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Changes in plasma hydroxyproline and plasma cell-free DNA concentrations after higher- versus lower-intensity eccentric cycling

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Running head: Hyp and cfDNA after eccentric cycling

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29 **ABSTRACT**

30 **Purpose:** We examined changes in plasma creatine kinase (CK) activity, hydroxyproline and
31 cell-free DNA (cfDNA) concentrations in relation to changes in maximum voluntary isometric
32 contraction (MVIC) torque and delayed-onset muscle soreness (DOMS) following a session of
33 volume-matched higher- (HI) versus lower-intensity (LI) eccentric cycling exercise.

34 **Methods:** Healthy young men performed either 5×1-min HI at 20% of peak power output
35 (n=11) or 5×4-min LI eccentric cycling at 5% of peak power output (n=9). Changes in knee
36 extensor MVIC torque, DOMS, plasma CK activity, and hydroxyproline and cfDNA
37 concentrations before, immediately after, and 24-72 h post-exercise were compared between
38 groups.

39 **Results:** Plasma CK activity increased post-exercise (141±73.5%) and MVIC torque decreased
40 from immediately (13.3±7.8%) to 48 h (6.7±13.5%) post-exercise (P<0.05), without significant
41 differences between groups. DOMS was greater after HI (peak: 4.5±3.0 on a 10-point scale)
42 than LI (1.2±1.0). Hydroxyproline concentration increased 40-53% at 24-72 h after both LI and
43 HI (P<0.05). cfDNA concentration increased immediately after HI only (2.3±0.9 fold,
44 P<0.001), with a significant difference between groups (P=0.002). Lack of detectable
45 methylated *HOXD4* indicated that the cfDNA was not derived from skeletal muscle. No
46 significant correlations were evident between the magnitude of change in the measures, but the
47 cfDNA increase immediately post-exercise was correlated with the maximal change in heart
48 rate during exercise (r=0.513, P=0.025).

49 **Conclusion:** Changes in plasma hydroxyproline and cfDNA concentrations were not
50 associated with muscle fiber damage, but the increased hydroxyproline in both groups suggests
51 increased collagen turnover. cfDNA may be a useful metabolic-intensity exercise marker.

52

53 **Keywords:** eccentric exercise; maximal voluntary isometric contraction; muscle damage;

54 delayed onset muscle soreness; connective tissue; extracellular matrix

55

ABBREVIATIONS

56

cfDNA	cell-free DNA
CK	Creatine kinase
ddPCR	droplet digital PCR
DOMS	Delayed-onset muscle soreness
HI	Higher intensity
HR	Heart rate
Hyp	Hydroxyproline
LI	Lower intensity
MVIC	Maximal voluntary isometric contraction
PPO	Peak power output
RPE	Rate of perceived effort

57

58

59 **INTRODUCTION**

60 Unaccustomed exercise consisting of eccentric (lengthening) muscle actions (i.e.,
61 eccentric exercise) has been shown to induce muscle damage represented by delayed onset
62 muscle soreness (DOMS), prolonged decreases in muscle function, muscle swelling, and
63 increases in intramuscular proteins such as creatine kinase (CK) in the blood (Clarkson et al.
64 1992). Eccentric cycling is an eccentric exercise modality in which knee extensor muscles
65 perform submaximal eccentric muscle actions when resisting to backward rotations of a motor-
66 driven ergometer. The number of studies investigating eccentric cycling have increased in the
67 last 20 years, with many reporting positive effects on muscle mass and strength (LaStayo et al.
68 2000; Julian et al. 2018).

69 It has been reported that DOMS is more associated with damage to and inflammation
70 within the muscular connective tissues, rather than to muscle fibers themselves (Crameri et al.
71 2007; Paulsen et al. 2010). Some studies have shown increases in collagen breakdown markers
72 such as hydroxyproline (Hyp) in urine (Brown et al. 1997) and blood (Brown et al. 1999) after
73 maximal eccentric knee extensor exercise. Since intramuscular connective tissues transmit
74 force and thus strongly influence muscle force output (Grounds et al. 2005), it is possible that
75 a prolonged decrease in muscle strength after eccentric exercise is also associated with damage
76 to connective tissues.

77 Our previous study showed a greater extent of DOMS after work-matched higher- than
78 lower-intensity eccentric cycling without significant differences in maximal voluntary
79 isometric contraction (MVIC) strength and plasma CK activity changes between the two
80 protocols (Mavropalias et al. 2020). If DOMS is more associated with connective tissue
81 damage than muscle fiber damage, it may be that greater connective tissue breakdown is
82 observed after higher- than lower-intensity eccentric cycling. Hyp is a non-proteinogenic
83 amino acid and a major component of collagen, critical to the stabilization of the collagen triple

84 helix (Kotch et al. 2008). Therefore, an increase in blood or urine Hyp concentration is assumed
85 to indicate muscle collagen breakdown (Murguia et al. 1988). Since mechanical impact due to
86 gravity is minimal during cycling (Woodward and Cunningham 1993), it seems likely that
87 increased Hyp concentration in the blood is from muscle connective tissue rather than bone
88 origin. It is possible that changes in blood Hyp concentration and the magnitude of muscle
89 functional loss and DOMS after eccentric cycling are highly associated, which would provide
90 greater insight on the involvement of the muscle connective tissues to these eccentric exercise-
91 induced phenomena.
92 Some studies have used blood cell-free DNA (cfDNA) as a muscle damage marker
93 (Ferrandi et al. 2018; Andreatta et al. 2018). cfDNAs are circulating cell-unbound, double-
94 stranded DNA fragments (Breitbach et al. 2012), normally present in a small amount in the
95 blood, but shown to increase after intense exercises such as running, cycling, and resistance
96 training (Atamaniuk et al. 2004, 2008, 2010; Tug et al. 2017a; Andreatta et al. 2018). For
97 example, Andreatta et al. (2018) compared high- (80% of one-repetition maximum) and low-
98 intensity (40%) leg press exercise (30 and 75 repetitions, respectively), and reported that serum
99 cfDNA concentration increased 1.6-fold at immediately after the high-intensity exercise only,
100 and the increase was correlated with the magnitude of decrease in squat and counter-movement
101 jump height. The authors concluded that cfDNA levels were sensitive to the exercise intensity
102 and that they could serve as a promising muscle damage marker (Andreatta et al. 2018).
103 However, the resistance exercise performed in the study by Andreatta et al. (2018) consisted
104 of both concentric and eccentric actions, and to the best of our knowledge no previous study
105 has examined changes in cfDNA after exercise consisting of eccentric-only muscle actions, to
106 investigate possible associations with muscle damage phenomena. Haller et al. (2018) reported
107 that increases in blood cfDNA concentration were positively correlated with the rate of
108 perceived exertion, and increased progressively with increasing running duration and intensity.

