Muscle-tendon unit mechanobiological responses to consecutive high strain cyclic loading

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Summary statement: Frequent high cyclic mechanical loading of a tendon over a short period of time may diminish the tendon's tolerance to high tensile loading and predispose it to overuse injury.

Abstract

In response to a mechanical stimulus, tendons have a slower tissue renewal rate compared to muscles. This could over time lead to a higher mechanical demand (experienced strain) for the tendon, especially when a high strain magnitude exercise is repeated without sufficient recovery. The current study investigated the adaptive responses of the triceps surae (TS) muscle-tendon unit (MTU) and extracellular matrix turnover-related biomarkers to repetitive high tendon strain cyclic loading. Eleven young male adults performed a progressive resistance exercise over 12 consecutive days, consisting of high Achilles tendon (AT) strain cyclic loading (90% MVC) with one leg once a day (Leg_{T1}) and the alternate leg three times a day (Leg_{T3}). Exercise-related changes in TS MTU mechanical properties and serum concentrations of extracellular matrix turnover-related biomarkers were analysed over the intervention period. Both legs demonstrated similar increases in maximal AT force (~10%) over the 12-day period of exercise. A ~20% increase in maximal AT strain was found for Leg_{T3} ($p<0.05$) already after 8 consecutive exercise days, along with a corresponding decrease in AT stiffness. These effects were maintained even after a 48h rest period. The AT mechanical properties for Leg_{T1} were unaltered. Biomarker analysis revealed no sign of inflammation, but altered collagen turnover and delayed increase in the collagen type I synthesis rate. Accordingly, we suggest that tendon is vulnerable to frequent high-magnitude and volume of cyclic mechanical loading, as accumulation of micro-damage can potentially exceed the rate of biological repair, leading to increased maximal tendon strain.

Introduction

Tendons are crucial components of musculotendinous units (MTUs) with the primary function to transmit generated muscle forces to the skeletal system in order to create motion. In particular, leg-extensor tendons need to withstand frequent cyclic loading during various daily and sporting activities, such as walking, running or jumping (Alexander, 1995; Kharazi et al., 2021; Lichtwark and Wilson, 2005). The largest lower extremity tendons (the Achilles and patellar tendon) demonstrate superior fatigue resistance i.e. the ability to withstand cyclic or sustained mechanical loading (Thorpe et al., 2017). However, the tensile loads to which they are subjected can often lead to acute or degenerative injuries, such as tendinopathies or even ruptures (Kannus and Natri, 1997). Remarkably, more than half of elite athletes experience tendinous tissue injuries over the course of their career (Cassel et al., 2018; Kujala et al., 2005), with the exact cause of these injuries still largely unknown and regarded as multifactorial tissue disorders (Millar et al., 2021).

From a tissue adaptation viewpoint, compared to muscles which respond well to a variety of mechanical and metabolic stimuli (Lambrianides et al., 2022), for long-term adaptations, tendons require a specific strain threshold to be exceeded in a particular time-dependent manner (Arampatzis et al., 2007; Bohm et al., 2014; Kongsgaard et al., 2007). If mechanical loading characteristics provide suitable conditions only for muscular strength adaptation, a discrepancy within the muscle-tendon unit can occur (Mersmann et al., 2016). Given that mechanical loading matches requirements for tendon adaptation, the response of tendinous tissue still appears to be less sensitive (Heinemeier et al., 2007), delayed in time (Langberg et al., 1999) and to peak after a certain loading magnitude and volume have been reached (Magnusson et al., 2010). This could explain observations of different adaptation rates between the two tissues over typical 12-14 week resistance training interventions (Kubo et

al., 2010; Kubo et al., 2012). In such situations, increments in muscle strength may precede adaptive increments in tendon stiffness even more than several weeks, potentially increasing the experienced tendon strain during muscular contractions (Arampatzis et al., 2020). Interestingly, tendon strain has been observed to be greater in tendinopathic tendons (Arya and Kulig, 2010; Wang et al., 2012) and to fluctuate more over time in athletes across different ages (Karamanidis and Epro, 2020; Mersmann et al., 2016). Considering, that the ultimate tendon strain can be regarded rather constant (LaCroix et al., 2013), operating closer to its ultimate strain increases the risk of tissue failure (Wren et al., 2003), and therefore an increase in the experienced tendon strain is proposed to indicate a higher mechanical demand for the tendon (Arampatzis et al., 2020).

Acute repetitive cyclic mechanical loading or prolonged loading at constant stress in both *ex vivo* and *in vivo* conditions has led to a continuous increase in tendon strain (Fung et al., 2010; Wang et al., 1995; Wren et al., 2003). One prominent mechanism for this as well as the development of chronic tendinous overuse injury is proposed to be an accumulation of structural "micro-damage" or "sub-ruptures" (Fung et al., 2010; Riley, 2008; Zitnay et al., 2020). Indeed, several *in vitro* animal studies have demonstrated progressive nanoscale damage of tendon collagen fibrils, denaturation of collagen triple-helical structure and increased collagen proteolysis through excessive cyclic overloading (Willett et al., 2007; Zitnay et al., 2020). Tendinous collagen is renewed at higher rates in tendinopathy in humans compared to healthy controls (De Mos et al., 2007; Heinemeier et al., 2018). This is evident from considerably higher activity of matrix metalloproteinases (the regulators of collagen turnover) along with increased content of denatured collagen and a relatively immature collagenous matrix (De Mos et al., 2007). These findings in aggregate indicate substantial accumulation of structural micro-damage in the tendinous tissue and could explain observed

elevation of tendon strain in tendinopathic tendons (Arya and Kulig, 2010; Wang et al., 2012).

