

DISCUSSION

The main purpose of this study was to investigate 1) how plasma myomiR levels are modified by the RBE and 2) whether plasma myomiR levels can predict the long-lasting deficit (> 24 h) of MVC torque (i.e., the magnitude of EIMD). We observed an increase in several miRNA levels (hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, and

hsa-miR-206) after both *exercise bouts 1* and 2. The levels of certain myomiRs (hsa-miR-1-3p and hsa-miR-133a-3p) were less affected after the second bout of exercise than after the first, thus confirming our first hypothesis. However, we did not confirm our second hypothesis that the association of myomiR levels with the largest MVC torque loss measured beyond 24 h after the exercises would be stronger than the association between MVC force loss and CK activity/Mb concentration.

Circulating Levels in Response to Downhill Walking Exercises

As expected, the repetition of exercise bouts induced a RBE: MVC torque loss was less decreased after *exercise bout 2* than after *exercise bout 1*. Moreover, CK activity and the Mb concentration increased less after *exercise bout 2* than after *exercise bout 1*. This result is consistent with that of previous studies (15, 17, 18), which reported an attenuation of several symptoms of EIMD after the second eccentrically biased bout of exercise relative to the first. Importantly, our study obtained original results concerning the evolution of miRNA levels after two eccentric exercise bouts.

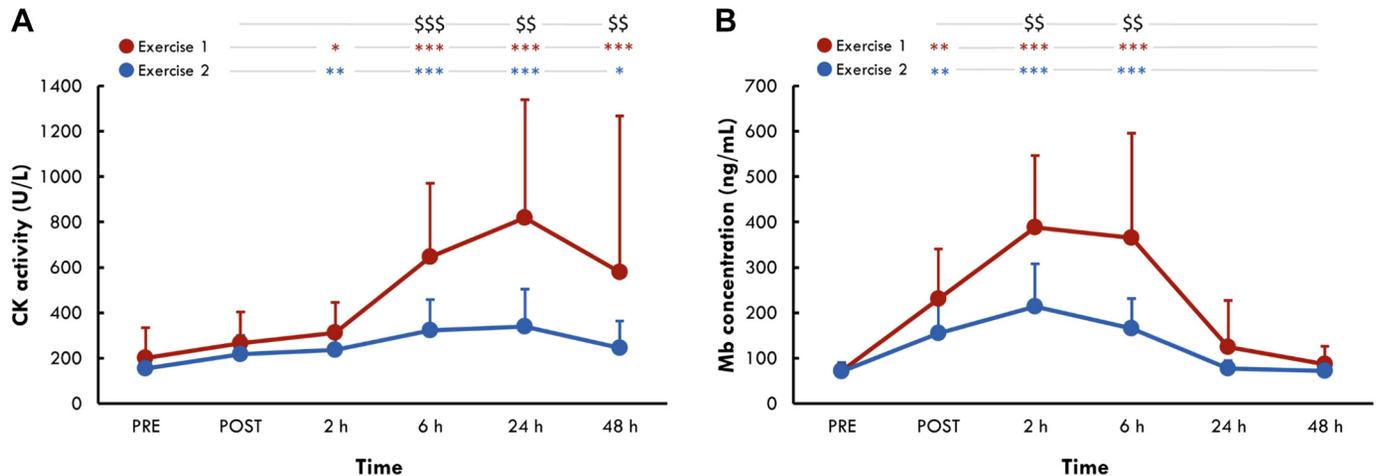


Figure 4. Time course of changes in (A) creatine kinase (CK) activity and (B) Myoglobin (Mb) concentration from PRE to 48 h after *exercise bout 1* [PRE (1), POST (1), 2 h (1), 6 h (1), 24 h (1), and 48 h (1)] and *exercise bout 2* [PRE (2), POST (2), 2 h (2), 6 h (2), 24 h (2), and 48 h (2)]. Data are expressed as relative abundance arbitrary units (AU). Significant differences from the PRE value are indicated: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Significant differences between *exercise bout 1* and *exercise bout 2* are indicated: \$\$ $P < 0.01$ and \$\$\$ $P < 0.001$. These P values were extracted from Friedman test and Dunn's multiple comparisons ($n = 19$). Data are expressed as means \pm SD.