109 Thus, comparison between higher- and lower-intensity eccentric cycling with a matched total
110 mechanical work may clarify whether post-exercise increases in cfDNA blood concentrations
111 are more related to muscle damage or metabolic load. The origin of cfDNA has been a matter
112 of speculation, but it has been hypothesized that it originates either from neutrophils (Breitbach
113 et al. 2014; Beiter et al. 2014) or skeletal muscle cells (Atamaniuk et al. 2010; Ferrandi et al.
114 2018). It is of interest to examine whether eccentric cycling induces increase in plasma cfDNA
115 concentration, and whether its changes are associated with the magnitude of changes in muscle
116 strength, muscle soreness and plasma CK activity, together with the source of increased plasma
117 cfDNA.

118 Given the above, the purpose of this study was to compare between higher- and lower-
119 intensity eccentric cycling with the same total mechanical work for changes in plasma Hyp and
120 cfDNA concentrations in relation to other indirect muscle damage markers such as plasma CK
121 activity, muscle strength, and DOMS. Moreover, we evaluated whether the plasma cfDNA
122 originated from skeletal muscle by targeting an epigenetic marker, and whether any
123 associations exist between changes in plasma cfDNA or Hyp concentrations and both muscle
124 strength loss and DOMS.

125

126 **METHODS**

127 **Participants**

128 The sample size was estimated using G*Power (Version 3.1.9.2, Universitat Kiel,
129 Germany) based on the study by Paschalis et al. (2005), who compared responses to high- and
130 low-intensity eccentric knee extensions, showing a greater decrease in MVIC torque after the
131 high- than low-intensity eccentric exercise with an effect size of 1.34. With a power level of
132 0.8, and an alpha level of 0.05, it was found that a total of 18 participants were required, thus
133 at least 9 participants were required in each group.

134 Based on this, 20 men who were unaccustomed to eccentric exercises and free from
135 lower limb injuries for at least 6 months prior to the study were recruited for this study. The
136 participants were instructed not to deviate from their regular dietary patterns and to not perform
137 any exercise during the experimental period. They were randomly allocated to either a higher-
138 intensity eccentric cycling group (HI, n=11) or a lower-intensity eccentric cycling group (LI,
139 n=9). No significant differences between the HI and LI groups were found for age (25.3 ± 3.6
140 vs 24.7 ± 5.9 y, p = 0.781), height (181.2 ± 7.2 vs 178.6 ± 8.6 cm, p = 0.467), and body mass
141 (83.3 ± 11.4 kg vs 75.7 ± 10.2 kg, p = 0.139). Ethical approval from the Edith Cowan
142 University human research ethics committee was provided before study commencement, and
143 every participant gave informed consent before participating in the study. The collection of
144 blood samples was conducted in accordance with all applicable laws, guidelines, and
145 regulation.

146

147 **Eccentric cycling**

148 Using an eccentric cycle ergometer (Grucox Eccentric Trainer, Grucox, South Africa),
149 participants completed a 2-min concentric cycling warm-up at 50 W after an appropriate seat
150 position was determined by the investigator in relation to their leg length. Peak power output
151 during a maximal 10-s isokinetic concentric cycling at 60 rpm (PPO) was determined for each
152 participant to set the intensity of the eccentric cycling. This was used in the previous study
153 (Mavropalias et al. 2020), and peak power output measure during eccentric cycling was not
154 used, because of potential muscle damage and the repeated bout effect that could affect the
155 outcome measures (Peñailillo et al. 2013). Participants in HI performed eccentric cycling at
156 20% PPO for 5 sets of 1 min with a 1-min rest between sets, and those in LI performed eccentric
157 cycling at 5% of PPO for 5 sets of 4 min with a 1-min rest between sets in order to achieve
158 equal total mechanical work. Our pilot studies showed that 20% PPO required high effort but

159 was a feasible target workload for participants who were unaccustomed to eccentric cycling to
160 cycle at 60 rpm for 1 min, while 5% PPO was chosen as an intensity that was largely different
161 (4-fold) from the 20% PPO, but still induced muscle damage following an unnacustommed
162 bout (Mavropalias et al. 2020). Each participant performed 1-min of eccentric cycling
163 familiarization immediately before performing HI or LI eccentric cycling, during which
164 eccentric cycling was started from 30 rpm reaching 60 rpm at the end of the minute at the target
165 power output. The participants were instructed to resist the pedals by using the lower limbs to
166 perform smooth backward rotations while maintaining their target power, which was shown on
167 a computer screen set in front of the ergometer. The pedal straps were removed from the
168 ergometer to ensure the participants only pushed against the pedals and did not pull when the
169 pedals were moving away from them to generate power using their knee and hip flexors.
170 Moreover, visual feedback was provided by the ergometer computer in relation to the muscle
171 action type (concentric: green bar vs eccentric: blue bar) according to the forces applied to its
172 pedals and the angle of the crank to ensure the participants were not performing concentric
173 actions. The investigator constantly monitored the screen to ensure the participants only
174 performed eccentric muscle actions. In addition, the use of a motorized ergometer allowed the
175 knee extensors to shorten passively during the muscle shortening phase.

176 Heart rate (HR) during cycling was continuously recorded by a HR monitor (Polar
177 S810i, Polar Electro, Finland). Rating of perceived effort (RPE) was assessed at the end of each
178 set using a modified version of Borg's category-ratio scale (0 –10; 0: nothing at all, 10:
179 maximal effort) (Borg 1998), as it has been reported that measuring effort during eccentric
180 cycling is more appropriate than exertion (Peñailillo et al. 2018). At the end of each set, the
181 participants were asked to rate their physical effort required by their knee extensors to maintain
182 the target power.

183

184 **Maximal voluntary isometric contraction (MVIC) torque**

185 An isokinetic dynamometer (System 3, Biodex Medical Systems, USA) was used to
186 assess MVIC torque, before, immediately after, and 24, 48, and 72 h after exercise. During
187 measurements, visual feedback of torque was displayed on a computer screen via a computer
188 software (LabVIEW, National Instruments, Australia). MVIC torque was measured from the
189 right knee extensors at knee joint angles of 20° and 70° after several submaximal contractions
190 as warm-up. Three 4-s maximal voluntary isometric knee extensions were performed at each
191 joint angle, separated by 1 min of passive rest. Participants were advised not to perform any
192 countermovement motion before the muscle action. The highest torque value from each angle
193 was used as a percentage of change compared to the baseline of the respective test, and the
194 average of those values for each angle at each timepoint was used for further analysis.

