European Journal of Applied Physiology
https://doi.org/10.1007/s00421-025-05936-6

ORIGINAL ARTICLE

Urinary N-terminal titin fragment concentration as a non-invasive biomarker of exercise-induced muscle damage in males and females

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Received: 13 May 2025 / Accepted: 31 July 2025
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Abstract

Purpose To examine the effects of muscle-damaging exercise on urinary N-terminal fragments of titin (UTF) in males and females, and its association with markers of exercise-induced muscle damage.

Methods 27 males ($n = 16$) and females ($n = 11$) (height: 1.74 ± 0.10 m; body mass: 72.2 ± 11.4 kg; age: 22 ± 3 years) performed 200 eccentric contractions of the knee extensor on an isokinetic dynamometer. Urine and serum samples were collected pre-, post- and 48 h post-exercise to quantify UTF and creatine kinase (CK). Additionally, knee extensor maximal voluntary isometric force (MVIF), voluntary activation (VA), time to peak twitch (TTP), evoked maximal rate of force development (RFD_{max}), potentiated twitch force (Tw_{pot}), and delayed-onset muscle soreness (DOMS) were recorded.

Results UTF (2.3 ± 1.8 to 3.3 ± 3.4 nmol/mg/dL) and CK (9.7 ± 4.8 to 14.5 ± 8.7 units/L) concentrations were elevated 48 h after exercise ($p < 0.01$). DOMS was greater at all post-exercise time points vs. pre-exercise ($p < 0.01$). MVIF, evoked RFD_{max}, VA, and Tw_{pot} all decreased after exercise ($p < 0.01$). The pre- to 48 h post-exercise change in UTF strongly correlated with CK ($r_s = 0.73$; $p < 0.01$), TTP ($r_s = -0.77$; $p < 0.01$) and evoked RFD_{max} ($r_s = -0.62$; $p < 0.01$) and moderately correlated with MVIF ($r_s = -0.45$; $p < 0.01$). Moderate strength correlations were found between the pre- to 48 h post-change in CK with DOMS ($r = 0.47$; $p = 0.03$). There were no sex differences for any variables ($p > 0.05$).

Conclusion UTF was similarly increased post- and 48 h post-exercise in males and females and was moderately to strongly correlated to CK and some markers of neuromuscular function, but not DOMS.

Keywords Muscle damage · Titin · Creatine kinase · Exercise · Biomarker

Introduction

Exercise-induced muscle damage (EIMD) is loosely defined as a set of symptoms typically evoked by strenuous, unaccustomed, and eccentric-heavy exercise. While EIMD can be measured directly by examining myofibrillar disruptions from muscle biopsy (Friden et al. 1983; Fridén and Lieber 2001), obtaining muscle samples is highly invasive. As such, EIMD is often measured indirectly as declines in muscle force production and an increase in intramuscular proteins in the blood, where changes often peak 24–72 h post-exercise (Howatson and Van Someren 2008). Several of these intramuscular proteins, most notably creatine kinase (CK), are significantly elevated following muscle-damaging exercise

Communicated by Mark Elisabeth Willems.

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(Hyldahl and Hubal 2014). As such, CK has been widely adopted as a biomarker of EIMD, especially in the absence of neuromuscular function testing (Warren et al. 1999; Brancaccio et al. 2010). However, the use of CK as a marker of EIMD has been questioned, since it is influenced by several other factors, including biological sex, hydration status, training status, and fibre type distribution (Nosaka and Clarkson 1996; Seifert et al. 2005; Brancaccio et al. 2007). Moreover, CK, and other proteins such as myoglobin, do not correlate with myofibrillar damage or declines in muscle force production (Margaritis et al. 1999; Fridén and Lieber 2001), the latter of which is considered the gold standard marker of EIMD (Warren et al. 1999). Collection of these intramuscular proteins also requires invasive procedures such as venepuncture, making them difficult to collect in field settings or in participants with a fear of needles. Hence, there is growing interest in the effects of exercise on other intramuscular proteins that could be less invasive and better reflect the magnitude of EIMD.

One such protein is titin (also known as connectin) which is a giant protein located in the sarcomere of striated muscle that enables the contraction of actin-containing thin filaments and myosin-containing thick filaments. Thus, titin serves as a molecular spring that provides elasticity and passive stiffness to myofibers (Herzog 2018). When muscle is damaged, titin is cleaved by proteolytic calpain and matrix metalloprotease enzymes, forming N-terminal titin fragments that are excreted in urine (Ali et al. 2010; Charton et al. 2015). Following the development of a highly sensitive enzyme-linked immunosorbent assay (ELISA) to quantitatively measure the concentration of urinary titin fragments (UTF) (Maruyama et al. 2016), recent studies have shown that UTF is elevated after muscle-damaging exercise (Kanda et al. 2017; Yamaguchi et al. 2020c, b; Tanabe et al. 2021a, b), and in clinical populations with muscle atrophy (Matsuo et al. 2019; Nakanishi et al. 2021).

Kanda et al. (2017) were the first to report that strenuous exercise (100 eccentric-biased calf raises) increased UTF concentrations in males, leading the authors to recommend UTF as a potentially useful biomarker to detect EIMD. More recently, Yamaguchi et al. (2020c) demonstrated elevated UTF levels in males after 30 maximal isokinetic eccentric contractions of the elbow flexor and reported moderate negative correlations between peak UTF levels and decreases in neuromuscular function (peak maximal voluntary isometric force (MVIF)) 24–144 h post-exercise. Tanabe et al. (Tanabe et al. 2021b) similarly reported elevated UTF concentrations 48 h following 30 maximal isokinetic eccentric contractions of the elbow flexor, with peak values strongly positively correlated with serum CK activity and transverse relaxation time. Furthermore, there were moderate negative correlations between peak UTF and MVIF and range of motion decline rate. Another study by Tanabe et al. (2021a) also found that a 90 min soccer match in male collegiate players elevated UTF 48 h post-exercise, with concentrations moderately negatively correlated with a decline in countermovement jump (CMJ) performance.

While previous studies suggest that UTF concentrations may serve as a non-invasive biomarker for detecting EIMD, current evidence is limited by small sample sizes ($n = 8$–17), comparison to a narrow range of neuromuscular assessments (Kanda et al. 2017; Yamaguchi et al. 2020c, Yamaguchi et al. 2020b; Tanabe et al. 2021a), and a lack of sex-based comparisons—despite indications that EIMD may be attenuated in females compared to males (Kendall and Eston 2002; Penailillo et al. 2011). Importantly, no study to date has examined the relationship between UTF and laboratory-based markers of EIMD such as evoked force and voluntary activation (VA). Accurate interpretation of maximal force loss following exercise requires consideration of central drive, yet central activation failure is rarely accounted for in EIMD research (Warren et al. 1999). As reductions in force could stem from impaired voluntary neural input rather than structural muscle damage alone, the use of twitch interpolation is recommended to objectively distinguish between central and peripheral contributions to muscle function loss (Byrne et al. 2004; Millet and Lepers 2004). Accordingly, we have incorporated this technique to provide a more comprehensive assessment of the neuromuscular response to EIMD and to better evaluate the sensitivity of UTF as a biomarker.

