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Running head: Hyp and cfDNA after eccentric cycling

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ABSTRACT

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

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

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

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

Keywords: eccentric exercise; maximal voluntary isometric contraction; muscle damage; delayed onset muscle soreness; connective tissue; extracellular matrix

ABBREVIATIONS

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

INTRODUCTION

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

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

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

helix (Kotch et al. 2008). Therefore, an increase in blood or urine Hyp concentration is assumed to indicate muscle collagen breakdown (Murguia et al. 1988). Since mechanical impact due to gravity is minimal during cycling (Woodward and Cunningham 1993), it seems likely that increased Hyp concentration in the blood is from muscle connective tissue rather than bone origin. It is possible that changes in blood Hyp concentration and the magnitude of muscle functional loss and DOMS after eccentric cycling are highly associated, which would provide greater insight on the involvement of the muscle connective tissues to these eccentric exercise-induced phenomena.

Some studies have used blood cell-free DNA (cfDNA) as a muscle damage marker (Ferrandi et al. 2018; Andreatta et al. 2018). cfDNAs are circulating cell-unbound, double-stranded DNA fragments (Breitbach et al. 2012), normally present in a small amount in the blood, but shown to increase after intense exercises such as running, cycling, and resistance training (Atamaniuk et al. 2004, 2008, 2010; Tug et al. 2017a; Andreatta et al. 2018). For example, Andreatta et al. (2018) compared high- (80% of one-repetition maximum) and low-intensity (40%) leg press exercise (30 and 75 repetitions, respectively), and reported that serum cfDNA concentration increased 1.6-fold at immediately after the high-intensity exercise only, and the increase was correlated with the magnitude of decrease in squat and counter-movement jump height. The authors concluded that cfDNA levels were sensitive to the exercise intensity and that they could serve as a promising muscle damage marker (Andreatta et al. 2018). However, the resistance exercise performed in the study by Andreatta et al. (2018) consisted of both concentric and eccentric actions, and to the best of our knowledge no previous study has examined changes in cfDNA after exercise consisting of eccentric-only muscle actions, to investigate possible associations with muscle damage phenomena. Haller et al. (2018) reported that increases in blood cfDNA concentration were positively correlated with the rate of perceived exertion, and increased progressively with increasing running duration and intensity.

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

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

METHODS

Participants

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

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

Eccentric cycling

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

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

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

Maximal voluntary isometric contraction (MVIC) torque

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

Muscle soreness

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

Blood sampling

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

10 min and supernatant-aliquoted to several microtubes and stored at -80° C for subsequent Hyp and cfDNA analyses.

Plasma creatine kinase (CK) activity

A 30-µL whole blood sample was pipetted from the EDTA tube and loaded to a strip for CK activity analysis using a Reflotron (Roche Diagnosis, Switzerland). This analysis provides plasma CK activity.

Plasma hydroxyproline (Hyp) concentration

For the assessment of plasma Hyp concentration, 125 µl of plasma sample was mixed with 125 µl of 12 N hydrochloric acid and then hydrolyzed for 24 h at 95°C. The mixture was left to cool, and subsequently filtered through a 0.22 µm PVDF syringe filter unit. The supernatants were analyzed based on the manufacturer's guidelines using a colorimetric kit (Cell Biolabs, Inc., USA), which allowed the determination of Hyp concentration through the reaction of oxidized hydroxyproline with 4-(dimethylamino) benzaldehyde.

