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Composition and adaptation of human myotendinous junction and neighboring muscle fibers to heavy resistance training

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The myotendinous junction (MTJ) is a common site of strain injury and yet understanding of its composition and ability to adapt to loading is poor. The main aims of this study were to determine the profile of selected collagens and macrophage density in human MTJ and adjoining muscle fibers, and to investigate whether heavy exercise loading would alter this profile. Fifteen individuals scheduled for anterior cruciate ligament repair surgery were randomized into three groups: control, acute or 4 weeks heavy resistance training. MTJ samples were collected from the semitendinosus and gracilis muscles and were sectioned and stained immunohistochemically for collagen types I, III, VI, XII, XIV, XXII, Tenascin-C and CD68. Macrophage density and distribution was evaluated and the amount of each collagen type in muscle and MTJ was graded. Collagen XXII was observed solely at the MTJ, while all other collagens were abundant at the MTJ and in muscle perimysium or endomysium. The endomysial content of collagen XIV, macrophages and Tenascin-C increased following 4 weeks of training. These findings illustrate the heterogeneity of collagen type composition of human MTJ. The increase in collagen XIV following 4 weeks of training may reflect a training-induced protection against strain injuries in this region.

Hamstring strain injuries are prevalent in several sports, especially soccer, and a considerable financial expense for elite clubs (Ekstrand et al., 2011; Eirale et al., 2013). From several studies investigating how these injuries best can be prevented, the most promising results are seen in the protocols consisting of heavy resistance exercise, including the Nordic Hamstring exercise (Mjølsnes et al., 2004; Arnason et al., 2008; Petersen et al., 2011; Schache, 2012; Nichols, 2013). With regard to the site of strain injury, the myotendinous junction (MTJ) as the interface between muscle and tendon is known to be the weakest link in the muscle-tendon chain (Nikolaou et al., 1987; Tidball, 1991), and ultrasonographic visualization of muscle strain injury in humans indicates that tissue damage involves the MTJ. Microscopy of experimental load-to-failure strain injuries in animals has revealed that the actual site of rupture is rarely the muscle-tendon interface, but rather the A-band of the distal sarcomeres close to the MTJ (Sharafi et al., 2011). In general, understanding of the structure and composition of the human MTJ as a basis for development of prevention and treatment strategies is poor. Study of the MTJ is challenging, however, since the structure is most clearly seen at the level of electron microscopy, where a typical pattern of tendon-muscle interdigitations with the shape as finger-like processes, is seen. Recently, in a three-dimensional electron microscopy study of human MTJ, we showed that these finger-like processes are in fact ridge-like protrusions of collagen-rich tendon inserting into furrow-like indentations of the muscle (Knudsen et al., 2015), the main implication of this being an even greater surface area between muscle and tendon through which force is transmitted. Little is known however regarding the composition of the matrix constituting the MTJ and endomysium surrounding the adjoining fibers.

Studies in animals have shown that both fibrillar collagen types I and III are present at the MTJ (Jozsa et al., 1991; Jarvinen et al., 1992). Furthermore, collagen type XXII, a member of the FACIT (Fibril Associated Collagens with Interrupted Triple helices) collagen family, has also been shown to be

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expressed preferentially in animal MTJ (Koch et al., 2004). The fact that this collagen type seems to be expressed exclusively at tissue junctions, such as MTJ, and not elsewhere in the muscle or tendon (Koch et al., 2004), suggests that the MTJ is a specialized construction, and also that collagen XXII could be a valuable tool in the study of MTJ. It is currently not known if this collagen type is expressed in human MTJ.

In muscle, the endomysium is rich in collagen types I and III (Light & Champion, 1984; Mackey et al., 2005) and VI (Kuo et al., 1997), and collagen VI in particular has been shown to play a key role in muscle regeneration and maintenance of the size of the muscle stem cell pool (Urciuolo et al., 2013). Besides these large fibrillar collagens, collagen types XII and XIV have also been found in animal muscles (Wälchli et al., 1994). As well as being members of the FACIT family of collagens (Wälchli et al., 1994), these collagen types do not form higher order of organization in fibrils or networks but are thought to function as linkage proteins between the fibrillar collagens and other components of the extracellular matrix (Nishiyama et al., 1994; Koch et al., 1995). They have both been reported to be expressed to a great extent in the perimysium and in tendon, but they are not highly expressed in the endomysium in the resting state (Chiquet et al., 2014; Zou et al., 2014). However upon loading, there is an upregulation in the expression of collagen type XII and the mechano-sensitive Tenascin-C in the endomysium (Trachslin et al., 1999; Fluck et al., 2003). Similarly, the tissue content of macrophages, which are known to have a vital role in tissue repair (Tidball & Villalta, 2010; Rigamonti & Zordan, 2014), has been reported to increase following exercise (McLennan, 1996) and could play an important role in the adaptation of human MTJ. Given that force transmission is concentrated at the MTJ, we hypothesized that collagen types XII, XIV and XXII would be strongly expressed in human MTJ and that types XII and XIV would be evident in the endomysium of muscle fibers close to the MTJ, and that heavy resistance training would result in an increased number of macrophages and amount of collagens. Thus, the main aims of this study were to describe the distribution of macrophages and matrix proteins in human MTJ and adjoining muscle fibers and to investigate the influence of heavy resistance exercise on this distribution.

Materials and methods

This study was a randomized controlled trial approved by The Research Ethics Committees of the Capital Region of Denmark (ref. H-4-2011-089) and conformed to the standards set by the Declaration of Helsinki. All volunteers gave written informed consent before inclusion. We enrolled 15 patients (mean age 34 ± SD 8 years) who were healthy except for an isolated anterior cruciate ligament (ACL) rupture and scheduled for ACL reconstruction surgery using a hamstrings-graft (gracilis and semitendinosus tendons). None of the subjects reported hamstrings injury and all were able to perform daily activities so could not be classified as inactive. The subjects were randomized, by drawing lots, into three groups: a control group (five males), an acute training group (four males, one female) or a long training group (heavy resistance exercise, HRE; two males, three females). The control group did not train, the acute training group trained once prior to the surgery and the long training group trained three times weekly for 4 weeks. The subjects were told not to do other resistance exercise or hard aerobic exercise during the study period. The acute group did one training session 2–3 days before their surgery. This period was chosen because acute increases in concentrations of macrophages and Tenascin-C are detectable a few days after a training session (Paulsen et al., 2010; Mackey et al., 2011). Each group consisted of five subjects initially, and we excluded one patient in the acute training group from the analyses because his training session was only 1 day before his surgery.

