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► **To cite this version:**

Emeric Chalchat, Martin Vincent, Keyne Charlot, Cyprien Bourrilhon, Stéphane Baugé, et al.. Circulating microRNA levels after exercise-induced muscle damage and the repeated bout effect. *AJP - Regulatory, Integrative and Comparative Physiology*, 2022, 324 (1), pp.R58-R69. 10.1152/ajpregu.00096.2022 . hal-03888370v1

HAL Id: hal-03888370

<https://uca.hal.science/hal-03888370v1>

Submitted on 7 Dec 2022 (v1), last revised 28 Feb 2024 (v2)

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1 **Circulating microRNA levels after exercise-induced** 2 **muscle damage and the repeated bout effect**

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- 27 **Running title:** Circulating microRNA levels after EIMD

28 **Abstract**

29 **Introduction:** The neuromuscular system is able to quickly adapt to exercise-induced muscle
30 damage (EIMD), such that it is less affected by subsequent damaging exercise, a
31 phenomenon known as the repeated bout effect (RBE). Circulating muscle-specific
32 microRNAs (myomiRs) may be able to potentially predict the long-lasting maximal voluntary
33 contraction (MVC) torque deficit (>24h), an indicator of EIMD. We aimed to investigate: (1)
34 how plasma myomiR levels are modified by the RBE and (2) whether plasma myomiRs can
35 predict the long-lasting MVC torque deficit.

36 **Methods:** Nineteen participants performed two identical bouts of loaded downhill walking
37 separated by two weeks. MVC torque, creatine kinase (CK) activity, myoglobin (Mb)
38 concentration, and myomiR levels were measured before and up to 48h after exercise.
39 Correlation and multiple regression analyses were performed to assess the ability of these
40 markers to predict the largest MVC torque loss beyond 24h post-exercise.

41 **Results:** Similar to MVC torque, CK activity, and the Mb concentration, the relative
42 abundance of certain myomiRs (hsa-miR-1-3p, hsa-miR-133a-3p) were less affected after the
43 second bout of exercise relative to the first bout. The CK activity, Mb concentration and level
44 of several myomiRs (hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-206) correlated with long-
45 lasting MVC torque loss. Multiple regression showed that the best combination of markers
46 to predict the long-lasting deficit of MVC torque included several myomiRs, Mb, and CK.

47 **Conclusion:** Certain myomiR levels increased less after exercise bout 2 than after exercise
48 bout 1, indicating the presence of the RBE. The measurement of myomiR levels in
49 combination with Mb concentrations and CK activity could improve the prediction of the
50 long-lasting MVC torque deficit.

51 **Keywords:** MyomiRs, creatine kinase, myoglobin, eccentric exercise, biomarkers

52 **New & Noteworthy**

53 The present study is the first to show that plasma myomiR levels can be modified by the RBE,
54 as their levels increased less after the second exercise bout relative to the first. This study is
55 also the first to suggest that myomiR levels could be used to partially predict MVC torque
56 loss at 24 h post-exercise (i.e., the magnitude of EIMD). Interestingly, the combined
57 measurement of certain myomiR levels with those of Mb and CK improved the predictive
58 value.

59 Introduction

60 Performing unaccustomed exercise may result in muscle damage, especially when
61 muscles are activated while lengthening (eccentric contractions). Exercise-induced muscle
62 damage (EIMD) results in the disruption of structural proteins in muscle fibers and/or
63 connective tissues, characterized by a long-lasting deficit (> 24 h) of force generating
64 capacity (1). In the clinical and/or sports context, it is important to monitor and accurately
65 quantify the magnitude of EIMD to apply a recovery strategy as early as possible and/or
66 modify training/rehabilitation programs. Long-lasting maximal voluntary contraction (MVC)
67 torque deficit (i.e., > 24 h) is generally considered to be a reliable and valid marker for the
68 magnitude of EIMD (1, 2). However, short-term (e.g. < 2 h) MVC torque loss does not provide
69 an accurate estimation of EIMD due to the confounding influence of muscle fatigue (2).
70 Therefore, increases in plasma levels of intramyocellular constituents (e.g., creatine kinase,
71 myoglobin) are often used to evaluate the magnitude of EIMD. However, the ability of these
72 markers to predict the magnitude of the long-lasting MVC torque deficit (i.e., magnitude of
73 EIMD) is limited (3). It is therefore necessary to search for alternative markers that increase
74 rapidly after the end of exercise (i.e., within the first hours) and accurately predict the
75 magnitude of MVC torque loss at 24 h post-exercise to adapt the recovery strategy and
76 training loads as soon as possible.

77 MicroRNAs (miRNAs), which are small noncoding RNAs involved in the post-
78 transcriptional regulation of gene expression, could potentially serve as alternative markers
79 of EIMD (4-6). Indeed, muscle-specific or muscle-enriched miRNAs (also called myomiRs),
80 such as miR-1, -133a, -133b, -208a, -208b, and -499a (7), can be actively secreted by or
81 passively leak out of injured myocytes and measured in the blood (5, 6). A large increase in

82 plasma myomiRs has been reported in response to various muscle damage models, such as
83 toxic muscle injury in rats (6) or after prolonged exercise, such as marathon (8-10) and ultra-
84 marathon runs (5). It has been found that the ability of myomiRs to distinguish damaged
85 from nondamaged muscle is better than that of creatine kinase (CK) (4, 6), suggesting that
86 myomiRs could more correctly diagnose muscle damage. However, the ability to predict the
87 magnitude of EIMD, as evaluated by the long-lasting MVC torque deficit, was not evaluated
88 in these studies.

89 Recently, we reported that myomiRs could better represent the magnitude of height
90 jump loss at 24 h post-exercise (i.e., magnitude of EIMD) than CK or myoglobin (Mb) after a
91 24-h ultramarathon in 11 elite runners (5). However, whether these observations from an
92 extreme ultra-endurance exercise can be transposed to the monitoring of milder muscle
93 damage in physical active people is unknown. An interesting paradigm to gain insight into
94 the accuracy of myomiRs to predict the long-lasting (> 24 h) MVC torque deficit is the
95 repeated bout effect (RBE) (11). The RBE refers to adaptations in which a single "damaging
96 exercise bout" (e.g., eccentrically-biased exercise) confers protection against EIMD from
97 subsequent bouts. Consequently, a repeat bout within the first weeks after the initial bout
98 results in reduced symptoms of EIMD (e.g., torque loss, CK activity, Mb concentration) and
99 enhanced recovery (11). Although it is known that a second eccentrically-biased exercise
100 bout can result in a reduced level of EIMD, it is not currently known if circulating myomiR
101 levels may be lower than after the first bout, thus potentially providing an alternative
102 marker for the RBE.

103 The aim of the present study was to examine the response of circulating myomiRs
104 after two identical bouts of eccentrically-biased exercise to investigate: (1) how plasma
105 myomiR levels are modified by the RBE and (2) whether plasma myomiR levels can predict

106 the long-lasting MVC torque deficit (> 24 h). We hypothesized that circulating myomiR levels
107 would: (1) increase less after the second bout than after the first of eccentric exercise and
108 (2) display a stronger association with MVC torque loss than conventional markers (i.e., CK
109 activity and Mb concentration).

110 **Materials and methods**

111 **Participants**

112 Nineteen young males (age: 31.3 ± 5.6 years, body mass: 78.0 ± 10.7 kg, height: 1.80
113 ± 0.06 m, body mass index: 24.0 ± 2.3 kg.m⁻², and fat mass: 16.0 ± 5.1 %) were fully informed
114 of the experimental procedures, gave their written informed consent, and agreed to
115 participate in this study. Participants performed regular activity (between 3 and 8 h.w⁻¹), had
116 no recent history of muscular, joint, or bone disorders, and did not take any medication that
117 could affect neuromuscular responses. None of them had experienced strenuous mountain
118 trekking and/or downhill walking and none had been involved in a resistance-training
119 program in the previous six months. Participants were instructed not to perform
120 unaccustomed activities or any interventions that could affect recovery, such as massage,
121 icing, nutritional supplementation, or nonsteroidal anti-inflammatory drugs, during the
122 experimental period. Breakfast was standardized throughout the experiment. Each
123 participant participated in an inclusion session, consisting of a complete medical
124 examination, including the collection of anthropometric data and complete familiarization
125 with the experimental procedures. The study was conducted in accordance with the
126 Declaration of Helsinki (12) and was approved by the Regional Ethics Committee (CPP Ile-de-
127 France 8, France, registration number: 2019-A01210-57, SMILE).

128 **Protocol**

129 Participants performed two bouts of forward downhill walking on a treadmill (pulsar
130 3p 4.0, HP cosmos, Warwickshire, UK) for 45 min while carrying a load equivalent to 30 % of
131 their body mass (carrying: a weighted vest of 10 % of the body mass and a backpack of 20 %
132 of the body mass) separated by two weeks (Figure 1). The treadmill gradient was set to -25
133 % and the velocity to 4.5 km.h⁻¹ and the participants were instructed to walk at their own
134 preferred stride length and frequency. This downhill walking exercise was performed by the
135 participants to induce EIMD especially on the quadriceps muscles, due to the high
136 proportion of active lengthening contractions. Five participants were unable to finish the
137 walking bout and performed the first exercise to exhaustion (mean ± SD: 30.2 ± 8.6 min).
138 These participants performed the same exercise for the same duration during the second
139 exercise. MVC torque was assessed before (PRE), and 24 and 48 h after the two bouts. Blood
140 samples were obtained at PRE, POST, and 2, 6, 24, and 48 h after the two exercise bouts.