The primary aim of this study was therefore to determine whether UTF is increased by muscle-damaging exercise and whether the response differs between males and females. A secondary aim was to correlate changes in UTF with changes to an array of neuromuscular function markers and muscle soreness. We also measured CK and correlated exercise-induced changes with neuromuscular function and muscle soreness for comparisons to UTF correlations. We hypothesised that muscle-damaging exercise would increase UTF and CK, but to a lesser extent in females, and that these changes would correlate with decrements in neuromuscular function and muscle soreness after 48 h, albeit more strongly with UTF than CK.

Methods

Ethical approval

The study received ethical approval from the Loughborough University Ethics Approvals (human participants) Sub-Committee (REF: 4955). Participants were fully informed of the risks and discomforts associated with the experimental procedures prior to providing written informed consent.

Table 1 Group differences in physical characteristics and total work completed during exercise

	Males ($n=16$)	Females ($n=11$)	p
Age (years)	23 ± 3	23 ± 4	0.82
Height (m)	1.81 ± 0.05	1.64 ± 0.07	< 0.01
Body mass (kg)	78.4 ± 9.9	63.0 ± 5.9	< 0.01
Total work (J/kg BM)	549 ± 85	458 ± 128	0.04
MVIF (N/kg BM)	10.6 ± 1.8	9.3 ± 1.7	0.07

MVIF, maximal voluntary isometric force per kg of body mass (BM) at baseline

Participants

27 participants (height: 1.74 ± 0.10 m; body mass: 72.2 ± 11.4 kg; age: 22 ± 3 years; see Table 1 for physical characteristics), including males ($n=16$) and females ($n=11$), volunteered to participate in this study. All participants were recreationally strength trained (defined as undertaking resistance training 1–4 days per week for the past 6 months) and deemed healthy following examination of a health screening questionnaire; this meant participants (1) had not experienced any muscle injuries in the past 6 months (2) were not using medication, (3) had a BMI between 18.5 and 30.0 kg/m^2, (4) and had no known underlying health conditions. Female participants were only eligible for inclusion if they were either naturally menstruating or using a combined oral contraceptive pill. Trials were completed during the early follicular phase of the menstrual cycle or the first week of the active pill cycle. The data presented in this manuscript formed part of a larger study examining the effects of a dietary intervention on EIMD. As there were no significant differences in the outcomes between the two interventions, data from both groups was used for analysis.

Study design

Data were collected across three laboratory visits: a familiarisation trial, an exercise trial, and a 48 h follow-up trial. During familiarisation, anthropometric measurements were collected, and participants were familiarised with the muscle soreness and neuromuscular function tests; for the latter, they experienced peripheral femoral nerve stimulation. Due to the discomfort of nerve stimulation, participants were given the opportunity to opt out of this aspect for subsequent visits. On the day of the exercise, data was collected pre- and immediately post-exercise. Participants returned to the laboratory 48 h post-exercise. On both visits, participants arrived at the laboratory in the morning following an overnight fast and were hydrated following consumption of 500 mL water. The 48 h post-exercise time point was chosen for follow-up because systemic changes in myofiber proteins, and urinary titin, do not tend to increase significantly until this time point (Warren et al. 1999; Clarkson and Hubal 2002; Kanda et al. 2017; Yamaguchi et al. 2020a). Participants were instructed to avoid exercise outside of study procedures 36 h prior to all trials. Nutritional supplements and therapeutic interventions aimed to enhance recovery (e.g. compression garments, cooling devices) were also restricted from 7 days prior to exercise until completion of the study.

Muscle-damaging exercise protocol

To induce muscle damage, participants performed 200 dominant-leg eccentric knee flexions on an isokinetic dynamometer (IKD) (HUMAC NORM, CSMi, Stoughton (MA), United States). Participants were seated at a hip angle of 125° and strapped securely across the chest and inguinal fold. The IKD range of motion limits were set individually with a total range of motion of ~ 90°. Contractions were performed in 20 sets of 10, with 1 min between-set rest, and at a constant angular velocity of 60°·s^{-1}. Participants initiated the contraction by 'kicking' from a 10° joint angle and subsequently resisting the action of the IKD lever arm as it moved to the participants' flexed position. During the concentric phase of action, participants were instructed to relax as the lever arm returned their leg to the start position. Participants were encouraged to perform each contraction maximally by the same investigator, and visual feedback was provided via a linked PC. Total work completed (J) was recorded and used for analysis.

Neuromuscular function

Neuromuscular function was evaluated using knee flexion dynamometry on the dominant leg; this was performed on a custom-built isometric strength testing chair tailored to each participant's anthropometric measurements. Participants were seated with the knee and hip positioned at 115° and 125° of extension, respectively. Movement of the upper body and hips was restricted using adjustable straps secured across the torso and hips. The dominant leg's shin was fastened with a cuff and reinforced canvas webbing above the lateral malleolus, at approximately 15% of the tibial length. The cuff, aligned perpendicular to the tibia, was connected in series with a calibrated S-beam strain gauge (Force Logic, Swallowfield, UK). After signal amplification (× 370), force data was sampled and recorded at 2000 Hz using an external analogue–digital converter (Micro 1401; CED Ltd., Cambridge, UK) and Spike2 PC software (CED Ltd., Cambridge, UK).

Participants performed a standardised warm-up consisting of ~ 3 s submaximal isometric knee extensions at 50% (× 3), 75% (× 3) and 90% (× 1) of perceived maximal effort,

with each contraction separated by 30 secs of rest. After the warm-up, participants rested for 2 min, then performed 5 sets of ~3 s MIVCs separated by 30 secs of rest to assess MVIF.

15 participants (male: $n=9$; female: $n=6$) consented to undertake peripheral femoral nerve stimulation. Prior to being seated in the testing chair, two self-adhesive electrodes (PALS879100, Axelgaard, Fallbrook (CA), USA) were attached to the participant's' dominant leg (anode: greater trochanter of the femur; cathode: over the femoral triangle/intersection of adductor longus, inguinal ligament and sartorius). Electrical stimulus intensity was standardised across participants via calibration on each test occasion prior to the neuromuscular warm-up. Stimulation was initially delivered at 20 mA, before being increased in 20mA stepwise increments until a plateau in knee extensor evoked twitch was obtained. Stimulus intensity for maximal voluntary isometric contraction (MVIC) efforts was set at 130% of this value to ensure supramaximal delivery.

