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Larger interface area at the human myotendinous junction in type 1 compared with type 2 muscle fibers

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The myotendinous junction (MTJ) is structurally specialized to transmit force. The highly folded muscle membrane at the MTJ increases the contact area between muscle and tendon and potentially the load tolerance of the MTJ. Muscles with a high content of type II fibers are more often subject to strain injury compared with muscles with type I fibers. It is hypothesized that this is explained by a smaller interface area of MTJ in type II compared with type I muscle fibers. The aim was to investigate by confocal microscopy whether there is difference in the surface area at the MTJ between type I and II muscle fibers. Individual muscle fibers with an intact MTJ were isolated by microscopic dissection in samples from human semitendinosus, and they were labeled with antibodies against collagen XXII (indicating MTJ) and type I myosin (MHCI). Using a spinning disc confocal microscope, the MTJ from each fiber was scanned and subsequently reconstructed to a 3D-model. The interface area between muscle and tendon was calculated in type I and II fibers from these reconstructions. The MTJ was analyzed in 314 muscle fibers. Type I muscle fibers had a 22% larger MTJ interface area compared with type II fibers ($p < 0.05$), also when the area was normalized to fiber diameter. By the new method, it was possible to analyze the structure of the MTJ from a large number of human muscle fibers. The finding that the interface area between muscle and tendon is higher in type I compared with type

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II fibers suggests that type II fibers are less resistant to strain and therefore more susceptible to injury.

KEYWORDS
confocal microscopy, injury prevention, muscle injury, muscle physiology, myotendinous junction

1 | INTRODUCTION

The myotendinous junction (MTJ) transmits force between muscle and tendon. The contact area between the termination of each muscle fiber and the collagen fibrils from the tendon has a highly specialized morphology, as the muscle membrane is intensively folded, forming invaginations of collagen-rich protrusions from the tendon—in 2-D resembling fingers, penetrating into the muscle fiber[1] (Figure 1).

Collagen type XXII is a specific marker of the MTJ,[2] and transmission electron microscopy-imaging (TEM) with immunogold labelling has confirmed that this particular collagen is present in the protrusions from the tendon into the skeletal muscle.[2] The direction of force transmission is different in an interface with these protrusions compared with a smooth interface.[3,4] Consequently, a higher proportion of force is transmitted as shear- or sliding-force, which is protective in relation to retaining the tissue structure, compared to tensile- or pulling-force.[3] Theoretically, the large area for force transmission as well as the reduced angle between the tissues lowers the stress on the MTJ.

Despite this advantageous structure, the MTJ is a common site of strain injuries.[5] These are among the most frequent non-contact injuries in many sports, especially in the hamstrings.[6–9] Eccentric exercise of the hamstring muscles (e.g., Nordic Hamstring) is very effective to reduce the risk of strain injuries.[10–14] It has been hypothesized that the size of the foldings and thereby the area of the interface might affect the risk of injury.[4,15] In animals, it has been reported that loading regimes result in a larger size of the foldings and of the overall interface area of MTJ, when measured on TEM-images.[15–19] Theoretically, this training effect optimizes the ability of the MTJ to transfer force and potentially it lowers the risk of strain injury.[3,4] In atrophic or unloaded muscle, the opposite occurs[20–23]: the foldings decrease in size, and this reduces the overall area through which force is transmitted. There seems to be an inverse correlation between how much the MTJ is loaded through activity and the risk of strain injury, meaning that following periods with lower loading the risk of strain injury is increased when high loading is resumed, for instance, at the beginning of a sports season. This implies that plasticity of the foldings at the MTJ may be a key factor for the risk of strain injury.[24]

Clinical studies have suggested that a fiber type distribution with a higher proportion of type II compared with type I fibers is associated with an increased risk of hamstring strain injury.[25,26] This could be because type II fibers are fast-twitch, supporting quick, powerful movements leading to differences in activity between individuals with different fiber type distributions. It could also be explained by a fiber-type specific difference in the tolerance of MTJ to sustain load. Animal studies have indicated that there might be differences in the structure of the foldings of the MTJ, dependent on muscle fiber type.[27,28] While this is of less importance in studies on mice, in which most muscles consist almost exclusively of type I or II fibers, it is relevant for analysis of human muscles which are composed of both fiber types in no fixed ratio. Unfortunately, it is not easy to distinguish between fiber types by TEM.