141 **MVC torque**

142 MVC torque was measured using an isokinetic dynamometer (Cybex Norm, Lumex,
143 Ronkonkoma, NY, United States). Participants were comfortably positioned on an adjustable
144 chair with the hip joint flexed at 70° (0° = neutral supine position). The axis of rotation of the
145 dynamometer was aligned with the femoral condyles of the femur and the lever arm was
146 attached 1 to 2 cm above the malleolus with a Velcro strap. Measurements were taken from
147 the participant's right leg, which was fixed at 90° (0° = knee fully extended). Torque data
148 were corrected for gravity, digitized, and exported at a rate of 2 kHz to an external analog-
149 to-digital converter (Powerlab 16/35; ADInstrument, New South Wales, Australia) driven by
150 Labchart pro 8.1 software (ADInstrument, New South Wales, Australia). The participants

151 performed two isometric MVCs of the knee extensors with two minutes of rest and were
152 instructed to grip the lateral handles of the seat to stabilize the pelvis and strongly
153 encouraged by the investigator. The MVC torque was determined as the peak torque
154 reached during the maximal effort. This was continuously monitored during the
155 experiments. When the level of variability was higher than 5%, a third MVC was performed.
156 The best of the two/three values was used for further analysis. Before the MVCs, the
157 participants performed a warm-up consisting of 8 contractions at submaximal and
158 progressive intensities (2 contractions at 20 %, 40 %, 60 % and 80 % of their perceived
159 maximal voluntary contraction).

160 **Muscle Soreness**

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

165 **Biological measurements**

166 Blood was drawn from the antecubital vein into two separate tubes (5 mL, Becton
167 Dickinson vacutainer, Franklin Lakes, USA), one containing EDTA and the other being a
168 serum-separating tube (SST). Blood in the SST was allowed to clot at ambient temperature
169 for 30 min and centrifuged (2,000 x g, 4°C, 10 min). Serum from the STT and EDTA plasma
170 was aliquoted and frozen at -80°C for further analysis. Serum CK activity and Mb
171 concentration were analyzed using an autoanalyzer (Advia 1800; Siemens Healthineers,
172 Erlangen, Germany) and plasma miRNAs were analyzed by real-time quantitative PCR (RT-
173 qPCR).

174 **Plasma miRNA extraction**

175 Total RNA was extracted from plasma as previously described (4-6). Briefly, total RNA
176 was extracted from 200 μ l plasma using the mirVana PARIS Kit (Ambion, Austin, USA) and
177 low-binding tubes (1.5 ml; DNA LoBind Tubes, Eppendorf, Hamburg, Germany) according to
178 the manufacturer's protocol. An additional precipitation step was performed as follows.
179 Column elution was performed with 180 μ l sterile water. Then, 18 μ l 3M sodium acetate
180 (Sigma-Aldrich, Saint-Quentin-Fallavier, France), 396 μ l 100% ethanol, and 1 μ l GlycoBlue
181 (Ambion) were added. Tubes were vortexed and the RNA was allowed to precipitate for 20
182 min at -20°C . After centrifugation (12,000 g, 4°C , 10 min), the pellet was washed with 400 μ l
183 70 % ethanol and allowed to dry for 30 min. The RNA was finally suspended in 12 μ l RNase-
184 free water, incubated for 10 min at 50°C , and frozen at -80°C until cDNA synthesis.

185 **Reverse transcription**

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

189 **Real-time quantitative qPCR**

190 RT-qPCR was performed on a 96-well LightCycler 480 instrument (Roche Applied
191 Science, Mannheim, Germany) with 4 μ l cDNA (diluted 1:40 in sterile water), 1 μ l PCR primer
192 mix, and 5 μ l 2x miRCURY SYBR Green Master Mix (Qiagen) in a 10 μ l reaction volume. We
193 measured hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, hsa-miR-206, hsa-miR-208a-3p,
194 hsa-miR-208b-3p, hsa-miR-378a-3p, and hsa-miR-499a-5p. We chose these miRNAs due to
195 their muscle-specific expression (13) and their documented responses to toxic (6) and
196 exercise-related muscle damage (4, 5). The PCR protocol consisted of one activation cycle

197 (95°C, 2 min), 52 amplification cycles (denaturation 95°C, 10 s, and annealing/amplification
198 56°C, 1 min), and a melting curve analysis step, as recommended by the manufacturer.
199 Undetectable values were handled as described by De Ronde et al. (14) by adding one
200 quantification cycle (Cq) to the highest Cq observed for a miRNA.

201 The stability of reference miRNAs (hsa-miR-16-5p, hsa-miR-20a-5p, hsa-miR-21-5p,
202 hsa-miR-103a-3p, hsa-miR-185-5p, hsa-miR-192-5p, and hsa-miR-320a-3p) was assessed
203 using geNorm, BestKeeper, NormFinder, the Δ CT method, and RefFinder. GeNorm
204 determined that all reference miRNAs were necessary for optimal normalization of the
205 target miRNAs. Final quantification is expressed as arbitrary unit (AU) and consisted of the
206 geometrical mean of the quantification performed with each reference miRNA. qPCR primer
207 information is supplied for the target and reference miRNA in supplementary spreadsheet 1
208 (doi: <https://doi.org/10.5281/zenodo.6938518>).

209 **Index of protection**

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

220 **Statistical analysis**

221 Statistical analyses were performed to assess: (1) the changes between the PRE and,
222 POST, 2, 6, 24, and 48 h post-exercise for both downhill walking bouts and (2) the effect of
223 exercise (i.e., downhill walking bout). Due to the absence of a normal distribution according
224 to the Shapiro-Wilk test, we performed a non-parametric Friedman analysis of variance to
225 assess the main effect of time on muscle soreness, miRNA, CK activity, and Mb concentration
226 levels. When a significant main effect was identified, Dunn's multiple comparisons were
227 used to identify which time points were significantly different to baseline for these variables.
228 Statistical differences between matched time points (i.e., exercise 1 vs. exercise 2) were
229 assessed using Mann-Whitney U test. Each p-value was adjusted to account for multiple
230 comparisons. MVC torque results were analyzed by two-way ANOVA (time × exercise) with
231 repeated measures for paired values. When an interaction between time and exercise was
232 found by the two-way ANOVA, Holm post-hoc tests were applied to test for differences
233 between the means. All time points (24 and 48 h) were compared to PRE and matched time
234 points were compared between exercise 1 and exercise 2 (e.g. 24 h after the exercise 1 vs.
235 24 h after the exercise 2).

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

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

261 **Results**

262 **PRE exercise measurements**

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

265 **MVC torque**

266 A significant interaction (time \times exercise, $p < 0.001$) was found for MVC torque
267 (Figure 2). MVC torque was below the baseline value up to 48 h after exercise bout 1,
268 whereas no significant changes were observed the days after the second bout of exercise
269 (Figure 2).

270 **Muscle soreness**

271 A significant effect of time was found for muscle soreness ($p < 0.001$). Indeed, muscle
272 soreness was increased after the first exercise (baseline: 2 ± 6 mm) at 24 h (46 ± 22 mm; $p <$
273 0.001) and 48 h post-exercise (53 ± 26 mm; $p < 0.001$). Similarly, muscle soreness was
274 increased after the second exercise (baseline: 2 ± 9 mm) at 24 h (21 ± 16 mm; $p < 0.001$) and
275 48 h post-exercise (18 ± 19 mm; $p < 0.001$). However, muscle soreness was significantly
276 lower after the second bout of exercise than after the first at 24 h ($p < 0.001$) and 48 h ($p <$
277 0.001) post-exercise.

278 **Circulating miRNA levels**

279 A significant effect of time was found for hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-
280 133b, and hsa-miR-206 levels ($p < 0.001$ for all these myomiRs) (Figure 3) but not for those of
281 hsa-miR-208a-3p (Exercise 1: $p = 0.321$; Exercise 2: $p = 0.908$), hsa-miR-208b-3p (Exercise 1 :
282 $p = 0.475$; Exercise 2: $p = 0.553$), hsa-miR-378-3p (Exercise 1: $p = 0.333$; Exercise 2: $p =$
283 0.823), or hsa-miR-499a-5p (Exercise 1: $p = 0.062$; Exercise 2: $p = 0.228$) (Supplementary
284 Table 1, doi: <https://doi.org/10.5281/zenodo.6458050>). After the first bout of exercise, the
285 levels were significantly higher for hsa-miR-1-3p (Figure 3a), hsa-miR-133a-3p (Figure 3b),
286 hsa-miR-133b (Figure 3c), and hsa-miR-206 (Figure 3d) after 2 and 6 h. After the second bout
287 of exercise, the levels were significantly higher for hsa-miR-206 (Figure 3d) at POST, for hsa-
288 miR-1-3p (Figure 3a) and hsa-miR-133b (Figure 3c) after 2 h, and for hsa-miR-133a-3p (Figure

289 3b) and hsa-miR-133b (Figure 3c) after 6 h. Moreover, the levels were significantly lower for
290 hsa-miR-1-3p (Figure 3a) and hsa-miR-133a-3p (Figure 3b) 6 h after the second bout of
291 exercise than after the first.