Circulating miRNA Levels in Response to the First Bout of Eccentric Exercise

We found hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, and hsa-miR-206 to increase after *exercise bout 1*. These results are consistent with those of several studies (4, 5, 8, 9, 19), which found an increase in the level of these myomiRs after downhill walking or a marathon or ultramarathon run. Conversely, we did not observe significant increases in the hsa-miR-208a-3p, hsa-miR-208b-3p, hsa-miR-378-3p, or hsa-miR-499a-5p levels in the present study, whereas their circulating levels were shown to increase after backward downhill walking (4), marathon (8, 9), and/or ultramarathon runs (5). Discrepancies between circulating myomiR profiles following a session of eccentric exercise have already been reported (4, 9) and could involve several factors. One possible explanation is that the observed discrepancies are due to differences in basal circulating levels between miRNAs, as previously suggested (4, 5). Here, we found that the circulating levels of certain myomiRs (hsa-miR-208a-3p, hsa-miR-208b-3p, and hsa-miR-499a-5p) were too low to be accurately detected both at baseline levels and in response to moderate damaging exercise. Among the 228 samples (12 time points for 19 subjects), miRNAs were detected in 223 for hsa-miR-1-3p (97.8%), 207 for hsa-miR-133a-3p (90.8%), 213 for hsa-miR-133b (93.4%), 188 for hsa-miR-206 (82.5%), 30 for hsa-miR-208a-3p (13.2%), 43 for hsa-miR-208b-3p (18.9%), 218 for hsa-miR-378-3p (95.6%), and 104 for hsa-miR-499a-5p (45.6%). This result is consistent with those of several studies (4, 20), such as that of Banzet et al. (4), which reported low levels of hsa-miR-208a-3p (not detectable), hsa-miR-208b-3p (not detectable in 6 of 9 participants), and hsa-miR-499a-5p after 30 min of a downhill walking exercise. A second factor that could explain the observed differences in circulating profiles after EIMD may be linked to the differential expression of myomiRs according to the muscle type, as well as skeletal muscle fiber contractile phenotype. Indeed, hsa-miR-208a-3p is specific to cardiac muscle, explaining why we did not find a significant increase in this myomiR after

the bouts of downhill walking, in which a low level of cardiac stress is expected. Interestingly, the two participants who showed the largest increases in hsa-miR-208a-3p levels in the present study were among the five participants who performed the exercise to exhaustion, suggesting that this myomiR may be an indicator of cardiac stress. However, as this was not the aim of the present study, we neither evaluated cardiac function nor measured other blood markers of cardiac stress. Hsa-miR-499a-5p has been shown to be more highly expressed in type I than in type II fibers (6). As type I fibers have been shown to be less susceptible to EIMD than type II fibers (21), this could partially explain why hsa-miR-499a-5p levels did not increase after the downhill walking exercise in the present study. Hsa-miR-378-3p levels did not significantly increase after downhill walking in the present study. This miRNA is not considered to be a myomiR but is among the most highly expressed miRNAs in muscle tissue (7, 22) and increases in its blood levels have been consistently reported after toxic muscle injury in the rat (6) or an ultra-marathon run (5) and in various animal models of Duchenne muscular dystrophy (DMD) (23–25). As hsa-miR-378-3p was already abundant in the plasma in the control condition (e.g., healthy organism at rest), it is possible that any muscle damage induced in the present study was not sufficient to raise the circulating level above the “background noise.”

Comparison of Circulating miRNA Levels between Exercise Bouts 1 and 2

Among the myomiRs that showed an increase in the level after the first bout of exercise (hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, and hsa-miR-206), only hsa-miR-1-3p and hsa-miR-133a-3p showed a differential profile, i.e., smaller increase, after the second. The fact that we observed different circulating profiles among miRNAs in response to one or two bouts of eccentric exercise is striking and could be due to a combination of several factors. Interestingly, we observed that the peak changes in CK activity and Mb