Taking into account that recovery of muscle function (force generation) can be achieved within several hours following most exercise modalities (Carroll et al., 2017), tendon is likely to be subject to increased strain since it cannot respond with matching recovery rate. This corresponds to previous proposals that repeated high loading magnitude exercise involving high tendon strain with relatively short resting periods can result in epochs where regeneration processes are overtaken by degradation of tendon matrix and an accumulation of "micro-damage" (Fung et al., 2010; Magnusson et al., 2010; Tran et al., 2020). This discrepancy in the recovery and adaptive processes within the MTU could increase the experienced tendon strain and hence diminish its tolerance to high tensile loading.

Previous *in vitro* investigations have clearly demonstrated that tendon damage (altered mechanical properties and cell morphology, severity of structural damage) is largely strain magnitude and cycle number dependent (Ros et al., 2019). Hence, the current study aimed to investigate, *in vivo*, whether an exercise involving high magnitude cyclic mechanical loading over 12 consecutive days with two different recovery periods between sessions leads to alterations in human Achilles tendon (AT) mechanical properties and extracellular matrix turnover-related biomarkers. We hypothesised that this particular form of exercise would allow muscle strength to regain between sessions and improve, but lead to a decrease in AT stiffness and elevated tendon strain dependent on exercise frequency and volume, which might potentially be related to time-dependent anabolic responses in collagen turnover.

Materials and Methods

Participants and experimental design

Eleven healthy young recreationally active male adults (age 26 ± 6 years; body mass 79 ± 7 kg; height 182 ± 7 cm; with moderate physical activity three times a week) were recruited to take part in the study. Exclusion criteria were any previous history of AT rupture, any acute AT pain, overuse injuries (e.g. tendinopathy) and other musculoskeletal impairments of the lower limbs (e.g. ankle joint pain) within the last six months. The study obtained ethical approval (no. 026/2013) from the local ethics committee and all participants gave written informed consent prior to the study, in accordance with the recommendations of the Declaration of Helsinki.

Each participant underwent 12 consecutive days of a resistance exercise intervention with high AT strain cyclic loading for both legs (Fig. 1): one leg once a day (~24h rest between each session; Leg_{T1}) and the contralateral leg three times a day (~2-3h rest between daily sessions; Leg_{T3}). Potential exercise-related changes in triceps surae (TS) MTU biomechanical properties were determined for both legs prior to the exercise intervention, every other day of the intervention and 48h after a non-contact retention period (Fig. 1). In addition, serum concentrations of biomarkers related to extracellular matrix turnover were assessed prior to the intervention, every fourth day of the intervention and 48h after the non-contact retention period (Fig. 1).

---- Please insert Figure 1 -----

Analysis of muscle and tendon mechanical properties

TS MTU mechanical properties (maximal ankle plantar flexion moment, maximal AT force, AT stiffness and maximal AT strain) were obtained in all participants using a custom-made

146 dynamometer (Fig. 2) synchronised with ultrasonography and motion capture as described in
147 more detail in our previous study (Karamanidis et al., 2016). The measurements were
148 performed on two separate days before the intervention (PRE1 and PRE2) and every other
149 day (day 2 to day 12) over the course of the intervention, as well as at 48h post last training
150 session (RET). On training days, measurements were performed 1h after the last training
151 session of the leg to allow some recovery prior the measurement. PRE1 and PRE2 (separated
152 by approximately 24h) were included in order to assess the reliability of the testing and to
153 determine baselines of individual participants (Base), with the RET measurement evaluating
154 retention of possible effects on TS MTU properties.

155 Briefly, each participant was seated with their knee joint fully extended and their foot
156 positioned on the dynamometer's foot plate perpendicular to the shank and thigh (the "neutral
157 position," Fig. 2). In order to minimize any joint rotation, the participant's foot was fixed
158 around the ankle and onto the foot plate using a custom-made fixation constructed from rigid
159 ski-boot buckles. The positioning of each participant with respect to the device was recorded
160 to allow exact reproduction of the setup for each individual at each measurement time point.
161 Prior to the measurement each participant performed individualised warm-up exercises (e.g.
162 jogging, hopping, stretching) followed by a standardized visually guided warm-up of 2–3
163 minutes of submaximal and three maximal isometric contractions with the aim of
164 "preconditioning" the tendon (Maganaris, 2003). Subsequently, the maximal ankle plantar
165 flexion moment and the force–elongation relationship of the tendon during the loading phase
166 were assessed by performing three maximal isometric voluntary ankle plantar flexion
167 contractions (MVCs) with verbal encouragement followed by three additional controlled
168 MVC ramp contractions. The latter were arranged with a three-second loading time guided by
169 visual feedback and with the instruction to hold the achieved joint moment for about 2-3
170 seconds (Arampatzis et al., 2007; Epro et al., 2017). Reaction forces during the contractions

were assessed by a custom-made force plate consisting of three strain gauge load cells (100 Hz). During each contraction the lower limb (the medial and lateral malleolus, the calcaneus and the head of the fibula) and the force plate (top and bottom of both sides) were tracked automatically using 8 light-emitting diodes (i.e. active markers) and two digital cameras (15 Hz, Basler, Germany; as in Karamanidis et al., 2016). The lever arm of the reaction force at the ankle joint during plantar flexion contractions was assessed from the point of force application under the foot and the ankle joint centre (the midpoint between malleoli). Inverse dynamics was used to calculate the resultant ankle joint moments, accounting for gravitational moments and compression forces from the fixation prior to each contraction using a passive measurement (muscles relaxed in the fixed position).