292 **Creatine kinase activity**

293 A significant effect of time was found for CK activity ($p < 0.001$). Indeed, CK activity
294 was increased from 2 h to 48 h after both exercises. CK activity was significantly lower 6 h,
295 24 h and 48 h after the second bout of exercise than after the first (Figure 4a).

296 **Myoglobin concentration**

297 A significant effect of time was found for Mb concentration ($p < 0.001$). Indeed, the
298 Mb concentration was significantly higher up to 6 h after both exercises (Figure 4b). The Mb
299 concentration was significantly lower 2 and 6 h after the second bout of exercise than after
300 the first (Figure 4b).

301 **Correlation between changes in myomiR levels from PRE exercise 1 and the** 302 **index of protection for MVC torque**

303 There was no correlation between peak changes in myomiR levels from PRE exercise
304 1 and the index of protection for MVC torque (Supplementary Table 2a, doi:
305 <https://doi.org/10.5281/zenodo.6458054>). Similarly, there was no correlation between
306 changes in myomiR levels from PRE exercise 1 to PRE exercise 2 and the index of protection
307 for MVC torque (Supplementary Table 2b, doi: <https://doi.org/10.5281/zenodo.6458054>).

308 **Correlation between CK activity, Mb concentration, and circulating myomiR** 309 **levels**

310 Peak changes in CK activity and Mb concentration significantly correlated with peak
311 changes in hsa-miR-1-3p (CK: $p < 0.001$, $\rho = 0.54$; Mb: $p = 0.005$, $\rho = 0.50$), hsa-miR-133a-3p
312 (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:
313 $p = 0.056$, $\rho = 0.35$), and hsa-miR-206 (CK: $p = 0.03$, $\rho = 0.35$; Mb: $p = 0.007$, $\rho = 0.48$).

314 **Correlation between markers and long-lasting (> 24 h) MVC torque loss**

315 There was no correlation between long-lasting (> 24 h) MVC torque loss and CK
316 activity (Figures 5a and 5c) or miRNA levels measured POST and 2 h post-exercise, whereas
317 there was a significant correlation with Mb concentration at 2 h post-exercise (Figure 5d).
318 Moreover, there was a significant correlation between long-lasting MVC torque loss and the
319 measurements at 6 h post-exercise for CK activity (Figure 5e), Mb concentration (Figure 5f),
320 and hsa-miR-1-3p (Figure 6a), hsa-miR-133a-3p (Figure 6b; nearly significant, $p = 0.053$), and
321 hsa-miR-206 (Figure 6d) levels. However, there was no significant correlation between long-
322 lasting MVC torque loss and hsa-miR-133b (Figure 6c), miR-208a, hsa-miR-208b, hsa-miR-
323 378, or hsa-miR-499a-5p levels (Table 2). At POST, multiple linear regression showed the
324 best combination of markers included: Mb, CK, hsa-miR-1-3p, hsa-miR-133b, and hsa-miR-
325 206 ($r^2: 0.485$, $p < 0.001$). The best combination included Mb and CK at 2 h post-exercise (r^2 :
326 0.440 , $p < 0.001$), whereas the combination of Mb and hsa-miR-1-3p ($r^2: 0.300$, $p = 0.002$)
327 was better at 6 h post-exercise.

328 **Discussion**

329 The main purpose of this study was to investigate (1) how plasma myomiR levels are
330 modified by the RBE and (2) whether plasma myomiR levels can predict the long-lasting
331 deficit (> 24 h) of MVC torque (i.e., the magnitude of EIMD). We observed an increase in

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

339 **Circulating levels in response to downhill walking exercises**

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

347 **Circulating miRNA levels in response to the first bout of eccentric exercise**

348 We found hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, and hsa-miR-206 to
349 increase after exercise bout 1. These results are consistent with those of several studies (4,
350 5, 8, 9, 19), which found an increase in the level of these myomiRs after downhill walking or
351 a marathon or ultra-marathon run. Conversely, we did not observe significant increases in
352 the hsa-miR-208a-3p, hsa-miR-208b-3p, hsa-miR-378-3p, or hsa-miR-499a-5p levels in the
353 present study, whereas their circulating levels were shown to increase after backward
354 downhill walking (4), marathon (8, 9) and/or ultra-marathon runs (5). Discrepancies between

355 circulating myomiR profiles following a session of eccentric exercise have already been
356 reported (4, 9) and could involve several factors. One possible explanation is that the
357 observed discrepancies are due to differences in basal circulating levels between miRNAs, as
358 previously suggested (4, 5). Here, we found that the circulating levels of certain myomiRs
359 (hsa-miR-208a-3p, hsa-miR-208b-3p and hsa-miR-499a-5p) were too low to be accurately
360 detected both at baseline levels and in response to moderate damaging exercise. Among the
361 228 samples (12 time points for 19 subjects), miRNAs were detected in 223 for hsa-miR-1-3p
362 (97.8 %), 207 for hsa-miR-133a-3p (90.8 %), 213 for hsa-miR-133b (93.4 %), 188 for hsa-miR-
363 206 (82.5 %), 30 for hsa-miR-208a-3p (13.2 %), 43 for hsa-miR-208b-3p (18.9 %), 218 for hsa-
364 miR-378-3p (95.6 %), and 104 for hsa-miR-499a-5p (45.6 %). This result is consistent with
365 those of several studies (4, 20), such as that of Banzet et al. (4), which reported low levels of
366 hsa-miR-208a-3p (not detectable), hsa-miR-208b-3p (not detectable in 6 of 9 participants),
367 and hsa-miR-499a-5p after 30 min of a downhill walking exercise. A second factor that could
368 explain the observed differences in circulating profiles after EIMD may be linked to the
369 differential expression of myomiRs according to the muscle type, as well as skeletal muscle
370 fiber contractile phenotype. Indeed, hsa-miR-208a-3p is specific to cardiac muscle,
371 explaining why we did not find a significant increase in this myomiR after the bouts of
372 downhill walking, in which a low level of cardiac stress is expected. Interestingly, the two
373 participants who showed the largest increases in hsa-miR-208a-3p levels in the present
374 study were among the five participants who performed the exercise to exhaustion,
375 suggesting that this myomiR may be an indicator of cardiac stress. However, as this was not
376 the aim of the present study, we neither evaluated cardiac function nor measured other
377 blood markers of cardiac stress. Hsa-miR-499a-5p has been shown to be more highly
378 expressed in type I than type II fibers (6). As type I fibers have been shown to be less

379 susceptible to EIMD than type II fibers (21), this could partially explain why hsa-miR-499a-5p
380 levels did not increase after the downhill walking exercise in the present study. Hsa-miR-378-
381 3p levels did not significantly increase after downhill walking in the present study. This
382 miRNA is not considered to be a myomiR but is among the most highly expressed miRNAs in
383 muscle tissue (7, 22) and increases in its blood levels have been consistently reported after
384 toxic muscle injury in the rat (6) or an ultra-marathon run (5) and in various animal models of
385 Duchenne muscular dystrophy (DMD) (23-25). As hsa-miR-378-3p was already abundant in
386 the plasma in the control condition (e.g., healthy organism at rest), it is possible that any
387 muscle damage induced in the present study was not sufficient to raise the circulating level
388 above the “background noise”.

389 **Comparison of circulating miRNA levels between exercise bouts 1 and 2**

390 Among the myomiRs that showed an increase in the level after the first bout of
391 exercise (hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, and hsa-miR-206), only hsa-miR-1-
392 3p and hsa-miR-133a-3p showed a differential profile, i.e., smaller increase, after the
393 second. The fact that we observed different circulating profiles among miRNAs in response
394 to one or two bouts of eccentric exercise is striking and could be due to a combination of
395 several factors. Interestingly, we observed that the peak changes in CK activity and Mb
396 concentration correlated moderately with peak changes in hsa-miR-1-3p, hsa-miR-133a-3p,
397 hsa-miR-133b, and hsa-miR-206 levels. Although still speculative, such a relationship
398 suggests that these myomiRs may share, at least partially, mechanisms of release to those of
399 CK and Mb (i.e., passive release). However, it is likely that this hypothesis cannot fully
400 explain the observed differences in circulating profiles reported here. First, it cannot be
401 excluded that the first bout of eccentric exercise led to modifications of the abundance of

402 myomiRs in the skeletal muscle tissue, as they are known to be involved in muscle
403 homeostasis and regeneration (26, 27). Despite the fact that no study has evaluated the
404 effect of an acute bout of eccentric exercise on muscle myomiR levels, several authors (28,
405 29) have shown that an acute bout of resistance exercise can reduce the expression of miR-
406 1, miR-133a, and miR-133b in skeletal muscle tissue 2 to 6 h post-exercise. Whether such
407 modifications are transient or persist for a longer period of time and whether this could
408 result in variations in their circulating levels is unknown. Circulating levels of members of the
409 let-7 miRNA family, abundantly expressed in muscle tissue, did not increase in response to
410 myofiber damage (24-26, 30), suggesting that active secretion mechanisms are involved. In a
411 recent review, Coenen-Stass et al. (30) discussed the clinical utility of extracellular miRNAs as
412 biomarkers of DMD, as well as their possible contribution to muscle physiology. These
413 authors proposed that the release of miRNAs in response to muscle damage may be due to a
414 combination of passive leakage resulting from myonecrosis and the impairment of the
415 sarcolemma, as well as an active secretion process that could accompany muscle
416 differentiation and regeneration. Interestingly, pioneering *in vitro* studies have shown that
417 miR-133a, miR-133b, miR-206, and miR-378 are actively released in extracellular vesicles
418 from myotubes (31) and myogenic progenitor cells (32) and may regulate myoblast
419 differentiation and extracellular matrix remodeling. Interestingly, these physiological
420 mechanisms have been identified to contribute to the RBE (11). Despite the different
421 context, we believe that the EIMD induced in our experimental model may share some of
422 these release mechanisms.