195

196 **Muscle soreness**

197 The magnitude of lower-limb soreness was assessed during movement at the same time
198 points as those of MVIC. Participants were then asked to sit down to and stand up from a chair
199 slowly (~3 s for each direction), and to report their perceived soreness in each direction using
200 a custom 0 (no soreness) – 10 (maximal soreness) scale. The average values of the two
201 directions of movement at each time point were used for subsequent statistical analysis.

202

203 **Blood sampling**

204 A venous blood sample was collected from an antecubital vein to two 6-mL EDTA
205 tubes at before, immediately after, as well as 24, 48, and 72 h after exercise, before other
206 measures were obtained. The tubes were centrifuged in a swing bucket rotor centrifuge
207 (Heraeus Multifuge 3 SR, Thermo Fisher Scientific, USA) at 1600 g for 10 min and the plasma
208 was separated to a 15-mL conical tube. The plasma was then further centrifuged at 2000 g for

209 10 min and supernatant-aliquoted to several microtubes and stored at -80° C for subsequent

210 Hyp and cfDNA analyses.

211

212 **Plasma creatine kinase (CK) activity**

213 A 30-µL whole blood sample was pipetted from the EDTA tube and loaded to a strip

214 for CK activity analysis using a Reflotron (Roche Diagnosis, Switzerland). This analysis

215 provides plasma CK activity.

216

217 **Plasma hydroxyproline (Hyp) concentration**

218 For the assessment of plasma Hyp concentration, 125 µl of plasma sample was mixed

219 with 125 µl of 12 N hydrochloric acid and then hydrolyzed for 24 h at 95°C. The mixture was

220 left to cool, and subsequently filtered through a 0.22 µm PVDF syringe filter unit. The

221 supernatants were analyzed based on the manufacturer's guidelines using a colorimetric kit

222 (Cell Biolabs, Inc., USA), which allowed the determination of Hyp concentration through the

223 reaction of oxidized hydroxyproline with 4-(dimethylamino) benzaldehyde.

224

225 **Plasma cfDNA concentration**

226 Between 2 and 5 mL of plasma were used for cfDNA isolation using the QIAamp

227 Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) as per the manufacturer's

228 instructions. Cell free DNA was quantified by droplet digital PCR (ddPCR) using QX200

229 AutoDG (Bio-Rad, Hercules, CA). Two DNA loci were used for quantification on the *AR* (X-

230 chromosome) and *BAP1* (Chromosome 3) genes using commercial assays for ddPCR (Bio-

231 Rad). Amplifications were performed using the following cycling conditions: 1 cycle of 95°C

232 (2.5C/s ramp) for 10 min, 40 cycles of 94°C (2.5C/s ramp) for 30 s and 57°C for 1 min,

233 followed by 1 cycle of 98°C (2.5C/s ramp) for 10 min. Copies of cfDNA per mL of plasma
234 were calculated based on the volume of plasma used for extraction and elution volume.
235
236 **cfDNA origin**
237 Five samples from the HI group with significant increase in DNA copies post exercise
238 were further quantified using the 2100 Bioanalyzer systems (Agilent, USA). To examine if the
239 cfDNA was derived from skeletal muscles, the presence of methylated *HOXD4* promoter was
240 examined. To evaluate the specificity of primers and probes, a primary human myoblast line
241 and melanoma cell lines (92.1, Mel 270) were used as biological positive and negative controls,
242 respectively. The primers and probes used are as follow:
243
244 Forward PCR primer: 5' - TTT TCC AAT TCT AAA ACT TAC TAC C – 3'
245 Reverse primer: 5' - TTT TCC AAT TCT AAA ACT TAC TAC C – 3'
246 Methylated probes (/56-FAM/AA GCG GTT T/ZEN/C GAA CGG TTT A/3IABkFQ/)
247 Unmethylated (/5HEX/AA GTG GTT T/ZEN/T GAA TGG TTT A/3IABkFQ/)
248
249 Mastermix reagents included: 1X ddPCR supermix (Bio-Rad), 250 nM probes, 900 nM
250 of each primer, 50 nM 7-Deaza (New England Biolabs, Ipswich, MA). Droplets were generated
251 using the Automatic Droplet generator QX200 AutoDG (Bio-Rad). PCR Cycle involved: 1
252 cycle of 95°C (2.5C/s ramp) for 10 min, 40 cycles of 94°C (2.5C/s ramp) for 30 s and 57°C for
253 1 minute, followed by 1 cycle of 98°C (2.5C/s ramp) for 10 min. Droplets were analyzed
254 through a QX200 Droplet Reader (Bio-Rad). QuantaSoft analysis software (Bio-Rad) was used
255 to acquire and analyze data.
256

257 **Statistical analyses**

258 Baseline values of all depedent variables were compared between groups using a
259 Student's t-test. A two-way (group × time) repeated-measures analysis of variance was used to
260 compare between HI and LI groups for changes in the dependent variables over time. The data
261 were assessed for assumptions of normality by analysing the standardized residuals using a
262 Shapiro-Wilk test, and for sphericity by a Mauchly's sphericity test. Partial eta squared values
263 (η^2_p) are also reported as a measure of factor variation size. Ordinal data (muscle soreness and
264 RPE) were rank-transformed before being analyzed through the repeated-measures analysis of
265 variance (Wobbrock et al. 2011). In the case of a significant interaction effect, a Holm's
266 sequential Bonferroni correction was performed to identify possible differences between
267 groups for each time point. Correlation analyses were performed using Pearson product-
268 moment correlation (r) for continuous data, whereas a Spearman correlation (ρ) was used for
269 ordinal data (muscle soreness and RPE). The significance level was set to $P \leq 0.05$. All
270 statistical testing was performed using Jamovi version 1.6.3 (Jamovi project, 2018). Data are
271 presented as mean ± standard deviation (SD).

272

273 **RESULTS**

274 **Heart rate and effort during eccentric cycling**

275 Average heart rate during eccentric cycling was greater ($P < 0.001$) in HI (130.4 ± 16.6
276 bpm) than LI (and 108.1 ± 18.7 bpm). Average RPE was also greater ($P < 0.05$) for HI (5.1 ±
277 2.2) than LI (3.0 ± 0.9).