The current study aimed to investigate whether there are differences in the surface area of human MTJ between type I and II fibers and introduce a novel method to analyze this area in whole muscle fibers, using immunohistochemistry (IHC) in combination with confocal microscopy. As type II fibers seems to be more exposed to strain injury than type I, it is hypothesized that type II fibers have a smaller MTJ interface area than type I fibers.

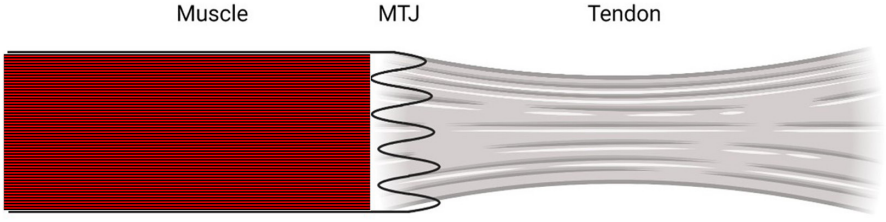

FIGURE 1 Schematic illustration showing the myotendinous junction (MTJ) positioned as the interface between the muscle and tendon. The unique structure is highlighted by the folded appearance of the junction. Created with BioRender.com

2 | METHODS

2.1 | Participants

Samples were collected from 10 human subjects (3 females and 7 males, mean age 27.1 ± 6.3, and BMI 24.3 ± 1.6) included between May 2019 and August 2020. All subjects were scheduled for ACL-reconstructive surgery and had not performed resistance exercise involving the hamstring muscles during the preceding 3 months. Activity level was not reported but in general the participants were non-athletes who were recreational active with sports such as running, cycling, and lighter aerobic activities. The study was approved by the local Research Ethics Committee and was performed according to the standards of the Helsinki Declaration. During surgery, the semitendinosus was harvested, and the tendon was used as graft. The excess tissue, containing MTJ, was used for this study. Immediately following dissection of the tissue, samples were prepared according to a single-fiber protocol, as described previously.[29] Briefly, samples were pinned in a silicone-coated dish and incubated in Krebs–Henseleit buffer with procaine, followed by incubation overnight in Zamboni fixative (2% paraformaldehyde, 15% picric acid in 0.1 M PBS; pH 7.2), and finally stored in 50% glycerol/PBS until dissection.

Using a stereo microscope, single muscle fibers were dissected from the tendon with a surgical scalpel. An effort was made to separate the tissues while keeping the tip of each muscle fiber intact. Fifty fibers were dissected from each subject and placed in NUNC well-plates for IHC staining.

2.2 | Staining protocol

The single fibers were incubated for 1 h in an immunobuffer (IB) (PBS containing 50 mM glycine, 0.03% saponin, 0.25% BSA, 0.05% sodium azide, and 0.1% Triton-X100 to increase the permeability of the fibers). A mixture of two primary antibodies was then added and incubated at 5°C for 3 days. Previous tests found this superior in relation to signal-to-noise ratio in the image analysis afterward compared with shorter incubation periods. The primary antibodies were as follows: Guinea-pig anti-collagen XXII diluted in IB 1:1000 (Manuel Koch University of Cologne, Germany) and mouse anti-myosin heavy chain 1 (MHC 1) (1:100, A4.951, Hybridoma Bank). Following washing in IB, the fibers were incubated with secondary antibodies overnight at 5°C (Goat Anti guinea pig Alexa 568, Goat Anti-mouse Alexa 647). Before mounting (Molecular Probes ProLong Gold anti-fade reagent with DAPI, cat. no. P36935; Invitrogen A/S), the fibers were washed in IB and once in PBS. When using a coverslip, it was seen that the fibers were compressed and that the level of compression was different between fibers. It was not possible to find a method to mount the coverslip in a standardized manner with an equal compression of all fibers, and it was decided not to use coverslips and scan the fibers directly with immersion oil on the objective. This was found not to affect the image quality.

