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1 **Circulating microRNAs after a 24-h ultramarathon run**
2 **in relation to muscle damage markers in elite athletes**

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26 **Abstract**

27 Ultra-endurance sports are growing in popularity but can be associated with adverse health
28 effects; such as exercise-induced muscle damage (EIMD), which can lead to exertional
29 rhabdomyolysis. Circulating microRNAs (miRNAs) may be useful to approach the degree of
30 EIMD. We aimed to: 1) investigate the relevance of circulating miRNAs as biomarkers of
31 muscle damage and 2) examine the acute response of skeletal/cardiac muscle and kidney
32 biomarkers to a 24-h run in elite athletes. **Eleven** elite athletes participated in the 24-h Run
33 World Championships. Counter-movement jump (CMJ), creatine kinase (CK), myoglobin
34 (Mb), creatinine (Cr), high-sensitive cardiac troponin T (hs-cTnT) and muscle-specific miRNA
35 (myomiR) levels were measured before, immediately after, and 24 and 48h after the race. CMJ
36 height was reduced immediately after the race ($-84.0\pm 25.2\%$, $p<0.001$) and remained low at
37 24h ($-43.6\pm 20.4\%$, $p=0.002$). We observed high CK activity ($53,239\pm 63,608$ U/L, $p<0.001$)
38 immediately after the race and it remained elevated 24h after ($p<0.01$). Circulating myomiRs
39 levels (miR-1-3p, miR-133a-3p, miR-133b, miR-208a-3p, miR-208b-3p, and miR-499a-5p)
40 were elevated immediately after the 24-h run (fold changes: 18–124,723, $p<0.001$) and
41 significantly ($p<0.05$) correlated or tended to significantly ($p<0.07$) correlate with the reduction
42 in CMJ height at 24h. We found no significant correlation between CMJ height loss at 24h and
43 CK ($p=0.23$) or Mb ($p=0.41$) values. All elite ultramarathon runners included in our study were
44 diagnosed with exertional rhabdomyolysis after the 24-h ultramarathon race. MyomiR levels
45 may be useful to approach the degree of muscle damage.

46

47 **Keywords:** exercise-induced muscle damage, rhabdomyolysis, ultra-endurance, muscle
48 function, cardiac stress, acute kidney injury, biomarkers.

49 **Introduction**

50 Ultra-endurance and ultramarathon events (> 42.195 km) have experienced considerable
51 growth in recent years. Their growing popularity, along with the constant pursuit of overcoming
52 one's limitations, has given rise to extremely challenging sports events. A 24-h run is a form of
53 ultramarathon in which a competitor runs as far as possible in 24 h on a short loop (400 – 2,500
54 m). The current World records are 303.5 km for men and 270.1 km for women, corresponding
55 to mean running speeds of ~12.6 and ~11.3 km/h, respectively. To achieve a good performance,
56 athletes must efficiently manage their exercise work rate by adopting a pacing strategy¹ and
57 managing their energy and fluid intake². On the other hand, the extreme duration and high
58 number of stretch-shortening cycles performed by exercising muscles over the 24-h race result
59 in a high level of exercise-induced muscle fatigue and damage, which translates into an acute
60 reduction in performance³.

61 The evidence that strenuous ultra-endurance exercise can cause alterations in
62 metabolism⁴ and the cardiovascular⁵ and musculoskeletal systems³ is well established. Prior
63 studies have shown that prolonged exercise, such as that encountered in ultra-endurance events,
64 is associated with cardiac stress⁶, exertional rhabdomyolysis, and sometimes acute kidney
65 injury⁷. Exertional rhabdomyolysis can manifest as myalgia, weakness, and edema and is
66 characterized by muscle breakdown and necrosis, resulting in leakage of intracellular muscle
67 constituents, such as electrolytes and sarcoplasmic proteins, including myoglobin (Mb) and
68 creatine kinase (CK), into circulation and the extracellular space. The accumulation of
69 sarcoplasmic proteins, specifically myoglobin, in the circulation is a potential complication for
70 the kidneys⁸. Acute kidney injury is a final-stage complication of massive rhabdomyolysis and
71 is defined as a rapid decline in renal functionality (hours to days)⁹. Although the impact of ultra-
72 endurance exercise on cardiac damage biomarkers and the risk of exertional rhabdomyolysis
73 and acute kidney injury has been largely evaluated⁷, it is known that training status can affect

74 the markers of muscle damage after exercise-induced muscle damage (EIMD)¹⁰. However, the
75 effect of ultra-endurance racing on elite runners is still poorly documented.

76 Although delayed loss of muscle function (i.e. 24-48 h) is considered to be the best
77 indirect marker for evaluating the magnitude of exercise-induced muscle damage¹¹, early
78 measurement (i.e. within a few hours after the end of exercise) may reflect a combination of
79 muscle damage and muscle fatigue rather than muscle damage alone^{11,12}. This marker is
80 therefore not suitable in clinical practice due to the necessity of immediate diagnosis of muscle
81 damage. Indeed, rapid countermeasures are required to prevent the risk of exertional
82 rhabdomyolysis and acute kidney injury. Serum CK and Mb levels are the most commonly used
83 biomarkers of exertional rhabdomyolysis¹³ but there is no accepted cut-off threshold for these
84 biomarkers (CK: 5 – 10X the upper limit range or > 5,000 – 10,000 U/L, Mb: 400 – 4000
85 ng/mL)^{14,15}. Indeed, the interpretation of these biomarkers is affected by high interindividual
86 variability and does not necessarily reflect the magnitude of delayed loss of muscle function¹⁶.
87 Moreover, there are currently no markers considered to be a “gold standard” for the evaluation
88 of the degree of exertional rhabdomyolysis¹⁷. It is therefore necessary to search for new relevant
89 biomarkers that increase within the first hours after exercise and accurately predict the
90 magnitude of muscle damage.

91 MicroRNAs (miRNAs), small noncoding RNA involved in the post-transcriptional
92 regulation of gene expression, may potentially serve as alternative biomarkers of muscle
93 damage^{18,19}. Indeed, some miRNAs (miR-1, -133a, -133b, -206, -208a, -208b, -486, and -499)
94 have been described as muscle-specific/enriched (cardiac and skeletal muscle), two of which
95 are specifically found in skeletal muscle (miR-133b and -206) and one in the cardiac muscle
96 (miR-208a)²⁰. These specific/enriched miRNAs (also called myomiRs) can be actively secreted
97 by or passively leak out of injured myocytes and measured in the blood. High levels of
98 serum/plasma myomiRs have been found in muscular dystrophy and myocardial infarction (MI)

99 patients relative to healthy subjects^{21,22}. In the healthy organism, elevation of myomiR has been
100 observed after toxic muscle injury in rats²⁰ and after marathon running¹⁹. Moreover, it has been
101 reported that the ability of myomiR to discriminate damaged from nondamaged muscle is
102 higher than that of CK^{18,20}, suggesting that myomiR may more accurately diagnose muscle
103 damage. Although no study has investigated the relationship between myomiR levels and
104 skeletal muscle function, Corsten et al.²³ found a good correlation between miR-499 and
105 troponin T levels in MI patients suggesting that this miRNA could be useful for determining
106 the degree of muscle damage.

107 Here, we aimed to: 1) investigate the relevance of circulating miRNA as biomarkers of
108 muscle damage and 2) examine the acute response of skeletal/cardiac muscle and kidney
109 biomarkers to the 24-h run World Championships in elite athletes. We hypothesized that the
110 present athletes would be diagnosed with exertional rhabdomyolysis, acute kidney injury and
111 present cardiac stress in response to the 24-h run. Moreover, myomiR levels would be more
112 representative of muscle damage than those of CK and Mb (i.e. more associated with muscle
113 function loss at 24 h).