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The elongation of the myotendinous junction (MTJ) of the m. gastrocnemius medialis during each plantar flexion contraction was recorded using a 7.5-MHz linear array ultrasound probe (73 Hz; α7, Aloka, Tokyo, Japan) and manually digitized with custom-made software in MATLAB (version 2020b; The MathWorks, Natick, MA, USA). The ultrasound probe was securely fixed on the shank longitudinally above the MTJ using a casing with adjustable straps to prevent any movement in relation to the skin (Fig. 2). The position of the probe was marked on the participant's skin using a permanent marker for the baseline measurement, which was reapplied daily to ensure identical probe placement at each measurement time point. Echo-absorbing tape attached to the skin was used to account for potential movement of the probe.

After preconditioning of the tendon, the length of the AT at rest (relaxed musculature) was determined as the distance between the MTJ of the m. gastrocnemius medialis and the most proximal point of the tuber calcanei (both defined using ultrasonography). The AT force during each contraction was determined by dividing the resultant ankle joint moment by the individual tendon moment arm, which was estimated as the perpendicular distance from the ankle joint's center of rotation (the average of medial and lateral malleoli) to the AT (Scholz et al., 2008). The effect of the inevitable ankle joint angular rotation on the measured tendon elongation during each contraction was taken into account by subtracting the elongation of the tendon caused by ankle joint changes (Muramatsu et al., 2001). The elongation of the tendon due to ankle rotation was estimated as the product of the AT moment arm and the ankle joint angular changes. Subsequently the AT stiffness was estimated using linear regression as the slope of the relation between calculated AT force and resultant tendon elongation over the range 50-100% of maximum AT force. The maximal plantarflexion moment (TS muscle strength) was calculated as the maximal value across all MVCs (three MVCs and the three controlled MVC ramp contractions. The force–elongation relationship of the tendon was determined using the mean value of three MVC ramp contractions. The ramp MVC contractions were preferred in order to account for potential effects of loading rate dependency due to tendon viscoelasticity.

Analysis of extracellular matrix turnover-related biomarkers

In order to estimate extracellular matrix turnover, biomarkers were analysed from a single venous blood sample obtained on six occasions over the exercise intervention in the mornings at 08.00-09.00h after fasting (Base and RET), or just after the first exercise session of the day at 08.00-10.00h (day 1, day 4, day 8 and day 12). Baseline measurement was performed on the first TS MTU mechanical properties baseline measurement day (PRE1). In total, 9.5 mL

of blood was collected using the Vacutainer blood withdrawal system (Becton Dickinson, Heidelberg, Germany). The blood samples were directly stored at 7°C for 30 minutes for deactivation of coagulation factors prior to centrifuging for 10 min at 1861 g and 4°C (Rotixa 50, Hettich Zentrifugen, Mühlheim, Germany). The collected serum was then stored at -80°C for subsequent analysis. Serum concentration levels of interleukin-6 (IL-6; inflammation marker), collagen type I propeptides (PICP and PINP; collagen type I synthesis rate markers), matrix metalloproteinases (MMP-2 and MMP-9; collagen protein turnover markers) were determined using ELISA kits (R&D Systems, Wiesbaden, Germany). The samples were analysed in duplicate and inter-assay variation was excluded by testing all samples for each participant on the same plate. In order to determine exercise-induced alterations in concentrations, assay values were normalised to the Baseline level.

Perceived pain

AT tendinopathy patients usually report significant levels of perceived tendon pain along with reduced tendon stiffness and elevated tendon strain in comparison to healthy individuals (Arya & Kulig, 2010). As we hypothesised an elevated tendon strain due to the implemented intervention, we expected that some participants might perceive some tendon pain along the exercise. Hence, each participant was asked to record any perceived pain using a numeric rating scale (NRS; Downie et al., 1978) on every second training day (following the day's last exercise session). The record related separately to each leg, to the ankle plantar flexor muscles and to the AT, as well as requesting information on any feeling of being "restricted in daily life". The NRS scale was rated from 0 to 10, with rating 0 defined as no pain or restrictions, ratings 1 to 3 as "mild", 4 to 6 as "moderate" and 7 to 10 as "severe".

Exercise intervention

Each participant underwent 12 consecutive days of a resistance exercise intervention with high AT strain cyclic loading of isometric plantarflexion contractions (Arampatzis et al., 2007). Each training session consisted of five sets of four repetitions of isometric plantarflexion contractions performed at 90% of MVC (3 s loading and 3 s relaxation) using the same custom-made dynamometer and visual feedback system as in the MTU measurements. Using the above-mentioned protocol, participants exercised with one leg once a day (Leg_{T1}, low exercise dose with ~24h rest between sessions and an expected peak in collagen synthesis rate; Magnusson et al., 2010)) and with the contralateral leg three times a day (Leg_{T3}, high exercise dose with ~2-3h rest between sessions within a day). The leg allocation for participants was randomised and the 90% MVC threshold was progressively increased every second day based on the individual MVC performed prior the first session of that training day. The chosen time frames, verified by prior pilot testing, arranged for necessary muscle strength recovery to allow training at the target 90% MVC threshold, hence ensuring constant high magnitude mechanical loading and tendon strain. The protocol allowed us to compare the influence of different resting periods (exercise frequencies) on days for which similar mechanical loading volumes were achieved (in relation to the participants' muscular capacities). Accordingly, it was possible to match the relative mechanical loading volume of day 12 of Leg_{T1} with day 4 of Leg_{T3}, which corresponded to a total of 12 exercise sessions (Fig. 1).

Statistics

The normality of distribution and homogeneity of variance of the data were analysed using Shapiro-Wilk and Levene's tests. A two-way repeated measures analysis of variance (ANOVA) was performed to detect potential differences between time points and legs.