During the final three MVICs, percutaneous single and paired electrical stimuli were delivered to the femoral nerve using square wave pulses (200 µs) via a constant current stimulator (DS7AH; Digitimer Ltd., Welwyn Garden City, UK). Pulses were delivered superimposed at the peak of maximal contraction (one paired pulse at 100 Hz) and post-contraction at rest interspersed by ~ 2–3 s (one 100 Hz paired pulse, one 10 Hz paired pulse, and a single pulse). Voluntary activation (VA) was calculated using the interpolated twitch technique (VA (%) = (1− (superimposed twitch/100 Hz paired potentiated twitch) × 100)). Evoked maximal rate of force development (RFD_{max}) was calculated from the single pulse potentiated twitch response—as the maximal slope of the force response calculated in 100 ms epochs. Potentiated twitch force (Tw_{pot}) and time to peak twitch (TTP) were determined using the 100 Hz single pulse.

These neuromuscular markers were chosen since most appear to be sensitive to muscle-damaging exercise and would therefore help to validate whether changes to UTF reflected EIMD. MVIF can be seen to be blunted for up to 48 h post-exercise (Prasartwuth et al. 2005; Turner et al. 2008; Power et al. 2010; Behrens et al. 2012; Doguet et al. 2016). VA was shown to decrease immediately post-exercise (Prasartwuth et al. 2005; Doguet et al. 2016). RFD_{max} was reported to drop immediately post-exercise, staying suppressed for up to 168 h (Molina and Denadai 2012; Jenkins et al. 2014; Farup et al. 2016; Vila-Chã et al. 2023). Janecki et al. (2014) reported that Tw_{pot} was diminished immediately post-muscle-damaging exercise and remained attenuated for up to 120 h. To our knowledge, no present study has examined TTP response to muscle-damaging exercise and thus it was of interest to evaluate these responses for the first time.

Muscle soreness

Delayed-onset muscle soreness (DOMS) was evaluated using a visual analogue scale. Participants performed a body weight squat to a 90° knee angle and were then asked to mark a point on a 200 mm line, where 0 mm indicated 'no soreness' and 200 mm indicated 'unbearably painful'. The mark was subjectively placed at the spot that best represented their perceived soreness in the lower limbs. The distance of the mark from the start of the line was measured in mm and recorded.

Sample collection and analysis

Spot urine and venous blood samples were collected at all time points; venous blood was collected from the antecubital fossa via venepuncture by a trained phlebotomist and was left to clot for 30 min at room temperature, followed by centrifugation at 3500 RCF for 10 min at 4°C to isolate serum. Urine and serum samples were then frozen at − 80°C until analysed.

UTF was analysed using a commercial sandwich ELISA (Titin N-terminal Fragment Assay Kit; Immuno-Biological Laboratories Co., Fujioka, Japan) in duplicate according to manufacturer's instructions. Briefly, urine samples were thawed and diluted between 1:20 and 1:50, then added to an antibody-coated 96-well plate where they underwent incubation at 37 °C for 1 h. After repeating a wash procedure four times, labelled antibodies were added to the plate then incubated at 37°C for 30 min. Following another five washes, tetramethylbenzidine substrate was added to wells and the plate was incubated at room temperature away from light exposure for 30 min. Upon colour development, the reaction was terminated by the addition of stop solution, and the absorbance of wells was read at 450 nm with a reference wavelength of 650 nm using a microplate reader. To account for transient changes in hydration across the time points, UTF (nmol/L) was normalised to creatinine concentrations (mg/dL) which were performed using a commercial assay (Creatinine Urinary Colorimetric Assay Kit, Cayman Chemicals, Ann Arbor, USA) in duplicate according to the manufacturer's instructions. UTF is therefore expressed as nmol/mg/dL. The intra-assay CV for the UTF and creatinine assays were 3.2% and 3.7%, respectively.

Serum CK was analysed using a commercial enzyme activity assay (CK Activity Assay Kit; Sigma-Aldrich, St. Louis, MA, USA) according to the manufacturer's instructions. CK activity in serum (units/L) was quantified by a coupled enzyme reaction resulting in the formation of NADPH that was measured at 340 nm. The formation of NADPH was subsequently proportionate to CK concentration, and 1 unit of CK is the amount of enzyme required to

transfer 1 µM of phosphate from phosphocreatine to ADP per minute.

Statistical analysis

Statistical analysis was performed using jamovi v2.3.26 (retrieved from https://www.jamovi.org). Normal distribution of data was confirmed with the Shapiro–Wilk test ($p > 0.05$) and by inspecting the Q–Q plots and histograms of the residuals. Independent samples t-tests were used to analyse biological sex differences in physical characteristics and total work performed during exercise. Linear mixed models (LMM; gamlj v2.6.6) (2 (males vs. females) × 3 (pre-, post-, 48 h post-exercise)) were performed on the UTF, CK, DOMS, and neuromuscular responses to exercise. Prior to LMM analysis, raw values were converted to per cent (%) change from pre-exercise to account for the anthropometric and physiological differences between male and female participants. Any significant main effects from the LMM were followed up with Holm–Bonferroni corrected post hoc tests. Effect sizes for LMM analysis are presented as partial eta squared (ηp^2: small: 0.01, medium: 0.06, large 0.14) (Cohen 1988).

Since UTF concentrations were not normally distributed, Spearman's ranked correlations were performed to examine associations between the change (from pre- to 48 h post-exercise) in UTF with the change in CK, DOMS, and neuromuscular function. Pearson's correlations were used to examine associations between CK, DOMS, and neuromuscular function. As in a previous study (Kanda et al. 2017), correlations were performed with % change data to standardise the changes between the two different outcomes (e.g. UTF and DOMS). Correlation analysis was not performed for pre-post changes, as UTF would not be expected to significantly increase immediately post-exercise as a result of muscle damage. Spearman's (r_s) and Pearson's (r) correlation coefficients were interpreted as follows: ±0.1 to ±0.3 *weak;* ±0.4 to ±0.6 *moderate;* ±0.7 to ±0.9 *strong* (Dancey and Reidy 2007). $p < 0.05$ was considered statistically significant. Figures were generated using GraphPad Prism (v9.4.1, Boston, USA). Data is presented as mean ± SD.

Results

Physical characteristics, total work completed during exercise, and MVIF at baseline between males and females are detailed in Table 1.

Changes in urinary titin fragments after exercise

Figure 1a presents the raw UTF concentrations after exercise for males and females. Figure 1b presents the % change in UTF after exercise for males and females. Main time effects were observed for UTF concentrations (time: $p = 0.02$; $\eta p^2 = 0.11$; Fig. 1b); UTF levels post-exercise were not different from pre-exercise ($p = 0.88$), but after 48 h, UTF was higher than pre-exercise ($p < 0.01$) and post-exercise ($p = 0.02$). No sex differences ($p = 0.88$; $\eta p^2 < 0.01$) or interaction effects ($p = 0.71$; $\eta p^2 < 0.01$) were found for UTF.