114

115 **Methods**

116 **Participants**

117 Athletes of the French national team (5 men and 6 women) gave their written informed
118 consent and agreed to participate in this study. The participant's characteristics are provided on
119 table 1. The study was conducted in accordance with the Declaration of Helsinki and was
120 approved by the Regional Ethics Committee (CPP Ile-de-France 8, France, registration number:
121 2019-A02445-52, Etude LemuR).

122 **Experimental design**

123 This study was conducted during the 2019 IAU 24H World Championships held in Albi
124 (France) from October 26-27, 2019. The race consisted of running the greatest distance possible
125 over 24 h (start of the race at 10:00 am the first day) on a loop of 1.491 km combining asphalt
126 (~75%) and tartan track (~25%; see Figure 1).

127 Vertical jump height (as an indicator of muscle function) and muscle soreness were
128 assessed 26 h before (between 7:00 am and 9:00 am, PRE), within 1 h after finishing (POST),
129 and 24 and 48 h after the race. Blood samples were obtained at the same time points for
130 biological analysis. A blood test was completed each morning in a fasted state. Dietary intakes
131 were recorded during the race (Lavoué et al.²). As the medical staff did not permit some
132 participants to performed CMJ due to medical monitoring, one participant (POST), two
133 participants (+24) and four participants (+48) did not perform the CMJ. Participants were asked
134 to refrain performing any physical activity the two days after the race.

135 **Functional measurements**

136 **Vertical jump**

137 Counter-movement jump (CMJ) height was assessed using an Optojump photocell
138 system (Microgate, Bolzano, Italy), and used as a measure of muscle function. Participants were
139 asked to keep their hands on their hips and jump as high as possible. They had two attempts to
140 achieve their best maximal jump height, which was used for further analysis. A self-determined
141 range of motion for the knee was permitted and they received verbal encouragement.

142 **Muscle soreness**

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

146 severe range of motion limitation observed immediately after the end of the race, the
147 participants performed a squat on a self-determined range motion, i.e. as flexed as possible.

148 **Biological measurements**

149 **Sampling**

150 Blood was drawn from the antecubital vein at each time point and collected into two
151 separate tubes (5 mL, Becton Dickinson Vacutainer, Franklin Lakes, USA), one containing
152 EDTA and the other lithium heparin. The collected blood was conserved at 4°C until plasma
153 was separated by centrifugation (2,000 x g, 10 min) within 1 h of collection. Lithium heparin
154 plasma was used within 2 h for standard biochemical analyses and EDTA plasma was aliquoted
155 and frozen at -20°C for further analyses.

156 **Biochemical analyses**

157 Plasma CK activity and Mb concentration were measured using a Roche Cobas c501
158 autoanalyzer and high-sensitive cardiac troponin T (hs-cTnT) and creatinine (Cr)
159 concentrations using a Roche Cobas e501/e601 autoanalyzer (Roche Diagnostics GmbH,
160 Mannheim, Germany). The estimated glomerular filtration rate (eGFR) was calculated using
161 the CKD-EPI (CKD Epidemiology Collaboration) equation. Before the race, several
162 participants presented Mb and hs-cTnT values below the detection limit of 21 ng/mL (5 of 11
163 PRE values) and 5 ng/L (8 of 11), respectively. For statistical analysis, such samples were given
164 the value of the detection limit.

165 ***Plasma miRNA extraction***

166 Total RNA was extracted from plasma as previously described^{18,20}. Briefly, total RNA
167 was extracted from 200 µL plasma using the mirVana PARIS kit (Ambion, Austin, USA) and
168 low-binding tubes (1.5 mL, DNA LoBind Tubes, Eppendorf, hamburg, Germany) according to
169 the manufacturer's protocol. An additional precipitation step was performed as follows.

170 Column elution was performed with 180 μ L sterile water. Then, 18 μ L 3M sodium acetate
171 (Sigma-Aldrich, Saint-Quentin-Fallavier, France), 400 μ L 70 % ethanol, and 1 μ L GlycoBlue
172 (Ambion) were added. Tubes were vortexed and the RNA allowed to precipitate for 20 min at
173 -20°C . After centrifugation (12,000 x g, 4°C , 10 min), the supernatants were carefully removed
174 and the pellets allowed to dry for 30 min. The RNA was finally suspended in 12 μ L sterile
175 water, incubated 10 min at 50°C , and frozen at -80°C until cDNA synthesis.

176 *Reverse transcription*

177 cDNAs were synthesized from 5 μ L total RNA diluted 1:6 in a 10 μ L reaction volume,
178 including the UniSp6 RNA spike-in using the miRCURY LNA RT kit (QIAGEN, Courtaboeuf,
179 France) according to the manufacturer's protocol.

180 *Real-Time Quantitative qPCR*

181 Real-Time Quantitative PCR (RT-qPCR) was performed on a 96-well LightCycler 480
182 instrument (Roche Applied Science, Mannheim, Germany) with 4 μ L cDNA (diluted 1:40 in
183 sterile water), 1 μ L PCR primer mix, and 5 μ L 2x miRCURY SYBR Green Master Mix
184 (QIAGEN) in a 10 μ L reaction volume. First, a quality control for extraction and reverse
185 transcription was performed by measuring endogenous Homo sapiens (hsa)-miR-103a-3p
186 (cutoff of ≤ 32 Cq) and synthetic UniSp6 RNA (cutoff of ≤ 20 Cq). Then, we measured hsa-
187 miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, hsa-miR-206, hsa-miR-208a-3p, hsa-miR208b-
188 3p, hsa-miR-378a-3p, and hsa-miR-499a-5p. We chose these miRNAs due to their muscle-
189 specific expression (Lee et al.²⁴) and their documented responses to toxic (Siracusa et al.²⁰) and
190 exercise-related muscle damage (Banzet et al.¹⁸; Mooren et al.²⁵; Baggish et al.¹⁹). PCR protocol
191 consisted in 1 activation cycle (95°C , 2 min), 52 amplification cycles (denaturation 95°C , 10 s,
192 and annealing/amplification 56°C , 1 min), and a melting curve analysis step, as recommended
193 by the manufacturer. Undetectable values were handled as described by De Ronde et al.²⁶ by
194 adding one quantification cycle (Cq) to the highest Cq observed for a miRNA.

195 The stability of potent reference miRNAs (hsa-miR-16-5p, hsa-miR-20a-5p, hsa-miR-
196 21-5p, hsa-miR-103a-3p, hsa-miR-185-5p, hsa-miR-192-5p, hsa-miR-210-3p, and hsa-miR-
197 320a-3p) was assessed using geNorm, BestKeeper, NormFinder, the Δ CT method, and
198 RefFinder. The optimal number of reference miRNAs was determined using geNorm. Five
199 reference miRNAs (hsa-miR-16-5p, hsa-miR-20a-5p, hsa-miR-21-5p, hsa-miR-185-5p, and
200 hsa-miR-320a-3p) were deemed sufficient for normalization. Final quantifications are
201 expressed as arbitrary unit (AU) and consisted of the geometrical mean of the quantification
202 performed with each reference miRNA. The raw Cq and qPCR primer information are supplied
203 for the target and reference miRNA in Supplementary Table 1.