Intraclass correlation coefficients (ICC; representing absolute agreement for single measures) were calculated to determine the between-day reliability for the two baseline measurements (PRE1 and PRE2) and for all analysed TS MTU mechanical properties (maximal AT force, maximal ankle plantarflexion moment, AT stiffness and maximal AT strain). ICC values were defined as representing reliability as: "poor" (<0.50), "moderate" (0.50-0.75), "good" (0.75-0.90) and "excellent" (>0.90) (Koo and Li, 2016). In order to investigate potential alterations in TS MTU mechanical properties, separate one-way repeated measures ANOVAs were performed for both legs (Leg_{T1} and Leg_{T3}) using measurement time point (Base, day 2, day 4, day 6, day 8, day 10, day 12, RET) as factor. Baseline (Base) for TS MTU mechanical properties was the average of PRE1 and PRE2 measurements. As the total training volume differed between legs (Leg_{T1} vs. Leg_{T3}) for a given testing day, we performed additional statistical analysis to investigate the potential different effects of the two exercise frequency paradigms on TS MTU properties at a time point of equal total volume of mechanical loading. Hence further separate one-way ANOVAs were used to compare mentioned TS MTU properties between the two legs [changes relative to baseline (%)] at time points of equal total volumes of mechanical loading (equal total numbers of sessions; day 12 for Leg_{T1} vs. day 4 for Leg_{T3}). For analysis of the extracellular matrix biomarker concentrations further one-way repeated measures ANOVAs were implemented to detect alterations across the 12-day exercise intervention (Base, day 1, day 4, day 8, day 12, RET). Baseline (Base) for these assessments was measured in the morning prior to the first TS MTU measurement session (PRE1). A further one-way repeated measures ANOVA was performed to analyse potential time-course changes in NRS pain. Bonferroni post-hoc comparisons were performed in situations for which significant main effects or interactions were detected. All statistical analyses were performed using custom MATLAB scripts (version 2020b; The MathWorks, Natick, MA, USA) or SPSS statistics software (version 26.0; IBM, Armonk, NY, USA).

Results in the text and figures are presented either as individual values, means and standard deviations (SDs) or boxplots (median, 25th percentile and 75th percentile). The level of significance was set consistently at $\alpha = 0.05$.

Results

Muscle and tendon mechanical properties

The two-way repeated measures ANOVA did not reveal any significant leg (Leg_{T1} vs. Leg_{T3}) or day (PRE1 vs. PRE2) effects or interactions at baseline (Base) measurements in maximal ankle plantarflexion joint moment (Leg_{T1} 214 ± 19 vs. 214 ± 17 Nm and Leg_{T3} 220 ± 21 vs. 218 ± 19 Nm, respectively for PRE1 vs. PRE2) and maximal AT force (Leg_{T1} 4.0 ± 0.3 vs. 4.0 ± 0.3 kN and Leg_{T3} 4.1 ± 0.3 vs. 4.1 ± 0.3 kN) or in the resulted maximal AT strain (Leg_{T1} 5.3 ± 0.7 vs. 5.3 ± 0.7 % and Leg_{T3} 5.1 ± 0.4 vs. 5.2 ± 0.5 %) and AT stiffness (Leg_{T1} 493 ± 82 vs. 489 ± 70 $N \cdot mm^{-1}$ and Leg_{T3} 533 ± 82 vs. 532 ± 83 $N \cdot mm^{-1}$). The intra-class correlation coefficient (ICC) analysis demonstrated "good" to "excellent" between-day reliability with values ranging from 0.781 to 0.960 across the analysed TS MTU mechanical properties.

The implemented one-way repeated measures ANOVAs revealed a statistically significant time effect for muscle strength: maximal AT force [$F(4.217,42.169)=9.614$, $P<0.001$ for Leg_{T1} and $F(3.493,34.930)=6.561$, $P=0.001$ for Leg_{T3}] and maximal ankle plantarflexion moment [$F(4.164,41.643)=9.389$, $P<0.001$ for Leg_{T1} and $F(3.450,34.505)=6.151$, $P=0.001$ for Leg_{T3}]. The post hoc comparisons revealed that the consecutive days of cyclic high AT strain loading led to significant ($0.001<P<0.047$) increase in the maximal ankle plantarflexion moment and maximal AT force in both legs following 6 days of training (Leg_{T1} and Leg_{T3}; Fig. 3) in comparison to Base (average of PRE1 and PRE2; on average about 11% increment). No further significant training-related increments in the muscle

strength were detected thereafter independent of the analysed leg, with the maximal ankle plantarflexion moment and maximal AT force remaining on average about 10% higher in relation to Base until the end of 12 consecutive days of cyclic high AT strain loading (Fig. 3). The training-induced increment in muscle strength values was retained after the 48h resting period in both legs ($0.011<P<0.022$; Fig. 3).

Concerning AT mechanical properties, the implemented one-way repeated measures ANOVAs revealed a statistically significant time point effect for AT stiffness [$F(3.663,36.626)=3.487$, $P=0.019$ for Leg_{T1} and $F(4.593,45.929)=11.832$, $P<0.001$ for Leg_{T3}] and maximal AT strain [$F(3.393,33.932)=3.943$, $P=0.013$ for Leg_{T1} and $F(3.703,37.029)=10.792$, $P<0.001$ for Leg_{T3}]. The post hoc comparisons showed a significant ($P=0.005$) decrease in AT stiffness for Leg_{T3} (high dose, 3 exercise sessions a day) in comparison to Base already following 8 days of cyclic high AT strain loading (Fig. 3), which was retained in the remaining training days until day 12 ($0.001<P<0.005$; Fig. 4) and even after the 48h resting period ($P=0.049$). Correspondingly, a significant ($P<0.001$) ca 19% increase was also detected for the maximal AT strain in the Leg_{T3} already following 8 days of exercise, with the heightened values maintained throughout the following training period until day 12 ($P=0.002$) and even after the 48h retention ($P<0.001$; Fig. 3). Whereas for the Leg_{T1} (low dose, one exercise a day) no significant changes were detected in AT stiffness as well as in maximal AT strain throughout the 12 consecutive days of cyclic high AT strain loading (Fig. 3). In addition, however it is notable to mention that for the Leg_{T1} a tendency towards decreased AT stiffness ($P=0.068$) and correspondingly an increased maximal AT strain ($p=0.073$) was detected at day 12 of consecutive days of cyclic high AT strain (Fig. 3).