Changes in serum creatine kinase after exercise

Serum samples could not be collected from $n = 4$; thus, CK was analysed for 23 participants. Figure 2a presents the raw

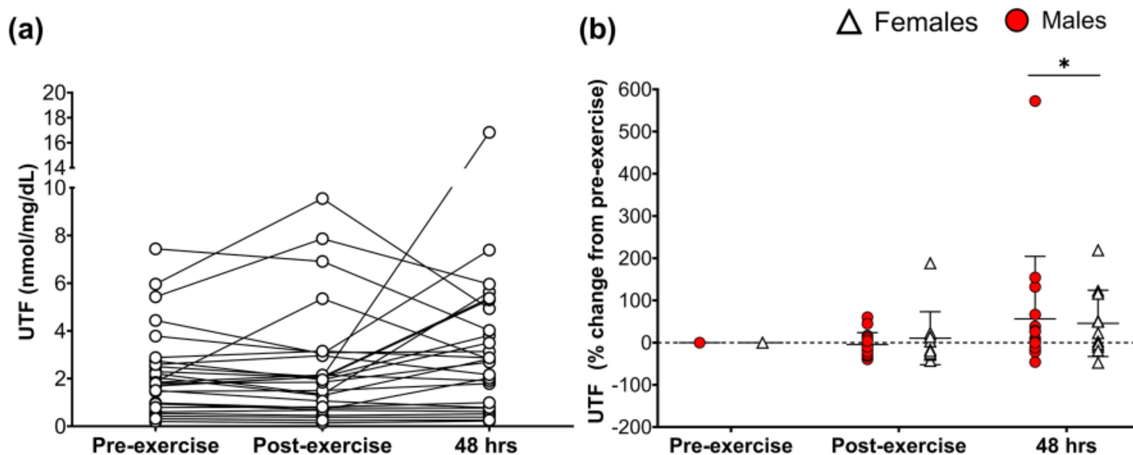

Fig. 1 Urinary titin fragment (UTF) responses following muscle-damaging exercise in males (red circles) and females (white triangles). **a** Individual raw UTF concentrations in all participants ($n = 27$). **b** % Change (from pre-exercise) of UTF at post- and 48 h post-exercise between males ($n = 16$) and females ($n = 11$). **b** Presents individual data points alongside mean and SD. * = significantly different from pre- and post-exercise

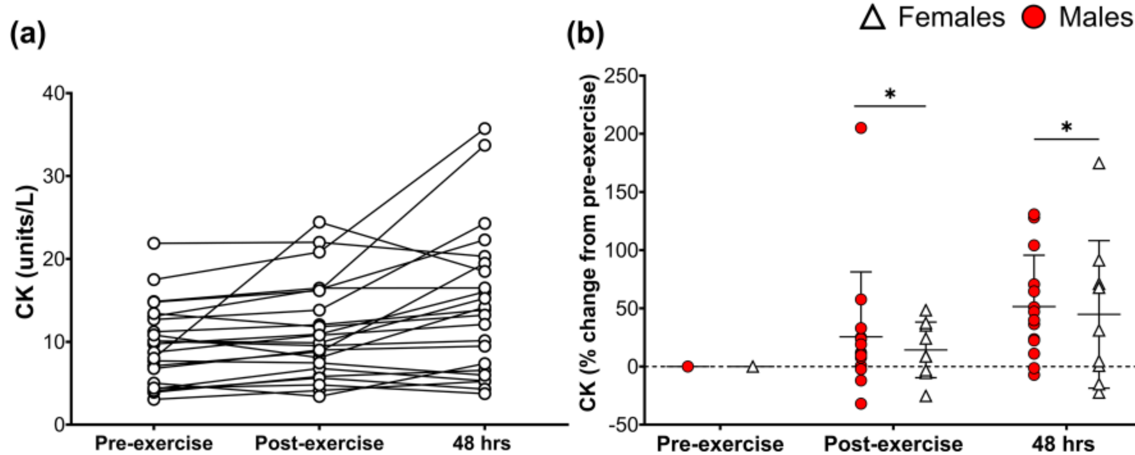

Fig. 2 Creatine kinase (CK) responses following muscle-damaging exercise in males (red circles) and females (white triangles). **a** Individual raw CK concentrations in all participants ($n = 23$). **b** % Change (from pre-exercise) of UTF at post- and 48 h post-exercise between males ($n = 14$) and females ($n = 9$). **b** Presents individual data points alongside mean and SD. * = significantly different from pre-exercise

CK concentrations after exercise for males and females. Figure 2b shows the % change of CK after exercise for males and females. Main time effects were found for CK ($p < 0.01$; $\eta p^2 = 0.32$; Fig. 2b); CK responses post-exercise and 48 h post-exercise were both elevated compared to pre-exercise, peaking 48 h post-exercise ($p < 0.01$) (Fig. 2b). No sex differences ($p = 0.62$; $\eta p^2 = 0.02$) or interaction effects ($p = 0.87$; $\eta p^2 < 0.01$) were observed for CK responses after exercise.

Changes in muscle damage markers after exercise

Changes in muscle damage markers are presented here as % change from pre-exercise; raw values can be found in Table S1. There were main time effects for MVIF ($p < 0.01$; $\eta p^2 = 0.77$; Fig. 3a), VA ($p < 0.01$; $\eta p^2 = 0.63$; Fig. 3b), Tw_{pot} ($p < 0.01$; $\eta p^2 = 0.93$; Fig. 3c), evoked RFD_{max} ($p < 0.01$; $\eta p^2 = 0.84$; Fig. 3d), TTP ($p < 0.01$; $\eta p^2 = 0.38$; Fig. 3e) and DOMS ($p < 0.01$; $\eta p^2 = 0.31$; Fig. 3f).

MVIF decreased post-exercise ($p < 0.01$) and remained attenuated after 48 h post-exercise ($p < 0.01$). VA, Tw_{pot} and evoked RFD_{max} all decreased post-exercise ($p < 0.01$ for all), but returned to their respective baseline values 48 h post-exercise (VA: $p = 0.58$; Tw_{pot}: $p = 0.12$; RFD_{max}: $p = 0.15$). TTP did not change post-exercise ($p = 0.20$), but immediately post-exercise, TTP was faster vs. 48 h post-exercise ($p < 0.01$). DOMS increased post-exercise ($p < 0.01$) and remained elevated 48 h post-exercise ($p < 0.01$).