Plasma cfDNA concentration

Between 2 and 5 mL of plasma were used for cfDNA isolation using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Cell free DNA was quantified by droplet digital PCR (ddPCR) using QX200 AutoDG (Bio-Rad, Hercules, CA). Two DNA loci were used for quantification on the *AR* (X-chromosome) and *BAP1* (Chromosome 3) genes using commercial assays for ddPCR (Bio-Rad). Amplifications were performed using the following cycling conditions: 1 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 1 min,

followed by 1 cycle of 98°C (2.5C/s ramp) for 10 min. Copies of cfDNA per mL of plasma were calculated based on the volume of plasma used for extraction and elution volume.

cfDNA origin

Five samples from the HI group with significant increase in DNA copies post exercise were further quantified using the 2100 Bioanalyzer systems (Agilent, USA). To examine if the cfDNA was derived from skeletal muscles, the presence of methylated *HOXD4* promoter was examined. To evaluate the specificity of primers and probes, a primary human myoblast line and melanoma cell lines (92.1, Mel 270) were used as biological positive and negative controls, respectively. The primers and probes used are as follow:

Forward PCR primer: 5' - TTT TCC AAT TCT AAA ACT TAC TAC C – 3'

Reverse primer: 5' - TTT TCC AAT TCT AAA ACT TAC TAC C – 3'

Methylated probes (/56-FAM/AA GCG GTT T/ZEN/C GAA CGG TTT A/3IABkFQ/)

Unmethylated (/5HEX/AA GTG GTT T/ZEN/T GAA TGG TTT A/3IABkFQ/)

Mastermix reagents included: 1X ddPCR supermix (Bio-Rad), 250 nM probes, 900 nM of each primer, 50 nM 7-Deaza (New England Biolabs, Ipswich, MA). Droplets were generated using the Automatic Droplet generator QX200 AutoDG (Bio-Rad). PCR Cycle involved: 1 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 1 minute, followed by 1 cycle of 98°C (2.5C/s ramp) for 10 min. Droplets were analyzed through a QX200 Droplet Reader (Bio-Rad). QuantaSoft analysis software (Bio-Rad) was used to acquire and analyze data.

Statistical analyses

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

RESULTS

Heart rate and effort during eccentric cycling

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

MVIC torque and muscle soreness

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

immediately (-13.3 ± 7.8%), 24 h (-9.7 ± 10.7%), and 48 h (-6.7 ± 13.5%) after eccentric cycling without a significant difference between HI and LI groups ($P = 0.173$, interaction $\eta^2_p = 0.091$). Muscle soreness increased at 24 – 72 h after exercise in both groups ($P = 0.016$, time $\eta^2_p = 0.413$), but maximal muscle soreness (mean of maximal values per group across all time points) was greater ($P < 0.05$, interaction $\eta^2_p = 0.225$) in HI (4.5 ± 3.0) than LI (1.2 ± 1.0).

Plasma CK activity

A significant increase in plasma CK activity from the baseline was found only at 24 h post-exercise (141 ± 73.5%, $P = 0.029$, time $\eta^2_p = 0.194$), but this increase did not differ significantly between the groups ($P = 0.268$, interaction $\eta^2_p = 0.070$). The average CK activity 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 IU/L at 72 h post-exercise.

Plasma Hyp concentration

Baseline Hyp concentration was 24.3 ± 5.9 μg/mL for HI and 27.9 ± 8.2 μg/mL for LI without difference between groups ($P = 0.286$). As shown in Figure 2, plasma Hyp concentration increased significantly at 24 – 72 h after cycling by 40 – 53% from baseline for both HI and LI ($P < 0.001$, time $\eta^2_p = 0.576$) with no significant difference between the groups at any time point ($P = 0.518$, interaction $\eta^2_p = 0.032$).

Plasma cfDNA concentration and its origin

Pre-exercise plasma cfDNA concentrations were similar between the groups, with 233 ± 129 copies/mL for HI and 203 ± 78.4 copies/mL for LI ($P = 0.552$). Plasma cfDNA concentrations increased from pre- to post-exercise in HI (Figure 3, $P < 0.001$, time $\eta^2_p = 0.464$), returning to baseline levels by 24 h post-exercise. HI showed a significantly greater increase in

plasma cfDNA concentration than LI group post-exercise (2.3 ± 0.9-fold, P = 0.002, interaction $\eta^2_p = 0.230$).