HRE training regime

Six exercises were selected to train quadriceps and hamstrings muscle groups. Quadriceps exercises included leg press and leg extensions. Hamstrings exercises included Nordic hamstring, lying leg curls, supine one-leg curls, and reverse hyperextensions (posterior chain exercise). The training sessions were all supervised and for all exercises subjects performed 3 sets of 6–8 repetitions. Full range of motion was endeavored and movement was controlled during both concentric and eccentric phases. For the Leg extensions, a suitable load was used in order for each subject to do 6–8 repetitions with maximum effort, approaching exhaustion. The starting position was set to a knee flexion angle of 120° and the end position was set to 0–10° before full extension. Nordic hamstring (Mjølsnes et al., 2004; Petersen et al., 2011; Schache, 2012; Nichols, 2013) was performed with the feet fixed under a step-bench. For lying leg curls, the load was adjusted each week and calculated as 70% of a weekly 1 RM test. Supine one-leg curls were performed with subjects supine in a bridge position with the passive leg extended and the working leg bent with the foot on a piece of fabric. The subjects extended and flexed the working leg by sliding the fabric back and forth on the floor. Reverse hyperextensions (kick-backs) were performed with the starting position of the upper body resting prone on a platform and the legs hanging down. From here, the subject kicked the legs back until they were parallel with the upper body.

Sample preparation

On the day of surgery, the surgeon stripped the semitendinosus and gracilis tendons, including the full MTJ of these two muscles. Samples of the MTJ were harvested for this study before the tendons were prepared as graft for the ACL-reconstruction. The MTJ samples were prepared in the operating theater and cut, if possible, into three pieces of 2 cm length, named 0, +2, or +4, representing the most distal sample (0) and increasingly proximal samples (+2 and +4). From each sample a piece of tissue consisting of both muscle and tendon was embedded in Tissue-Tech and frozen in liquid nitrogen-cooled isopentane and stored at −80 °C. Part of each sample was also prepared for a study of the MTJ by transmission

electron microscopy, as described previously (Knudsen et al., 2015). 10 μm thick frozen sections were cut from each frozen specimen using a cryostat, and placed on glass slides (Superfrost Plus, Menzel-Gläser, Braunsshweig, Germany), which were stored at −80 °C until analysis. The 0, +2, and +4 samples from each specimen were put on the same slide.

Immunohistochemical staining for confocal microscopy

Four triple-labelling protocols were performed to study the distribution of collagen type XXII in relation to the muscle sarcolemma (labeled with dystrophin) and several components of the muscle extracellular matrix. Sections were allowed to air dry and were fixed with 5% formaldehyde (Histofix; Histolab, Gothenburg, Sweden) before overnight incubation in the fridge with a mix of the three primary antibodies. A mix of three appropriate secondary antibodies was applied the following day for 45 min after which the slides were stained with Hoechst 33342 (2.5 μg/mL, cat. no. H1399; Invitrogen A/S, Taastrup, Denmark) and mounted (Molecular Probes ProLong Gold anti-fade reagent, cat. no. P36934; Invitrogen A/S). The primary antibody combinations used were: (a) Dystrophin and collagen types XII and XXII; (b) Dystrophin and collagen types XIV and XXII; (c) Collagen types III, XIV, XXII; and (d) Collagen types VI, XII, XXII, from the following primary antibodies: mouse anti-Dystrophin (cat. no. NCL-DYS1, Novocastra; Leica Microsystems A/S, Ballerup, Denmark), mouse anti-collagen III (cat. no. C7805; Sigma-Aldrich Denmark A/S, Copenhagen, Denmark), mouse anti-collagen VI (cat. no. 5C6; Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA), rabbit anti-collagen XII (KR74, made by Manuel Koch), and rabbit anti-collagen XIV (KR71, made by Manuel Koch), and guinea pig anti-collagen XXII (KG36, made by Manuel Koch). Correspondingly the secondary antibodies used were: for (1) and (2) Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 568 goat anti-guinea pig, and Alexa Fluor 647 goat anti-mouse (Molecular Probes cat. no. A11034, A11075, A21236, respectively; Invitrogen A/S); (3) and (4) Alexa Fluor 488 goat anti-mouse, Alexa Fluor 568 goat anti-guinea pig, and Alexa Fluor 647 goat anti-rabbit (Molecular Probes cat. no. 11029, A11075, A21245, respectively; Invitrogen A/S).

Confocal imaging

Images were acquired with a Zeiss LSM710 (Carl Zeiss, Oberkochen, Germany), using the following objectives: 20×/0.8 Plan-Apochromat, 40×/1.3 oil DIC EC Plan-Neofluar, 63×/1.4 oil DIC Plan-Apochromat. Hoechst, Alexa Fluor 488, Alexa Fluor 568, and Alexa Fluor 647 were excited by a 405 nm Diode laser, a 488 nm argon laser, a 543 nm HeNe laser, and a 633 nm HeNe laser, respectively. The colors presented in the figures are pseudo color (e.g., the nuclei are displayed as green) in order to make it easier to distinguish between the various markers.

Immunohistochemical staining for wide field fluorescence microscopy

Sections were allowed to air dry and were fixed with 5% formaldehyde (Histofix; Histolab) before incubation with the primary antibody overnight in the fridge. The relevant secondary antibody was applied the following day for 45 min, after which the slides were mounted with a mounting medium containing DAPI (Molecular Probes ProLong Gold anti-fade reagent, cat. no. P36935; Invitrogen A/S). Washing in two changes of TBS was performed after each step. The primary antibodies used were mouse anti-collagen I (cat. no. C2456; Sigma-Aldrich), mouse anti-collagen III (cat. no. C7805; Sigma-Aldrich), mouse anti-collagen VI (cat. no. 5C6; Developmental Studies Hybridoma Bank), rabbit anti-collagen XII (KR74 Manuel Koch), rabbit anti-collagen XIV (KR71 Manuel Koch), CD68 (cat. no. M0718; Dako Denmark A/S, Glostrup, Denmark), and mouse anti-tenascin-C (cat. no. NCL-Tenas-C, Novocastra; Leica Microsystems) and the secondary antibodies used were Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 568 goat anti-mouse (Molecular Probes cat. no. A11034 and A11031; Invitrogen A/S). All slides were blinded such that the person performing the analysis was blinded to the treatment group, muscle (gracilis and semitendinosus) and subject.

Grading of collagen types I, III, VI, XII, and XIV

The samples were viewed by one observer using fluorescence microscopy on an Olympus BX51 microscope. The sections were graded to estimate the amount of each of the different collagen types (I, III, VI, XII, and XIV) in the muscle, tendon, and MTJ regions of the sample. The MTJ region was considered to be the interface between the tendon and adjoining muscle fibers. To grade the amount of collagen the scale 0–2 was used, where 0 represents no stained tissue, 1 an intermediate amount and 2 a high amount, subjectively evaluated by the observer. Representative images were captured with a digital camera (Olympus DP71, Olympus Deutschland GmbH, Hamburg, Germany) mounted on the microscope, controlled by the software CellˆF (Olympus Soft Imaging Solutions, GmbH, Münster, Germany). Control and long training samples were analyzed.