423 In an attempt to evaluate whether the magnitude of changes in circulating miRNA
424 levels after exercise could be an indicator of the functional adaptations induced by the RBE,
425 we searched for correlates between peak changes in acute (< 48 h) and long-term (2 weeks;

426 PRE exercise 2) circulating myomiR levels and index of protection for torque change, but
427 failed to observe a significant correlation. Despite these negative results, a role for
428 circulating myomiRs in RBE adaptations cannot be excluded, as their abundance in the blood
429 compartment depends on complex and interrelated factors, such as varying half-lives (33),
430 kinetics, and mechanisms of release. Thus, it cannot be excluded that myomiRs may play a
431 role in RBE adaptations. However, further research is needed to obtain evidence to directly
432 support this hypothesis.

433 **Association between EIMD markers and long-lasting MVC torque loss**

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

448 concomitant measurement of circulating myomiR levels, Mb concentrations, and CK activity
449 could improve the diagnosis of EIMD.

450 **Perspectives and significance**

451 Circulating myomiRs may have a future interest in the diagnosis of muscle damage
452 both in sport medicine and in the monitoring of physical activity. However, further research
453 is needed for a better understanding of the release mechanisms and intercellular
454 communication roles of these circulating miRNAs. Moreover, the best combination of
455 markers to predict muscle damage should be determined in various exercises.

456

457 **Limitations**

458 Our study had several limitations. First, we included only men, limiting any
459 conclusions for women. However, our previous work has shown that myomiR levels increase
460 after toxic muscle injury in female rats (6) and after a 24-h ultra-marathon in women (5).
461 Siracusa et al. (6) also found similar circulating myomiR levels between male and female rats
462 in control conditions and in response to muscle damage. This is in accordance with the
463 observations of Duttagupta et al. (34), who reported that myomiRs were not mentioned
464 among miRNAs differently expressed between men and women. Second, as Ramos et al. (35)
465 showed that certain circulating myomiRs can be affected by the duration and intensity of
466 exercise, differences between participants could be a limitation. In the present study, the
467 relative intensity and duration of the exercise could have been different between the
468 participants, as five of the 19 participants performed the exercise to exhaustion and stopped
469 before the 45-min limit. However, all participants performed the same exercise two weeks
470 later, allowing comparison of circulating myomiR levels under similar conditions. Third, the

471 degree of adherence to the physical activity and nutritional guidelines was not assessed
472 using a questionnaire, despite these factors could affect the myomiRs circulating levels.
473 Therefore, the results could be influenced by the lack of adherence to these guidelines.
474 Finally, as plasma miRNA level assessment is a lengthy process (~6 h from RNA extraction
475 from the plasma to the end of RT-qPCR), this marker is not yet compatible with the clinical
476 and/or sports context, in which it is important to predict long-lasting MVC torque loss (i.e.,
477 quantify EIMD) as early as possible. Therefore, further research is needed for the
478 development of more rapid analytical methods that may be appropriate for point-of-care
479 tests.

480 **Conclusion**

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

494 after the second bout of eccentric exercise are yet to be identified, as well as their functional
495 relevance.

496 **Acknowledgments**

497 We would like to thank Phillippe Colin, Caroline Dussault, Walid Bouaziz, Pierre-Emmanuel
498 Tardo-Dino, Olivier Nespoulous, and Alexandra Malgoyre for their technical and medical
499 support during the data collection and the IRBA Biological Analyses and Molecular Biology
500 Units for their technical support for the data analysis.

501 **Disclosures**

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

503 **Authors' contributions**

504 EC, JS, SGV, and VM designed the study and drafted the manuscript. EC, JS, BL, and SGV
505 collected the data. EC, JS, SBau, SBo, AG, and BL participated in the data analysis. All the
506 authors contributed to the interpretation of the data and editing of the manuscript.

507 **Tables**

508 **Table 1.** Absolute PRE values of Exercise bout 1 and Exercise bout 2 for all variables. MVC:
 509 maximal voluntary torque, hsa: homo sapiens, CK: creatine kinase, Mb: myoglobin. Data are
 510 expressed as mean ± SD. Results with a P-value < 0.05 were considered significant.

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)	0.45 ± 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

511 **Table 2.** Correlations between the largest maximal voluntary contraction torque loss
 512 measured beyond 24 h after the end of the exercises and homo sapiens (hsa)-miR-1-3p, hsa-
 513 miR-133a-3p, hsa-miR-133b, hsa-miR-206, hsa-miR-208a-3p, hsa-miR-208b-3p, hsa-miR-378-
 514 3p, and hsa-miR-499a-5p levels, creatine kinase (CK) activity, and myoglobin (Mb)
 515 concentration. Significance (p) and Spearman rank correlation coefficient (ρ) values are
 516 shown. Results with a P-value < 0.05 were considered significant and are shown in bold.

	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

517 **Figure legends**

518 **Figure 1.** Design of the study. Participants performed two bouts of downhill walking on a
519 treadmill for 45 min while carrying a load equivalent to 30% of their body mass (treadmill
520 gradient: -25%, speed: 4.5 km.h⁻¹) separated by two weeks (left). Measurements (right) were
521 assessed at various time points as shown on the timeline (top).

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

527 **Figure 3.** Time course of changes in (a) homo sapiens (hsa)-miR-1-3p, (b) hsa-miR-133a-3p,
528 (c) hsa-miR-133b, and (d) hsa-miR-206 levels from PRE to 48 h after exercise bout 1 (PRE (1),
529 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),
530 6 h (2), 24 h (2), and 48 h (2)). Data are expressed as relative abundance arbitrary units (AU).
531 Significant differences from the PRE value are indicated: *p < 0.05, **p < 0.01, and ***p <
532 0.001. Significant difference between exercise bout 1 and exercise bout 2: [§]p < 0.05. These p-
533 values were extracted from Friedman test and Dunn's multiple comparisons (n=19). Data are
534 expressed as mean ± SD.

535 **Figure 4.** Time course of changes in (a) creatine kinase (CK) activity and (b) Myoglobin (Mb)
536 concentration from PRE to 48 h after exercise bout 1 (PRE (1), POST (1), 2 h (1), 6 h (1), 24 h
537 (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
538 (2)). Data are expressed as relative abundance arbitrary units (AU). Significant differences

539 from the PRE value are indicated: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Significant
540 differences between exercise bout 1 and exercise bout 2 are indicated: $^{\$}$ $p < 0.01$ and $^{\$ \$}$ $p <$
541 0.001. These p-values were extracted from Friedman test and Dunn's multiple comparisons
542 ($n=19$). Data are expressed as mean \pm SD.

543 **Figure 5.** Correlations between the largest maximal voluntary contraction (MVC) torque loss
544 measured beyond 24 h after the end of exercise and creatine kinase (CK) activity measured
545 at POST (a), 2 h (c), and 6 h (e) after exercise and myoglobin (Mb) concentration measured at
546 POST (b), 2 h (d), and 6 h (f) after the exercises. Significance (p) and Spearman rank
547 correlation coefficient (ρ) values are presented ($n=19$).