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Moreover, considering the individual data of the analysed subjects in both groups, it was evident that in comparison to the Base all participants demonstrated a considerable increase in tendon strain for Leg_{T3} (Fig. 5) with the majority maintaining this elevated state even after 48h of retention (8 out of 11 participants). On the other hand, for Leg_{T1} not all participants showed an increase and majority demonstrated decrease towards Base values after the 48h rest period (Fig. 5).

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When examining the potential differences in TS MTU properties between the legs at the time point of equal total volume of mechanical loading (following 12 exercise sessions), the performed one-way ANOVA revealed no significant differences of day 12 in Leg_{T1} vs. day 04 in Leg_{T3} in the absolute values nor in relative changes from Base in maximal ankle plantarflexion moment and maximal AT force (Leg_{T1} on average 11.5±5.4% higher vs. Leg_{T3} 8.0±7.1% higher in relation to Base; Fig. 3). Correspondingly, no significant leg-differences were detected in the relative change from Base neither in AT stiffness or maximal AT strain (Fig. 6).

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Extracellular matrix turnover-related biomarkers

Concerning the extracellular matrix turnover-related biomarkers, the implemented ANOVA detected a time point effect [$F(1.202,12.023)=32.929$, $P<0.001$] for the circulating IL-6 with an initial increase observed after the first exercise session, which, however, did not reach significance. In the progress of the training regimen, the IL-6 concentration reduced

significantly below Base level ($0.001<P<0.048$; Fig. 7). The matrix metalloproteinase analysis revealed similarly a time effect [$F(2.506,25.064)=22.232$, $P<0.001$ for MMP-2 and $F(1.591,15.906)=39.527$, $P<0.001$ for MMP-9]. The post hoc comparisons identified a significant ($0.001<P<0.045$) increase in the MMP-2 and a decrease in MMP-9 activity ($P<0.001$) already following 4 days of cyclic high AT strain loading exercise, whereas 48h after the last exercise session the MMP-2 activity level returned to Base level (Fig. 7). Collagen type I propeptides investigation revealed also a time point effect [$F(2.448,24.476)=15.948$, $P<0.001$ for PICP and $F(2.644,26.442)=17.335$, $P<0.001$ for PINP]. The following post hoc comparisons showed relatively stable levels of circulating PICP and PINP, with significant increases in both compared to the Base level found only 48h after the last exercise session ($0.001<P<0.048$; Fig. 7).

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Perceived pain and restriction of daily life

At the Base none of the subjects reported any pain in the ankle plantar flexor muscles and AT, as well as having a feeling of being "restricted in daily life". The investigation of any perceived pain or discomfort of the involved ankle plantar flexor muscles revealed similarly a time point effect for both legs [$F(2.793,27.931)=4.468$, $P=0.012$ for Leg_{T1} and $F(3.660,36.599)=3.907$, $P=0.011$ for Leg_{T3}]. The following post hoc comparisons detected an increase ($0.005<P<0.037$) in NRS pain scale ratings (on average from 1.5±0.7 to 2.7±1.2, respectively from day 2 to day 12 for both legs), reaching a level corresponding to a "mild pain". After the 48h non-active retention period both legs showed reductions in pain (average 1.6±0.7 for Leg_{T1} and Leg_{T3}) towards the first training days. For the perceived pain in the AT however, the one-way repeated measures ANOVA revealed a statistically significant time

point effect for Leg_{T3} [F(4.177,41.765)=45.610, P<0.001], with the post-hoc comparisons showing significantly (P<0.001) higher NRS pain scale ratings from day 8 onwards in comparison to day 2 (Fig. 8). These values kept increasing until the end of the 12 consecutive days of cyclic high AT strain loading, reaching the levels of "moderate" pain or discomfort and were maintained even after 48h non-active retention period (no significant difference to day 12; Fig. 8). A time effect [F(3.751,37.509)=4.496, P=0.005] was detected also for Leg_{T1}, with a tendency for slightly increased AT pain (P=0.069) on the day 12 in comparison to the starting days of the intervention with reaching the levels of "mild" pain or discomfort (Fig. 8). Feeling of being "restricted in daily life" demonstrated similar to muscular pain scores a slight increase in both legs [F(1.986,19.856)=3.930, P=0.037 for Leg_{T1} and F(2.338,23.379)=11.544, P<0.001 for Leg_{T3}], reaching the highest value of "mild restriction" by the end of intervention at day 12 (average 3.1±2.1 for Leg_{T1} and Leg_{T3}).

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Discussion

Compared to muscles, tendons appear to have a slower tissue renewal rate in response to mechanical stimuli, which may cause a higher mechanical demand for the tendon, especially when high magnitude exercise is repeated with insufficient rest periods. The present study investigated, in healthy young adults, whether high strain cyclic loading of the TS MTU over consecutive days with different recovery periods led to alterations in its biomechanical properties and extracellular matrix turnover-related biomarkers. The findings supported our hypothesis that high strain exercise of this form would lead to decrease in tendon stiffness and elevated tendon strain in a manner dependent on exercise dose (amount of mechanical loading trials over a time period). These effects may be partly related to a time-delayed anabolic response in collagen turnover.