2.3 | Confocal microscopy

The images of the muscle fibers were obtained using an iMIC microscope equipped with a wide field and a confocal unit (Till Photonic, FEI Munich, Germany).[30] The microscope was equipped with appropriate filter settings for detecting DAPI, Texas Red/Alexa 561/594 and Cy5/Alexa 647. For confocal imaging, the iMIC used an Andromeda disk system (FEI, Germany) and a Hamamatsu 16-bit camera (Model C10600-10B-H, Hamamatsu Photonic) for recording. For wide field microscopy, the iMIC used a 12-bit camera ((Model C84484-03G02, Hamamatsu Photonic) and the following objectives: 10×, numerical aperture (NA) = 0.35 and 60×, NA = 1.46). Through a 10× objective a wide field overview image of the entire slide was made. The Alexa 647 staining was used to distinguish between type I and II fibers (Figure 2A) by the presence of MHC1 staining in type I fibers. In some muscle fibers, only some regions of the fiber showed immunoreactivity against the MHC1 antibody. Therefore, a type I fiber was defined as a fiber showing immunoreactivity against MHC1 in any part of the fiber (Figure 2A).

Z-stacks of individual fibers were created using a 561 nm laser to image the collagen XXII staining through oil immersion and the 60× objective. Based on the wavelength and the numerical aperture (NA) of the objective, a theoretical working resolution (smallest visual distance between two pixels) could be calculated: resolution = wavelength (λ/2* NA). In the current setup, the theoretical resolution was ~192 nm with a z-resolution of 200 nm. Each fiber was scanned from bottom to top with a z-distance between images of 0.2 µm using the same laser intensity, but varying exposure time (between 99 and 200 m) to get at least 10.000 gray values in all fibers (Figure 3).

All z-stack files were saved in the same folder and later re-arranged and re-labeled by a person not involved in the analysis, to blind the observer responsible for measuring the interface area. The 647 nm channel type was not included in the z-stack, and therefore both the fiber type and subject were blinded for the observer.