Collagen XXII

Since not all regions of muscle-tendon interface displayed immunoreactivity for collagen XXII, the proportion of MTJ that was positive for collagen XXII was determined. Images of an area with a long piece MTJ with adhering muscle and tendon were captured. One picture for every sample was taken and analyzed in ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA) . The length of MTJ containing collagen XXII was measured and divided by the length of the entire MTJ, defined as the interface between the tendon and muscle tissue.

Macrophages

To analyze macrophage content, CD68+ cells were counted manually on images in ImageJ and a cell count per mm^2 muscle was calculated. For each sample, the number of CD68+ cells located at the muscle-tendon interface (strict line between the tendon and muscle) was counted and divided by the length of the MTJ analyzed.

Statistics

Data for changes in collagen gradings of human MTJ samples are not available. However, assuming that a relevant increase in the major collagens is 0.6 with an SD of 0.3, then with significance level of 0.05 and a statistical power of 90%, we would require five subjects in each group. Since no differences were found between samples from the two muscles or the three positions (0, +2, or +4), the mean of the three positions for

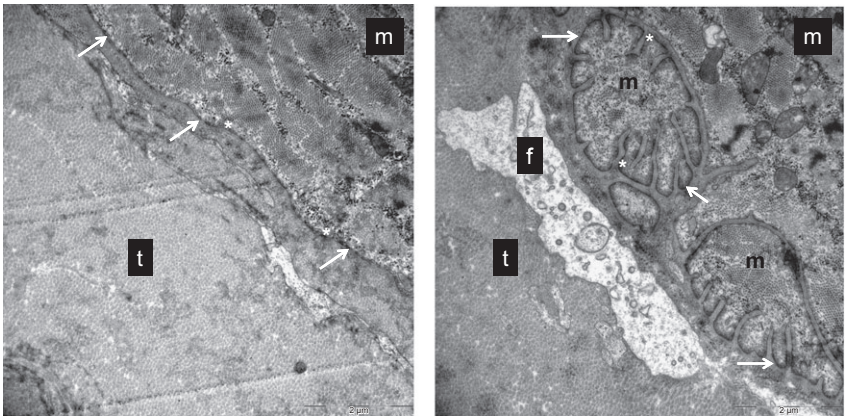


Fig. 1. Transmission electron microscopy. "Flat" MTJ (left) vs classic folded MTJ (right) illustrating differences in the appearance of human muscle-tendon interface (white arrows). In both images, collagen fibrils of the tendon (t) are discernable in the bottom left, with muscle myofibrils (m) visible in the top right. The muscle basement membrane (*) can be observed as a dark coating on the MTJ. The large white area is part of a cell – most likely a fibroblast (f). Both images are from the same human sample.

each muscle was calculated and the mean of the two muscles for each subject was then determined. This was performed for each variable. Unpaired two-tailed *t*-tests were used to compare data between control and acute or long training groups and the significance level was set to 0.05.

Results

Transmission electron microscopy

As shown in Fig. 1, within the same sample two different patterns of MTJ structure are evident, a flat interface between the muscle and tendon and the classic more folded complex interface with continuing interdigitations along the interface.

Confocal microscopy

Collagen XXII distribution

Most regions of the MTJ stained very intensely for collagen XXII, but some regions of intact muscle-tendon interface were negative. When stained regions of the MTJ were studied at higher magnification, collagen XXII was clearly visible along the regions of the myofiber border at the muscle-tendon interface but a fine staining was also often observed along other regions of the myofiber border, adjacent to other myofibers (Fig. 2). Collagen XXII immunoreactivity was also occasionally seen as very fine lines extending into some regions of the tendon (Fig. 3).

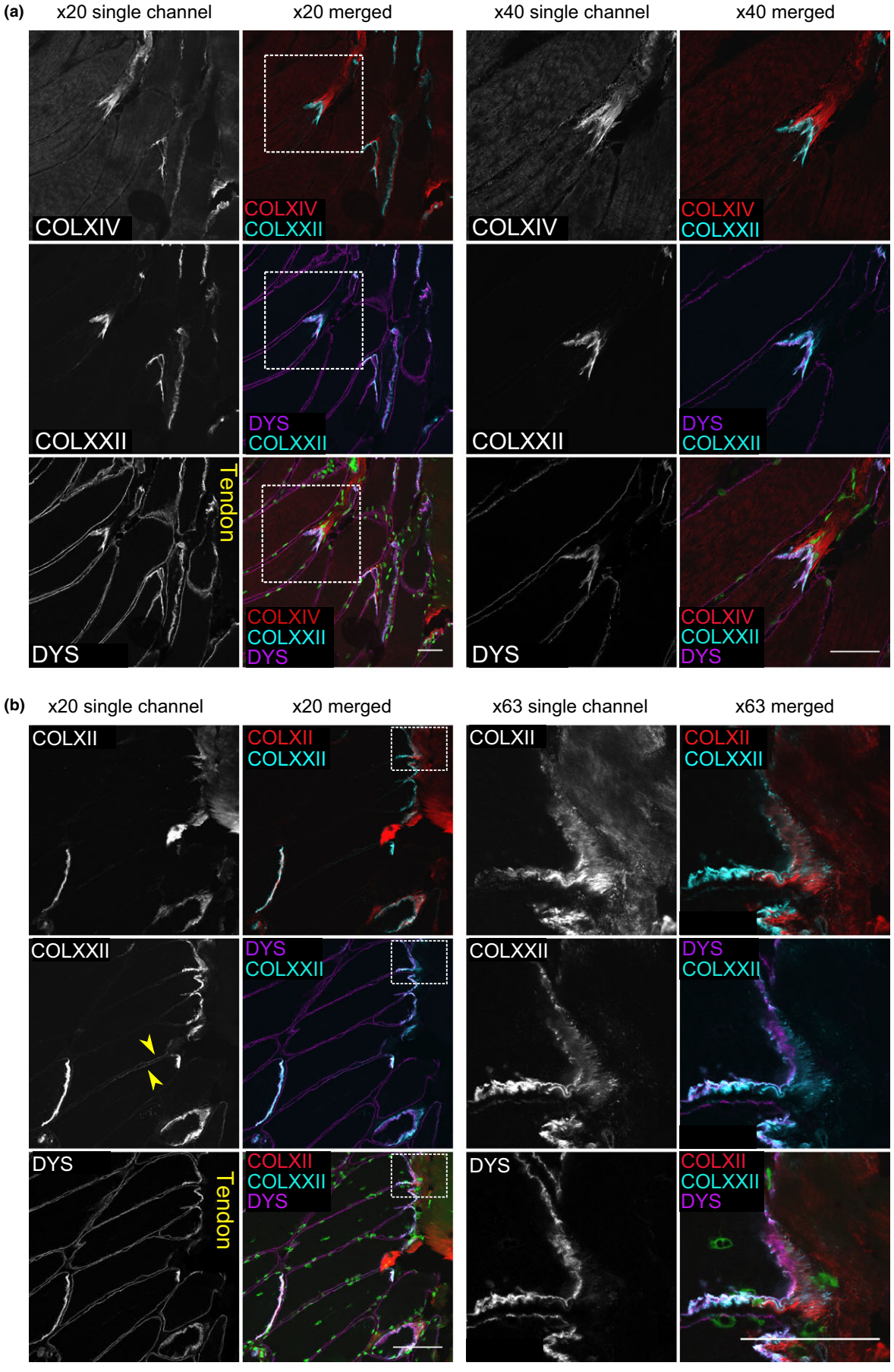
Using dystrophin as a marker of the myofiber membrane (sarcolemma), collagen XXII was observed to be "outside" the myofiber but in close association with dystrophin staining. In triple-labeling experiments with dystrophin, collagen XXII and collagen XIV, the general pattern for the order of appearance of these proteins from the sarcolemma toward the extracellular matrix was dystrophin, collagen XXII and collagen XIV (Fig. 2). Collagen XII and XIV displayed a similar distribution pattern.