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

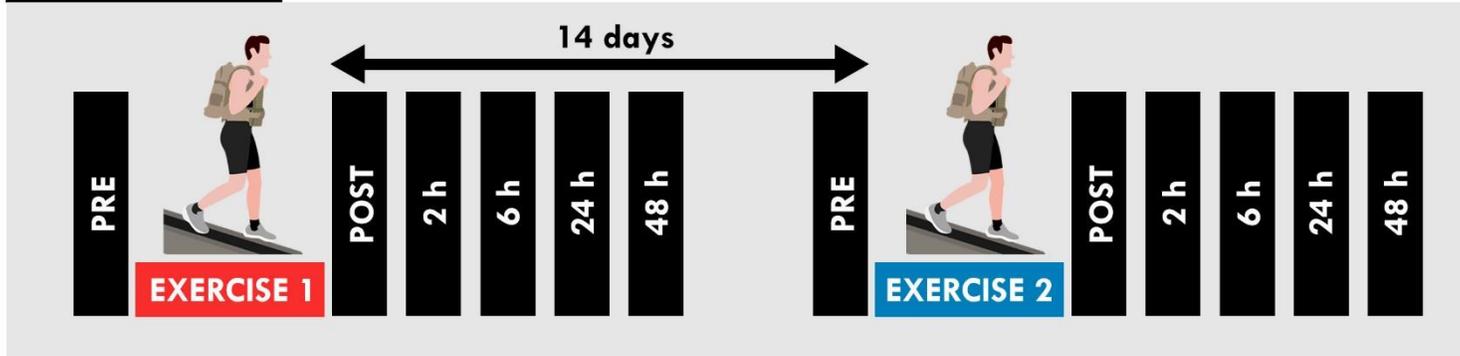
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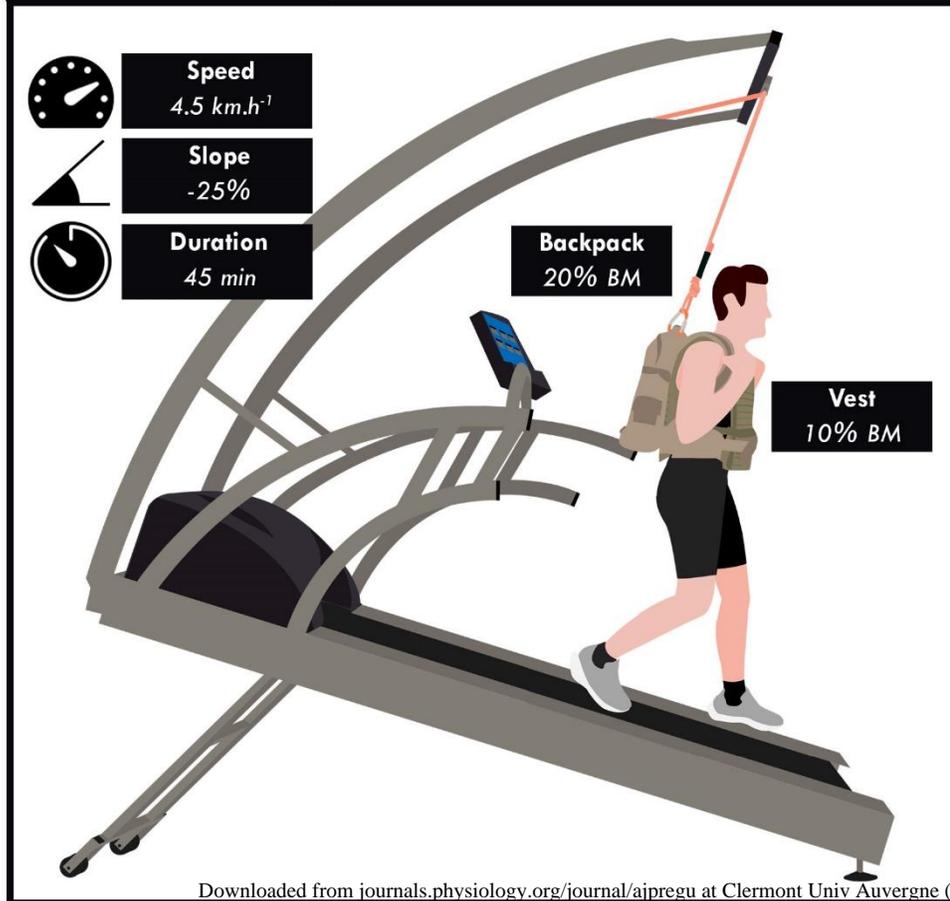
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PROTOCOL