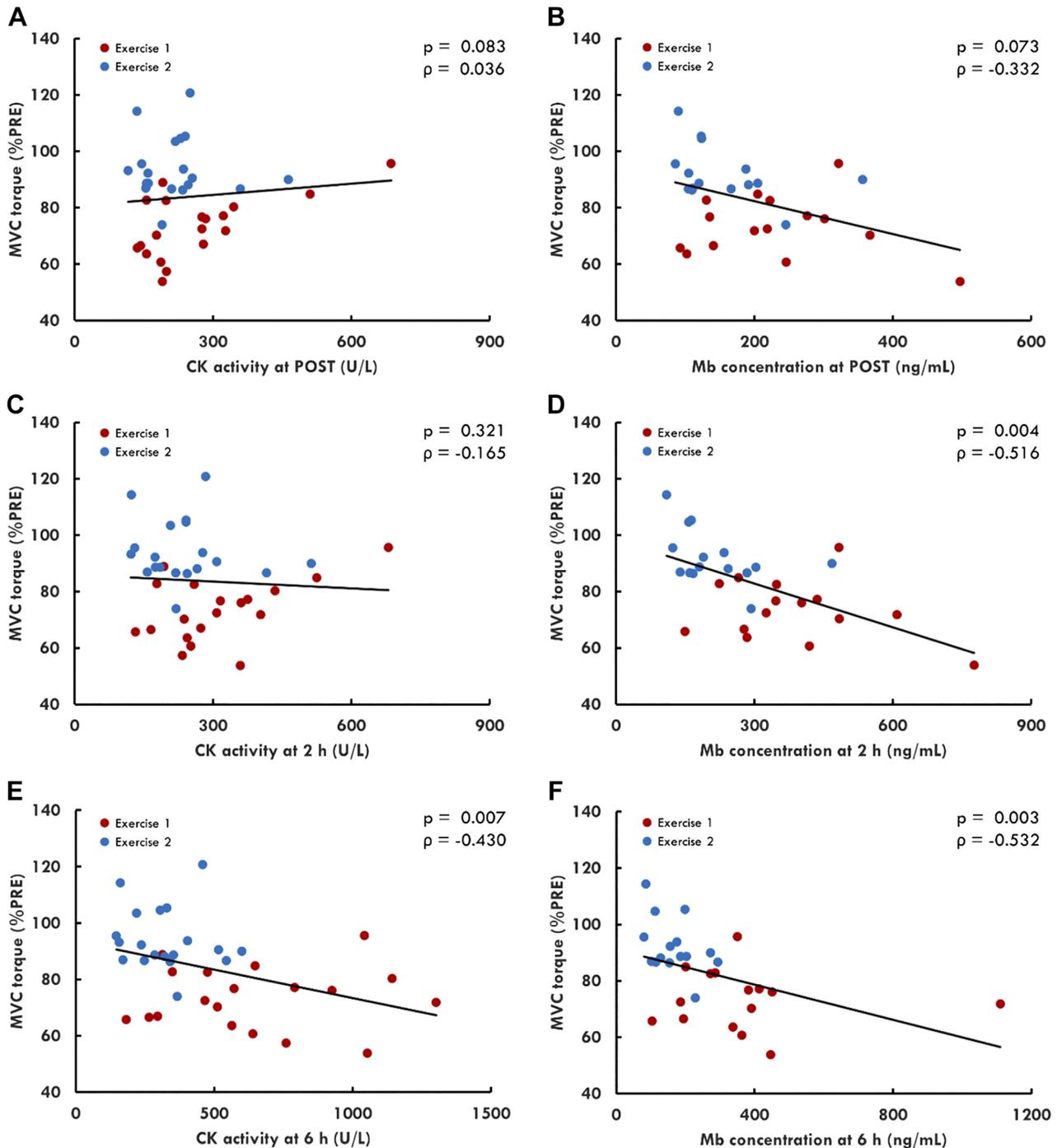


Figure 5. Correlations between the largest maximal voluntary contraction (MVC) torque loss measured beyond 24 h after the end of exercise and creatine kinase (CK) activity measured at POST (A), 2 h (C), and 6 h (E) after exercise and myoglobin (Mb) concentration measured at POST (B), 2 h (D), and 6 h (F) after the exercises. Significance (P) and Spearman rank correlation coefficient (ρ) values are presented ($n = 19$).

concentration correlated moderately with peak changes in hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, and hsa-miR-206 levels. Although still speculative, such a relationship suggests that these myomiRs may share, at least partially, mechanisms of release to those of CK and Mb (i.e., passive release). However, it is likely that this hypothesis cannot fully explain the observed differences in circulating profiles reported here. First, it cannot be excluded that the first bout of eccentric exercise led to modifications of the abundance

of myomiRs in the skeletal muscle tissue, as they are known to be involved in muscle homeostasis and regeneration (26, 27). Despite the fact that no study has evaluated the effect of an acute bout of eccentric exercise on muscle myomiR levels, several authors (28, 29) have shown that an acute bout of resistance exercise can reduce the expression of miR-1, miR-133a, and miR-133b in skeletal muscle tissue 2 to 6 h postexercise. Whether such modifications are transient or persist for a longer period of time and whether this could result in