No sex differences or interaction effects were observed for MVIF (group: $p = 0.19$; $\eta p^2 = 0.07$; interaction: $p = 0.30$; $\eta p^2 = 0.03$), VA (group: $p = 0.86$; $\eta p^2 < 0.01$; interaction: $p = 0.97$; $\eta p^2 < 0.01$), Tw_{pot} (group: $p = 0.98$; $\eta p^2 < 0.01$; interaction: $p = 0.80$; $\eta p^2 = 0.02$), evoked RFD_{max} (group: $p = 0.44$; $\eta p^2 = 0.05$; interaction: $p = 0.61$; $\eta p^2 = 0.04$), and DOMS (group: $p = 0.71$; $\eta p^2 < 0.01$; interaction: $p = 0.71$; $\eta p^2 = 0.01$).

Main group ($p = 0.02$ $\eta p^2 = 0.35$) and interaction ($p = 0.04$; $\eta p^2 = 0.22$) effects were evident for TTP, as males demonstrated quicker TTP than females. At 48 h post-exercise, TTP had recovered closer to baseline in males compared to females ($p = 0.03$).

Correlations between urinary titin fragments and muscle damage markers

Moderate negative correlations were observed between UTF and MVIF ($r_s = -0.45$; $p < 0.01$; Fig. 4a), whilst strong negative correlations were observed with Tw_{pot} ($r_s = -0.77$; $p < 0.01$; Fig. 4f) and evoked RFD_{max} ($r_s = -0.62$; $p < 0.01$; Fig. 4e). A strong positive correlation was found for UTF and CK ($r_s = 0.72$; $p < 0.01$; Fig. 4c). No significant correlations were observed between UTF and DOMS ($r_s = 0.22$; $p = 0.15$; Fig. 4b), VA ($r_s = -0.25$; $p = 0.19$; Fig. 4d) and TTP ($r_s = 0.33$; $p = 0.89$; Fig. 4g). Table S2 presents Spearman's correlation coefficients between UTF and soreness and neuromuscular function markers in males and females. In both males and females, strong negative correlations were found between UTF and Tw_{pot}.

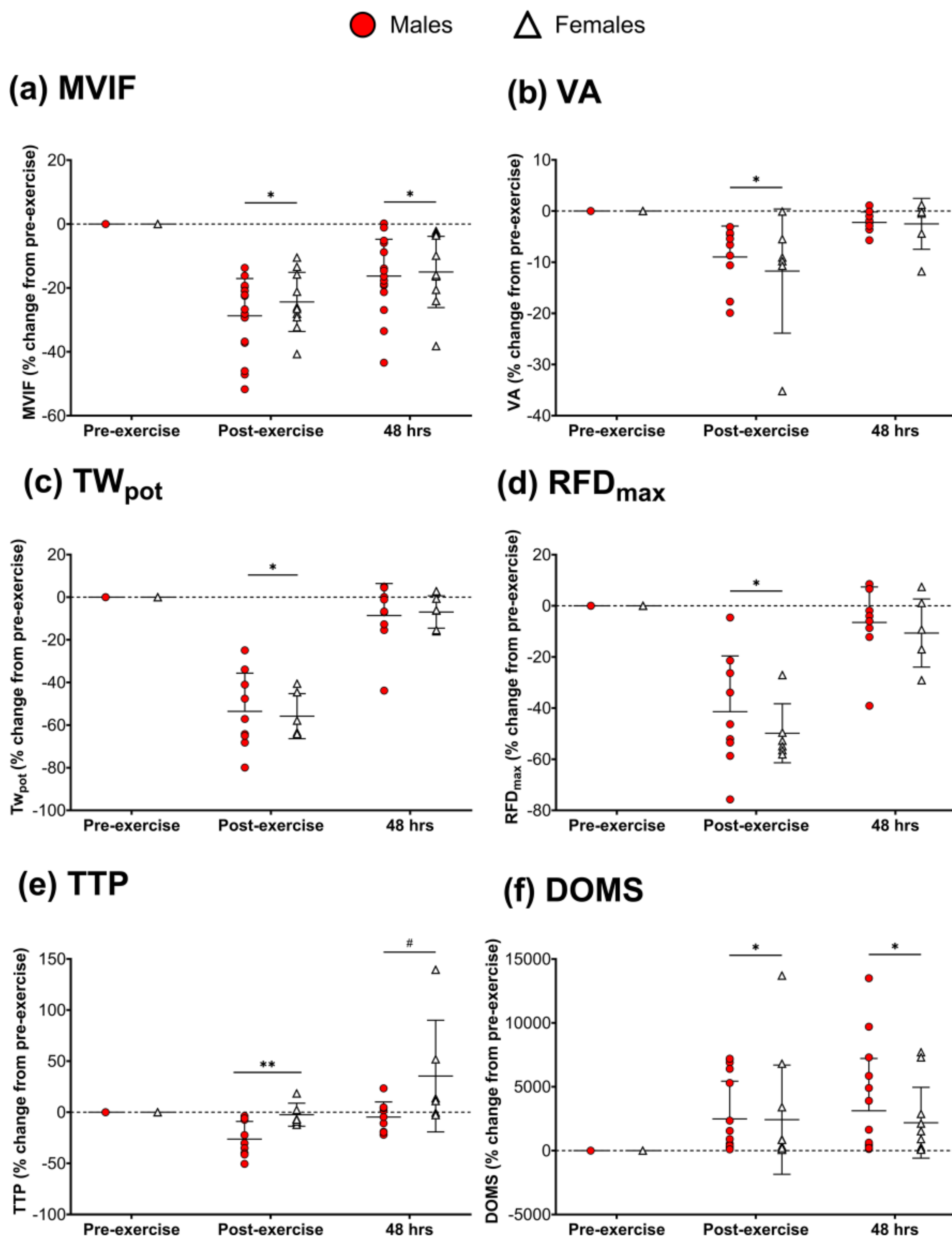

Fig. 3 Changes in neuromuscular function and soreness markers after exercise in males and females. **a** Maximal voluntary isometric force (MVIF) ($n = 27$); **b** voluntary activation (VA) ($n = 17$); **c** potentiated twitch force (Tw_{pot}) ($n = 17$); **d** evoked maximal rate of force development (RFD_{max}) ($n = 17$); **e** time to peak twitch (TTP) ($n = 17$); **f** delayed-onset muscle soreness (DOMS) ($n = 27$). All figures are presented as individual data points alongside mean and SD. * = different from pre-exercise. ** = significantly different from 48 h post-exercise. # = interaction effect