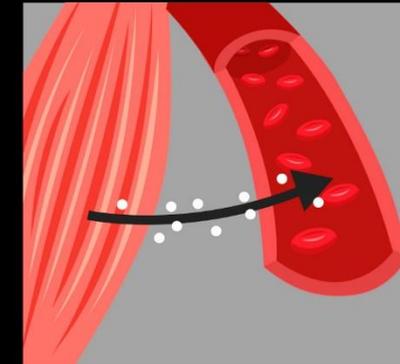


EXERCISE 1 & 2

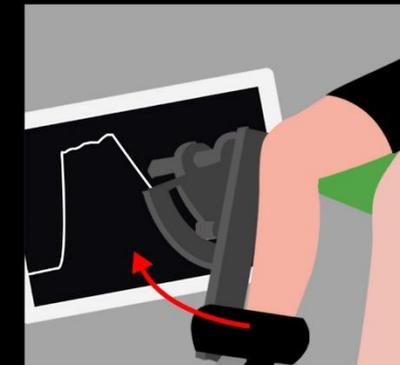


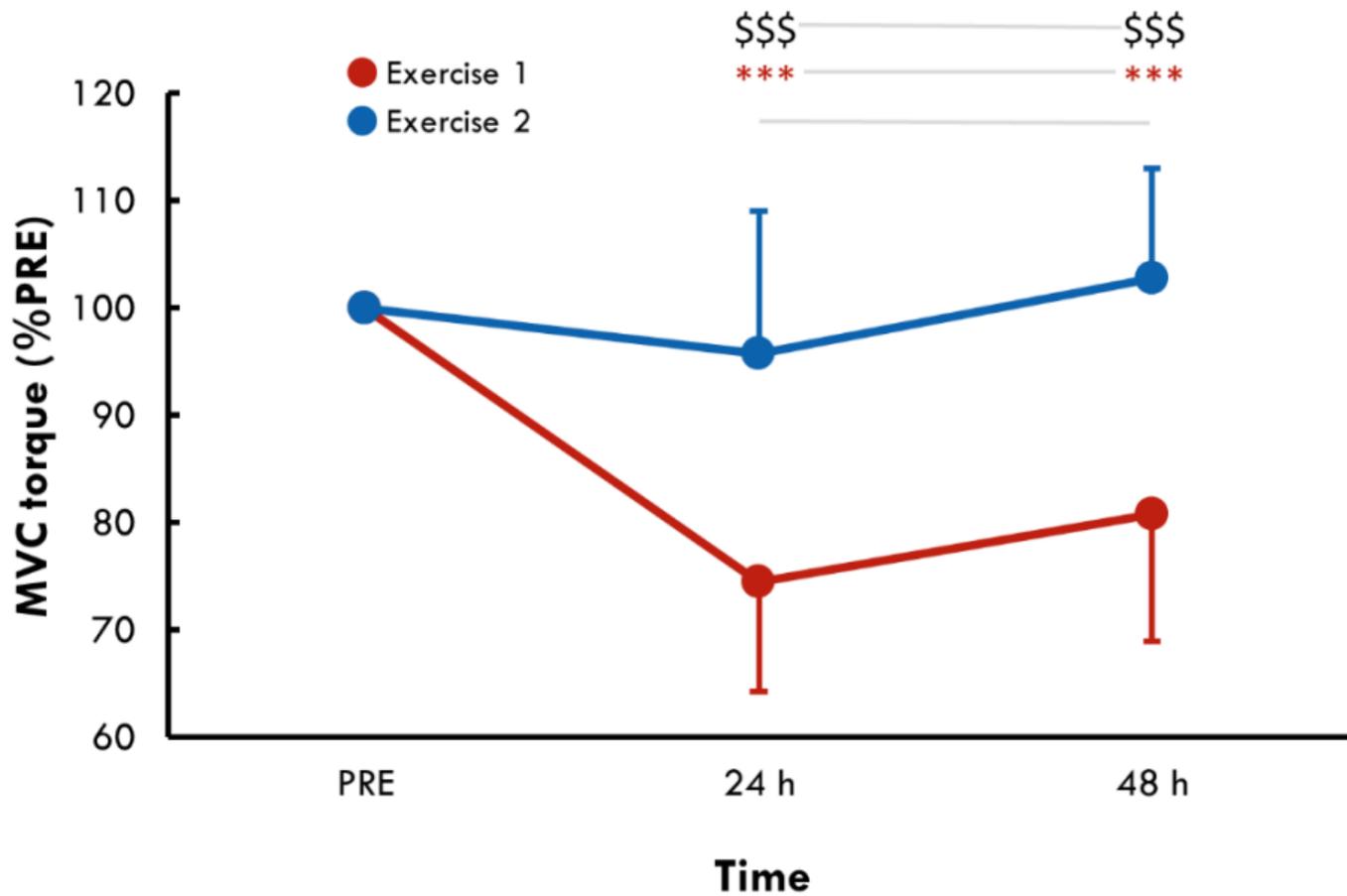
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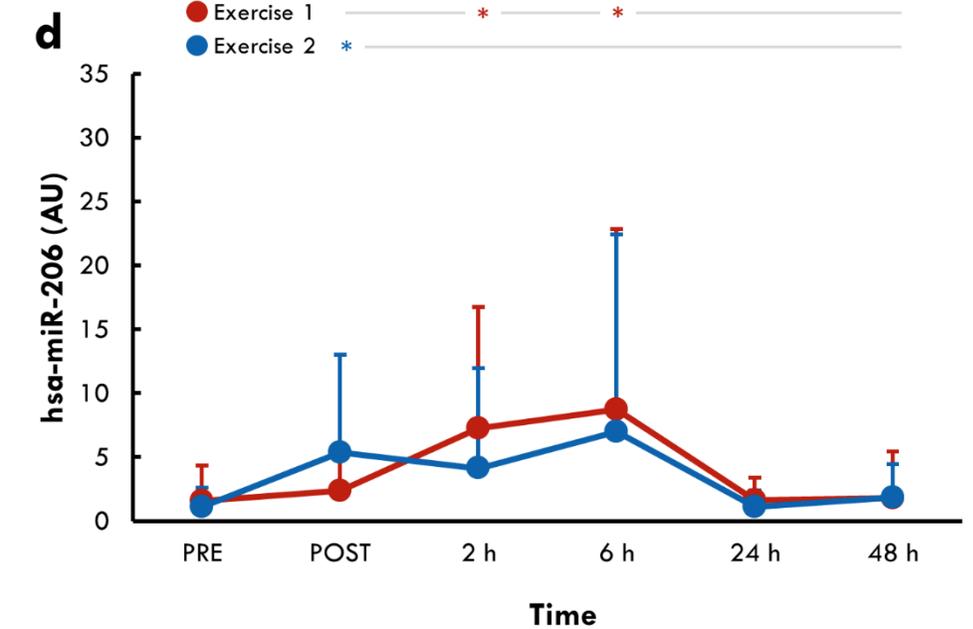
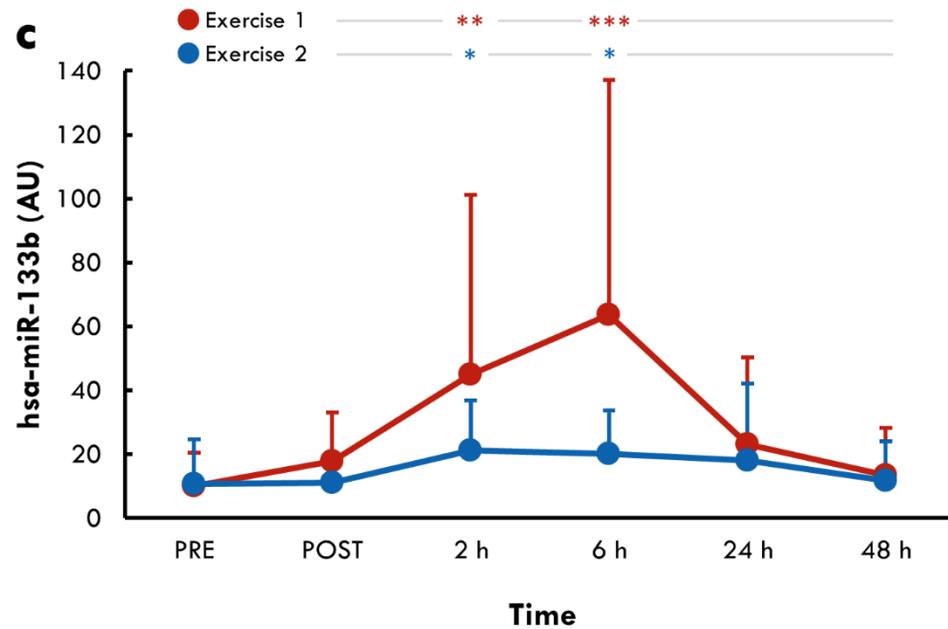
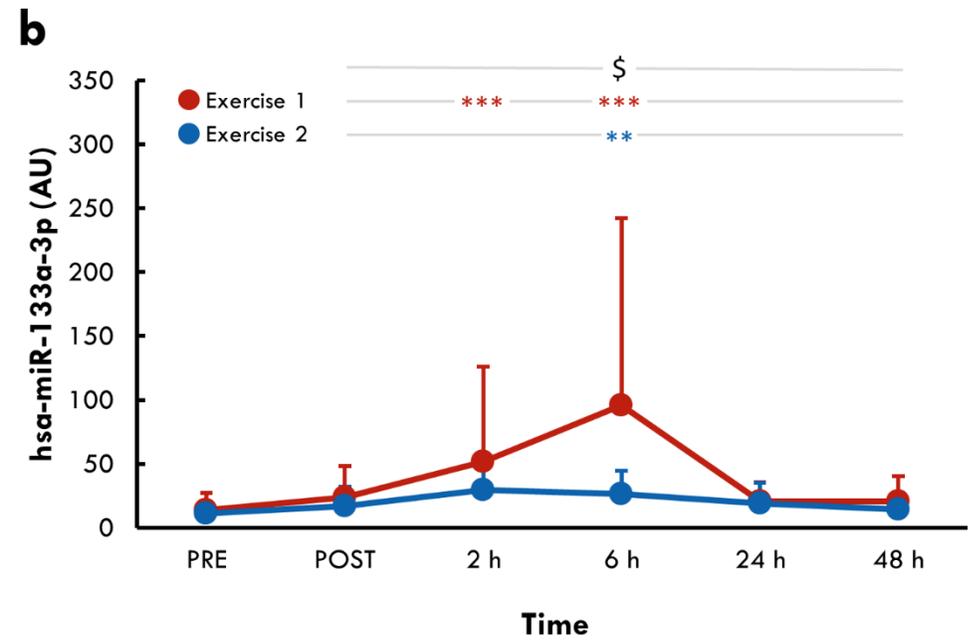
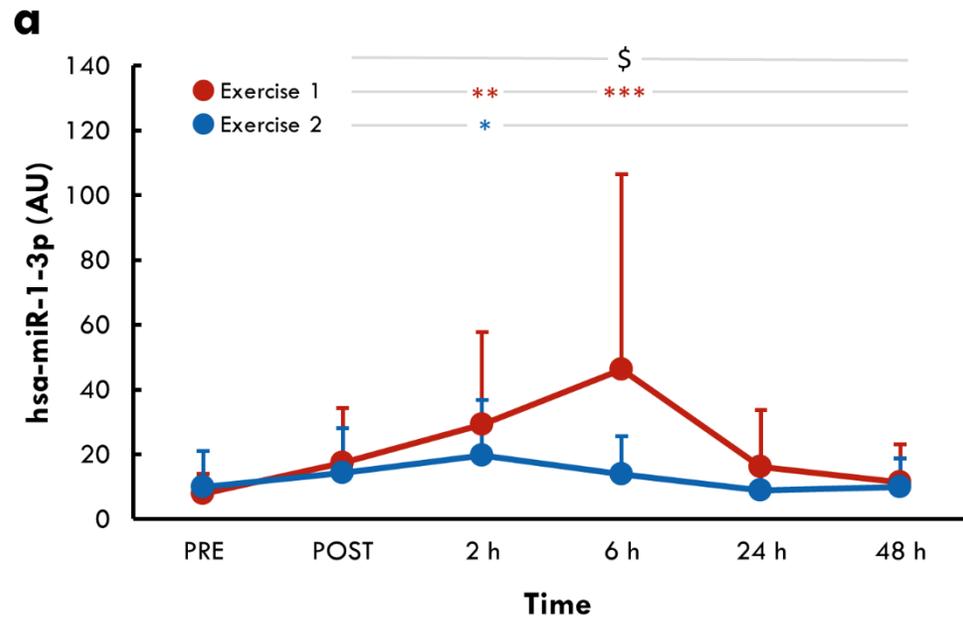
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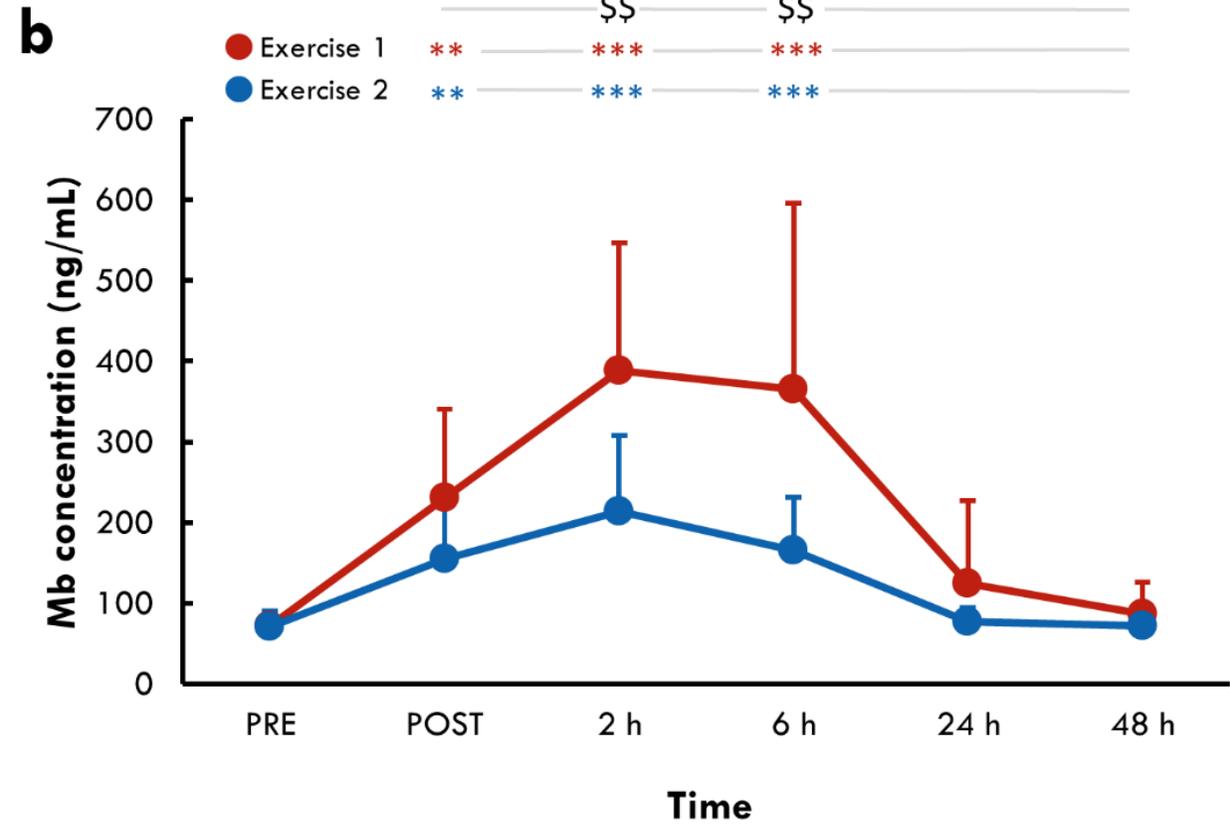
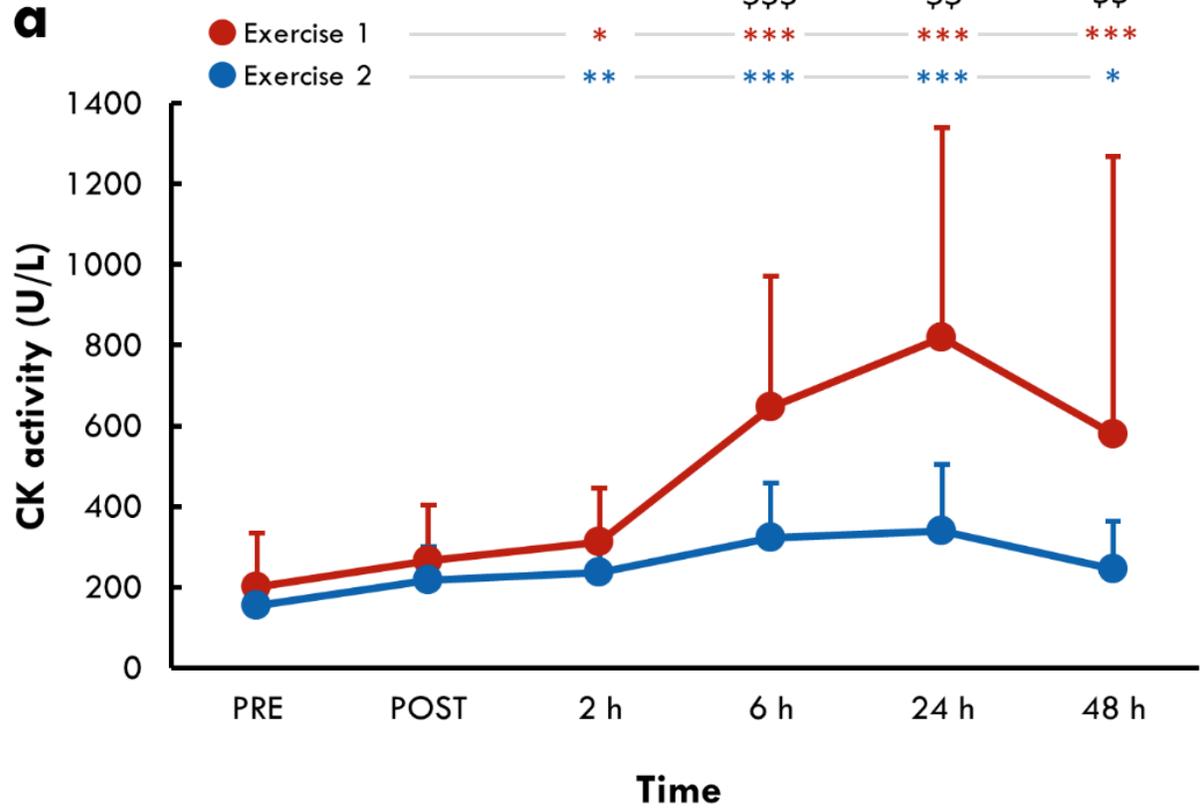