Five samples in the HI group with significant increase in DNA copies post exercise by ddPCR and with a high overall cfDNA yield via chip-based capillary electrophoresis (Bioanalyzer), were selected for downstream methylation analysis of *HOXD4*. The upstream region of *HOXD4* contains two myogenic hypermethylated sites with high specificity for muscle derived DNA (Ehrlich and Lacey 2013). We evaluated whether the increase on cfDNA was muscle derived using a ddPCR assay specific for this epigenetic change. However, methylated *HOXD4* was not detectable in the cfDNA of the five samples pairs analyzed (Figure 4).

Correlations

Figure 5 shows some of the correlation analysis results. No significant correlations were observed between the maximal decrease in MVIC torque at 24 to 72 h post-exercise and peak change in Hyp concentration (r = 0.114, P = 0.631), pre- to post-exercise change in cfDNA concentration (r = -0.425, P = 0.070), or maximal change in CK activity (r = -0.430, P = 0.058). No significant correlations were detected between maximal muscle soreness and maximal change in Hyp concentration (ρ = -0.111, P = 0.642), pre- to post-exercise change in cfDNA concentration (ρ = 0.354, P = 0.137), or maximal change in CK activity (ρ = 0.042, P = 0.859).

There were no significant correlations between maximal change in CK activity and maximal change in Hyp concentration (r = -0.015, P = 0.950) or between maximal change in Hyp concentration and pre- to post-exercise change in cfDNA concentration (r = -0.228, P = 0.347). Although the change in cfDNA concentration from pre- to post-exercise significantly correlated with maximal change in CK activity (r = 0.577, P = 0.010), it was no longer

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

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

DISCUSSION

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

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

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

represents connective tissue damage, and if so why no significant difference between HI and LI was evident for the changes.

Eccentric exercise-induced muscle damage increases muscle inflammation and release of matrix metalloproteinases (also known as matrix metallopeptidases), an enzyme family that degrades collagen and other extracellular matrix components, and triggers the subsequent phases of synthesis (Koskinen et al. 2002; Paulsen et al. 2010). The increase in plasma Hyp concentration observed in the current study at 24 – 72 h after the acute eccentric cycling session may reflect increased collagen turnover via metalloproteinases. In addition, the lack of difference in plasma Hyp concentration responses between the two intensities (HI vs LI) may suggest that eccentric exercise-induced collagen breakdown is dependent upon the total mechanical work rather than exercise intensity, when the intensity is submaximal. However, this speculation requires further studies to assess which mechanical factor is mostly responsible for collagen breakdown after eccentric exercise, and if there is a difference when comparing mechanical work-matched submaximal with maximal exercise intensity eccentric cycling protocols for changes in plasma Hyp concentration.

It was hypothesized that the post-exercise muscle functional loss and DOMS would be associated with collagen breakdown. However, no significant correlations between the magnitude of increase in plasma Hyp concentration and either muscle functional loss or DOMS were evident (Figure 5). It is therefore unlikely that collagen breakdown is the direct cause of the muscle functional loss and DOMS after eccentric cycling, however we acknowledge the limitation that our muscle damage model involved the entire lower limb, whereas our muscle function measure was only in knee extensors. Crameri et al. (2007) showed that tenascin C, a protein responsible for extracellular matrix de-adhesion from the cell membranes, increased similarly after both voluntary and electrically-stimulated maximal eccentric exercise with a 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