204 **Statistics**

205 Statistical analyses were performed to assess the changes between the PRE, POST, and
206 24 and 48 h measurements. Due to the limited sample size of our study and the absence of a
207 normal distribution of the data according to Shapiro-Wilk tests, we performed non-parametric
208 Friedman analysis of variance. Statistical differences between time points were assessed using
209 Dunn's multiple comparisons test. The Friedman test statistic χ^2 and the effect size (Kendall's
210 W test value) are reported. The Kendall's W coefficient value range from 0 (no relationship) to
211 1 (a perfect relationship). Missing data were imputed using the MissMDA R package

212 Correlations were performed between CMJ height loss at 24 h and changes in CK, Mb,
213 myomiRs and muscle soreness. Indeed, as muscle function (i.e. CMJ height) loss at 24h is
214 considered to be the best indirect marker for evaluating the magnitude of exercise-induced
215 muscle damage¹¹, any significant correlation suggests that the associated measure could be
216 useful to approach the magnitude of muscle damage. The level of association between
217 parameters was assessed using Spearman's rank correlation coefficient (ρ).

218 Significance was defined as $p < 0.05$. Analyses were performed using GraphPad Prism
219 (Version 8.4.3, GraphPad Software, CA, USA) and R (version 3.6.1, R Core Team (2017)). R:

220 A Language and Environment for Statistical Computing, available at: [https://www.R-](https://www.R-project.org/)
221 [project.org/](https://www.R-project.org/)). All data are presented as the means \pm standard deviation (SD) throughout the
222 manuscript.

223

224 **Results**

225 **Functional measurements**

226 **Vertical jump**

227 The Friedman test showed a significant effect of time on CMJ height ($P < 0.001$, $\chi^2 =$
228 28.64, Kendall's $W = 0.87$). The initial jump height was 19.6 ± 3.7 cm (Table 2). CMJ height
229 was reduced by ~84% (2.8 ± 4.1 cm, $p < 0.001$) immediately after the race with five of the
230 participants unable to jump (Figure 2). CMJ height remained low, by ~44% (10.9 ± 5.0 cm, p
231 $= 0.002$; Figure 2) 24 h after the end of the race. Despite not being statistically different from
232 the PRE values ($p = 0.19$), jump height was still reduced by ~30% at 48 h (13.0 ± 4.6 cm; Figure
233 2).

234 **Muscle soreness**

235 The Friedman test showed a significant effect of time on quadriceps muscle soreness (p
236 < 0.001 , $\chi^2 = 19.36$, Kendall's $W = 0.587$). The muscle soreness of the athletes was significantly
237 higher immediately and 24 h after the end of the race (Figure 2).

238 **Biological measurements**

239 **Biochemical analyses**

240 The Friedman test showed a significant effect of time on CK ($P < 0.001$, $\chi^2 = 31.91$,
241 Kendall's $W = 0.97$), Mb ($P < 0.001$, $\chi^2 = 30.00$, Kendall's $W = 1.0$) (Figure 3A), hs-cTnT (P
242 < 0.001 , $\chi^2 = 28.90$, Kendall's $W = 0.88$), Cr ($P < 0.001$, $\chi^2 = 23.53$, Kendall's $W = 0.71$), and

243 eGFR ($P < 0.001$, $\chi^2 = 24.63$, Kendall's $W = 0.75$). CK activity was significantly higher
244 between PRE (137 ± 112 U/L; Table 2) and POST ($53,239 \pm 63,608$ U/L, $p < 0.001$) and 24 h
245 ($19,432 \pm 20,947$ U/L, $p = 0.003$) but there was no significant difference between the PRE and
246 48 h ($9,456 \pm 13,767$ U/L, $p = 0.29$) values (Figure 3A). Mb concentrations were significantly
247 higher between PRE (34 ± 19 ng/mL; Table 2) and POST ($9,748 \pm 7997$ ng/mL, $p < 0.001$) and
248 24 h (669 ± 585 ng/mL, $p = 0.002$) but were not significantly different between PRE and 48 h
249 (242 ± 255 ng/mL, $p = 0.42$) values (Figure 3B). The hs-cTnT values were significantly higher
250 in POST (34.1 ± 18.3 ng/L) compare to PRE (6.3 ± 2.3 ng/L, $p < 0.001$; Table 2) but there was
251 no significant difference between the PRE and 24 h (14.6 ± 8.0 ng/L, $p = 0.06$) or 48 h ($8.3 \pm$
252 6.9 ng/L, $p = 1.00$) values (Figure 3C). There were no significant differences in Cr levels
253 between PRE (75.6 ± 14.3 $\mu\text{mol/L}$; Table 2) and POST (89.7 ± 15.0 $\mu\text{mol/L}$, $p = 0.12$), 24 h
254 (75.0 ± 18.1 $\mu\text{mol/L}$, $p = 1.00$), or 48 h (67.2 ± 13.8 $\mu\text{mol/L}$, $p = 0.10$), but the values were
255 significantly lower at 24 h and 48 h than at POST (24 h: $p = 0.008$, 48 h: $p < 0.001$). Similarly,
256 there were no significant differences in the eGFR between PRE (93.8 ± 9.9 ml/min/1.73 m²)
257 and POST (78.0 ± 12.8 ml/min/1.73 m², $p = 0.12$), 24 h (94.9 ± 13.0 ml/min/1.73 m², $p = 1.00$),
258 or 48 h (102.6 ± 8.9 ml/min/1.73 m², $p = 0.06$), but the values were significantly lower at 24
259 and 48 h than at POST (24 h: $p = 0.01$, 48 h: $p < 0.001$).

260 **Circulating miRNA levels**

261 The Friedman test showed a significant effect of time on the levels of hsa-miR-1-3p (P
262 < 0.001 , $\chi^2 = 23.18$, Kendall's $W = 0.70$), hsa-miR-133a-3p ($P < 0.001$, $\chi^2 = 24.49$, Kendall's
263 $W = 0.74$), hsa-miR-133b ($P < 0.001$, $\chi^2 = 24.05$, Kendall's $W = 0.73$), hsa-miR-206 ($P < 0.001$,
264 $\chi^2 = 22.75$, Kendall's $W = 0.69$), hsa-miR-208a-3p ($P < 0.001$, $\chi^2 = 20.78$, Kendall's $W = 0.63$),
265 hsa-miR-208b-3p ($P < 0.001$, $\chi^2 = 26.02$, Kendall's $W = 0.79$), hsa-miR-378-3p ($P < 0.001$, χ^2
266 $= 21.87$, Kendall's $W = 0.66$), and hsa-miR-499a-5p ($P < 0.001$, $\chi^2 = 28.96$, Kendall's $W =$
267 0.88). We observed a significant increase in circulating miRNA levels (hsa-miR-1-3p, hsa-miR-

268 133a-3p, hsa-miR-133b, hsa-miR-206, hsa-miR-208a-3p, hsa-miR-208b-3p, hsa-miR-378-3p,
269 and hsa-miR-499a-5p) immediately after the 24-h run (fold changes to PRE: 18 to 124,723, p
270 < 0.001). Circulating levels of hsa-miR-499a-5p remained elevated ($p = 0.02$) and hsa-miR-
271 208b-3p tended to remain elevated ($p = 0.08$) 24 h after the end of the run (Figure 4).

272 **Correlations with reduced jump height**

273 Circulating levels of hsa-miR-208a-3p and 208b-3p at the end of the 24-h ultramarathon
274 significantly correlated with the reduction in CMJ height at 24 h ($p < 0.05$) and approached
275 significance for hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-133b, and hsa-miR-499a-5p ($p <$
276 0.07) (Figure 5). However, none of these myomiRs were correlated with CMJ height measured
277 immediately after the race. We found no significant correlation between the reduction in CMJ
278 height at 24 h and CK ($p = 0.23$) or Mb ($p = 0.31$) levels measured at the end of the 24-h
279 ultramarathon (Figure 5). Finally, no significant correlations were found between CK, Mb,
280 cTnT, muscle soreness and myomiRs at each time points.