278

279 **MVIC torque and muscle soreness**

280 MVIC torque and DOMS values are shown in Figure 1. MVIC torque (average torque
281 of knee joint angles of 20° and 70°) decreased from the baseline ($P < 0.01$, time $\eta^2_p = 0.321$)

282 immediately (-13.3 ± 7.8%), 24 h (-9.7 ± 10.7%), and 48 h (-6.7 ± 13.5%) after eccentric
283 cycling without a significant difference between HI and LI groups (P = 0.173, interaction η^2_p
284 = 0.091). Muscle soreness increased at 24 – 72 h after exercise in both groups (P = 0.016, time
285 η^2_p = 0.413), but maximal muscle soreness (mean of maximal values per group across all time
286 points) was greater (P < 0.05, interaction η^2_p = 0.225) in HI (4.5 ± 3.0) than LI (1.2 ± 1.0).
287
288 **Plasma CK activity**
289 A significant increase in plasma CK activity from the baseline was found only at 24 h
290 post-exercise (141 ± 73.5%, P = 0.029, time η^2_p = 0.194), but this increase did not differ
291 significantly between the groups (P = 0.268, interaction η^2_p = 0.070). The average CK activity
292 of both groups was 248.2 ± 272.5 IU/L at 24 h, 176.5 ± 172.8 IU/L at 48 h, and 142.1 ± 112.9
293 IU/L at 72 h post-exercise.
294
295 **Plasma Hyp concentration**
296 Baseline Hyp concentration was 24.3 ± 5.9 μg/mL for HI and 27.9 ± 8.2 μg/mL for LI
297 without difference between groups (P = 0.286). As shown in Figure 2, plasma Hyp
298 concentration increased significantly at 24 – 72 h after cycling by 40 – 53% from baseline for
299 both HI and LI (P < 0.001, time η^2_p = 0.576) with no significant difference between the groups
300 at any time point (P = 0.518, interaction η^2_p = 0.032).
301
302 **Plasma cfDNA concentration and its origin**
303 Pre-exercise plasma cfDNA concentrations were similar between the groups, with 233
304 ± 129 copies/mL for HI and 203 ± 78.4 copies/mL for LI (P = 0.552). Plasma cfDNA
305 concentrations increased from pre- to post-exercise in HI (Figure 3, P < 0.001, time η^2_p = 0.464),
306 returning to baseline levels by 24 h post-exercise. HI showed a significantly greater increase in

307 plasma cfDNA concentration than LI group post-exercise (2.3 ± 0.9-fold, $P = 0.002$, interaction

308 $\eta^2_p = 0.230$).

309 Five samples in the HI group with significant increase in DNA copies post exercise by

310 ddPCR and with a high overall cfDNA yield via chip-based capillary electrophoresis

311 (Bioanalyzer), were selected for downstream methylation analysis of *HOXD4*. The upstream

312 region of *HOXD4* contains two myogenic hypermethylated sites with high specificity for

313 muscle derived DNA (Ehrlich and Lacey 2013). We evaluated whether the increase on cfDNA

314 was muscle derived using a ddPCR assay specific for this epigenetic change. However,

315 methylated *HOXD4* was not detectable in the cfDNA of the five samples pairs analyzed (Figure

316 4).

317

318 **Correlations**

319 Figure 5 shows some of the correlation analysis results. No significant correlations were

320 observed between the maximal decrease in MVIC torque at 24 to 72 h post-exercise and peak

321 change in Hyp concentration ($r = 0.114$, $P = 0.631$), pre- to post-exercise change in cfDNA

322 concentration ($r = -0.425$, $P = 0.070$), or maximal change in CK activity ($r = -0.430$, $P = 0.058$).

323 No significant correlations were detected between maximal muscle soreness and maximal

324 change in Hyp concentration ($\rho = -0.111$, $P = 0.642$), pre- to post-exercise change in cfDNA

325 concentration ($\rho = 0.354$, $P = 0.137$), or maximal change in CK activity ($\rho = 0.042$, $P = 0.859$).

326 There were no significant correlations between maximal change in CK activity and

327 maximal change in Hyp concentration ($r = -0.015$, $P = 0.950$) or between maximal change in

328 Hyp concentration and pre- to post-exercise change in cfDNA concentration ($r = -0.228$, $P =$

329 0.347). Although the change in cfDNA concentration from pre- to post-exercise significantly

330 correlated with maximal change in CK activity ($r = 0.577$, $P = 0.010$), it was no longer

331 significantly correlated ($r = 0.285$, $P = 0.251$) when one outlier was removed who showed an
332 increase of 686 IU/L (919% increase).

333 There were no significant correlations between maximal change in CK activity and
334 maximal change in HR ($r = 0.251$, $P = 0.287$) or maximal RPE ($\rho = 0.372$, $P = 0.106$), or
335 maximal change in Hyp concentration and maximal change in HR ($r = 0.157$, $P = 0.522$).
336 However, maximal change in Hyp concentration was significantly correlated with maximal
337 RPE ($\rho = 0.678$, $P = 0.001$), and the pre- to post-exercise change in cfDNA concentration was
338 significantly correlated with the maximal change in HR ($r = 0.513$, $P = 0.025$) and maximal
339 RPE ($\rho = 0.473$, $P = 0.041$).

340

341 **DISCUSSION**

342 The present results revealed a significant increase in plasma Hyp concentration at 24 –
343 72 h after exercise with no detectible differences between HI and LI eccentric cycling groups.
344 Plasma cfDNA concentration increased immediately after HI eccentric cycling only and
345 returned to the baseline by 24 h post-exercise, however the lack of methylated *HOXD4*
346 indicated that this increase in cfDNA was not derived from skeletal muscle. No significant
347 correlations were evident between either Hyp or cfDNA change and changes in muscle strength
348 or muscle soreness. Nevertheless, the magnitude of increase in cfDNA was significantly
349 correlated with both maximal heart rate increase and rate of perceived effort during the
350 eccentric cycling, indicating that cfDNA concentrations in the blood were most likely
351 influenced by eccentric exercise intensity.

352 Hyp is a non-proteinogenic amino acid and a major component of collagen, and its main
353 function is to stabilize the collagen triple helix (Kotch et al. 2008). Therefore, concentration
354 increases of this structural molecule in either blood or urine have been used as an indicator of
355 muscle collagen breakdown (Murguia et al. 1988; Virtanen et al. 1993; Brown et al. 1997,