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

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

Regarding the origin of cfDNA, damage and apoptosis of skeletal muscle cells or leukocytes have been previously considered to be candidate sources (Atamaniuk et al. 2008). The lack of methylated *HOXD4* cfDNA in the plasma samples suggests that the increased cfDNA did not originate from skeletal muscle (Figure 4). Our results do not support the hypothesis that post-exercise increase in cfDNA in the blood are derived from skeletal muscle cells undergoing apoptosis or necrosis (Atamaniuk et al. 2004, 2010; Ferrandi et al. 2018). Moreover, based on our findings it seems unlikely that there was DNA 'leakage' from exercised muscle cells due to increased membrane permeability or increased release of extracellular vesicles (Helmig et al. 2015), or that there was an increased rate of clearance of DNA fragments originating from muscle cells that stuck on cell membranes or lymph due to increased blood perfusion (Breitbach et al. 2012). Instead, one plausible explanation is that both baseline and post-exercise levels of cfDNA largely originated from haematopoietic-derived cells, as reported in a previous study (Tug et al. 2015). Even though the precise physiological event that causes the increase in plasma cfDNA concentration is not yet fully understood, Beiter et al. (2014) speculated that activated neutrophils might primarily contribute to exercise-evoked cfDNA levels by releasing neutrophil extracellular traps. Neutrophils respond to exercise by forming neutrophil extracellular traps which is thought to contribute to an increased hypercoagulable exercise-induced state (Beiter et al. 2015). Future research should examine the source of the exercise-induced increases in plasma cfDNA concentration.

Based on the findings from the present study, it appears that plasma Hyp concentration increases after eccentric cycling possibly as a result of collagen breakdown, but the increase was not associated with decreases in muscle function or DOMS. Plasma cfDNA concentration does not appear to be a marker of muscle damage, as there was no detectable methylation of *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.

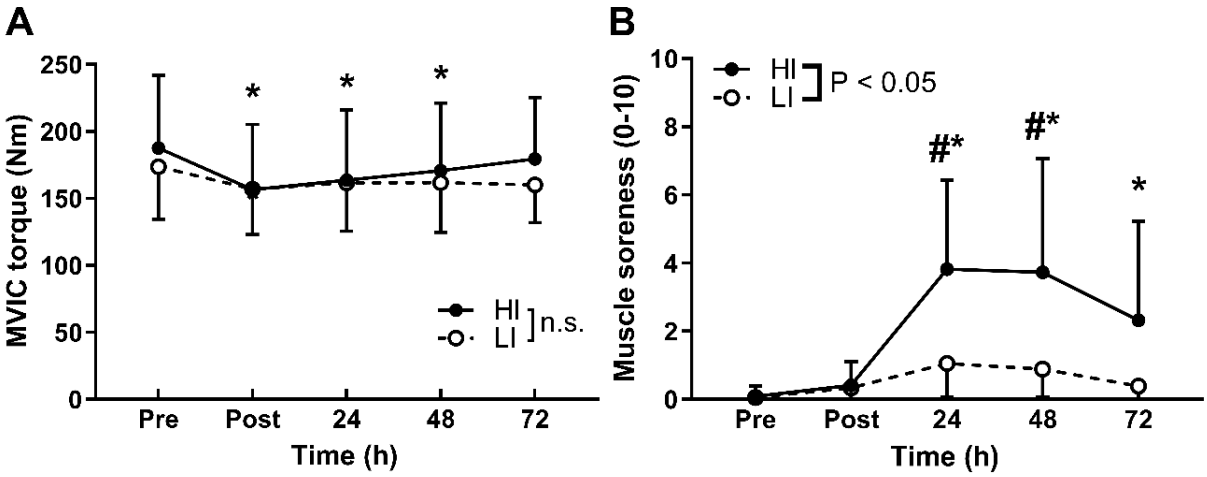
Figure 3. Changes (mean ± SD) in plasma cfDNA concentration before (Pre), immediately after (Post), and 24, 48 and 72 h after higher-intensity (HI) and lower-intensity (LI) eccentric cycling.
†: significant ($P < 0.001$) difference of the HI group from the baseline value. #: significant ($P < 0.05$) 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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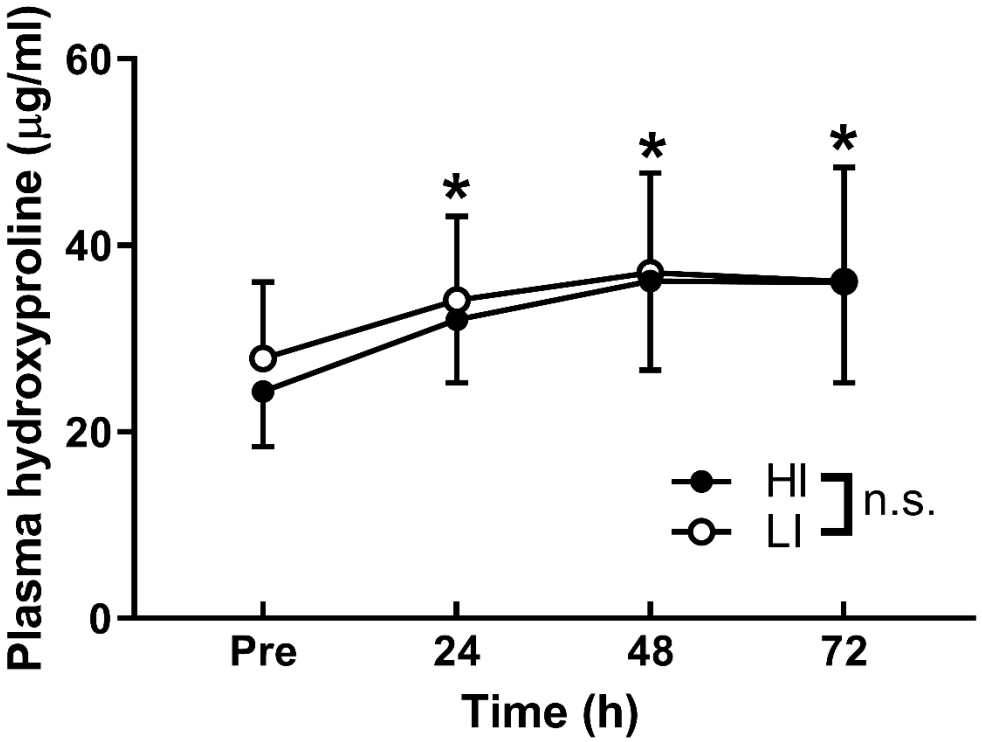
684 **Figure 2**