281

282 **Discussion**

283 The main purpose of this study was to investigate the relevance of circulating miRNA
284 levels as biomarkers of muscle damage. We observed an increase in the level of all our target
285 muscle-specific miRNAs immediately after the end of the exercise, which often correlated with
286 muscle function measured 24 h after the end of the ultramarathon race. By contrast, CK and
287 Mb levels did not correlate with muscle function, confirming the hypothesis that myomiR levels
288 may be more representative of muscle damage than most commonly used biomarkers. Our
289 second objective was to examine the acute response of skeletal/cardiac muscle and kidney
290 biomarkers to the 24-h run World Championships in elite athletes. The results confirm the
291 hypothesis that all participants would be diagnosed with exertional rhabdomyolysis

292 immediately after the race, based on increases in CK and Mb levels¹⁴. Hs-cTnT values measured
293 immediately after the race were higher than the 99th percentile upper reference limit (URL),
294 suggesting a high level of cardiac stress. The normal Cr and eGFR values reported after the race
295 suggest that no significant deterioration of kidney function was induced by the 24-h
296 ultramarathon run. Moreover, despite the high level of sarcoplasmic proteins into the
297 bloodstream, kidney function did not seem to be significantly affected.

298 **Acute effect of an ultramarathon on muscle and kidney**

299 In our study, muscle function measured 24 h after the end of the exercise was reduced
300 by ~40% and was still diminished by ~30% 48 h post-exercise. Only one study has previously
301 described the recovery of lower-limb muscle function in the days following an ultramarathon
302 run¹². The authors¹² assessed muscle function differently from our study and reported a smaller
303 decrease in muscle function (a loss of ~10 - 15% of knee extension and plantar flexion torque
304 at 48 h post-exercise). This smaller reduction in muscle function is consistent with the smaller
305 CK activity (~16,000 U/L vs. ~43,000 U/L) and Mb concentration (~1,400 ng/mL vs. ~9,700
306 ng/mL) measured immediately after the 166-km mountain ultramarathon¹² relative to the results
307 reported here, which suggests a higher degree of muscle damage in our study.

308 Indeed, all our participants were diagnosed with exertional rhabdomyolysis (CK >
309 10,000 U/L) immediately after the race and 8 of 11 still had CK levels above 10,000 U/L 24 h
310 later. A 24-h ultramarathon run is known to induce exertional rhabdomyolysis. However, the
311 CK and Mb responses were higher in our study than those of Martin et al.³ and Waśkiewicz et
312 al.⁴. The higher distance covered by our athletes relative to those of the above-mentioned
313 studies^{3,4} (~230 km vs. 150-170 km) may explain the higher CK activity. Indeed, the duration
314 of exercise may increase the degree of muscle damage²⁷. Accordingly, the CK activity observed
315 after the race in our study is consistent with the values reported after the 246-km Spartathlon
316 running race²⁸. On the other hand, despite the higher²⁸ level of muscle damage markers observed

317 after the 24-h ultramarathon relative to those reported by Millet et al.¹², our participants reported
318 a similar level of muscle soreness, suggesting that elite athletes are less susceptible to muscle
319 soreness.

320 Despite the large increase of intramuscular protein content in the blood, in particular
321 Mb, only 1 of 11 participants showed a significant change in acute kidney injury biomarker
322 levels immediately after the race (absolute increase in Cr: 29 μmol , eGFR: 60 mL/min/1.73
323 m^2). These biomarker values were not beyond the pathological limit (defined by an absolute
324 increase in Cr $> 26.5 \mu\text{mol}$ and eGFR $< 60 \text{ mL/min/1.73 m}^2$)⁹ for the 10 other athletes and
325 returned to normal 24 h after the ultramarathon for all runners. The transient and low-to-
326 moderate alterations of kidney function we observed in the elite athletes are different than those
327 observed by Hoffman & Weiss²⁹. Indeed, they reported that more than 40% of the 585 runners
328 were diagnosed with acute kidney injury, whereas none of our participants met the criterion
329 (1.5X baseline Cr values). Although our study was not designed to assess this question, external
330 (e.g. race characteristics, environmental conditions) or internal factors (e.g. training level,
331 genetic profiles, sex) may at least partially explain these discrepancies⁷.

332 All our participants presented a hs-cTnT value higher than the 99th percentile URL (10
333 ng/g for women and 15 ng/L for men³⁰) at this time point, whereas none of them showed any
334 such values before the race. Our results thus suggest a high level of cardiac stress immediately
335 after the 24-h ultramarathon race. This finding is consistent with the increase in cardiac specific
336 troponin T levels observed immediately after another 24-h ultramarathon run⁵. Although the
337 troponin assay was of a different generation in this study, the values for well-trained athletes
338 ($\sim 0.01 \text{ ng/mL}$ ⁵) were comparable to our values (34 ng/L). Indeed, Sandoval et al.³⁰ reported
339 that a hs-cTnT value of 30 ng/L is similar to a fourth-generation troponin T value of 0.01 ng/mL.
340 However, the values we measured immediately after the race were lower than those reported
341 after a marathon run ($\sim 60 \text{ ng/L}$ vs. 34 ng/L³¹). This is probably due to the lower exercise

342 intensity during an ultra-long distance run than during a marathon ³². In our study, the hs-cTnT
343 values measured 24 and 48 h after the end of the race decreased relatively rapidly (Figure 3C).
344 Only 6 of the 11 participants still had values above the 99th percentile URL at 24 h and only
345 three of them at 48 h. Consequently, the relatively small increase in hs-cTnT levels relative to
346 the MI cut-off threshold (> 100 ng/L³⁰), the rapid recovery kinetics (Figure 3C), and the absence
347 of symptoms in our study suggest the non-pathological release of cTnT that is likely explained
348 by the release of unbound cytosolic cTnT rather than cardiomyocyte necrosis ³².

349 **Change in circulating miRNA levels**

350 The levels of all our target plasma miRNAs significantly increased immediately after
351 the race relative to baseline values, suggesting that myomiR levels are sensitive to ultra-
352 endurance exercise. This response to exercise has been already observed under various
353 conditions of exercise^{18,25,33-35}. Although no study has investigated circulating myomiR levels
354 after ultra-endurance races (known to be a good model of muscle damage ^{3,12}), several have
355 shown an increase after marathon runs^{25,34,35}. Similar to our results, these studies described an
356 increase in circulating miR-1, miR-133a, miR-206, miR-208b, and miR-499a immediately after
357 the exercise. However, the magnitude of the increase was smaller than that reported here, maybe
358 due to shorter exercise duration and therefore less exercise-induced muscle damage and/or a
359 shorter duration of release. Indeed, these studies ^{25,34,35} reported lower CK responses (~600 U/L,
360 ~4X baseline value, ~2,000 U/L) than observed in our study. Circulating miRNA has often been
361 reported to have a short half-life in the blood and a large body of evidence suggests rapid
362 clearance from the circulation following acute tissue injury (skeletal and cardiac muscles and
363 liver), possibly through renal elimination³⁶ or tissue uptake/biodistribution³⁷. However, the
364 clearance mechanisms of circulating miRNA are poorly understood and it cannot be excluded
365 that the rate of miRNA liberation into the blood exceeds the rate of clearance in the context of
366 EIMD extended over 24 h. On the other hand, not all previous studies that investigated miRNA

367 responses after exercise interpreted elevated levels of circulating miRNA as being indicative of
368 muscle damage. For example, Mooren et al.²⁵ suggested that myomiRs could be biomarkers of
369 aerobic exercise capacity. Ramos et al.³³ showed that circulating miR-1 and miR-133a levels
370 were related to the intensity and duration of endurance running, suggesting that miRNAs could
371 mediate physiological adaptation to exercise. Indeed, Guescini et al.³⁸ showed that extracellular
372 vesicles originating from the skeletal muscle may be enriched in myomiRs, which are known
373 to be involved in muscle homeostasis³⁹.