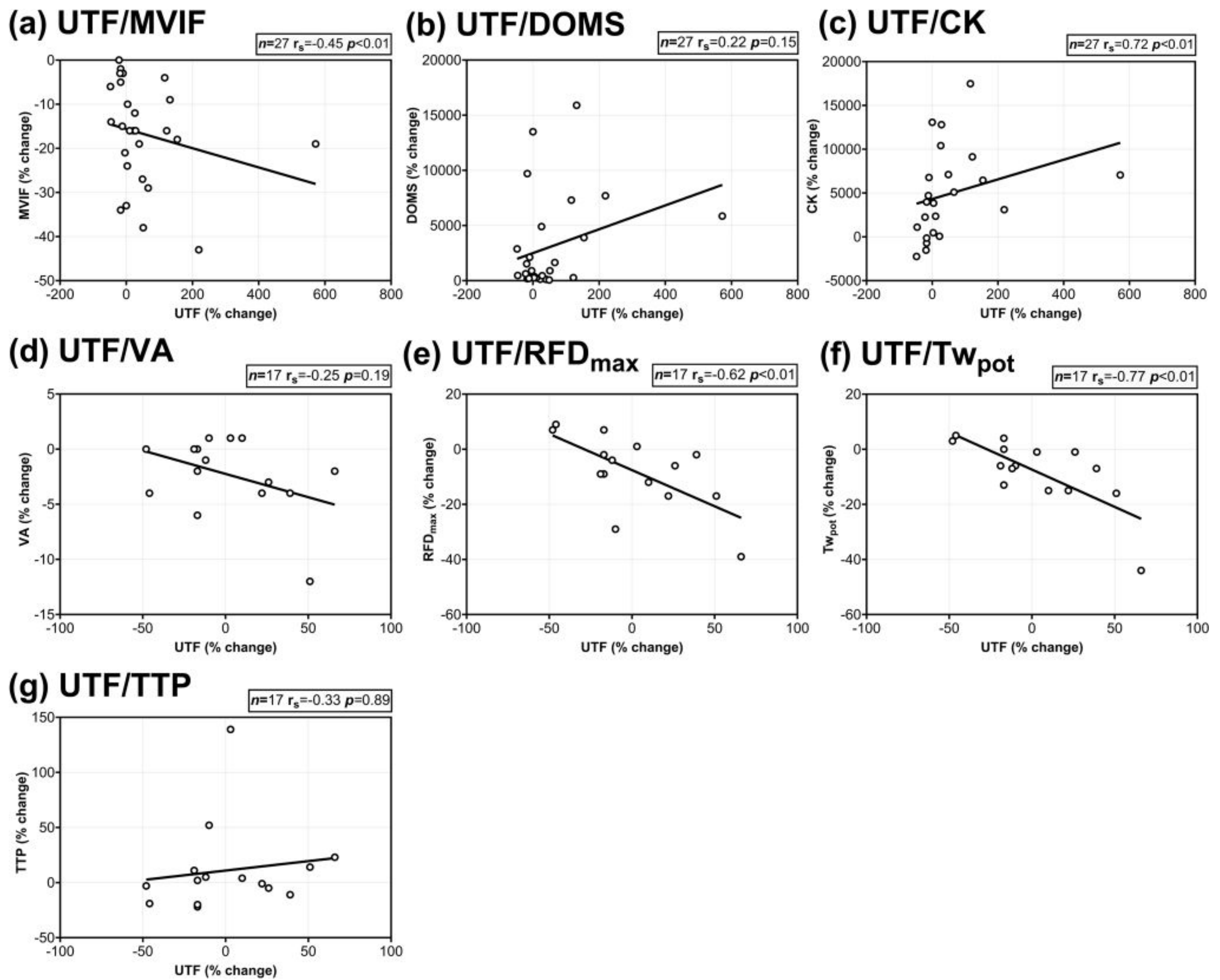

Fig. 4 Spearman's correlations coefficients (r_s) between the per cent (%) change (from pre- to 48 h post-exercise) of urinary titin fragments (UTF) and **a** maximal voluntary isometric force (MVIF) ($n=27$), **b** delayed-onset muscle soreness (DOMS) ($n=27$), **c** creatine kinase (CK) ($n=27$), **d** voluntary activation (VA) ($n=17$), **e** evoked maximal rate of force development (RFD_{max}) ($n=17$), **f** potentiated twitch force (Tw_{pot}) ($n=17$), **g** time to peak twitch ($n=17$)

Correlations between serum creatine kinase and muscle damage markers

Moderate strength correlations were found between the % change in CK and DOMS ($r=0.47$; $p=0.03$; Fig. 5b) and evoked RFD_{max} ($r=-0.47$; $p=0.10$; Fig. 5d). No significant correlations were found between CK and MVIF ($r=-0.06$; $p=0.78$; Fig. 5a), VA ($r=-0.09$; $p=0.78$; Fig. 5c), Tw_{pot} ($r=0.28$; $p=0.34$; Fig. 5e), and TTP ($r=0.01$; $p=0.97$; Fig. 5f).

Discussion

This study explored whether (a) muscle-damaging exercise increased UTF in both males and females; (b) there were any sex differences in post-exercise UTF responses, and (c) whether changes in UTF levels 48 h after exercise correlated with various indirect markers of EIMD. The main findings indicate that UTF concentrations are similarly elevated 48 h after muscle-damaging exercise in both males and females, and increases in UTF are more strongly correlated with decrements in neuromuscular function than CK. In contrast, increases in CK are more closely correlated to DOMS, but there is a strong positive correlation between UTF and CK. These findings suggest that UTF may be a useful non-invasive, indirect, biomarker