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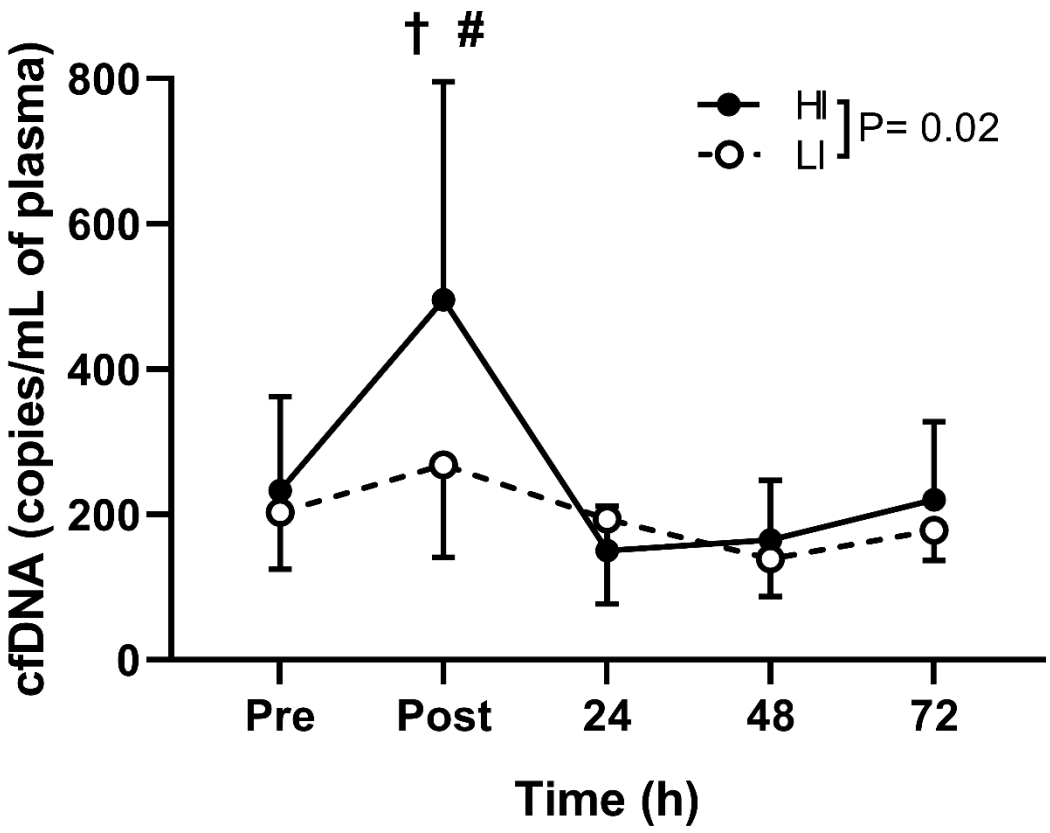
687 **Figure 3**