374 Whether the circulating miRNAs quantified in our study are released in the blood due
375 to active secretion or passively leaked from injured myocytes due to EIMD cannot be
376 determined with the current experimental setting. However, post-exercise fold changes reported
377 in previous studies^{19,25,33} were considerably lower than those reported here (fold changes: 18–
378 124,723). Ramos et al.³³ found that miR-1 and miR-133a fold changes were up to 5 after 90
379 min running and other authors (Baggish et al.¹⁹; De Gonzalo-Calvo et al.⁴⁰) found that miR-1,
380 miR-133a, miR-133b and miR-499 fold changes were up to 18.5 after a marathon run. Thus, the
381 large increases in circulating miRNA levels that we observed were unlikely solely due to
382 exercise-induced secretion³³ but also to leakage from injured myocytes¹⁸. Indeed, the increase
383 in circulating miRNA levels observed after the 24-h ultramarathon in our study is higher than
384 that reported after toxic muscle injury in rats²⁰. High circulating miRNA levels have also been
385 reported in human studies on pathological^{21,22} and physiological¹⁸ muscle damage. Banzet et
386 al.¹⁸ reported an elevation in the level of several circulating myomiRs in the plasma after an
387 eccentric exercise known to induce EIMD but not after a concentric exercise. A positive
388 correlation was found between miR-133a and CK levels²⁵ and between miR-1/miR-133a and
389 CK/troponin T levels³⁵, suggesting that these myomiRs may be useful for the diagnosis of
390 muscle damage and cardiac stress. Although we did not find a correlation between myomiR
391 and CK/troponin T levels in our study, the positive correlations observed between the level of

392 some myomiRs and muscle function loss 24 h after the race suggest that circulating myomiRs
393 were representative of the level of muscle damage. However, whether the release was actively
394 secreted by or passively leaked out of injured myocytes remains to be documented.

395 **Interest of biomarkers of delayed muscle function loss**

396 In the present study, the CK activity and Mb concentration measured immediately after
397 the end of exercise did not correlate with the muscle function measured 24 h later, suggesting
398 that these biomarkers do not reflect the magnitude of exercise-induced muscle damage. These
399 results are consistent with those of Damas et al.¹⁶, who observed high interindividual variability
400 of CK activity relative to the degree of muscle function loss after eccentric elbow flexor
401 contractions. Our results are also consistent with the lack of an accepted cut-off threshold for
402 CK activity (e.g. 5-10X the upper limit range or > 5,000 – 10,000 U/L)¹⁴ and Mb concentration
403 (400 to 4,000 ng/mL)¹⁵ to determine a pathological state. Thus, although these commonly used
404 biomarkers of exertional rhabdomyolysis are still of great utility to detect the presence of
405 muscle damage their ability to quantify the magnitude of muscle damage appears to be limited.

406 We found significant or nearly significant correlations between the level of most of our
407 target circulating miRNAs measured immediately after the 24-h ultramarathon and muscle
408 function measured 24 h later. However, we found no correlations between loss of muscle
409 function at 24 h and CK activities or Mb levels, suggesting that myomiRs may be more relevant
410 than CK or Mb to represent the magnitude of muscle damage in our study (Figure 5). Although
411 more studies are required to confirm this assumption, these results are consistent with those of
412 Siracusa et al.²⁰, who reported that the accuracy in discriminating damaged from nondamaged
413 muscle in rats was lower for CK than myomiRs. Moreover, our results are also concordant with
414 those of another study¹⁸, which reported that circulating myomiR levels are able to discriminate
415 between uphill and downhill walking exercises, whereas CK activity and Mb concentration
416 were not, suggesting that myomiRs may be more sensitive and specific to muscle damage.

417 In addition, hsa-miR-206, hsa-miR-208b-3p and hsa-miR-499a-5p may be of additional
418 interest due to their low circulating levels, as they are often undetectable in the absence of
419 muscle damage (Figure 4)^{18,20}. Indeed, it has been shown that hsa-miR-206 was undetectable
420 after low-level muscle damage (loss of ~15% of muscle function at 24 h¹⁸), whereas a high level
421 was detected after a marathon run²⁵ and in the present study. Moreover, hsa-miR-208b-3p and
422 hsa-miR-499a-5p appear to have additional utility relative to other myomiRs because their
423 plasma levels were still elevated 24 h after the end of the 24-h ultramarathon (Figure 4). We
424 found no significant correlation between the circulating levels of these miRNAs measured 24
425 h after the end of the ultramarathon and muscle function, suggesting limited utility for
426 monitoring recovery after muscle injury (24 - 48 h). Previous work has shown that some
427 myomiRs could remain elevated up to 24 h after tissue injury. For instance, miR-1 was still
428 elevated at 24 h in the serum of rats after an acute myocardial infarctus (Cheng et al.⁴¹). Similar
429 patterns were also reported in the plasma of rats following an acute myotoxic injury, where
430 miR-208b remained elevated at 24 h but not the others myomiRs (Siracusa et al.²⁰). However,
431 there are no strong arguments to explain these kinetics. MyomiRs appear to exert various role
432 in muscle biology. For instance, miR-1 and miR-206 target Pax3 during the process of
433 myogenesis (Goljanek-Whysall et al.⁴²), while miR-208b and miR-499 have a redundant role
434 in maintaining slow myofiber and repressing fast myofiber phenotype (Van Rooij et al.⁴³).
435 Given that the circulating levels of all our myomiRs correlated well with each other, it may not
436 be necessary to simultaneously measure them all in clinical practice. As suggested by Siracusa
437 et al.²⁰, assessment of cardiac- and skeletal muscle-specific miRNA (i.e. hsa-miR-208a, hsa-
438 miR-208b, and hsa-miR-133b or miR-206) could provide sufficient information to diagnose
439 muscle damage.

440 Surprisingly, hsa-miR-208a-3p strongly correlated with muscle function in the present
441 study, although this myomiR is cardiac-specific²² and is not detected after toxic muscle injury

442 in rats²⁰ and downhill walking in humans¹⁸. It is possible that subjects exhibiting high levels of
443 cardiac stress were also those who underwent a high degree of muscle damage. Covariates, such
444 as exercise intensity or training experience, may affect both muscle damage⁴⁴ and cardiac
445 stress³² and may therefore explain this surprising correlation. We found no significant
446 correlation between hs-cTnT levels measured immediately after the race and CMJ measured 24
447 h later, which does not support this hypothesis. However, no correlation has been found
448 between miR-208a, troponin, and cardiac function parameters in acute myocardial infarction
449 and coronary heart disease⁴⁵. Consequently, more studies are required to interpret the magnitude
450 in the change of the levels of cardiac-damage biomarkers.