356 1999; Tofas et al. 2008). For example, Brown et al. (1997) observed an increase (69%) in urine
357 Hyp concentration at 2 days after 50 maximal eccentric knee extensions and subsequently
358 concluded that the result indicated the breakdown of collagenous connective tissues.
359 Additionally, Tofas et al. (2008) found that plasma Hyp concentration increased at 24 – 72 h,
360 and peaked (80% increase) at 48 h after 200 plyometric jumps, indicating a strong effect on
361 connective tissues. In contrast, Virtanen et al. (1993) did not detect a change in serum Hyp
362 concentration for 96 h after 50 maximal concentric bilateral knee extensions, potentially
363 indicating that non-damaging concentric muscle work did not trigger Hyp release. Bone
364 turnover could increase Hyp concentrations in the blood after exercise (Maïmoun et al. 2006;
365 Kish et al. 2015), thus increased Hyp concentration in the blood or urine does not exclusively
366 reflect collagen breakdown originating from the muscle connective tissues of exercised
367 muscles. However, since gravity-induced mechanical impact is minimal during cycling
368 (Woodward and Cunningham 1993), and the muscle force generated during eccentric cycling
369 protocol was not necessarily high in relation to maximal capacity, it seems likely that the origin
370 of the increased Hyp in the blood was the connective tissue surrounding muscle fibers
371 (endomysium), fascicle (perimysium) and/or muscle fascia (epimysium) rather than the bone.
372 We hypothesized that increases in plasma Hyp concentration would be greater after HI than
373 LI, since we observed higher DOMS values after HI than LI. Baseline Hyp values were higher
374 than those in previous studies, however the magnitude of increase (40 – 53%) and time course
375 of the changes were comparable to those reported in the previous studies (Brown et al. 1997;
376 Tofas et al. 2008). It should be noted that the changes in plasma Hyp concentration were similar
377 between HI and LI (Figure 2). Thus, eccentric exercise intensity does not appear to be a critical
378 factor influencing muscle collagen breakdown, since the 4-fold intensity difference between
379 the conditions was not a significant factor for triggering increased collagen breakdown. Further
380 research is warranted to examine whether increased plasma Hyp concentration indeed

381 represents connective tissue damage, and if so why no significant difference between HI and
382 LI was evident for the changes.
383 Eccentric exercise-induced muscle damage increases muscle inflammation and release
384 of matrix metalloproteinases (also known as matrix metallopeptidases), an enzyme family that
385 degrades collagen and other extracellular matrix components, and triggers the subsequent
386 phases of synthesis (Koskinen et al. 2002; Paulsen et al. 2010). The increase in plasma Hyp
387 concentration observed in the current study at 24 – 72 h after the acute eccentric cycling session
388 may reflect increased collagen turnover via metalloproteinases. In addition, the lack of
389 difference in plasma Hyp concentration responses between the two intensities (HI vs LI) may
390 suggest that eccentric exercise-induced collagen breakdown is dependent upon the total
391 mechanical work rather than exercise intensity, when the intensity is submaximal. However,
392 this speculation requires further studies to assess which mechanical factor is mostly responsible
393 for collagen breakdown after eccentric exercise, and if there is a difference when comparing
394 mechanical work-matched submaximal with maximal exercise intensity eccentric cycling
395 protocols for changes in plasma Hyp concentration.
396 It was hypothesized that the post-exercise muscle functional loss and DOMS would be
397 associated with collagen breakdown. However, no significant correlations between the
398 magnitude of increase in plasma Hyp concentration and either muscle functional loss or DOMS
399 were evident (Figure 5). It is therefore unlikely that collagen breakdown is the direct cause of
400 the muscle functional loss and DOMS after eccentric cycling, however we acknowledge the
401 limitation that our muscle damage model involved the entire lower limb, whereas our muscle
402 function measure was only in knee extensors. Crameri et al. (2007) showed that tenascin C, a
403 protein responsible for extracellular matrix de-adhesion from the cell membranes, increased
404 similarly after both voluntary and electrically-stimulated maximal eccentric exercise with a
405 similar DOMS response in both conditions, even though the electrically-stimulated muscle

406 induced more pronounced muscle fiber damage. The authors concluded that an increase in
407 tenascin C expression provides further evidence of a potential role of the extracellular matrix
408 in the development of DOMS. It will be of interest to determine whether plasma Hyp
409 concentration increases following a repeated bout of eccentric cycling that induces less DOMS
410 and smaller changes in muscle function and other indirect markers of muscle damage.

411 The present study was the first to examine changes in plasma cfDNA concentration
412 following eccentric cycling, and revealed a 2-fold increase in plasma cfDNA concentration
413 immediately after HI but not LI (Figure 3). Increases in plasma cfDNA concentration
414 immediately post-exercise have been reported after many different exercises such as half-
415 marathon (18.6-fold) (Atamaniuk et al. 2004), ultra-marathon (5.25-fold) (Atamaniuk et al.
416 2008), weightlifting (3.15-fold) (Atamaniuk et al. 2010), exhaustive rowing (2.5-fold) (Velders
417 et al. 2014), exhaustive stationary cycling (5.6-fold (Frühbeis et al. 2015), and 4.4-fold (Tug et
418 al. 2017a)), high-intensity leg press exercise (1.62-fold) (Andreatta et al. 2018), repeated 40-m
419 sprints with 1-min (1.9-fold) or 5-min (2.8-fold) inter-sprint rests (Haller et al. 2018), whole
420 body resistance-training (1.6-fold) (Tug et al. 2017b), high-intensity interval treadmill running
421 (~1.8-fold) (Ferrandi et al. 2018), and exhaustive treadmill running (2 – 15-fold) (Fatouros et
422 al. 2010; Beiter et al. 2011, 2014; Tug et al. 2015; Helmig et al. 2015; Stawski et al. 2017;
423 Haller et al. 2018). However, none of these studies clearly addressed whether eccentric muscle
424 actions could trigger increases in cfDNA without concentric actions, significant fatigue, or
425 exhaustion. In the present study, we observed a statisticaly significant increase in plasma
426 cfDNA concentration; however, the magnitude of increase was smaller than that reported in
427 many previous studies. This may indicate that metabolic demand is a key factor influencing
428 cfDNA concentration as the metabolic demand of the eccentric cycling in the present study
429 would have been far lower than that of the exercise in most previous studies. This hypothesis
430 is consistent with the finding of a significant correlation between the cfDNA increase

431 immediately post-exercise and maximal change in heart rate during eccentric cycling, and the
432 lack of statistical increase in plasma cfDNA after LI eccentric cycling. In fact, significant
433 correlations between the magnitude of increase in blood cfDNA concentration and blood
434 lactate levels, perceived exertion, and HR have been reported after incremental treadmill
435 running (Beiter et al. 2011; Breitbach et al. 2014; Haller et al. 2017) and intermittent sprints of
436 different rest periods (Haller et al. 2018). No correlations between plasma cfDNA
437 concentration and muscle functional loss or DOMS were evident in the present study (Figure
438 5), so it appears that post-exercise increases in blood cfDNA concentration are not strongly
439 associated with muscle damage. It is of future interest to investigate plasma cfDNA responses
440 to eccentric versus concentric cycling with the same metabolic demand (e.g., the same level of
441 oxygen consumption).