Studies of triple-labeling with collagen XXII, collagen XII (or XIV) and collagen VI (Fig. 3) or collagen III (Fig. 4) appear to place the endomysial types III and VI further away from type XXII than types XII and XIV. It is likely that types XII and XIV are closely associated with the endomysium or tendon on one side and the myofiber basement membrane on the other.

Widefield microscopy

As displayed in Table 1, all collagen types studied were abundant at the MTJ. Sample images for each collagen type are displayed in Fig. 5. Additional images of collagen XIV staining are included to illustrate the complexity of the distribution pattern. The collagen types observed to be present to the greatest extent in muscle endomysium were types III and VI, with lower levels of staining of the collagen forms observed in tendon. Collagen types I and XII demonstrated stronger staining of the tendon than the muscle. Collagen type XIV exhibited stronger

Fig. 2. Collagen XXII & dystrophin. Representative confocal images of healthy human gracilis (control) myotendinous junction (MTJ) stained with COLXXII, dystrophin (DYS) and either COLXIV (a) or COLXII (b). The tendon is to the right of the MTJ in these images. Single channel and merged images are displayed with nuclei shown in green. The dotted line boxes indicate the areas imaged at higher magnification. The muscle fibers are delineated by dystrophin and the ends of most of the fibers at the MTJ demonstrate intense immunoreactivity for COLXXII. Yellow arrowheads indicate fine COLXXII staining at myofiber-myofiber borders. It is clear that COLXXII is positioned between DYS and COLXIV, and is closely associated with DYS in particular. The same pattern was observed for COLXII. Scale bars 50 μm.


(a)
x20 single channel
x20 merged
x40 single channel
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COLXIV
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staining of the muscle endomysium than the tendon and also stained the endomysium of fibers close to the MTJ more strongly than fibers further away. Interestingly, whereas the MTJ content of collagen types I and XII was similar to tendon, the content of collagen III in the MTJ was similar to muscle. With regard to the effect of training, no differences were found for collagen types I, III, VI, or XII, when the training group was compared with the control group. The expression of collagen XIV in muscle tissue was found to be elevated in the training vs control group ($P = 0.014$).

Collagen XXII

A mean of 660.9 μm (± 148.5) equivalent to 56.7% (± SD 9.6) of the entire MTJ length in the control samples contained collagen XXII, and 722.7 μm (± 156.3) equivalent to 52.7% (± 13.3) in the long trained subjects.

Tenascin-C

Tenascin-C was found to be expressed to a greater extent in the acute trained muscle tissue (1.1 ± 0.3 arbitrary units; $P = 0.024$) and in the long trained muscles (1.2 ± 0.4; $P = 0.013$) when compared to control samples (0.4 ± 0.4) (Fig. 6).

Macrophages

The mean area of muscle cross-section analyzed was 1.43 ± SD 0.25 mm^2 for the control samples, 1.46 ± 0.28 mm^2 for the acute trained, and 1.44 ± 0.29 mm^2 for the long trained samples. As shown in Fig. 7, the acute training group had similar numbers of CD68+ cells infiltrated in the muscle tissue to that observed in the controls. However, a greater concentration was seen in the long trained muscles compared to controls ($P = 0.016$). At the MTJ, macrophages were found to a similar extent in all groups.