451 **Limitations of the study**

452 Our study had several limitations. First, we included a relatively small number of
453 participants due to the limited number of athletes on the French national team, which did not
454 allow to test a potential effect of sex. However, Duttagupta et al.⁴⁶ showed that myomiRs were
455 not mentioned among miRNAs differently expressed between male and female. Nielsen et al.⁴⁷
456 found an effect of sex in myomiRs expression (mice; 133a and 133b) in resting skeletal muscle
457 in relation to testosterone, however they also found that aerobic exercise training seems to
458 override any effects of testosterone. Therefore, it seems that the potential difference in myomiR
459 expression in skeletal muscle does not seem to translate into a different circulating myomiR
460 level. Moreover, Siracusa et al.²⁰ found similar circulating myomiR levels between female and
461 male rats at baseline and in response to muscle damage. Taken together, these results suggest
462 that circulating myomiRs were likely not substantially affected by sex in our study. Second, the
463 experimental group of this study was restricted to elite athletes in a world championship
464 context. On one hand, more studies are required to determine whether the present miRNA
465 results may be replicated and apply to a wider population. On the other hand, this kind of
466 competitive event may lead to an important physiological stress. Therefore, it could induce

467 health problems requiring rigorous medical monitoring⁷. In this context, the assessment of
468 scientific measures could be secondary and therefore reduce the access to the athletes. Third,
469 as dietary intakes were not controlled during the present race because it is impossible in the
470 context of ultra-endurance competitions (Lavoue et al.²), interindividual differences could have
471 affected circulating miRNA levels (Mantilla-Escalante et al.⁴⁸). **Fourth**, despite the use of a
472 reliable and reproducible RT-qPCR normalization technique⁴⁹, it is impossible to compare
473 results from different studies without considering control values (i.e. healthy resting subject
474 values). Therefore, no clinical recommendation can be provided with this type of normalization.
475 **Finally, as plasma miRNA levels assessment is a long process (~6 h; including RNA extraction**
476 **and RT-qPCR), this biomarker is not compatible with medical emergency (e.g. exertional**
477 **rhabdomyolysis, acute kidney injury). Therefore, further research is needed to the development**
478 **of faster analytic methods that may be appropriate for point-of-care tests.** Recently developed
479 novel PCR-free techniques⁵⁰ may allow more widespread use of circulating miRNA as
480 biomarkers of muscle damage in the future.

481

482 **Perspectives**

483 All elite ultramarathon runners included in our study were diagnosed with exertional
484 rhabdomyolysis (CK > 10,000 U/L) after the 24-h run World Championships. However, despite
485 the high level of sarcoplasmic proteins into the bloodstream, the deterioration of kidney
486 function was relatively moderate and transient in this population. Further research is needed to
487 investigate factors explaining the potential lower susceptibility of world-class/elite athletes to
488 acute kidney injury. The elevation of cardiac biomarkers was likely due to exercise-related
489 cytosolic release rather than cardiac necrosis. Importantly, the present study is the first to show
490 that muscle-specific miRNAs were more associated with the magnitude of muscle function loss
491 than CK and Mb, suggesting that myomiRs may be useful to approach the magnitude of muscle

492 damage. Specifically, assessment of both cardiac- and skeletal muscle-specific miRNA such as
493 hsa-miR-208a, hsa-miR-208b and hsa-miR-133b or hsa-miR-206 could be a good approach to
494 diagnose muscle damage.

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

499

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505 designing the figures.

506

507 **Conflict of interest**

508 The authors declare no conflicts of interest, financial or otherwise. Furthermore, the
509 authors declare that the results of the study are presented clearly, honestly, and without
510 fabrication, falsification, or inappropriate data manipulation.

511

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- 636

637 SUPPLEMENTAL DIGITAL CONTENT

638 Supplementary Table 1.xlsx

639

640 FIGURE LEGENDS

641 **Figure 1. Aerial view of the race loop.** The aerial view was extracted from ®Google Maps.

642

643 **Figure 2. Changes in counter movement jump (CMJ) height (A) and muscle soreness of**
644 **the quadriceps (B) after the 24-h ultramarathon run.** CMJ height and muscle soreness
645 values are expressed as %PRE and mm (on 0-100mm scale), respectively. Data are displayed
646 as interquartile ranges (25th and 75th percentiles, colored boxes), medians (horizontal white
647 bars), minimum-maximum (whiskers), and individual values (dots). *: $p < 0.05$, ** $p < 0.01$,
648 *** $p < 0.001$.

649

650 **Figure 3. Changes in creatine kinase activity (A), myoglobin concentration (B), and high-**
651 **sensitive cardiac troponin T (hs-cTnT) concentration (C) after the 24-h ultramarathon**

652 **run.** Data are expressed as interquartile ranges (25th and 75th percentiles, colored boxes),
653 medians (horizontal white bars), minimum-maximum (whiskers), and individual values (dots).
654 *p < 0.05, **p < 0.01, ***p < 0.001.

655

656 **Figure 4. Changes in plasma miRNA levels after the 24-h ultramarathon run for homo**
657 **sapiens (hsa)-miR-1-3p (A), hsa-miR-133a-3p (B), hsa-miR-133b (C), hsa-miR-206 (D),**
658 **hsa-miR-208a-3p (E), hsa-miR-208b-3p (F), hsa-miR-378a-3p (G), and hsa-miR-499a-5p**
659 **(H).** Data are expressed as the relative abundance arbitrary unit (AU) and are displayed as
660 interquartile ranges (25th and 75th percentiles, colored boxes), medians (horizontal white bars),
661 minimum, maximum (whiskers), and individual values (dots). *p < 0.05, **p < 0.01, ***: p <
662 0.001.

663

664 **Figure 5. Correlation between the magnitude of the reduction of counter movement jump**
665 **(CMJ) height measured 24 h after the end of the 24-h ultramarathon run and plasma**
666 **miRNA levels measured immediately after the end of the race.** Data are displayed for homo
667 sapiens (hsa)-miR-1-3p (A), hsa-miR-133a-3p (B), hsa-miR-133b (C), hsa-miR-206 (D), hsa-
668 miR-208a-3p (E), hsa-miR-208b-3p (F), hsa-miR-378a-3p (G), and hsa-miR-499a-5p (H).
669 CMJ height loss values are expressed as %PRE, plasma miRNA levels as relative abundance
670 arbitrary unit (AU), creatine kinase activity as U/L, and myoglobin concentration as (ng/mL).

671

672 **TABLES**

673

674 **Table 1- Participant characteristics**

	Sex	Age	Height	Weight	BMI	Fat mass	Performance*	Ranking**	Experience in UM	Training	
#	M or F	years	cm	kg	kg.m ⁻²	% of weight	km (% vs PB)		years	number of	h.week ⁻¹
										24-h	
1	M	39	181	75	22,9	4,2	272 (+7)	4	20	1	15
2	M	53	170	60	20,8	6,2	259 (-2)	12	9	20	27
3	M	53	172	63,1	21,3	8,9	248 (-1)	19	5	4	6
4	M	46	188	73,8	20,9	14,5	236 (-6)	31	9	6	15
5	M	50	175	69,5	22,7	9,7	236 (-6)	34	4	8	14
6	F	37	160	42,9	16,8	10,1	241 (+12)	7	4	1	12
7	F	52	166	53,1	19,3	23,5	222 (-7)	21	13	16	6
8	F	45	160	51,9	20,3	19,1	219 (+4)	26	6	3	8
9	F	46	160	62,9	24,6	18,6	209 (-7)	36	13	2	10
10	F	31	171	58	19,8	12	201 (-4)	49	8	3	10
11	F	52	169	61,4	21,5	22,2	193 (-9)	58	9	2	8
Mean (M)		48.2 ± 5.9	177 ± 7	68.3 ± 6.6	21.7 ± 1.0	8.7 ± 3.9	250 ± 15	20 ± 13	9.4 ± 6.3	7.8 ± 7.3	15 ± 8
Mean (F)		43.8 ± 8.4	164 ± 5	55.0 ± 7.4	20.4 ± 2.6	17.6 ± 5.4	214 ± 17	33 ± 19	8.8 ± 3.6	4.5 ± 5.7	9 ± 2
Mean		45.8 ± 7.4	170 ± 9	61.1 ± 9.6	21.0 ± 2.1	13.5 ± 6.5	231 ± 24	27 ± 17	9.1 ± 4.8	6.0 ± 6.4	12 ± 6

675 BMI: body mass index, UM: ultramarathon, PB: personal best previous performance in 24-h

676 races * Values into brackets represent the difference between the performance in this race and

677 their previous best performance (PB). ** 214 and 147 participants started the race in male and

678 female category, respectively.