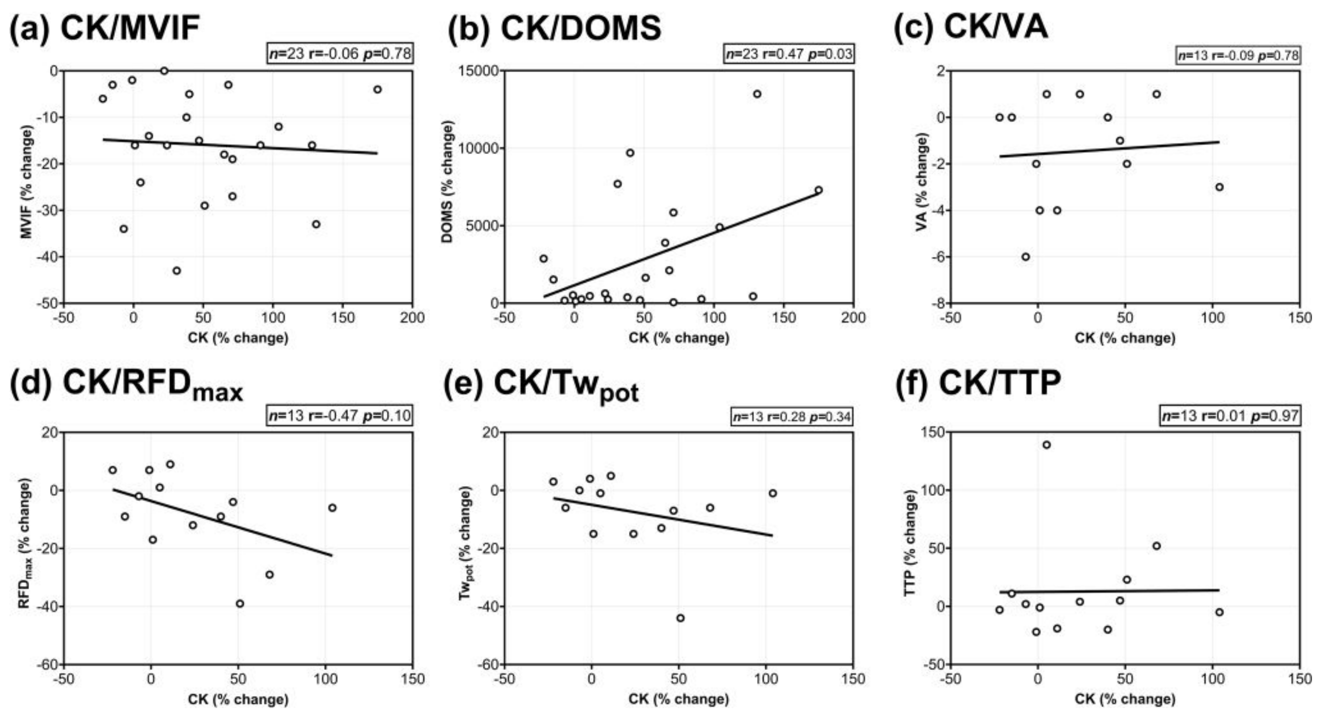

Fig. 5 Pearson's correlations coefficients (r) between the per cent (%) change (from pre- to 48 h) of creatine kinase (CK) and **a** maximal voluntary isometric force (MVIF) ($n=23$); **b** delayed-onset muscle soreness (DOMS) ($n=23$), **c** voluntary activation (VA) ($n=13$), **d** evoked maximal rate of force development (RFD_{max}) ($n=13$), **e** potentiated twitch force (Tw_{pot}) ($n=13$), **f** time to peak twitch (TTP) ($n=13$)

of myofibrillar disruption after strenuous eccentric exercise in males and females.

Several recent studies suggest that UTF may serve as a non-invasive biomarker for detecting EIMD in healthy individuals, as it reliably increases following damaging exercise and correlates with declines in muscle function and elevations in DOMS (Kanda et al. 2017; Yamaguchi et al. 2020c, Yamaguchi et al. 2020b; Tanabe et al. 2021a, Tanabe et al. 2021b). Notably, UTF concentrations rise more substantially following eccentric-biased exercise compared to concentric-biased protocols, reinforcing the idea that its elevation is specifically linked to mechanical muscle damage rather than general exercise-induced physiological stress (Yamaguchi et al. 2020a). Additionally, one study reported that UTF responses are attenuated following a repeated bout of exercise, suggesting that UTF may be influenced by training status and the repeated bout effect (Yamaguchi et al. 2020b). Collectively, these findings point to UTF as a potentially more sensitive and specific marker of EIMD than traditional biomarkers such as CK, whose validity has been increasingly questioned (Brancaccio et al. 2007). Furthermore, the clinical utility of UTF is supported by its use as a biomarker in muscle-related pathologies, including Duchenne muscular dystrophy and idiopathic inflammatory myopathies (Ishii et al. 2023; Sun et al. 2023), underscoring its relevance in conditions characterised by elevated muscle damage and catabolism.

In agreement with previous studies (Kanda et al. 2017; Yamaguchi et al. 2020c; Tanabe et al. 2021b), the present study demonstrates that high-volume, maximal eccentric contractions elevated UTF concentrations in some, but not all, participants 48 h post-exercise. Eccentric contractions are well known to induce structural damage to skeletal muscle structures (Cermak et al. 2012; Deyhle et al. 2016). Specifically, repeated overstretching during eccentric loading leads to morphological disruption of sarcomeres, including damage to Z-lines (Friden et al. 1983)—the anchoring regions where titin connects to the M-line and contributes to sarcomerogenesis and myofibrillar assembly (Prado et al. 2005). Direct evidence of titin disruption comes from Trappe et al. (2002), who reported an approximate 30% reduction in titin content in vastus lateralis biopsies following a single bout of high-intensity eccentric exercise. Although the precise mechanisms underlying titin breakdown remain inconclusive, it is widely theorised that focal sarcomeric damage is followed by an uncontrolled Ca^{2+} influx into the myofibrillar cytosol (Gissel 2005), which activates calcium-dependent proteolytic enzymes, such as calpains and matrix metalloproteinases, leading to the degradation of key structural proteins, including titin (Beckmann and Spencer 2008; Ali et al. 2010). The activation of calpains, alongside the

release of pro-inflammatory mediators, further compromises sarcolemmal integrity and sarcomeric stability (Letavernier et al. 2012; Fatouros and Jamurtas 2016). It is therefore plausible that the rise in UTF observed here and in other studies reflects the proteolytic degradation of titin, combined with increased sarcolemma permeability, enabling titin fragments to enter the bloodstream and be excreted in urine via glomerular filtration.

Nonetheless, as shown in Fig. 1(a), UTF concentrations did not increase in all participants following exercise, indicating that — like CK — UTF exhibits considerable inter-individual variability. While differences in training status were controlled for by recruiting only strength-trained individuals, several biochemical mechanisms may explain this variability. Firstly, some individuals may have inherently more robust sarcomeric structures, potentially due to differential expression of titin isoforms. Specifically, a higher proportion of the more compliant N2BA isoform, as opposed to the stiffer N2B variant, could confer greater sarcomere elasticity, reducing mechanical strain and titin disruption under eccentric loading (Lewinter and Granzier 2010). Secondly, the proteolytic activity of calpains, which degrade titin, is highly Ca^{2+} dependent (Gissel 2005). Therefore, individual differences in calcium homeostasis—influenced by factors such as sarcoplasmic reticulum function, ion channel behaviour, and mitochondrial buffering capacity—could modulate calpain activation and, consequently, titin degradation. These mechanisms together may account for the non-responder phenotype observed in some participants and subsequently may be a limiting factor for using UTF as a biomarker for detecting EIMD.

EIMD induces a prolonged reduction in muscle force-generating capacity that can last for several days (Byrne et al. 2004). As such, changes in neuromuscular function are viewed as the most appropriate marker of EIMD (Warren et al. 1999; Paulsen et al. 2012; Damas et al. 2016). Hence, we measured an array of neuromuscular responses to determine whether the IKD exercise protocol induced EIMD, and whether changes in UTF reflected EIMD. In this study, we observed that exercise elicited changes in markers of neuromuscular function; however, after 48 h, only MVIF remained attenuated. A reduction in MVIF without concurrent impairments in VA, RFD_{max}, or twitch potentiation does not necessarily indicate that EIMD was absent but rather suggests that the damage may have been relatively mild, predominantly mechanical rather than neural, and that recovery processes were likely already underway—particularly among more trained individuals. Additionally, there was considerable inter-individual variability in neuromuscular responses: while some participants returned to baseline by 48 h, others exhibited persistent deficits across multiple markers. It is also important to acknowledge that the sample size for MVIF ($n = 27$) was larger than that for VA, Tw_{pot}, TTP, and RFD_{max} ($n = 17$), which may have increased the sensitivity to detect changes in MVIF relative to the other neuromuscular measures.