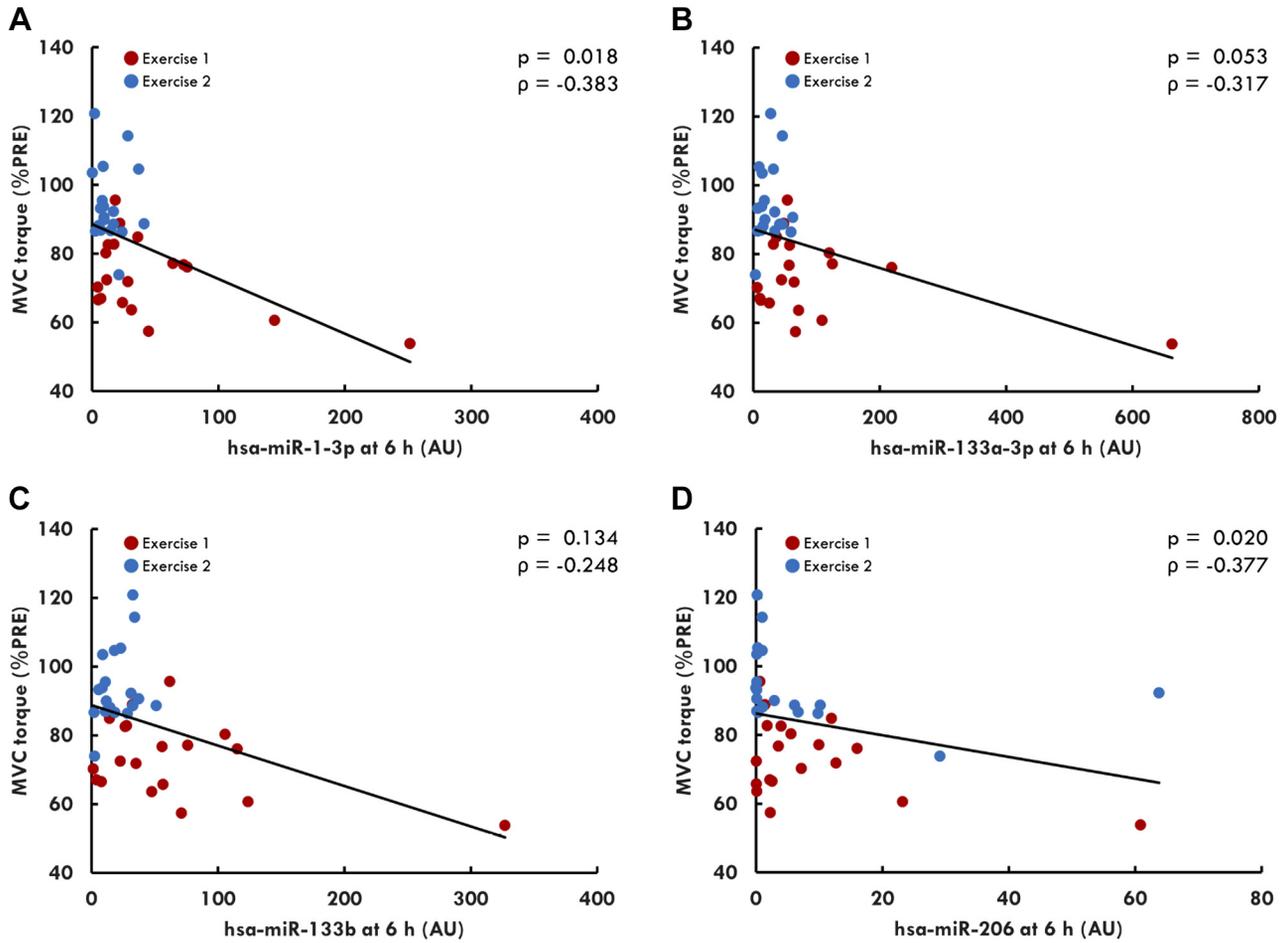


Figure 6. Correlations between the largest maximal voluntary contraction (MVC) torque loss measured beyond 24 h after the end of exercise and plasma miRNA levels measured 6 h after the end of the exercise. Data are displayed for homo sapiens (hsa)-miR-1-3p (A), hsa-miR-133a-3p (B), hsa-miR-133b (C), and hsa-miR-206 (D). MVC torque loss values are expressed as %PRE and plasma miRNA levels as relative abundance arbitrary units (AU). Significance (P) and Spearman rank correlation coefficient (ρ) values are presented (n = 19).