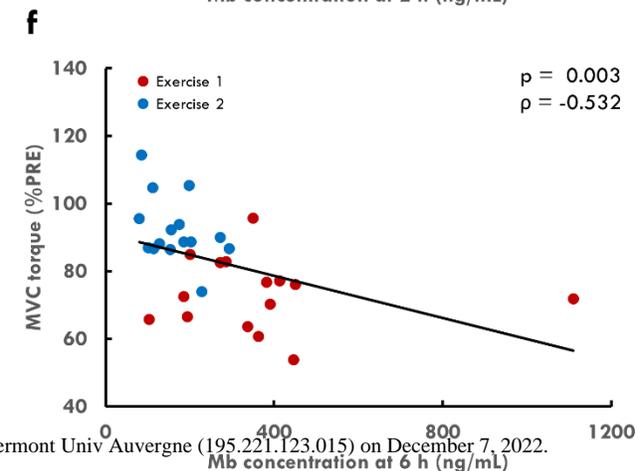
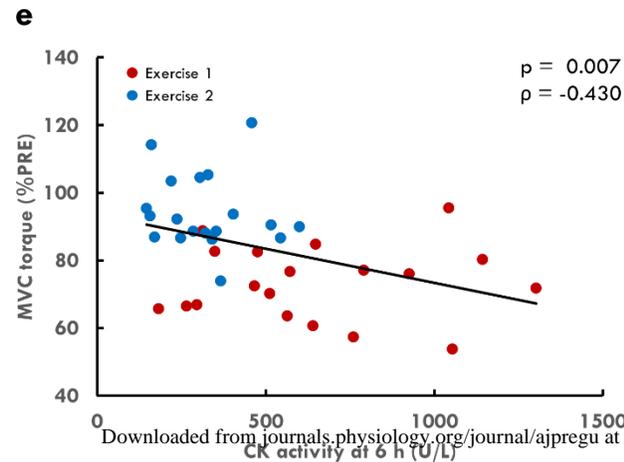
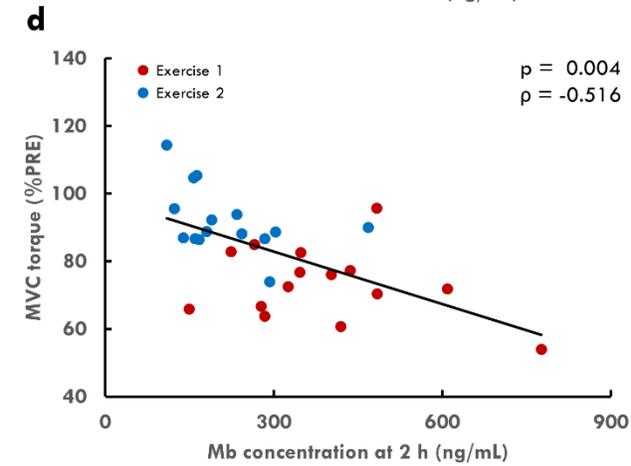
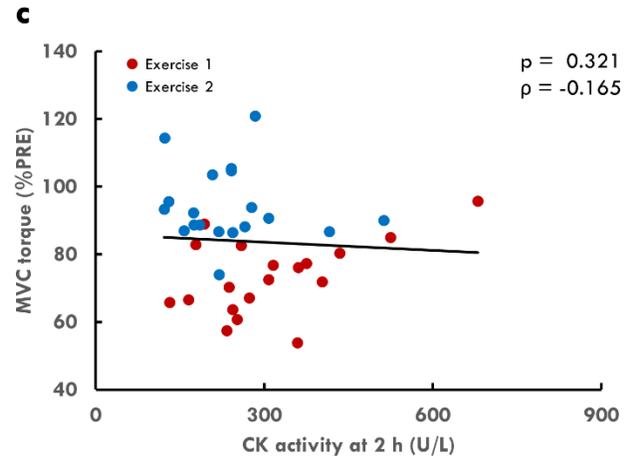
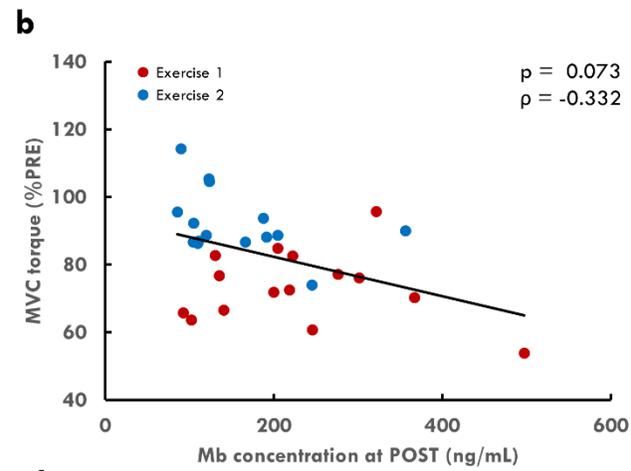
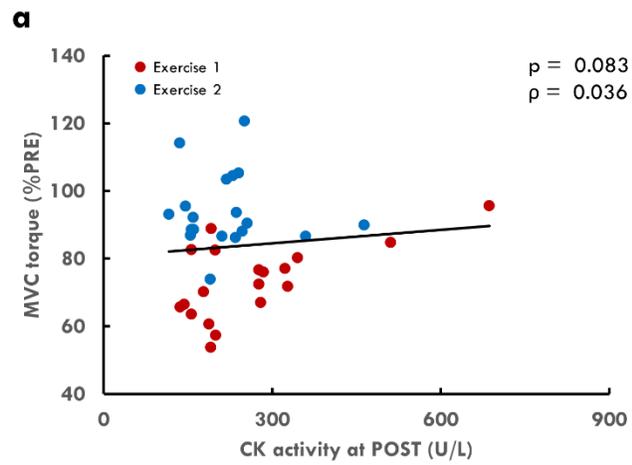
MAXIMAL VOLUNTARY CONTRACTION