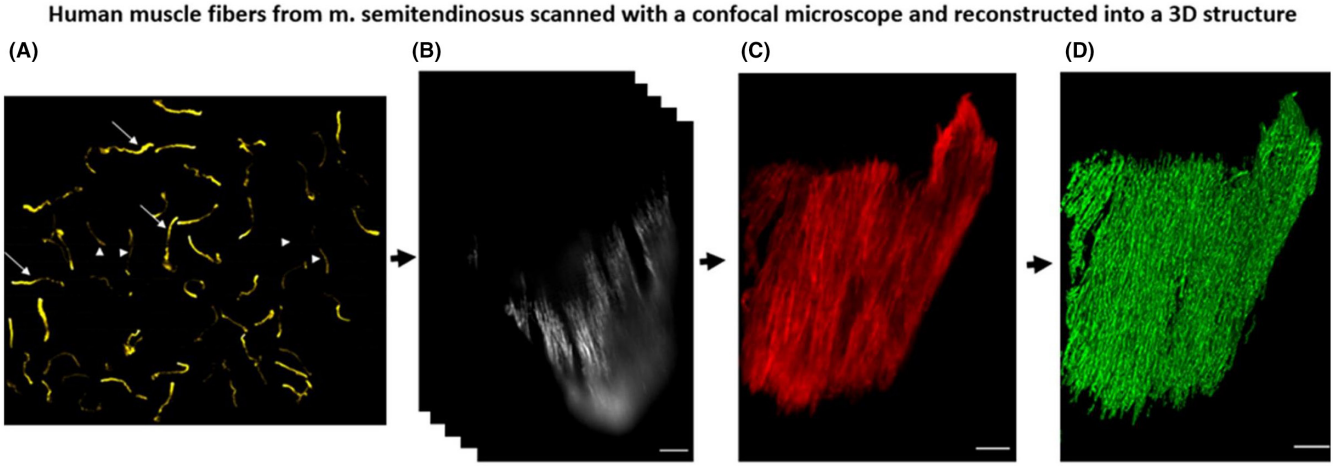

FIGURE 2 Figure shows the workflow of the study, starting with (A) a widefield fluorescent image of all fibers from a subject. Type I MHC is labeled, from which the fiber types can be identified (type I arrow, type II arrowhead) and used for the later analyses. Each fiber is also labeled with collagen XXII antibody and the fiber end is scanned using the confocal part of the microscope, providing images through the whole depth of the fiber, as shown in (B). These confocal images are merged in a z-stack (image C) which is used to create the 3D surface (D) from which the measurements are made. Scale bars are 10 μm

2.4 | Image analysis

The z-stack of individual muscle fibers was analyzed using Imaris Bitplane 9.5 (RRID:SCR_007370; Bitplane) using the "Surfaces" plug-in to create an artificial surface based on a thresholding of the collagen XXII staining from which the area could be measured (see Figure 2). The settings for creating the surfaces were identical for all fibers from all subjects. The fiber typing was distinguished by myosin heavy chain 1 antibody (Figure 2). Individual fibers were imaged using an iMIC spinning disc confocal microscope and stacked into z-stacks creating a 3D-structure. Representative images through the z-stack are shown in Figure 3.

To test the reproducibility of the method, multiple blinded measurements of the same fibers were made, showing a variation of less than 1%.

During the dissection, staining and imaging some fibers were either lost or in a condition that made analysis impossible. Some fibers appeared to have lost the end which could have happened during dissection or in the staining process. In some fibers, the signal to noise ratio was too low for the software to create a useful surface and these fibers were therefore excluded. From a total of 500 dissected fibers (50 fibers from each subject), 314 fibers were found suitable for image analysis.

2.5 | Statistics and power calculation

A pilot experiment was performed to obtain data to estimate the necessary sample size to distinguish a relevant difference in surface area between fiber types. Twenty-eight fibers (14 of each type) from a single subject were analyzed, and the interface areas were found to be 27 382 μm^2 ($\pm$1460) for type I fibers and 23 732 μm^2 ($\pm$1821) for type II fibers. Based on this, at least 6 subjects were needed to enable confirmation that this is an actual difference with a power of 80% and significance level of 0.05. Since the fiber type distribution might vary between subjects and some fibers would be lost during preparation, we chose to include 50 fibers from each subject to be sure to have at least 10 fibers of each type from each subject.

Unfortunately, samples from two subjects did not have the minimum 10 fibers of each fiber type and had to be excluded from the analysis. From each of the remaining 8 subjects (3 females, 5 males), 15 type I (median, range 11–20) and 20 type II fibers (range 12–32) were analyzed.

In order to account for fiber size, the interface area was also expressed relative to the diameter of the fiber.

Due to large variation in the data, Wilcoxon signed rank test was chosen to analyze differences between fiber types. Significance level was set to $p < 0.05$. Possible connections between the size of interface area for each fiber type and age, BMI, height, weight, and time from ACL injury to sampling were analyzed by correlation analyses. Graphs and correlation analyses were made in GraphPad Prism 9.1.1 and Wilcoxon signed rank tests were made in SigmaPlot 14.