442 It should be noted that plasma cfDNA concentrations returned to the baseline at 24 h
443 post-exercise. This has been observed in previous studies, with return to baseline occurring in
444 as little as 30 – 120 min (Atamaniuk et al. 2004, 2008, 2010; Fatouros et al. 2010; Velders et
445 al. 2014; Andreatta et al. 2018). A possible cause of this rapid decrease could be the
446 accompanying increase in deoxyribonuclease-1 activity in healthy individuals in order to
447 preserve immune homeostasis (Velders et al. 2014; Beiter et al. 2014, 2015). Another
448 interesting observation was that a relatively short exercise time (only 5 min of exercise, 9 min
449 in total, including the resting periods) was needed to increase plasma cfDNA concentration in
450 the present study. Beiter et al. (2011) reported an increase in plasma cfDNA concentration
451 within 10 min after the onset of incremental treadmill running. In the present study, no blood
452 samples were taken between the immediate post-exercise sample and at 24 h post-exercise, but
453 based on previous investigations we assumed that cfDNA concentration would return to
454 baseline as soon as 120 min post-exercise (Atamaniuk et al. 2004, 2008, 2010; Fatouros et al.
455 2010; Velders et al. 2014; Andreatta et al. 2018).

456 Regarding the origin of cfDNA, damage and apoptosis of skeletal muscle cells or
457 leukocytes have been previously considered to be candidate sources (Atamaniuk et al. 2008).
458 The lack of methylated *HOXD4* cfDNA in the plasma samples suggests that the increased
459 cfDNA did not originate from skeletal muscle (Figure 4). Our results do not support the
460 hypothesis that post-exercise increase in cfDNA in the blood are derived from skeletal muscle
461 cells undergoing apoptosis or necrosis (Atamaniuk et al. 2004, 2010; Ferrandi et al. 2018).
462 Moreover, based on our findings it seems unlikely that there was DNA 'leakage' from
463 exercised muscle cells due to increased membrane permeability or increased release of
464 extracellular vesicles (Helmig et al. 2015), or that there was an increased rate of clearance of
465 DNA fragments originating from muscle cells that stuck on cell membranes or lymph due to
466 increased blood perfusion (Breitbach et al. 2012). Instead, one plausible explanation is that
467 both baseline and post-exercise levels of cfDNA largely originated from haematopoietic-
468 derived cells, as reported in a previous study (Tug et al. 2015). Even though the precise
469 physiological event that causes the increase in plasma cfDNA concentration is not yet fully
470 understood, Beiter et al. (2014) speculated that activated neutrophils might primarily contribute
471 to exercise-evoked cfDNA levels by releasing neutrophil extracellular traps. Neutrophils
472 respond to exercise by forming neutrophil extracellular traps which is thought to contribute to
473 an increased hypercoagulable exercise-induced state (Beiter et al. 2015). Future research
474 should examine the source of the exercise-induced increases in plasma cfDNA concentration.
475 Based on the findings from the present study, it appears that plasma Hyp concentration
476 increases after eccentric cycling possibly as a result of collagen breakdown, but the increase
477 was not associated with decreases in muscle function or DOMS. Plasma cfDNA concentration
478 does not appear to be a marker of muscle damage, as there was no detectable methylation of
479 *HOXD4* in the cfDNA molecules, and the cfDNA concentration increase after exercise seemed

to be more associated with the metabolic intensity of the exercise (i.e. heart rate increase and perceived effort).

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DECLARATION OF INTEREST

Competing interests: The authors declare there are no competing interests.

AUTHOR CONTRIBUTIONS

GM, AB and KN conceived and designed the study, and GM and LC conducted the experiments. GM collected, and GM, OB, WP, LC, MM, TK, and EG analyzed the data. GM drafted the manuscript with AB and KN. All authors read, edited and approved the manuscript.

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FIGURE LEGENDS

Figure 1. Changes (mean ± SD) in maximal voluntary isometric contraction (MVIC) torque at 70° and 20° knee flexion (average of the two angles) [A], and muscle soreness (average value reported during sitting up and down from a chair) [B], before (Pre), immediately after (Post), and 24 – 72 h after higher-intensity (HI) and lower-intensity (LI) eccentric cycling.
*: significant ($P < 0.05$) difference from the baseline for both groups. #: significant ($P < 0.05$) difference between groups. ANOVA results for interaction (group × time) effect are shown in the legend for each group.

Figure 2. Changes (mean ± SD) in plasma hydroxyproline concentration before (Pre), 24, 48 and 72 h after higher- (HI) and lower-intensity (LI) eccentric cycling.
*: significant ($P < 0.05$) difference from the baseline for both groups, n.s.: no significant group × time interaction effect.

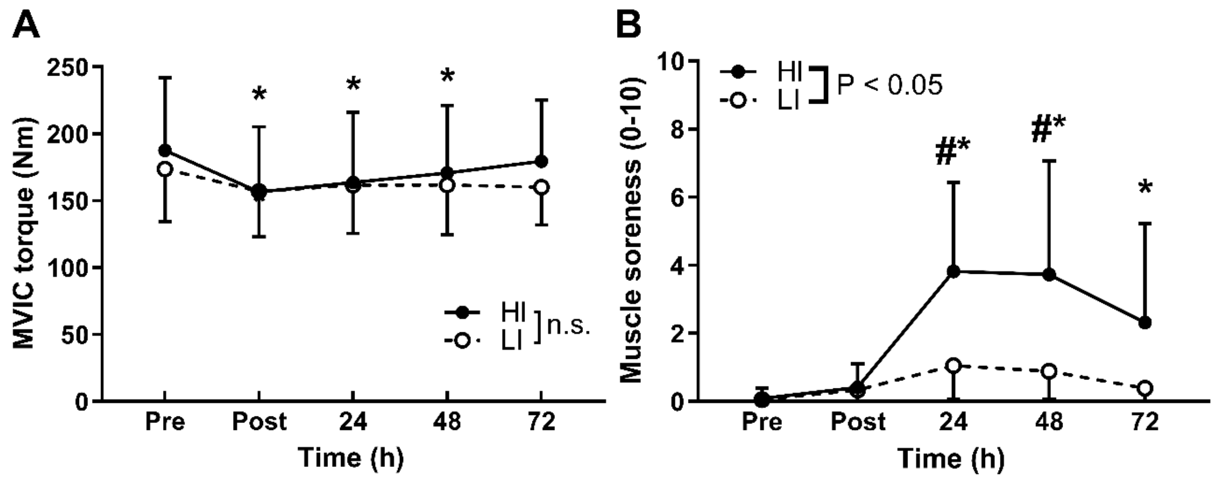
Figure 3. Changes (mean ± SD) in plasma cfDNA concentration before (Pre), immediately after (Post), and 24, 48 and 72 h after higher-intensity (HI) and lower-intensity (LI) eccentric cycling.
†: significant ($P < 0.001$) difference of the HI group from the baseline value. #: significant ($P < 0.05$) difference between groups. $P = 0.02$ shows the group × time interaction effect.