variations in their circulating levels is unknown. Circulating levels of members of the let-7 miRNA family, abundantly expressed in muscle tissue, did not increase in response to myofiber damage (24–26, 30), suggesting that active secretion mechanisms are involved. In a recent review, Coenen-

Stass et al. (30) discussed the clinical utility of extracellular miRNAs as biomarkers of DMD, as well as their possible contribution to muscle physiology. These authors proposed that the release of miRNAs in response to muscle damage may be due to a combination of passive leakage resulting from

Table 2. Correlation between delayed maximal voluntary contraction torque loss and circulating microRNA levels at POST, 2 h, and 6 h postexercise

	POST		2 h		6 h	
	P	ρ	P	ρ	P	ρ
hsa-miR-1-3p, AU	0.1426	-0.2424	0.0882	-0.2804	0.0176	-0.3831
hsa-miR-133a-3p, AU	0.1609	-0.2321	0.1581	-0.2336	0.0528	-0.3166
hsa-miR-133b, AU	0.1437	-0.2417	0.1022	-0.2692	0.1339	-0.2476
hsa-miR-206, AU	0.4619	0.1230	0.2375	-0.1963	0.0197	-0.3767
hsa-miR-208a-3p, AU	0.7094	-0.0625	0.6809	0.0689	0.7007	-0.0645
hsa-miR-208b-3p, AU	0.2831	-0.1787	0.9969	0.0007	0.7280	-0.0583
hsa-miR-378-3p, AU	0.4367	-0.1318	0.9917	-0.0018	0.7285	-0.0590
hsa-miR-499a-5p, AU	0.3134	-0.1680	0.8022	-0.0420	0.8108	0.0402
CK activity, U/L	0.8306	0.0359	0.3211	-0.1654	0.0071	-0.4295
Mb concentration, ng/mL	0.0729	-0.3321	0.0035	-0.5159	0.0025	-0.5315

Correlations between the largest maximal voluntary contraction torque loss measured beyond 24 h after the end of the exercises and homo sapiens (hsa)-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, hsa-miR-206, hsa-miR-208a-3p, hsa-miR-208b-3p, hsa-miR-378-3p, and hsa-miR-499a-5p levels, creatine kinase (CK) activity, and myoglobin (Mb) concentration are presented. Significance (P) and Spearman rank correlation coefficient (ρ) values are shown. Results with a P < 0.05 were considered significant and are shown in bold.

myonecrosis and the impairment of the sarcolemma, as well as an active secretion process that could accompany muscle differentiation and regeneration. Interestingly, pioneering *in vitro* studies have shown that miR-133a, miR-133b, miR-206, and miR-378 are actively released in extracellular vesicles from myotubes (31) and myogenic progenitor cells (32) and may regulate myoblast differentiation and extracellular matrix remodeling. Interestingly, these physiological mechanisms have been identified to contribute to the RBE (11). Despite the different context, we believe that the EIMD induced in our experimental model may share some of these release mechanisms.

To try to evaluate whether the magnitude of changes in circulating miRNA levels after exercise could be an indicator of the functional adaptations induced by the RBE, we searched for correlates between peak changes in acute (<48 h) and long-term (2 wk; *PRE exercise bout 2*) circulating myomiR levels and index of protection for torque change but failed to observe a significant correlation. Despite these negative results, a role for circulating myomiRs in RBE adaptations cannot be excluded, as their abundance in the blood compartment depends on complex and interrelated factors, such as varying half-lives (33), kinetics, and mechanisms of release. Thus it cannot be excluded that myomiRs may play a role in RBE adaptations. However, further research is needed to obtain evidence to directly support this hypothesis.

Association between EIMD Markers and Long-Lasting MVC Torque Loss

We found that the long-lasting MVC torque deficit correlated moderately with the levels of hsa-miR-1-3p, hsa-miR-133a-3p (tendency; $P = 0.053$), hsa-miR-206, CK activity, and Mb concentration measured at 6 h postexercise. However, we did not confirm our hypothesis that the association between myomiR levels and the largest MVC torque loss measured beyond 24 h after the bouts of exercise would be stronger than the association between MVC torque loss and CK activity/Mb concentration. These results are not consistent with those of our previous study (5), which showed that myomiR levels were more highly correlated with the height jump loss measured at 24 h than CK activity or Mb concentration after a 24-h ultra-marathon run in elite athletes. These discrepancies could be explained by differences in exercise duration and intensity, as well as in the training status of the participants between studies (elite versus physically active in the present study). Furthermore, our results also suggest that a combination of several markers, including Mb, CK, and certain myomiRs (hsa-miR-1-3p, hsa-miR-133b, and hsa-miR-206) could improve our ability to predict MVC torque reduction in the days following exercise. Therefore, the concomitant measurement of circulating myomiR levels, Mb concentrations, and CK activity could improve the diagnosis of EIMD.