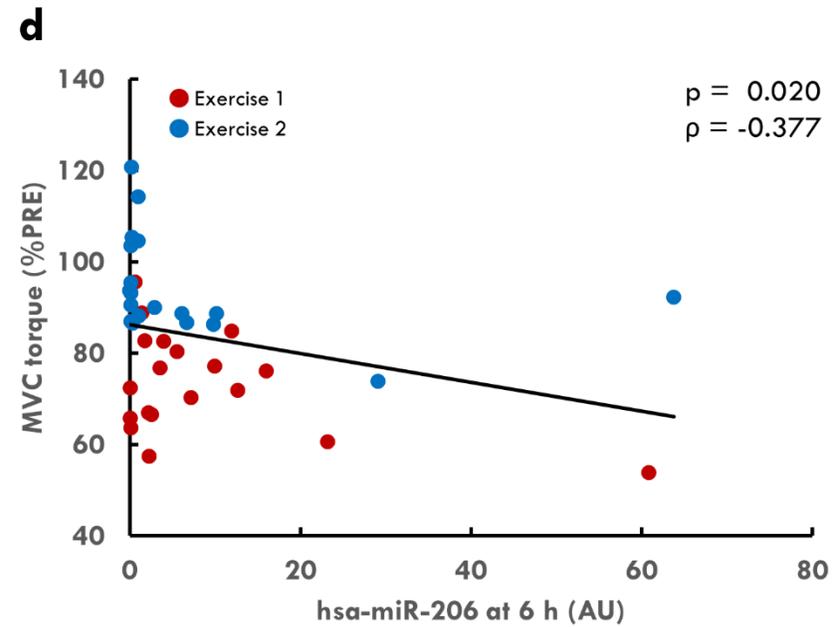
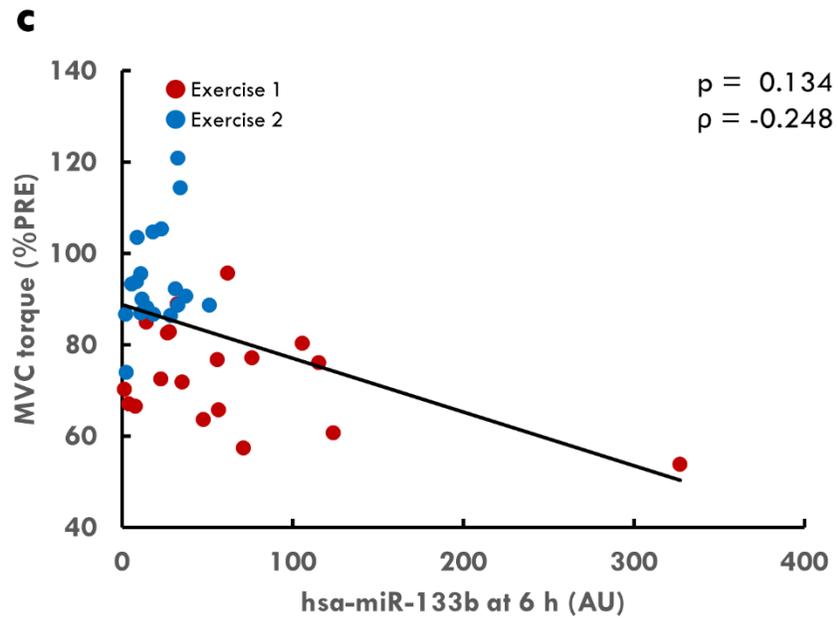
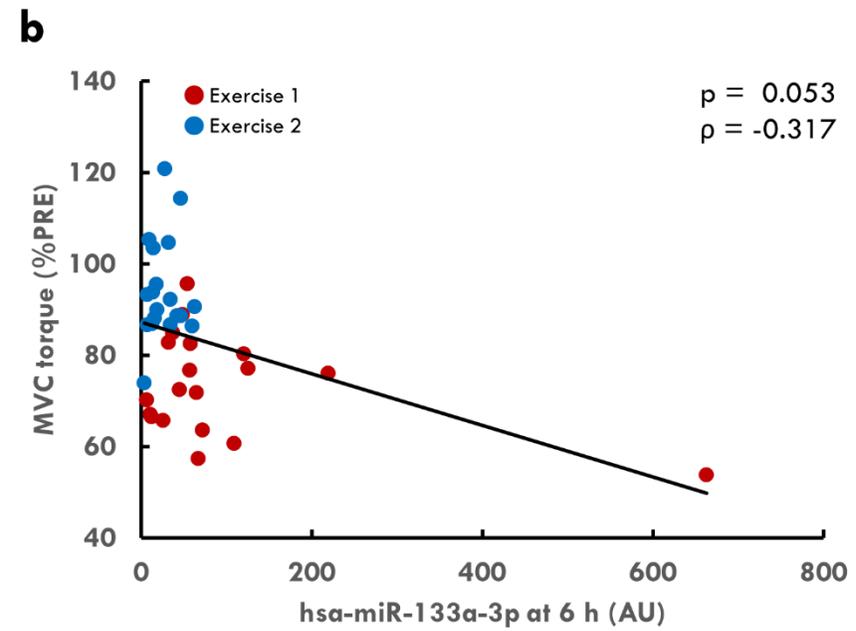
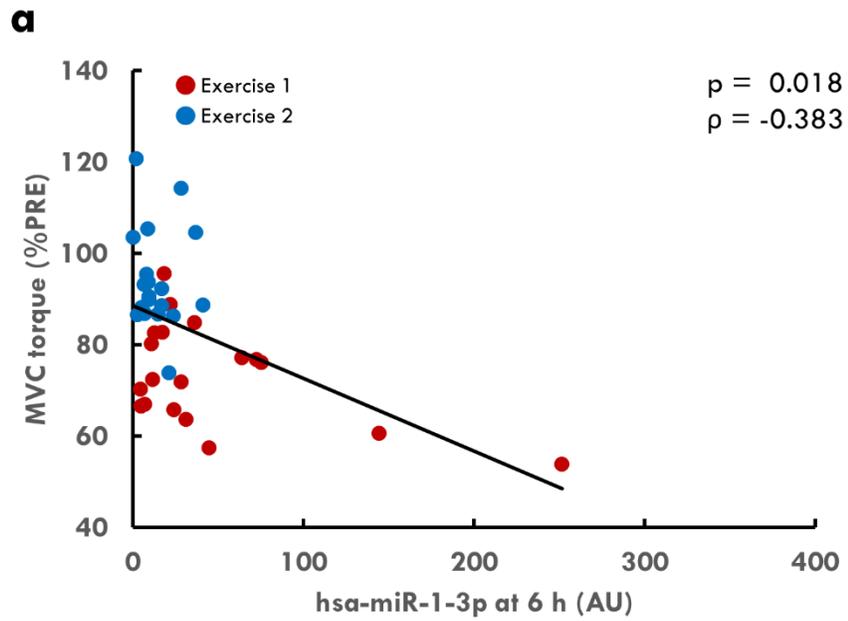












Absolute PRE values	Exercise bout 1	Exercise bout 2	p
MVC torque (N.m)	312 ± 51	311 ± 67	0.98
hsa-miR-1-3p (AU)	7.9 ± 6.2	10.0 ± 10.9	0.95
hsa-miR-133a-3p (AU)	14.1 ± 13.0	11.3 ± 9.0	0.70
hsa-miR-133b (AU)	10.0 ± 10.4	10.6 ± 14.0	0.79
hsa-miR-206 (AU)	1.55 ± 2.78	1.09 ± 1.53	0.98
hsa-miR-208a-3p (AU)	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)	0.45 ± 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

Table 1. Absolute PRE values of Exercise bout 1 and Exercise bout 2 for all variables. MVC: maximal voluntary torque, hsa: homo sapiens, CK: creatine kinase, Mb: myoglobin. Data are expressed as mean ± SD. Results with a P-value < 0.05 were considered significant.

	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

Table 2. 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. Significance (p) and Spearman rank correlation coefficient (ρ) values are shown. Results with a P-value < 0.05 were considered significant and are shown in bold.