Discussion

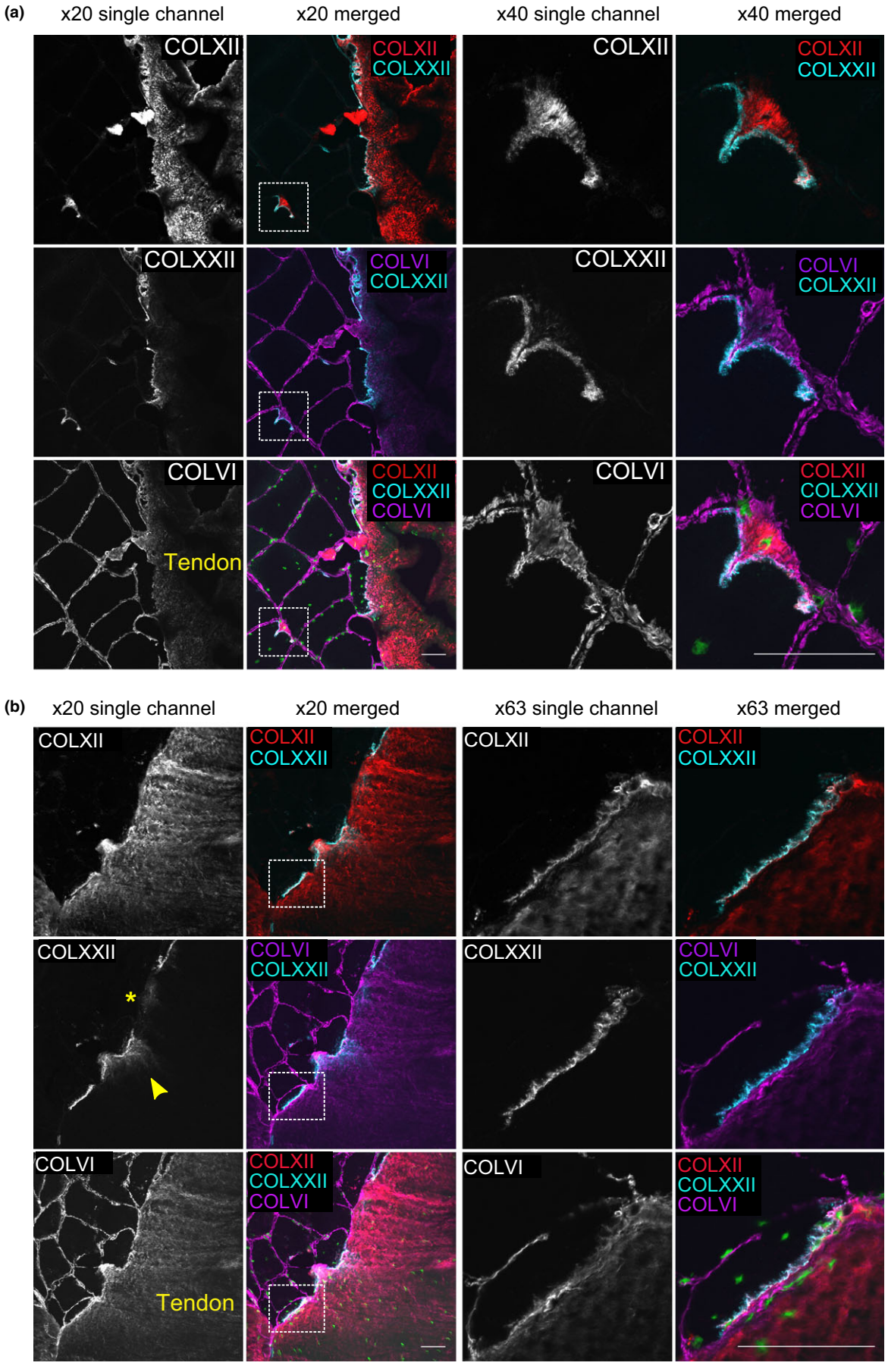
This study is the first to investigate, in trained and untrained humans, the distribution of macrophages and matrix proteins in samples containing MTJ and adjoining muscle and tendon. The main findings were that, when compared to control samples, 4 weeks of regular training was associated with elevated levels of macrophages and expression of tenascin-C and collagen XIV in muscle endomysium close to the MTJ. Also, collagen XXII was observed to be strongly expressed exclusively in MTJ.

We investigated the location of the collagen types near the MTJ in relation to dystrophin, as a marker of the myofiber plasma membrane. The confocal images revealed that collagen XXII coated the muscle fiber membrane in close proximity to dystrophin. Collagen XXII is the "inner" collagen type whereas collagens III, VI, XII, and XIV are located further away from the myofiber membrane. Interestingly, collagen type XXII was found exclusively at the MTJ and not elsewhere in the muscle or tendon, aside from some occasional fine lines extending into the tendon, in line with the finding in animal studies that collagen XXII is expressed exclusively at tissue junctions (Koch et al., 2004).

The function of collagen XXII is unknown, but Charvet et al. (2013) reported a muscular dystrophy phenotype and decreased force production, due to the destabilization of the myosepta, in collagen XXII deficient zebra fish (Charvet et al., 2013). These findings suggest that this collagen is important for the structural integrity and stabilization of the MTJ. Our findings of collagen XXII in human MTJ and not in muscle or tendon support the usefulness of collagen XXII as a marker of human MTJ.

However, collagen XXII was not seen expressed along the entire length of the muscle-tendon interface. As we also report here, when viewed at the electron microscopy level, not all regions of the MTJ display the classic complex folding pattern but instead display a completely flat interface between muscle and tendon. It is possible that these flat regions are structurally weaker and further work could investigate whether these flat regions correspond to the regions lacking collagen XXII. It should be noted however that light microscopy studies of the MTJ are limited by the difficulty in distinguishing the tendon tissue constituting the MTJ on one side and the endomysium of the adjoining muscle fiber on the other side. As such, the detection of specific collagen types in this region merely reflects the composition of both tendon and endomysium at their point of attachment. In addition when viewing MTJ preparations with myofibers cut cross-sectionally, it would appear that many fibers adjacent to the tendon have not reached the point of terminating and as such do not form true MTJ connections. It is therefore also possible that collagen XXII does in fact stain all regions of true MTJ and the unstained muscle-tendon interface simply represents these

Fig. 3. Collagen XXII, VI & XII. Representative confocal images of two samples (a, b) healthy human semitendinosous (control) myotendinous junction (MTJ), stained with COLXXII, COLVI, and COLXII. Single channel and merged images are displayed with nuclei shown in green. The dotted line boxes indicate the areas imaged at higher magnification. COLVI was strongly present in the endomysium and COLXXII is strongly present at the ends of the most of the myofibers at the MTJ. Scale bars 50 μm.