DOMS is another commonly reported manifestation of EIMD, typically appearing 12–48 h post-exercise and peaking between 24 and 72 h (Jones et al. 1989; Clarkson et al. 1992; Cleak and Eston 1992). Although widely used as an indirect marker of EIMD, DOMS exhibits a poor temporal relationship with histological evidence of ultrastructural muscle damage (Clarkson et al. 1992). Nonetheless, previous studies have reported positive correlations between UTF concentrations and DOMS following muscle-damaging exercise (Kanda et al. 2017; Yamaguchi et al. 2020c). In the present study, DOMS was significantly elevated at 48 h post-exercise, suggesting that EIMD may have occurred despite a lack of consistent responses in some neuromuscular markers. However, this increase in DOMS did not correlate with changes in UTF. Several factors may explain the discrepancy between our findings and earlier studies reporting a positive relationship between UTF and DOMS, including: (1) differences in the muscle groups targeted (quadriceps in the present study vs. gastrocnemius (Kanda et al. 2017) or elbow flexors (Yamaguchi et al. 2020c)) (2) participant sex, as previous studies included only males; (3) variation in the assessment tools used to quantify DOMS (e.g. 200 mm vs. 100 mm visual analogue scales); and (4) differences in participant training status, as those in the current study had prior resistance training experience, whereas the training backgrounds of participants in earlier studies were not reported (Kanda et al. 2017; Yamaguchi et al. 2020c).

Correlations were performed to determine whether UTF could serve as a valid intramuscular biomarker for detecting the functional manifestations of EIMD (DOMS and neuromuscular function). Given that neuromuscular function is considered the gold standard marker of EIMD (Warren et al. 1999), a strong correlation with these markers would suggest UTF could be an appropriate biomarker of EIMD, which is less invasive than the most commonly measured intramuscular protein, serum CK. Overall, we found that changes in UTF correlated more strongly with changes in neuromuscular function markers than changes in CK 48 h after exercise. Specifically, changes in UTF were negatively correlated with MVIF, Tw_{pot} and evoked RFD_{max}, whilst changes in CK correlated with DOMS and evoked RFD_{max}. Although this is the first study to examine and establish an association between UTF and TW_{pot} and evoked RFD_{max}, the temporal relationship between UTF and MVIF has been reported elsewhere; Yamaguchi et al. (2020c) similarly found a strong negative correlation between these markers from 48 to 144 h post-EIMD, whilst Tanabe et al. (2021b) reported a moderate negative correlation between peak MVIF and peak UTF. This close association is likely explained by titin's key role in generating passive force in skeletal muscle and therefore

overall force production (Freundt and Linke 2019). As reported previously (Yamaguchi et al. 2020c), UTF was also strongly correlated with CK. Collectively, our findings add to the growing literature that suggests UTF is a potentially useful biomarker of myofibrillar damage following strenuous exercise. Given the strong correlation between UTF and CK at 48 h post-exercise, UTF could be the preferred marker for the indirect detection of damage to intramuscular proteins.

This is the first study to examine exercise-induced UTF levels in females. We hypothesised that females would have lower UTF responses to exercise given that some studies found that females (from human and rodent models) experience less severe symptoms of EIMD after eccentric exercise compared to males (Bär et al. 1988; Amelink et al. 1988; Dernbach et al. 1993; St Pierre Schneider et al. 1999; Carter et al. 2001). However, evidence remains inconsistent (Clarkson and Hubal 2001). The rationale for a blunted EIMD response in females is attributed to various factors, notably increased levels of oestrogen, which may have a protective effect on muscle damage by modulating inflammation (Carter et al. 2001). Furthermore, males have a higher proportion of type II muscle fibres, which are more susceptible to damage from eccentric exercise (Dannecker et al. 2012). However, this study suggests that exercise-induced UTF concentrations are not different between males and females. Other than the small differences in TTP at 48 h post-exercise between males and females, there was no evidence of sex differences in markers of EIMD. Thus, our findings suggest that UTF could be used as a marker of EIMD in both males and females.

This study has several limitations that should be acknowledged. Firstly, we did not include a concentric-biased, non-damaging exercise control group which would have enabled us to confirm whether increases in UTF were specifically attributable to EIMD rather than other mechano-physiological responses to exercise. However, Yamaguchi et al. (2020a) have previously demonstrated that UTF levels increase to a greater extent following eccentric compared to concentric exercise, thereby supporting the link between UTF elevation and EIMD. Secondly, measurements were only taken at 48 h post-exercise due to limited resources which limited our ability to replicate the findings by Kanda et al. (2017) that highlighted UTF levels tended to peak at 96 h post-exercise. This meant that we missed an opportunity to assess temporal dynamics, including potential sex differences or stronger correlations, which may have emerged at later time points. Additionally, the data presented here are part of an exploratory analysis within a larger study, and an a priori power calculation was not conducted specifically for this sub-analysis. As such, the study may have been underpowered to detect smaller effects or interactions. Notwithstanding these limitations, this study had a larger sample size than all previous investigations examining UTF responses to exercise and, importantly, was the first to characterise UTF responses in females. Furthermore, the inclusion of a comprehensive range of neuromuscular function markers, which have not previously been evaluated alongside UTF, strengthens the novelty and translational relevance of our findings. Together, these data provide new insights into the potential utility of UTF as a biomarker of EIMD in physically active males and females.

Conclusion

In conclusion, strenuous, eccentric-heavy exercise significantly increased UTF concentrations in some individuals 48 h post-exercise. Notably, this study is the first to demonstrate that UTF increases similarly in both males and females. Compared to CK, UTF showed a stronger association with exercise-induced deficits in neuromuscular function, although not with DOMS. However, similar to CK, UTF responses exhibited substantial inter-individual variability. These findings suggest that while UTF may better reflect the extent of EIMD than CK, its variable responsiveness currently limits its reliability as a standalone biomarker.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00421-025-05936-6.

Author contributions Conceptualisation: E.J.H, T.C, J.T. Collection, analysis, or interpretation of data: E.J.H, J.T. Writing—original draft preparation: E.J.H, T.C, K.M.R, J.S., L.J.J, J.T. Writing—review and editing: E.J.H, T.C, K.M.R, J.S., L.J.J, J.T. All authors have read and agreed to the published version of the manuscript.

Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interests There are no conflicts of interest to declare.

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