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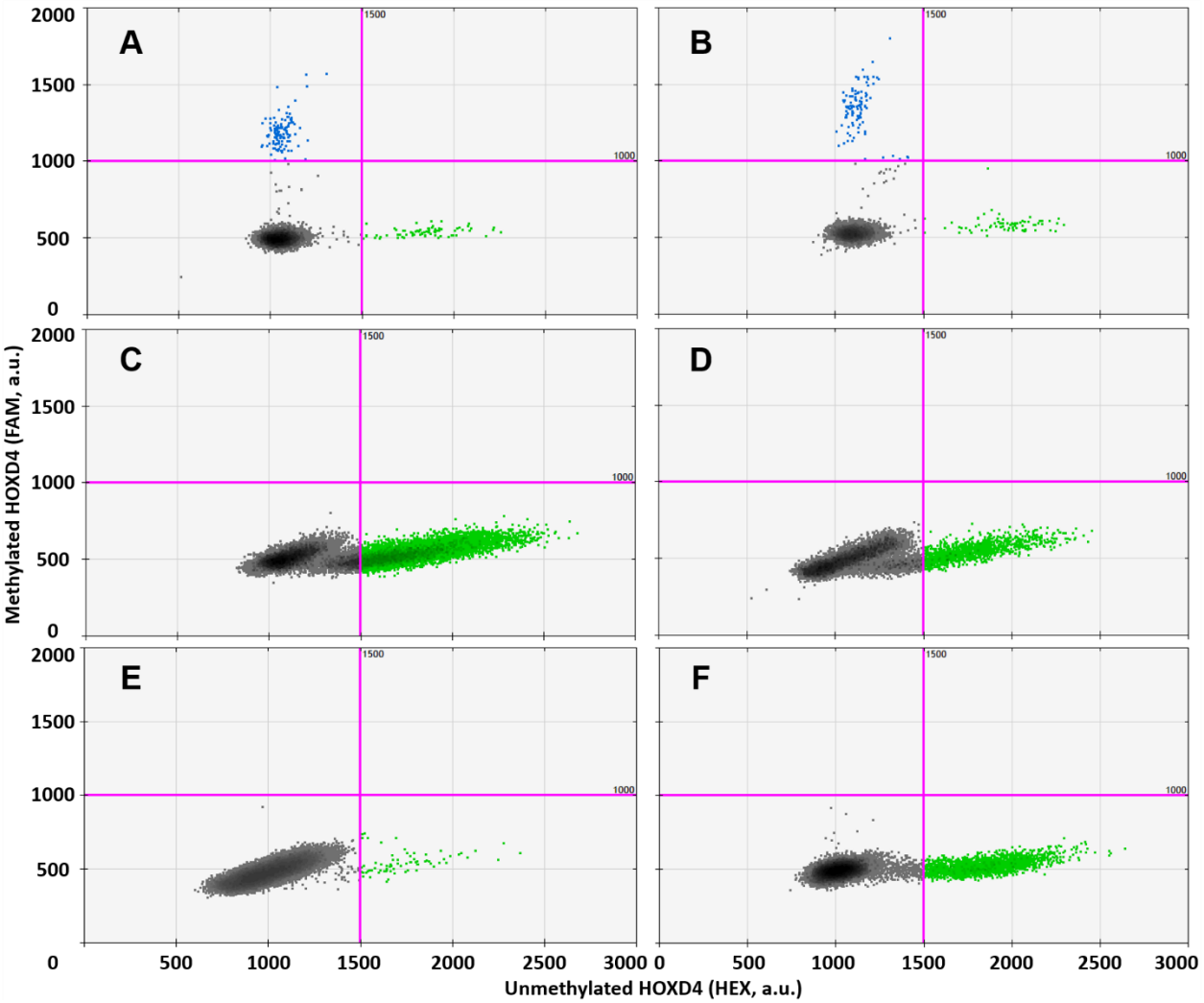