679

680 **Table 2 – Initial absolute values of the different measurements.**

CMJ (cm)	19.6 ± 3.7
Creatine kinase (U.L ⁻¹)	137.3 ± 112.4
Myoglobin (ng.mL ⁻¹)	34.2 ± 18.7
Hs-cTnT (ng.L ⁻¹)	6.3 ± 2.3
Creatinine (µmol.L ⁻¹)	75.6 ± 14.3

681 Means ± SD. CMJ : Counter-movement jump; Hs-cTnT : high-sensitive cardiac troponin T.

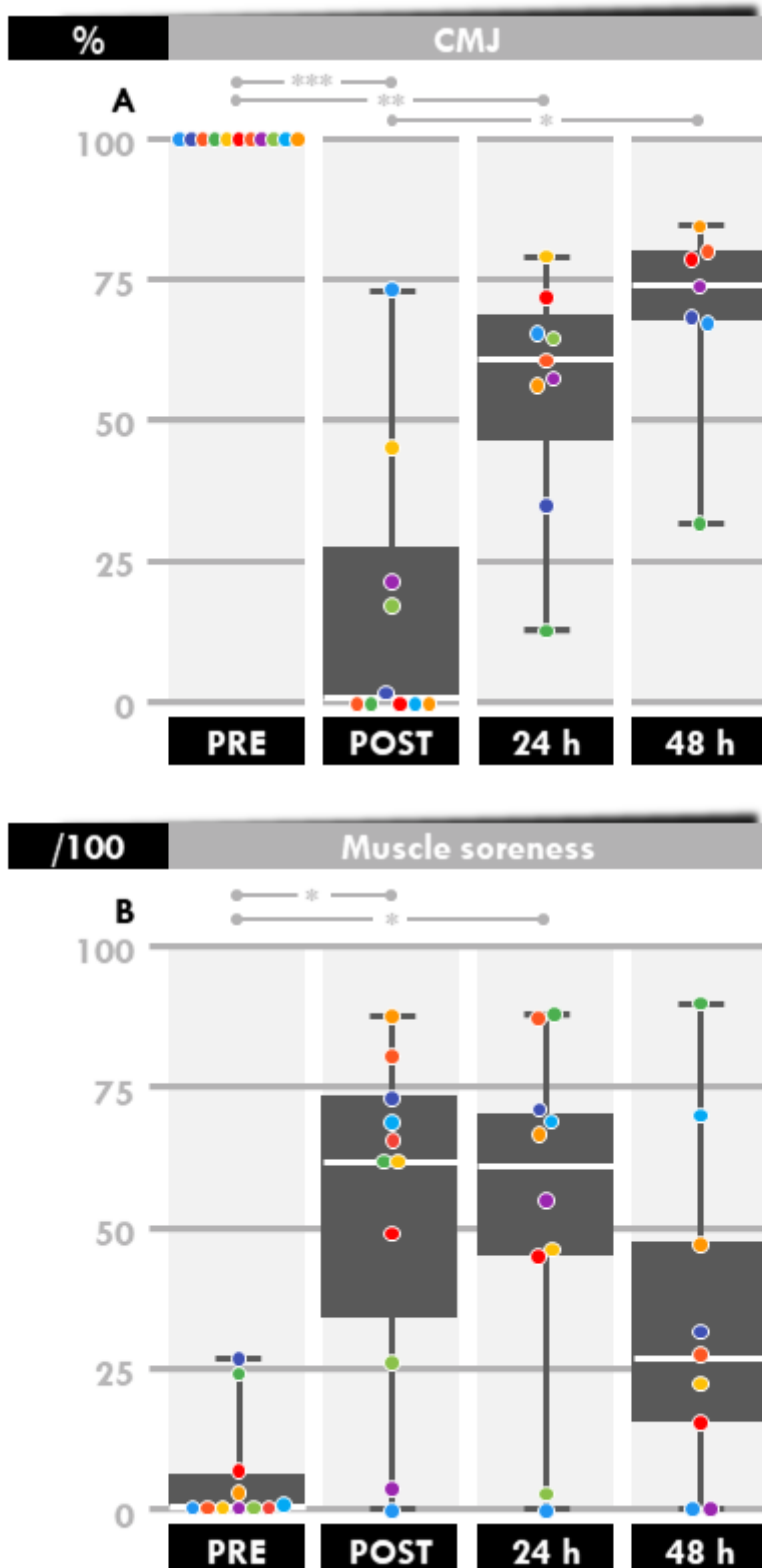
682 Figure 1.