3 | RESULTS

There was a high complexity of the interface area related to the length and number of foldings on all individual

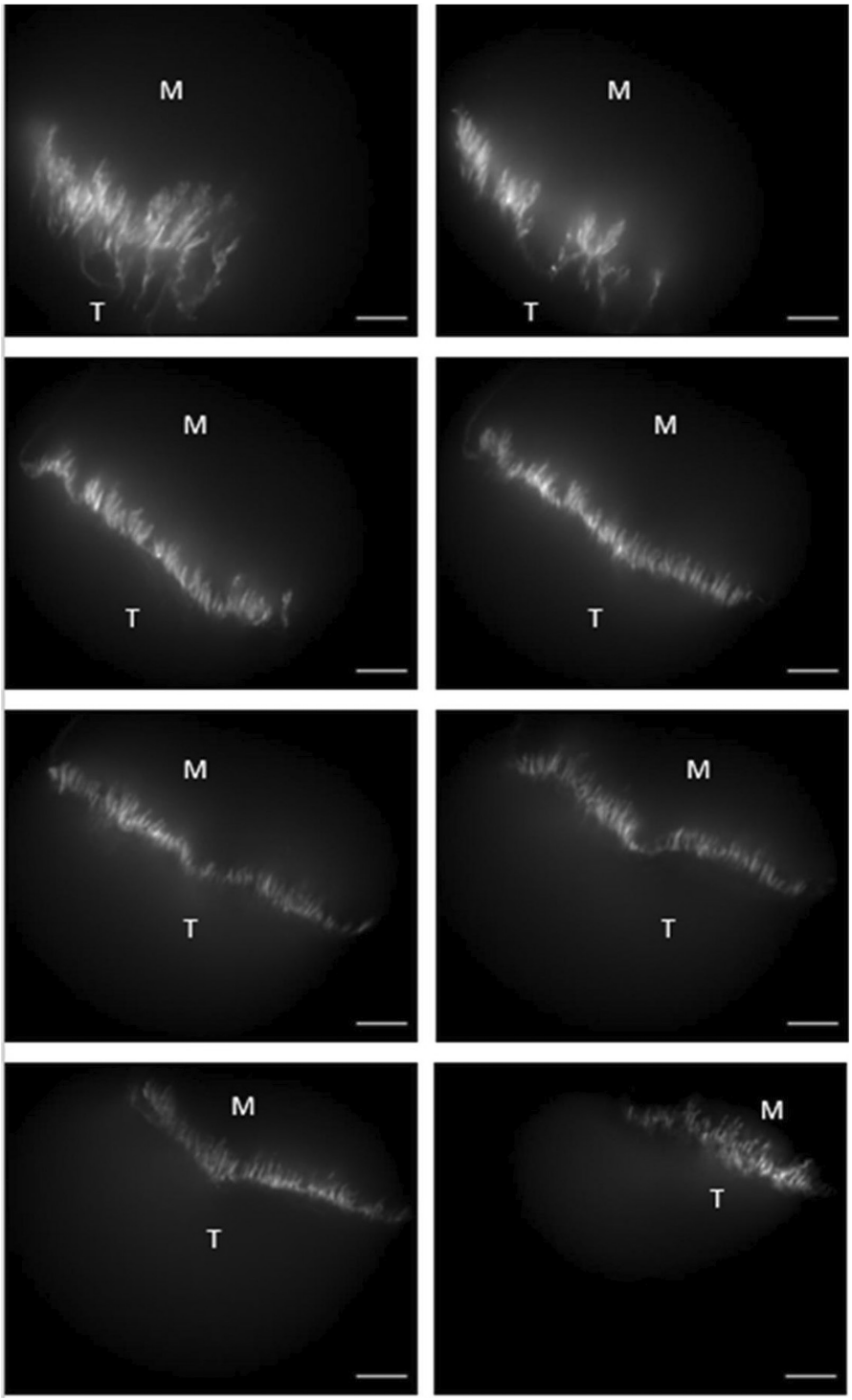

FIGURE 3 Representative confocal images of the collagen XXII labeled MTJ separating the muscle (M) from the tendon (T). The images are randomly picked from the z-stack containing the 300 images that are used to make the 3D reconstruction. Scale bars are 10 μm

images but to an even greater extent on the collection of images in 3D z-stacks (Figure 4). Based on the z-stacks, a reconstruction of the surface was created (Figure 4) from which the size of interface area between muscle and tendon was measured. In 314 muscle fibers collected from eight subjects the entire MTJ was imaged and analyzed. Generally, the appearance of the foldings at the fiber end was very heterogenous between subjects (Figure 5). On average, a 4–6-fold difference was seen between the size of the smallest and largest fiber of same fiber typing from same individuals. In one subject, more than a 9-fold difference was seen between the smallest and largest fiber of same fiber typing. This was also seen when the size of the interface area was expressed relative to the diameter of the fiber. The female participants had the smallest interface area, however, this gender difference disappeared when the data were normalized to fiber diameters.