(a)
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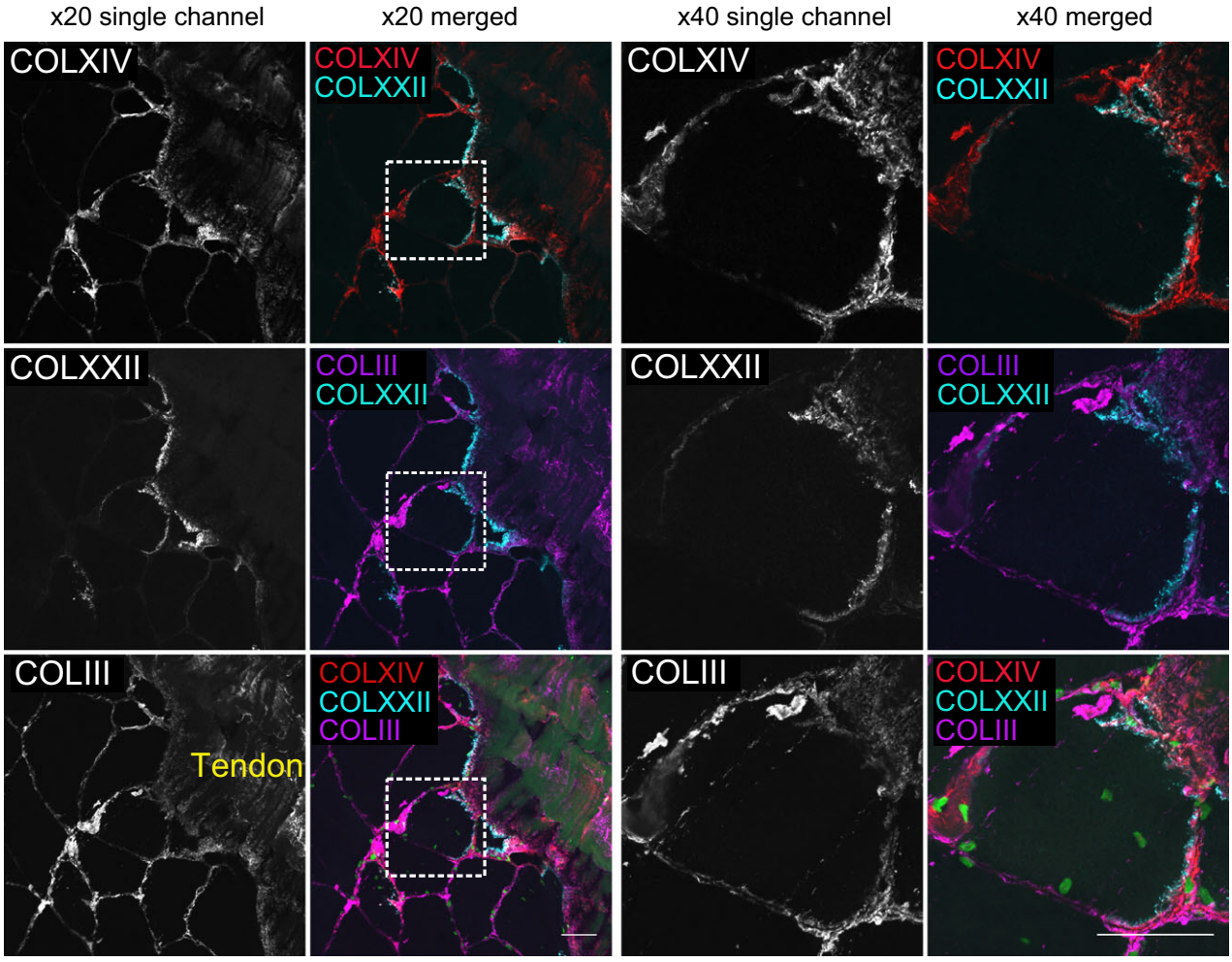

Fig. 4. Collagen XXII, III & XIV. Representative confocal images of healthy human semitendinosous (control) myotendinous junction (MTJ), stained with COLXXII, COLIII, and COLXIV. Single channel and merged images are displayed with nuclei shown in green. The dotted line boxes indicate the areas imaged at higher magnification. COLIII clearly stains the endomysium and COLXXII is strongly present at the ends of the most of the myofibers at the MTJ. Scale bars 50 μm.

Table 1. The effect of 4 weeks training on collagen in human MTJ and adjoining tendon and muscle

	Collagen I	Collagen III	Collagen VI	Collagen XII	Collagen XIV
Tendon control	1.9 ± 0.2	0.7 ± 0.6	1.2 ± 0.8	1.6 ± 0.5	1.1 ± 0.2
Tendon trained	1.8 ± 0.2	0.7 ± 0.3	1.0 ± 0.5	1.8 ± 0.2	1.0 ± 0.8
Muscle control	0.7 ± 0.4	2.0 ± 0.0	1.9 ± 0.2	1.0 ± 0.6	0.6 ± 0.3
Muscle trained	1.3 ± 0.6	2.0 ± 0.0	2.0 ± 0.1	1.3 ± 0.5	1.2 ± 0.4*
MTJ control	1.9 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	1.9 ± 0.1	1.9 ± 0.2
MTJ trained	2.0 ± 0.2	1.7 ± 0.4	1.6 ± 0.1	1.7 ± 0.2	1.7 ± 0.4

Values are means (± SD) of visual gradings performed on human MTJ material and its adjoining muscle and tendon in a control group ($n = 5$) and a group performing 4 weeks of training ($n = 5$). The samples were graded 0, 1, or 2, with 0 representing no staining and 2 the highest level of staining.

*$P < 0.05$ vs control.

fibers that have not been sectioned at the point of termination at the MTJ.

Confocal imaging of our samples confirmed the presence of collagen VI at the MTJ, located in the muscle ECM peripheral to collagen XXII staining. Our widefield images clearly show that the endomysium in human skeletal muscle contains collagen III, VI, and XIV. This is consistent with previous studies on animal muscles (Light & Champion, 1984; Wälchli et al., 1994; Kuo et al., 1997; Mackey et al., 2005). While the function of collagen VI has not yet been clarified, it is known that deficiencies in this collagen in humans are associated with muscle disorders such as Bethlem Myopathy and Ullrich Congenital muscular