418 The current study implemented a high-magnitude AT strain cyclic loading protocol
419 (Arampatzis et al., 2007) over 12 consecutive days using two recovery modalities: either
420 ~24h rest between sessions (Leg_{T1}; once per day) or ~2-3h rest between sessions (Leg_{T3}; three
421 times per day). Previous investigations (Ackermans et al., 2016; Mademli and Arampatzis,
422 2008; Peltonen et al., 2012) attempted to alter *in vivo* human tendon compliance with acute
423 fatiguing mechanical loading paradigms, whereas the current study aimed to maintain muscle
424 force generation capacity over the entire exercise period to keep AT strain at a constant high
425 level (high mechanical demand on the tendon). Both legs demonstrated a gradual increase in
426 muscle strength (~10% over 12 days in maximal ankle joint moment and AT force; Fig. 3)
427 suggesting that the rest periods did not lead to clear fatigue for the ankle plantar flexors
428 irrespective of differences in total loading volume. This rapid increase in muscle strength
429 could be explained as predominantly a neural adaptation (Moritani and DeVries, 1979).
430 Accordingly, one might suggest that the regeneration of muscular performance capacity has
431 surpassed the recovery processes in tendinous tissues in both legs (Magnusson et al., 2010).
432 Indeed, as hypothesised, allowing a shorter recovery of ~2-3h between daily sessions (Leg_{T3})
433 led to a clear alteration of AT mechanical properties already after 8 consecutive days of
434 loading (Fig. 3). By the end of the 12 days of training a clear shift in the force-elongation
435 relationship was detected (Fig. 4), with a ~24% reduction in AT stiffness and a ~19%
436 increase in maximal AT strain (Fig. 3). Hence the increase in maximal tendon strain can be
437 predominately explained by structural changes within the tendon leading to a decrease in
438 tendon stiffness, rather than to an increase in muscle strength due to training. It is interesting
439 that, despite both training modalities allowing muscle force generation capacity to recover
440 faster than the tendon, no significant alterations in AT mechanical properties (tendon stiffness
441 or maximal tendon strain) were identified for loading with ~24h rest between training
442 sessions. After the last day of exercise there was merely a tendency towards reduced AT

443 stiffness (P=0.068) and increased maximal AT strain (P=0.073) for Leg_{T1} (Fig. 3). This was
444 reflected in almost identical force-elongation relationships between Base and day 12 (Fig. 4).

445 The above findings are in accordance with previous *ex vivo* and *in vivo* studies that
446 demonstrated that cyclic mechanical loading or prolonged application of a constant load led
447 to a continuous decrease in tendon stiffness and to a corresponding increase in tendon strain
448 (Fung et al., 2010; Wang et al., 1995; Wren et al., 2003). Moreover, that these modifications
449 in tendon compliance (from high cyclic loading with short recoveries) persisted even after the
450 48h no-exercise retention period (RET; Fig. 3) supports the idea of accumulation of tendinous
451 tissue damage. This conception is reinforced through investigation of the data of individual
452 participants. The leg trained at high exercise dose (Leg_{T3}) demonstrated an increase in
453 maximal AT strain in all participants by the end of day 12, an increase maintained over the
454 48h non-contact retention period in the majority of participants (Fig. 5). On the other hand,
455 for some participants, even training once a day (Leg_{T1}) led to large changes in maximal AT
456 strain (Fig. 5), emphasising the individuality of MTU mechanical properties and the
457 importance of monitoring and management of exercise-loading, as proposed previously
458 (Arampatzis et al., 2020; Karamanidis and Epro, 2020).

459 In order to examine potential differential effects on TS MTU properties of the two exercise
460 frequency paradigms, we took into account the distinct training volume between the legs by
461 comparing the time point of equal total volume of mechanical loading (following 12 exercise
462 sessions): Leg_{T1} at day 12 and Leg_{T3} at day 4. Nevertheless, no significant differences were
463 revealed between the legs in TS muscle strength (maximal ankle plantarflexion moment and
464 maximal AT force), AT stiffness or maximal AT strain following 12 sessions (Fig. 5).
465 Accordingly, with the current study design we were unable to establish whether different
466 recovery durations influence changes in AT mechanical properties for given exercise volume.
467 Possibly the chosen exercise duration of 12 consecutive days was too short to address this

question since Leg_{T3} first showed significant alterations in tendon stiffness and maximal strain following 24 sessions (day 8; Fig. 3). Therefore, it appears that the total volume of high mechanical loading (total volume of tendon strain) is the more decisive factor for exercise-induced alterations in maximal AT strain and the primary risk factor for higher mechanical demand on the tendon. Whether and to which extent different recovery times for high AT cyclic loading influence observed changes in maximal tendon strain needs further investigation, for example using longer exercise periods. It is however interesting to note that Leg_{T1} demonstrated a tendency ($p=0.068$; Fig. 3) towards decreased AT stiffness by the end of 12 consecutive days of loading. This indicates that there could be a plateau in net collagen synthesis and tendon matrix regeneration after a certain loading volume might have been reached over a short time period, as suggested in previous studies (Magnusson et al., 2010).