683

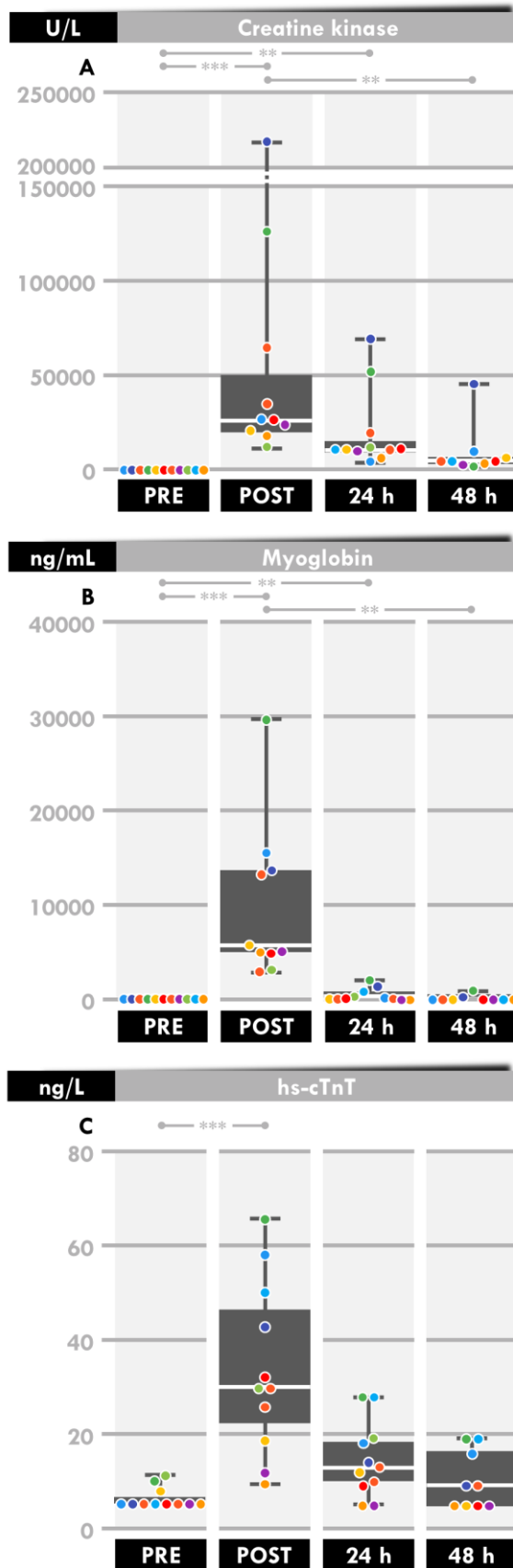
684

685 Figure 2.

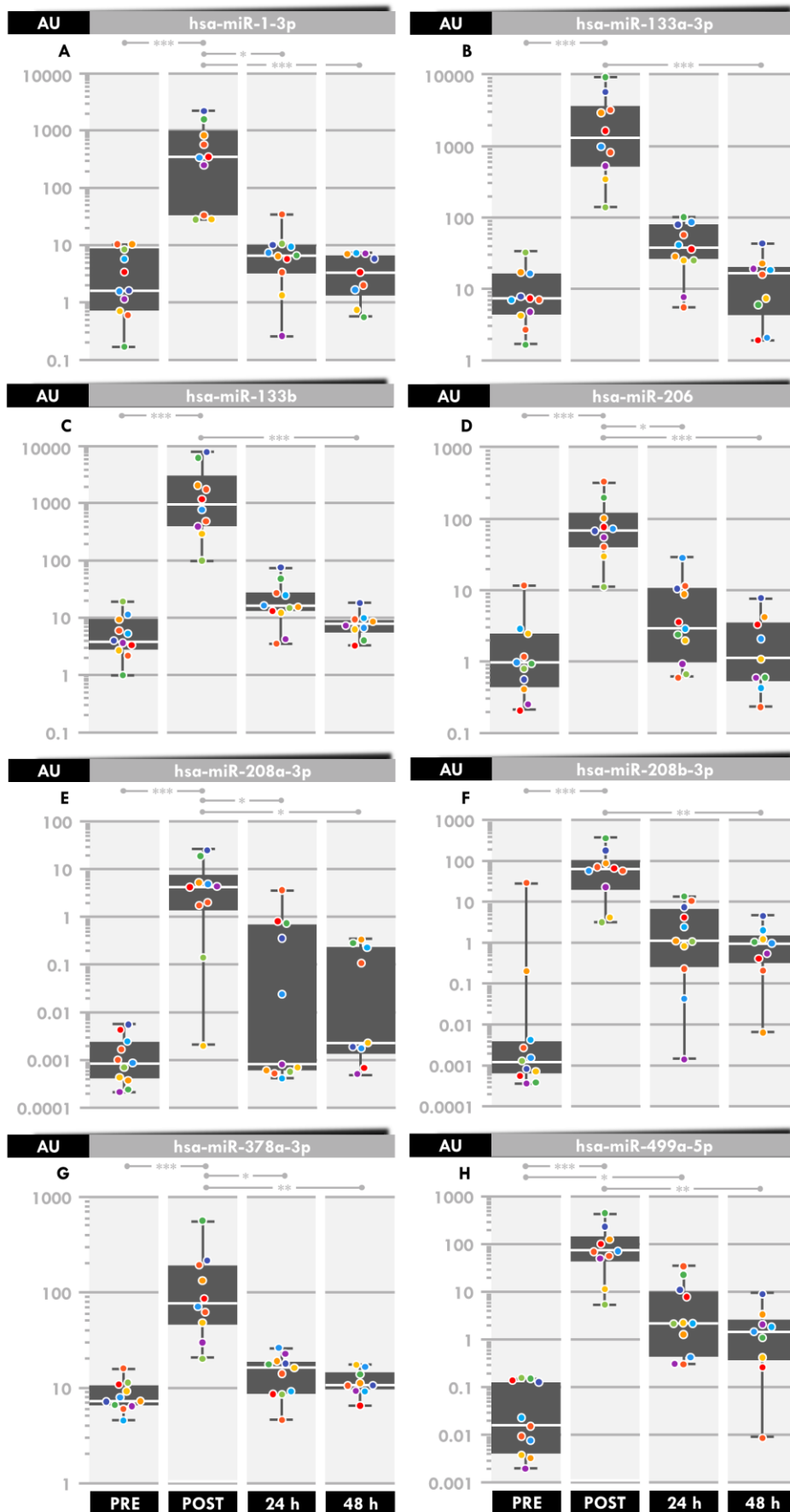


686

687 Figure 3.

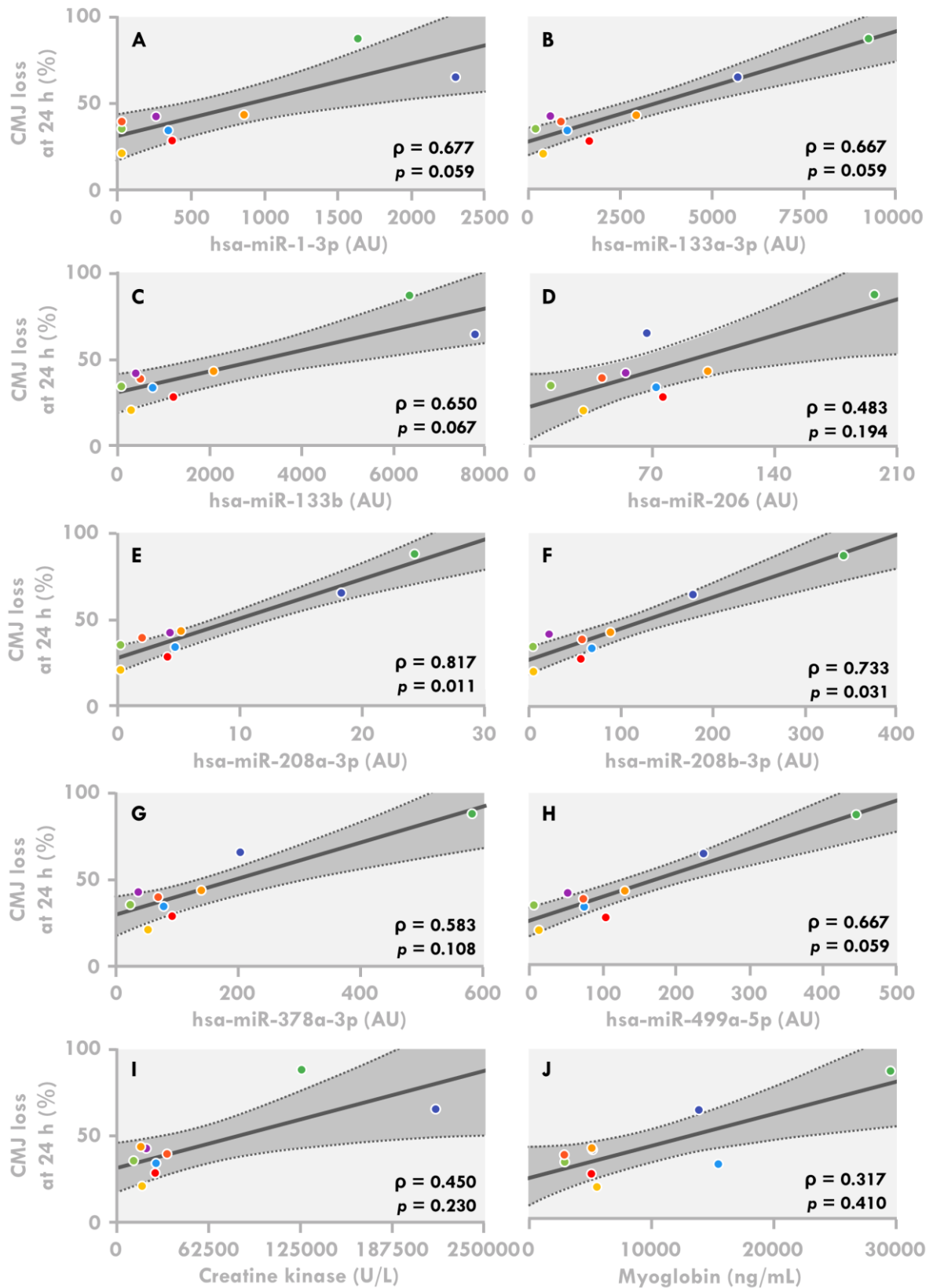


689 Figure 4.



690

691 Figure 5.



692