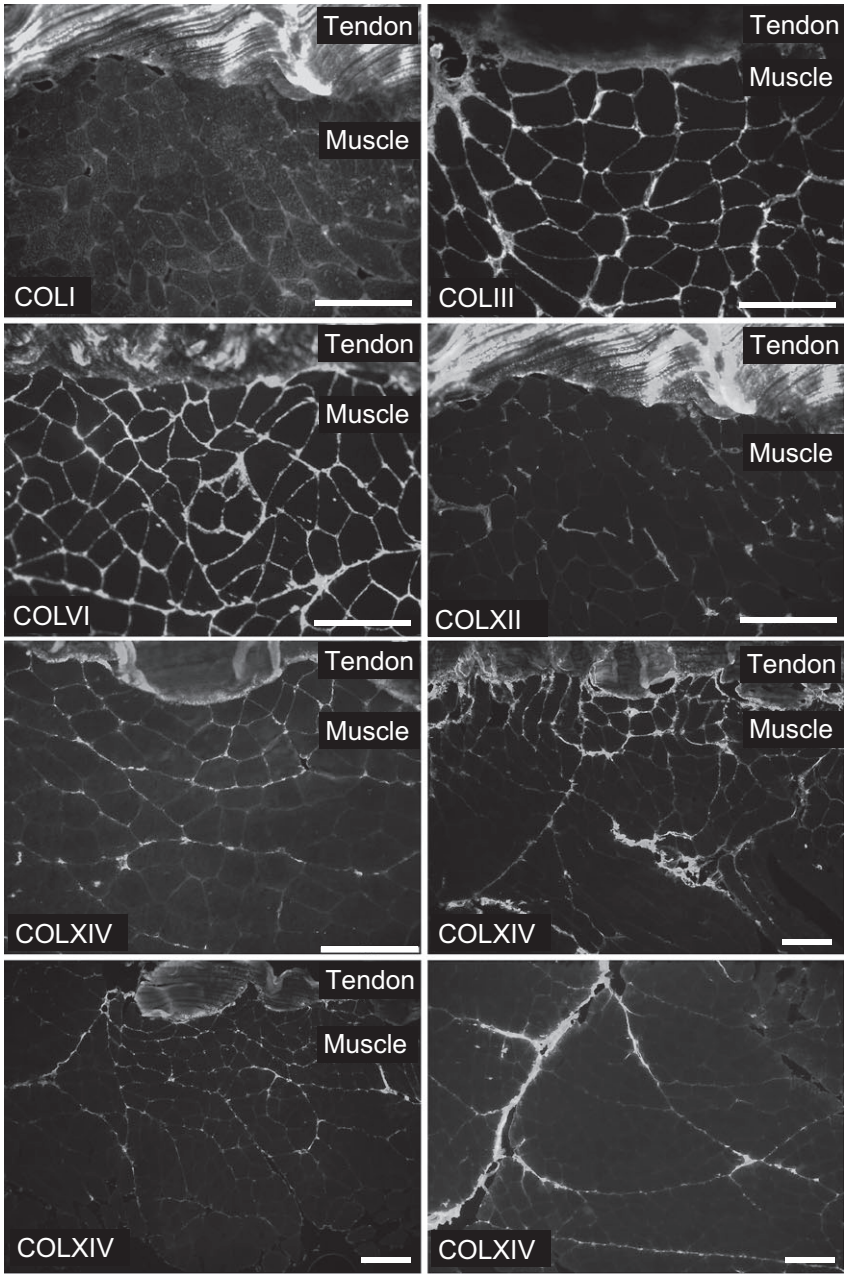

Fig. 5. Collagen distribution in human MTJ. Immunofluorescent widefield images of healthy human muscle and tendon with myotendinous junction (MTJ) stained for collagen I, III, VI, XII, XIV. Four images of collagen XIV are shown (from three individuals) at different magnifications to highlight the stronger endomysial staining around myofibers close to the MTJ and to illustrate the strong staining of the thick perimysium (bottom right), but not endomysium, further away from the MTJ. Scale bars 200 μm.

dystrophy (Lampe & Bushby, 2005) and in mice regeneration after injury is severely impaired in its absence (Urciuolo et al., 2013), highlighting a potential role in maintenance and restoration of muscle and thus the importance of its location at the MTJ. While we found no increase in Collagen VI after 4 weeks of training, it is possible that collagen accumulation would be apparent after a longer period. In support of this, there is evidence that 10 weeks of training is required before an effect of training on the risk of strain injury can be observed (Mjølsnes et al., 2004).

While collagen XIV was noted to be primarily expressed in the perimysium, it was also observed in some endomysial areas alongside collagen III and VI. This collagen is known to be smaller than the other collagens and seems to play a role during fibril fusions (Nishiyama et al., 1994; Tao et al., 2012). Interestingly, the amount of collagen XIV appeared to be greatest in the endomysium around the fibers closest to the MTJ and was almost completely confined to perimysium further away from the MTJ. Collagen XII, along with type XIV, belongs to the

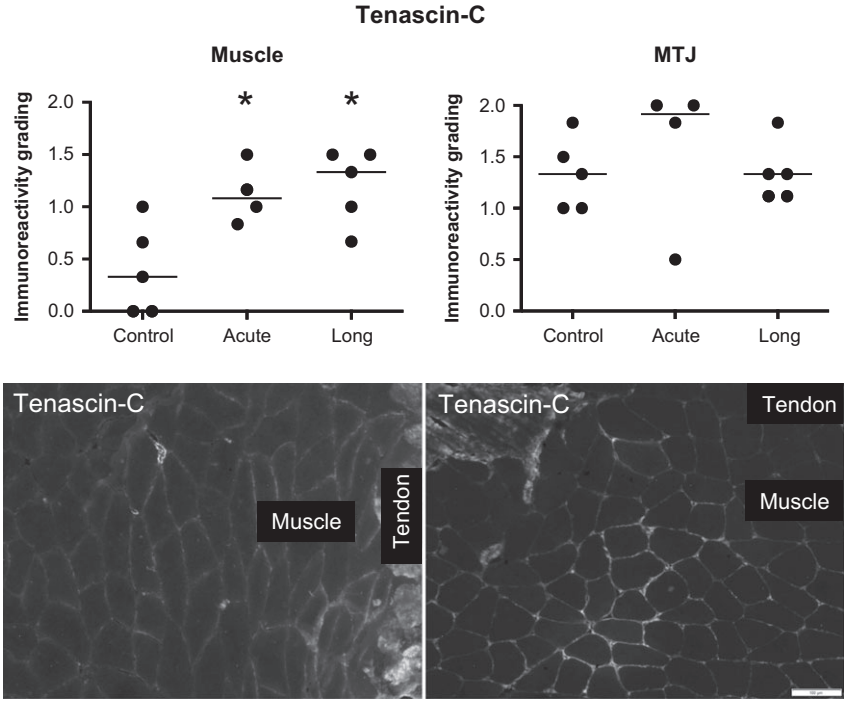

Fig. 6. Tenascin-C in muscle and MTJ. The figure shows the amount of tenascin-c immunoreactivity in muscle and MTJ in the three groups. Each dot represents the mean grading of tenascin-c from one subject. The line represents the median. *vs control group ($P < 0.05$). Also shown are representative images of samples containing low (left) and high (right) levels of Tenascin-C, from a control and long-trained individual, respectively. Scale bar 100 μm.

FACIT family of collagens and is known to be located throughout the body in dense connective tissues (Wälchli et al., 1994). While our distribution study would indicate that type XII is not found in the endomysium close to the MTJ to the same extent as type XIV, the confocal images indicate a similar distribution of these two proteins at the MTJ. Following 4 weeks of HRE, a greater amount of collagen XIV was seen in the endomysium vs controls, indicating that this collagen type might be important in strengthening the tissue junction. Collagen XIV, as well as collagen XII, has shown to contain domains responsible for connecting ECM structures like collagen fibrils and tenascin X (Nishiyama et al., 1994; Bohme et al., 1995; Tao et al., 2012). Therefore, collagen XIV can be thought of as a small anchor linking larger structures together into large ECM-sheaths. Several studies have indicated that the endomysium is important in force transmission, via a lateral force transmission pathway (Passerieux et al., 2006, 2007). In this pathway, most of the force is transmitted as shear stress, thereby decreasing the overall stress on the ECM and MTJ. It is possible that an increase in the amount of collagen XIV could strengthen the ECM-scaffold near the MTJ and possibly optimize the lateral force transmission pathway, thereby reducing the load on the MTJ and potentially protecting this structure from strain injuries. Alternatively, the elevation in collagen XIV with training may be transient and represent an ongoing process of remodeling of other matrix proteins. In support of this, no differences were seen between groups in any of the larger collagen types. Remodeling of muscle ECM is a slow process (Mackey et al., 2011), and it is possible that 4 weeks of training is not long enough to induce detectable changes at the protein level. However, it should also be noted that these collagens were present in abundance at the MTJ and our visual grading method may not have been sensitive enough to detect changes if they did occur. Furthermore, the impact of intensity and type of exercise, with focus on the extent of range of motion, on the remodeling responses of collagen remains a completely unexplored area and may provide valuable insight into understanding injury-preventive mechanisms at the human MTJ.