Along with examination of alterations in TS MTU biomechanical properties, the current study aimed also to investigate whether these were reflected in changes in circulating levels of biomarkers of extracellular matrix collagen turnover. Firstly, we investigated the level of IL-6 as a well-established biomarker for both inflammation (Pedersen and Febbraio, 2012) and acute stimulation of collagen synthesis in tendon (Andersen et al., 2011). Despite observing an initial increase in circulating IL-6 after the first exercise session, this change did not achieve significance. As AT cyclic loading progressed, the IL-6 concentration dropped to baseline level, and even below it, by the end of the exercise and the 48h retention periods (Fig. 7). This indicated that the applied AT loading paradigm did not necessarily lead to an inflammation at systemic level, suggesting that possible inflammatory influences on our collagen turnover data may have been low. These observations follow previous investigations demonstrating acute peaks in IL-6 mRNA expression following acute cyclic loading (Legerlotz et al., 2013). Inflammatory signalling does not appear to be of greater magnitude in tendinopathic tendons after acute mechanical loading (Pingel et al., 2013). In the short

493 term, elevation of IL-6 levels seems to support collagen synthesis (Andersen et al., 2011),
494 whereas long-term chronic elevation as seen in tendinopathy patients (Legerlotz et al., 2012)
495 has been related to a reduction in collagen I synthesis (Katsma et al., 2017).

496 Taking into consideration that long-lasting inflammatory influences in our loading paradigm
497 seemed low, we sought to investigate the activities of collagen breakdown markers, the
498 matrix metalloproteases MMP-2 and MMP-9, which have been shown to be altered in
499 response to physical exercise (Suhr et al., 2007; Suhr et al., 2010). Over the exercise period
500 we observed increased activities of MMP-2, whereas 48h after the last exercise session the
501 MMP-2 activity level dropped back to baseline (Fig. 7). In contrast, MMP-9 activity
502 immediately decreased after the first exercise session and did not increase over the
503 investigation period (Fig. 7). Therefore it seems that MMP-2 and MMP-9 are activated
504 independently and MMP-9 likely has less impact on tendon remodeling for short-term high
505 AT cyclic loading. These findings are supported by previous observations of elevated levels
506 of MMP-2 in response to exhaustive exercise or in chronic tendinopathic tendons and patients
507 with a history of AT rupture (De Mos et al., 2007; Riley, 2008).

508 As a consequence of alterations in MMP-2 and MMP-9 levels, collagen turnover may be
509 altered during consecutive days of cyclic loading. We gained insight from study of two
510 procollagen type I peptides, PICP and PINP (Langberg et al., 1999; Langberg et al., 2001)
511 and observed that both remained more or less stable over the training period, albeit with some
512 minor variations. However, 48h after the last exercise bout we found a ~20% increase in both
513 of their circulating levels compared to baseline (Fig. 7). This is in line with previous findings
514 (Langberg et al., 1999; Langberg et al., 2001) that 72 hrs after long-distance running and after
515 11 weeks of repetitive exercise collagen type I propeptides are significantly increased at the
516 peritendinous level, indicating anabolic balance in protein turnover in human tendons.
517 Considering that MMP-2 reached its peak at the last exercise session of day 12 (Fig. 7), the

induction of increased collagen type I propeptides 48h after the last exercise bout could be explained by a time-delayed adaptive response in collagen type I synthesis, since its turnover is processed over very slow time frames (Heinemeier et al., 2013). Accordingly, the macro-level alterations in maximal tendon strain and tendon stiffness throughout the consecutive high AT strain cyclic loading could be partly related to a time-delayed anabolic response in collagen turnover and potential accumulation of micro-damage over the course of cyclic loading that seemingly inhibits the regeneration processes. These findings provide some support for previous views that tendon overuse injuries (e.g. tendinopathy) should be characterized rather as a degenerative condition than an inflammatory process (Riley, 2008) and are primarily related to matrix turnover (Pingel et al., 2013).

Tendon overuse injuries such as tendinopathy are often in the longer term related to pain in tendinous structures and surrounding tissues, but not necessarily in the developing stages of overuse (Snedeker and Foolen, 2017). In the current study, we observed significantly higher AT pain for Leg_{T3} over this short exercise period (Fig. 8) as well as a tendency towards restricted daily living. There were no leg differences in perceived pain in the TS muscle, supporting identified alterations in the tendon and in analysed biomarkers. While an exact turning point to chronic tendon overuse (e.g. tendinopathy) is difficult to specify, it seems to be related to accumulated tissue damage and to accompany onset of long-lasting tendon pain (Snedeker and Foolen, 2017). In this way, the current study provides evidence for a potential relationship between the total volume of high magnitude tendon strain, the accumulation of tendon damage and related increases in maximal AT strain, which could be one of the primary risk factors for development of lasting tendon overuse injuries.

One might argue that AT moment arms were not measured using MRI and co-activation from antagonistic or synergistic muscles were not accounted for, which could in absolute terms affect our estimated AT force and correspondingly the AT stiffness. However, the

antagonistic moment contribution to generated plantarflexion moment during maximal isometric contractions seems rather low in healthy young adults (Mademli et al. 2004), and the co-activation decreases throughout exercise as the muscular co-ordination improves (Carolan and Carafelli, 1992). Hence, this would have a negligible influence especially on the outcomes in relative terms due to the intra-subject protocol used in this study and implementing the same joint configurations throughout our experiments. More importantly, the main observation that the maximal tendon strain (mechanical tendon demand) may increase due to frequent high mechanical loading over a short time period, is not affected by these limitations. A further critical point is linked to the biomarker analysis in the current study. Venous blood was collected rather than blood from tissue local to the tendon (i.e. peritendinous tissue or the tendon proper). Hence the biomarker findings need to be viewed with caution as they may not reflect localised inflammatory processes and tendon reactions to the exercise, and fail to allow differentiation between the two recovery paradigms due to the intra-individual study design. Nevertheless, since the participants refrained from any other physical exercise throughout the investigation period, whole body levels of the analysed biomarkers should give an approximate estimation of the inflammatory, degenerative and regenerative processes taking place within the system. This lack of knowledge does not alter the overall conclusion of the paper because we detected reasonable changes in MMP-2/MMP-9 and PICP/PINP levels at the systemic level. Since the collagen type I propeptides reflect predominantly collagen turnover in tendons, we can argue in a convincing manner that tendon tissue is remodelled in response to the intervention. We cannot say to what extent remodelling occurs, but observations of significant changes at the systemic level indicate relatively strong turnover. Future studies might investigate different loading paradigms either in separate groups or inspect processes at a more localised level. Furthermore, it is important to note that our investigation was limited to physically active young healthy male adults.