Perspectives and Significance

Circulating myomiRs may have a future interest in the diagnosis of muscle damage both in sport medicine and in the monitoring of physical activity. However, further research is needed for a better understanding of the release mechanisms and intercellular communication roles of these circulating

miRNAs. Moreover, the best combination of markers to predict muscle damage should be determined in various exercises.

Limitations

Our study had several limitations. First, we included only men, limiting any conclusions for women. However, our previous work has shown that myomiR levels increase after toxic muscle injury in female rats (6) and after a 24-h ultramarathon in women (5). Siracusa et al. (6) also found similar circulating myomiR levels between male and female rats in control conditions and in response to muscle damage. This is in accordance with the observations of Duttagupta et al. (34), who reported that myomiRs were not mentioned among miRNAs differently expressed between men and women. Second, as Ramos et al. (35) showed that certain circulating myomiRs can be affected by the duration and intensity of exercise, differences between participants could be a limitation. In the present study, the relative intensity and duration of the exercise could have been different between the participants, as 5 of the 19 participants performed the exercise to exhaustion and stopped before the 45-min limit. However, all participants performed the same exercise two wk later, allowing comparison of circulating myomiR levels under similar conditions. Third, the degree of adherence to the physical activity and nutritional guidelines was not assessed using a questionnaire, despite these factors could affect the myomiRs circulating levels. Therefore, the results could be influenced by the lack of adherence to these guidelines. Finally, as plasma miRNA level assessment is a lengthy process (~6 h from RNA extraction from the plasma to the end of RT-qPCR), this marker is not yet compatible with the clinical and/or sports context, in which it is important to predict long-lasting MVC torque loss (i.e., quantify EIMD) as early as possible. Therefore, further research is needed for the development of more rapid analytical methods that may be appropriate for point-of-care tests.

Conclusions

The levels of several myomiRs (hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, and hsa-miR-206) increased in response to loaded downhill walking, known to induce EIMD. The levels of two myomiRs (hsa-miR-1-3p and hsa-miR-133a-3p) measured 6 h postexercise increased less after *exercise bout 2* than *exercise bout 1*, suggesting that the levels of these circulating myomiRs could represent the adaptations conferred by the RBE, although these changes were not associated with functional adaptations of the neuromuscular system. The levels of several myomiRs (hsa-miR-1-3p, hsa-miR-133a-3p, and hsa-miR-206), CK activity, and Mb concentration measured at 6 h postexercise correlated with the long-lasting MVC torque deficit (>24 h) (i.e., the magnitude of EIMD), suggesting that myomiR levels were not better predictors of MVC torque reduction than conventional markers (e.g., CK and Mb). Finally, the combined measurement of certain myomiRs with myoglobin and creatine kinase could improve the prediction of long-lasting MVC torque loss. These results confirm that the RBE has an effect on myomiR levels. The mechanisms accounting for their limited release after the second bout of eccentric exercise are yet to be identified, as well as their functional relevance.

SUPPLEMENTAL DATA

Supplementary Spreadsheet S1: doi: <https://doi.org/10.5281/zenodo.6938518>.

Supplementary Table S1, doi: <https://doi.org/10.5281/zenodo.6458050>.

Supplementary Tables S2a and S2b: doi: <https://doi.org/10.5281/zenodo.6458054>.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

E.C., S.G., J.S., and V.M. conceived and designed research; E.C., S.G., J.S., C.B., S. Baugé, S. Bourdon, A.G., and B.L. performed experiments; E.C. and J.S. analyzed data; E.C., S.G., J.S., V.M., and K.C. interpreted results of experiments; E.C. and K.C. prepared figures; E.C. drafted manuscript; E.C., S.G., J.S., V.M., and S. Banzet edited and revised manuscript; E.C., S.G., J.S., V.M., K.C., C.B., S. Baugé, S. Bourdon, A.G., B.L., and S. Banzet approved final version of manuscript.

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