Our finding of greater tissue levels of the matricellular protein tenascin-C in muscle 2–3 days following a single bout of HRE provides support for this form of exercise as a strong initiator of matrix remodeling and also confirms that the training stimulus actually reached the region of tissue studied. The rapid response of tenascin-C detected in the acute trained group is in line with animal and human studies reporting dramatic protein increases at about 2 days post-exercise (Fluck et al., 2000; Tidball & Villalta, 2010; Mackey et al., 2011). The finding that tenascin-C protein was also elevated after 4 weeks of training further supports the collagen XIV data, together indicating strong ongoing

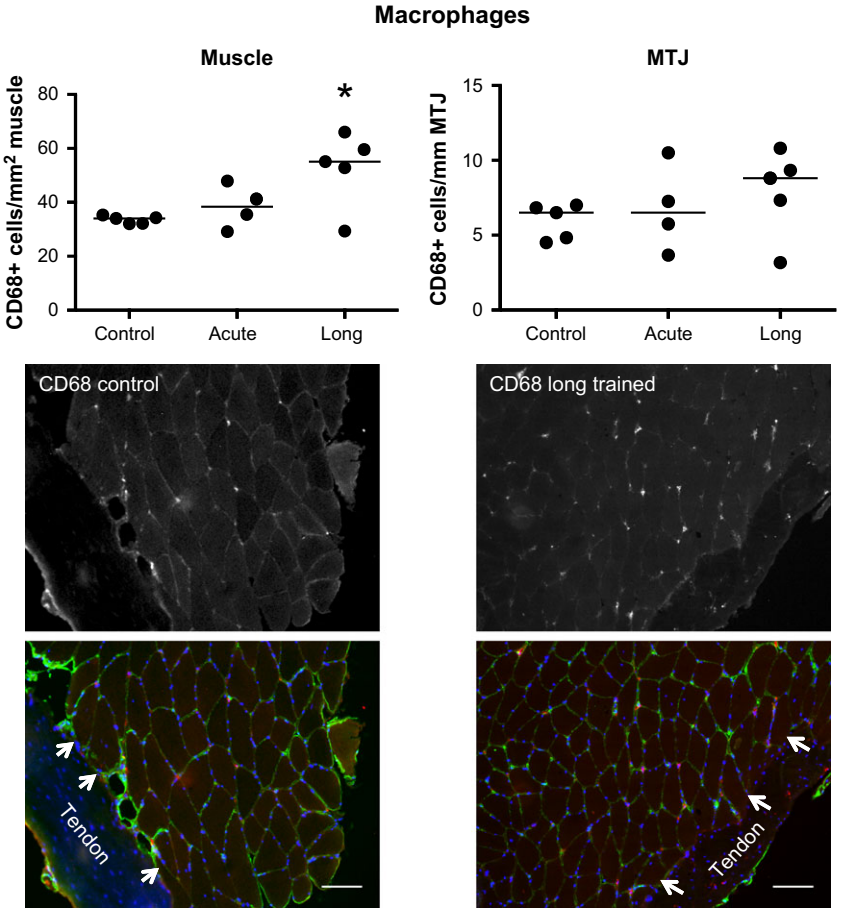

Fig. 7. CD68+ cells in muscle and MTJ. The figure shows the number of CD68+ cells presented as number of CD68+ cells/mm^2 muscle or as number of CD68+ cells/mm MTJ length in controls, after one recent training and 4 weeks training. Each dot represents the mean of each subject and the line the median. *vs control group ($P < 0.05$). The images are examples of the CD68 staining used in this assessment. CD68 staining is shown alone (greyscale, upper images) or merged as CD68 (red), laminin (green) and nuclei (blue), for a control sample (gracilis, left) and a long trained sample (semitendinosous, right). Arrows indicate the MTJ. Scale bars, 100 μm.

remodeling of muscle matrix in this region close to MTJ.

While we did not observe any differences between the acute trained and controls in macrophage content of muscle close to the MTJ, 4 weeks of HRE was found to result in an enhanced concentration of macrophages when compared to controls. An increase in macrophages number within a few days after hard exercise or injury has been reported for human skeletal muscle, when sampling the mid-region of the muscle by the needle biopsy method (Peterson et al., 2003; Paulsen et al., 2010; Mackey et al., 2011), but macrophages content in human muscle close to the MTJ, including changes after exercise, has not been previously reported. We found macrophages in perimysium and endomysium and clear evidence that macrophages adhere specifically to the MTJ in both control and training groups. This is in line with the requirement of macrophages to repair the MTJ region at rest and in response to loading. The density of macrophages observed in the muscle of the controls (2342 cells/mm^3) is not very different from pre-exercise values reported in other studies with values ranging between 700–2400 cells/mm^3 (Peterson et al., 2003; Mackey et al., 2011), indicating that the control group is a suitable reference for our trained groups when the tissue samples were harvested. Physical activity levels were also probably comparable in so far as participation in structured resistance training programmes was one of the exclusion criteria for these studies.

In conclusion, we report elevated content of collagen XIV and tenascin-C in the endomysium near MTJ following 4 weeks of HRE, indicating a matrix remodeling that may be important in the protection against strain injuries. In addition, we show that a greater concentration of macrophages in the muscle area near the MTJ following the same period, as well as detecting macrophages specifically at the interface of the MTJ, indicating a potential role in tissue remodeling at the MTJ. The presence of collagen XXII at human MTJ, but not in muscle or tendon,

supports its value as a tool for defining the human MTJ histologically.

Perspectives

Prevention of hamstring strain injuries remains a clinical challenge, where the Nordic Hamstring protocol appears to be the most promising form of training. This study is the first to investigate the morphologic effects of HRE on the MTJ and adjoining muscle tissue. Our findings of increased collagen XIV and macrophages content between muscle fibers at the MTJ indicate an important role for these adaptations in the strengthening of this tissue area. Further insight into the composition of human MTJ and loading-induced adaptation may contribute to the basis for development of strategies to strengthen this injury-prone region.

Key words: Injury prevention, Nordic Hamstring, extracellular matrix.

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