Hence findings may not be generalisable to females, pathological conditions or other age groups due to potential influences of metabolic and hormonal factors on muscle-tendon responsiveness to mechanical loading.

Conclusion

The current findings indicate that homeostasis of human AT is vulnerable to frequent high strain cyclic loading. The mechanical demand of the tendon with respect to its maximal strain can increase in response to a high magnitude and volume of cyclic tendon strain, which could be a result of delayed regeneration of tendinous tissue and accumulation of molecular damage within the tendon. Whether insufficient regeneration between exercise sessions plays a role in the alterations in AT mechanical properties could not be answered, but excessive loading over a relative short period of time seems to be one of the triggers for increased tendon strain, which could possibly predispose it to overuse injury.

List of symbols and abbreviations

AT	Achilles tendon
IL-6	interleukin-6
MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase-9
MTU	muscle-tendon unit
MVC	maximal voluntary contraction
PICP	procollagen type I carboxy-terminal propeptide
PINP	procollagen type I aminoterminal propeptide
RET	retention
TS	*triceps surae* muscle

US ultrasonography

Author contributions

GE and KK conception and design of research; GE performed experiments; GE, FS and KK analyzed data; GE, FS and KK interpreted results of experiments; GE prepared figures; GE and KK drafted manuscript; GE, FS and KK edited and revised manuscript; GE, FS and KK approved final version of manuscript.

Competing interests

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

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Data availability

All relevant data can be found within the article.

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609

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Figure legends

Figure 1: Experimental design of the triceps surae (TS) muscle-tendon unit (MTU) training and measurements. Participants (n=11) underwent consecutive 12 days of high Achilles tendon (AT) strain cyclic loading intervention for one leg once a day (Leg_{T1}; ~24h rest between each session) and for the contralateral leg three times a day (Leg_{T3}; ~2-3h rest between daily sessions) using a custom-made dynamometer. All participants underwent measurement sessions for TS MTU mechanical properties (MTU measurement), extracellular matrix turnover markers (Biomarker collection) and perceived pain questionnaire (NRS pain scale) on indicated specific time points: prior (PRE1 and PRE2; 48h before the first training session), during exercises days (D02 to D12) and following a 48h non-contact retention period (RET).

Figure 2: The setup for triceps surae (TS) muscle-tendon unit (MTU) measurements: (A) The participant was seated with their knee fully extended and shank perpendicular to the foot on the strain gauge load cell-based dynamometer (Force plate). (B) Two digital cameras were used to track the motion of four active LED markers on the lower extremity and four markers fixed on the force plate. (C) The displacement (ΔL) of the myotendinous junction (MTJ) of m. gastrocnemius medialis during maximal isometric plantar flexion ramp contractions was manually digitized from ultrasonography recordings between rest (0%) and 100% MVC.

Figure 3: Box plots and individual values (n=11) in maximal ankle plantarflexion (PF) moment, Achilles tendon (AT) stiffness, maximal AT strain at Baseline (Base; average of PRE1 and PRE2), after every second training day (D02 to D12) and following 48h retention (RET) in once a day trained leg (Leg_{T1}) and three times a day trained leg (Leg_{T3}). Data was

analysed using separate one-way repeated measures ANOVAs. *, statistically significant difference to Base (P<0.01).

Figure 4: Achilles tendon (AT) force-elongation relationship at Baseline (average of PRE1 and PRE2) and following 12 consecutive days (D12) of high AT strain cyclic loading in once a day trained leg (Leg_{T1}) and three times a day trained leg (Leg_{T3}). The solid lines illustrate the mean force-elongation relationship up to a common force level across participants and measurement time points. Maximal values are shown as means and SD (circles and error bars).

Figure 5: Individual values (n=11) in maximal AT strain at Baseline (Base), after the 12th training day (D12) and following 48h retention (RET) in once a day trained leg (Leg_{T1}) and three times a day trained leg (Leg_{T3}).

Figure 6: Box plots and individual values (n=11) in the relative change from Baseline (Base; average of PRE1 and PRE2) in Achilles tendon (AT) stiffness and maximal AT strain following 12 exercise sessions for once a day trained leg (Leg_{T1}; respectively day 12) and three times a day trained leg (Leg_{T3}; respectively day 04). Data was analysed using one-way ANOVAs.

Figure 7: Extracellular matrix turnover-related biomarkers. The relative change to baseline (Base) measurement in serum concentration levels of interleukin-6 (IL-6), collagen type I propeptides (PICP and PINP), matrix metalloproteinases (MMP-2 and MMP-9) at day 1 (D01), day 4 (D04), day 8 (D08), day 12 (D12) and retention (RET) measurement time

834 points. Data was analysed using separate one-way repeated measures ANOVAs. *,
835 statistically significant (P<0.05) difference to Base.

836

837 **Figure 8:** NRS pain scale (from 0 to 10) for perceived Achilles tendon (AT) pain and
838 discomfort following the last session of the day for once a day trained leg (Leg_{T1}) and three
839 times a day trained leg (Leg_{T3}) at day 02 to day 12 and following 48h rest (RET). Please note
840 that at Baseline all investigated subjects reported no pain (zero). Data was analysed using
841 one-way repeated measures ANOVAs. * statistically significant (P<0.05) difference to D02.