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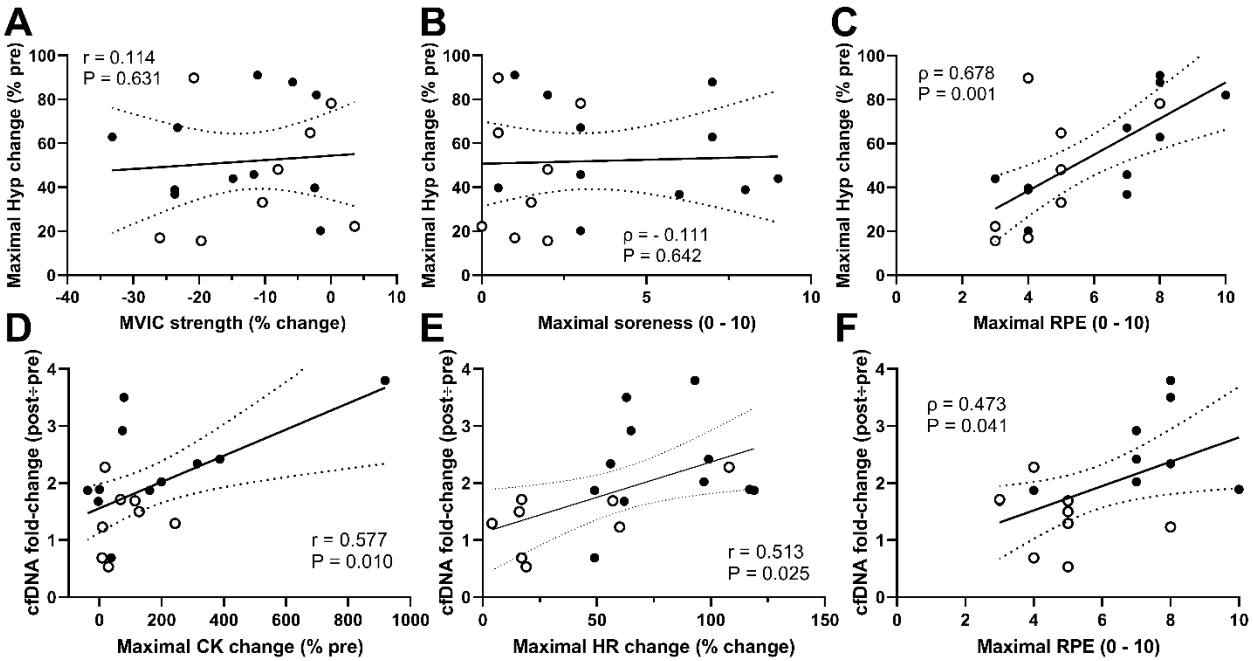
693 **Figure 5**



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