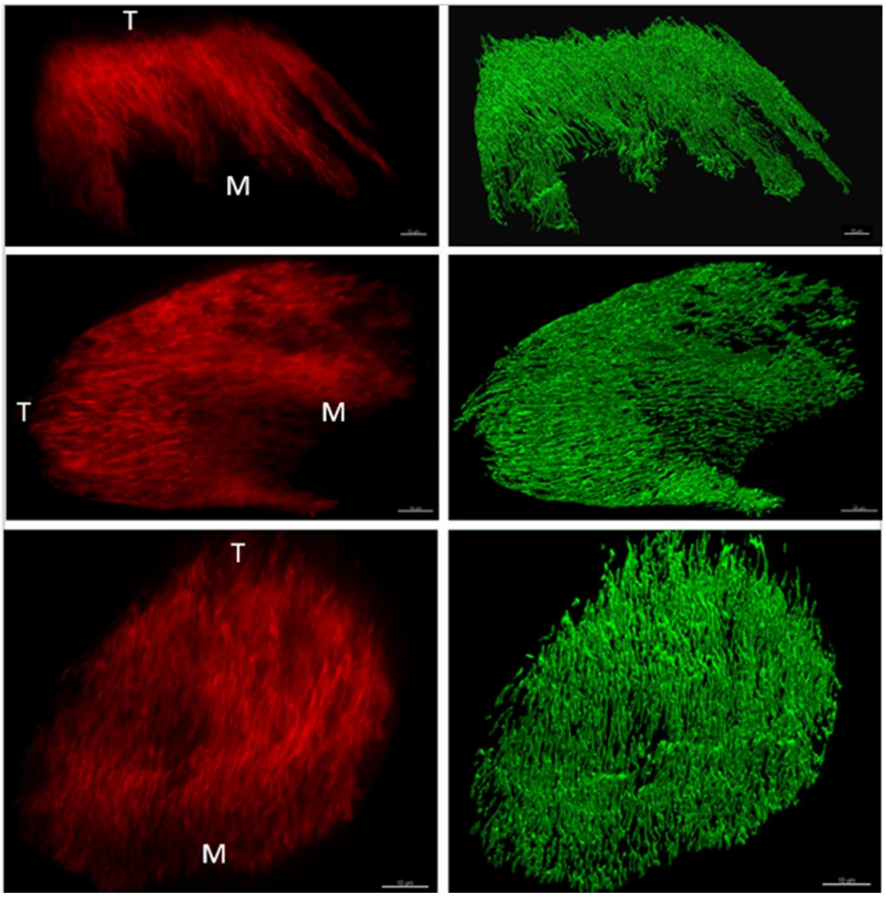

FIGURE 4 Left column shows z-stacks of the termination at the myotendinous junction of one whole muscle fiber with the muscle (M) and tendon sides (T) marked. The right column is the software constructed surface based on the z-stack from left column. Based on these constructed surfaces the interface area was calculated. Scale bars are 10 μm