Figure 4. *HOXD4* methylation analysis in positive control (100 and 0% methylated PCR gDNA controls in 50:50 ratio) [A], primary human myoblast cell line [B], gDNA [C] and supernatant cfDNA [D] of uveal melanoma cell line Mel270 (negative control), composite data from the five participants before [E] and immediately after [F] higher-intensity eccentric cycling. Diagram denotes fluorescence signal intensity (amplitude) of droplets containing methylated (Channel 1, FAM) and unmethylated (Channel 2, HEX) *HOXD4* DNA copies respectively. Pink lines denote assay thresholds, gray dots show empty droplets, green dots are *HOXD4* unmethylated DNA, and blue dots are *HOXD4* methylated DNA.

Figure 5. Correlations between the largest percent decrease in maximal voluntary isometric contraction (MVIC) torque at 24 – 72 h post-exercise and maximal increase in plasma hydroxyproline concentration (Hyp) [A], maximal muscle soreness scores and maximal increase in plasma Hyp [B], rate of perceived effort (RPE) and maximal increase in Hyp [C], maximal change in plasma creatine kinase (CK) activity and fold-change in plasma cell-free DNA (cfDNA) concentration from pre- to immediately post-exercise [D], maximal increase in heart rate (% change from rest) during exercise and fold-change in plasma cfDNA concentration from pre- to immediately post-exercise [E], and RPE and fold-change in plasma cfDNA concentration from pre- to immediately post-exercise [F]. Black (●) and white (○) circles represent the participants in the higher-intensity (HI) and lower-intensity (LI) eccentric cycling groups, respectively. Statistics for Pearson product moment (r), and Spearman correlation (ρ) and their respective P values are reported within each graph. Dotted lines demonstrate 95% CIs for the linear regression line.

680 Figures

681 **Figure 1**



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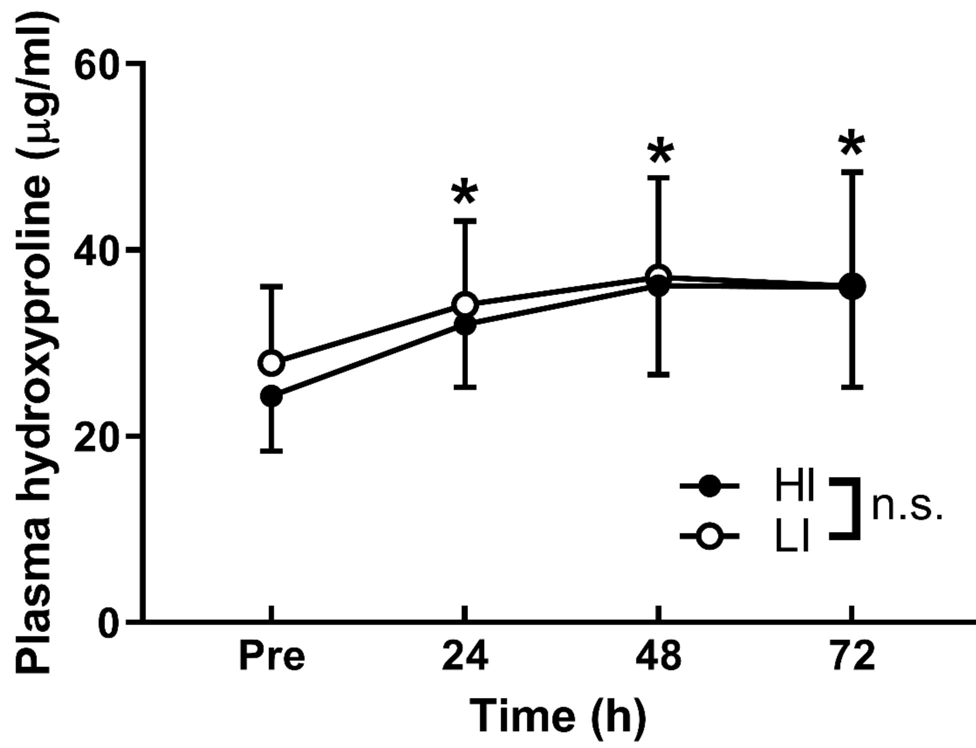
684 **Figure 2**



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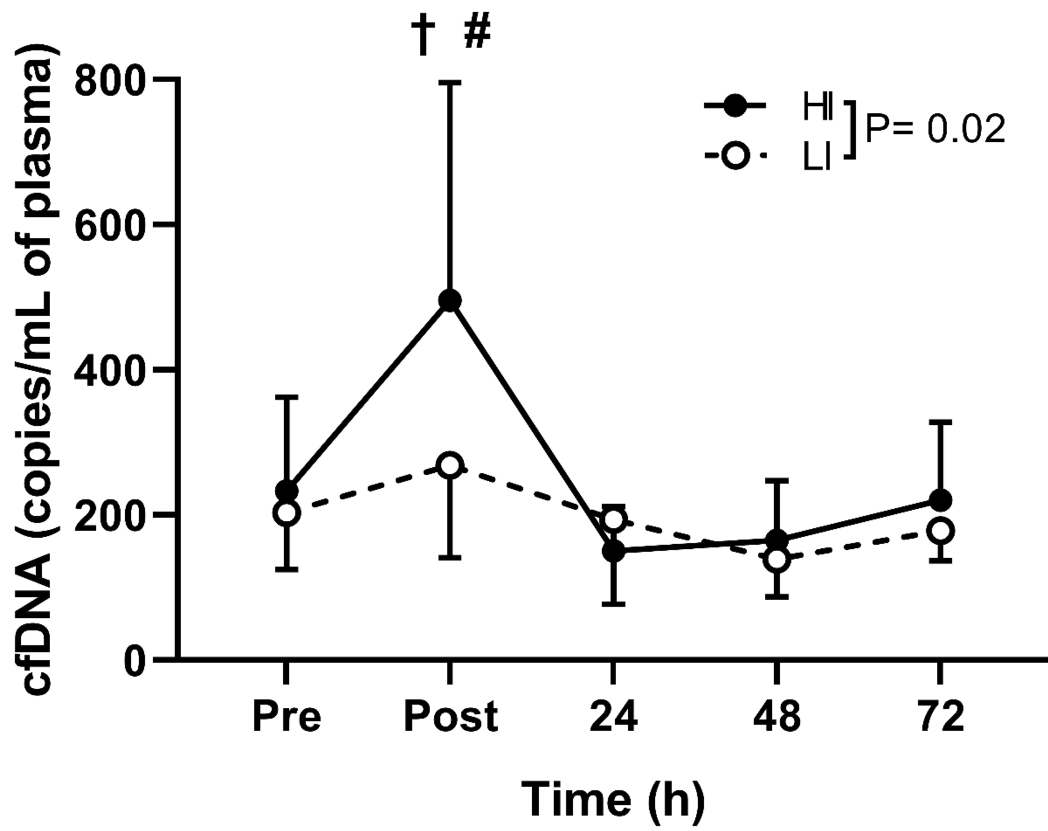
687 **Figure 3**



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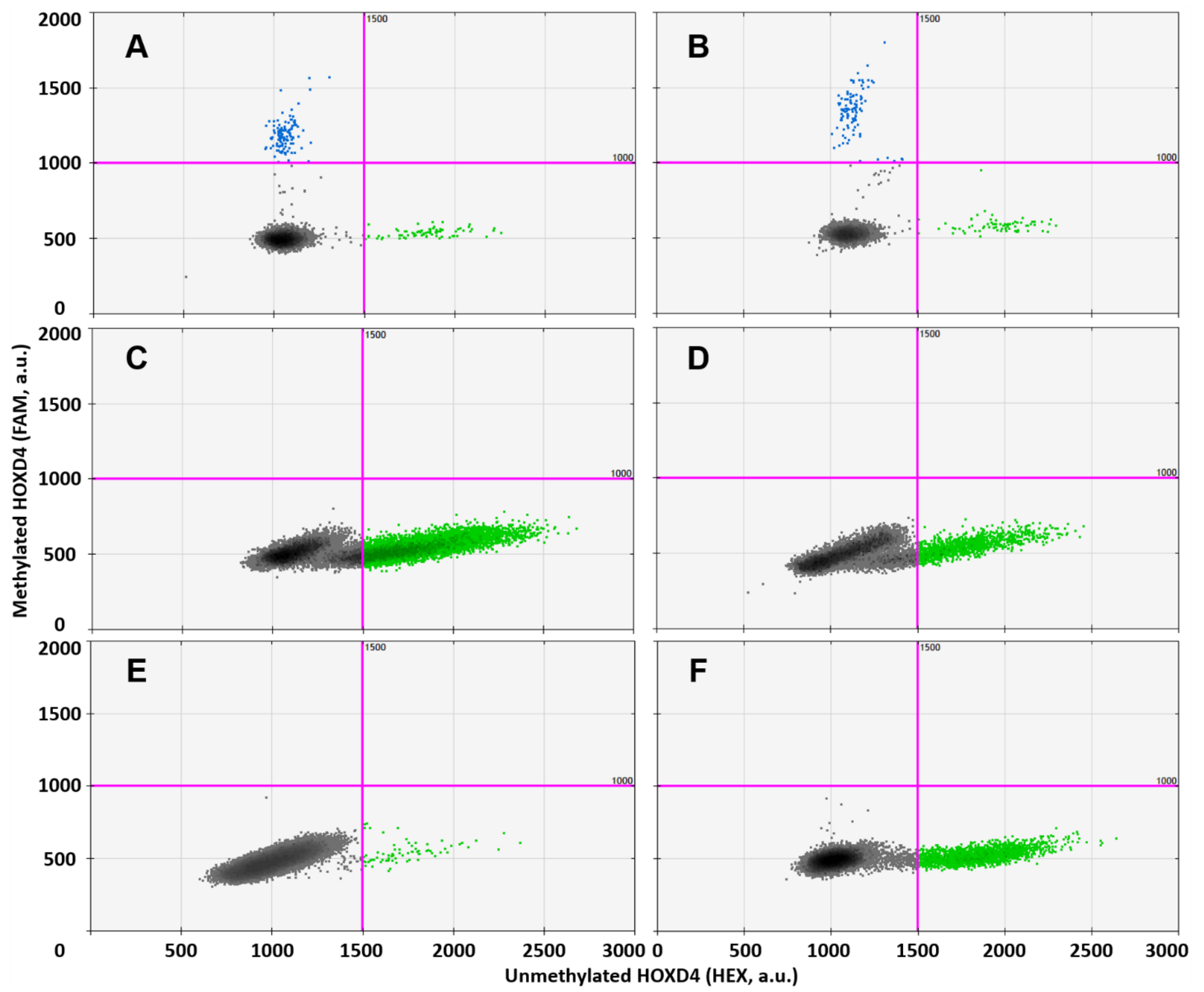
690 **Figure 4**



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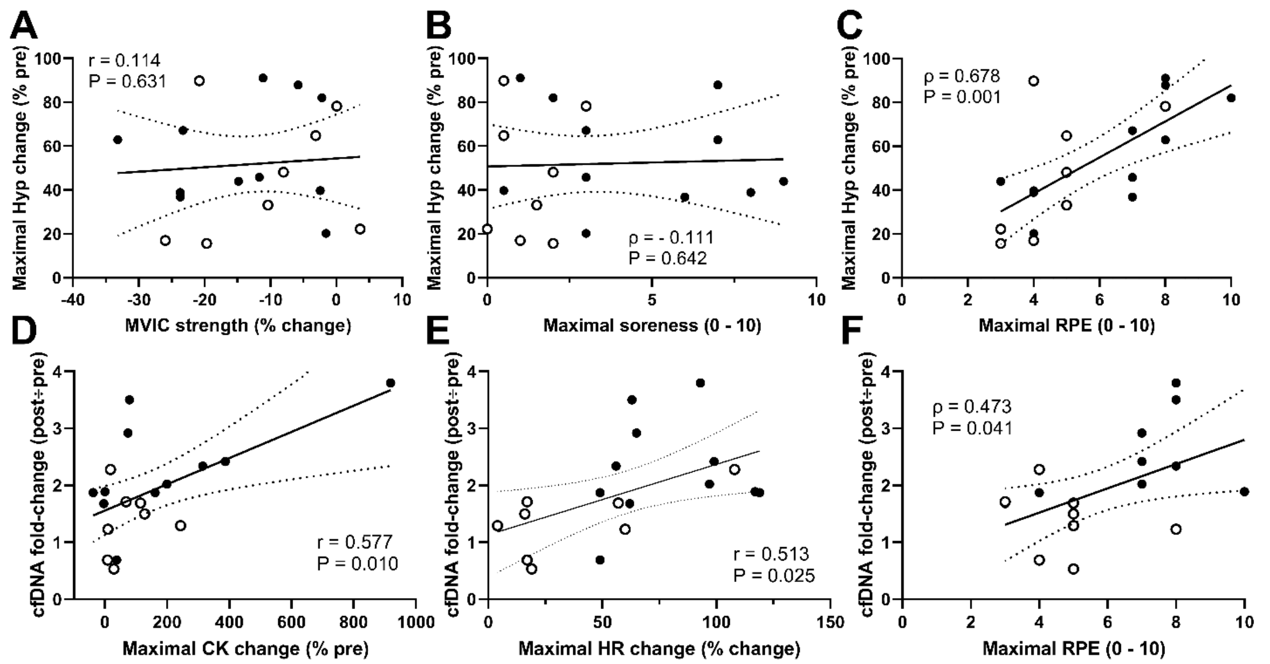
693 **Figure 5**



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