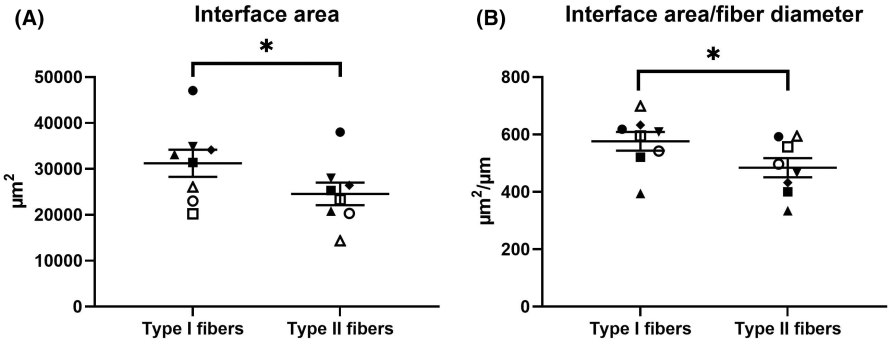

FIGURE 5 Interface area of MTJ type I and type II fibers. In (A) the interface area is plotted as individual median values as well as the median for all type I and II fibers. There is a significant difference between the fiber types ($p = 0.023$). In (B) the interface area is expressed relative to the muscle fiber diameter of each fiber type per subject, showing the medians for type I and II fibers. The difference between fiber types is significant ($p = 0.008$). Each subject is represented by an individual symbol. Females are indicated by open symbols and males by closed symbols

The average interface area of type I fibers was 22.3% larger than in type II fibers ($p = 0.023$) (Figure 5A). Also, when expressed relative to the fiber diameter, the interface area of type I fibers was significantly larger than type II ($p = 0.008$) (Figure 5B). The mean diameter of both fiber types was 53 μm and the distribution showed a higher number of type II (58%) compared with type I (42%) fibers. There was no correlation between the size of interface area in type I or type II fibers and time from ACL injury to sampling, age, BMI, height, or weight.

4 | DISCUSSION

The present study introduces a new method involving confocal microscopy to visualize and measure the folded surface area of human hamstring MTJ's. This novel measurement method can analyze the entire MTJ of a muscle fiber from a high number of fibers, which is useful for future studies examining the basic structure of the MTJ and changes that may be introduced to the structure

following loading and unloading. The study could confirm the hypothesis that type I fibers from human semitendinosus muscles have a larger MTJ interface area compared with type II fibers from the same muscle.

The unique structure of the MTJ has been the subject of several studies previously, mostly with transmission electron microscopy (TEM) as the imaging technique.[16–18,21,23,28] 3D reconstructions from TEM images of human MTJ samples have visualized the complexity of this surface.[31] However, one study analyzed the foldings of the MTJ by light microscopy.[32] The muscle membrane at the MTJ was identified through coloring for dystrophin, and the size of the tendon foldings into muscle was calculated from a 3-D reconstruction, based on the consecutive cross-sections of the muscle fibers approaching the MTJ. An advantage of TEM is that very small structures can be visualized due to the high resolution of this technique, compared with widefield light microscopy. The drawback is that these very small regions might not be representative of the whole fiber or tissue. Furthermore, the tissue preparation and image analysis are very time consuming compared with immunohistochemistry, meaning that it has only been possible to analyze the area of few fibers in previous studies. In the current study a spinning disc confocal microscope was used to scan individual muscle fiber ends. Collagen XXII, which is known to be exclusively located at MTJ and not elsewhere in skeletal muscle or tendon, was used to label the folded surface of each section, and the stack of sections was compiled into a 3D structure—this process is relatively fast and enables analysis of a large number of whole muscle fibers.

The stacked images were able to show a very detailed organization of the foldings. This revealed a large variation in both size and appearance between fibers. Interestingly, the interface area was significantly higher for type I fibers compared with type II. If a larger interface area lowers the risk of injury during force transmission, as suggested previously,[4,15,33] then, the present findings suggest that type I fibers could be more resistant to load-induced injury than type II fibers. Type II fibers are known to be capable of producing faster and stronger contractions compared with type I fibers, and this can result in a higher strain at the MTJ.[34–36] However, fatigue may also influence the risk of strain injuries, as it reduces the amount of energy that can be absorbed by the muscle.[35,37] Since type II fibers are easily fatigued, it has been suggested that a higher proportion of type II fibers in a muscle leads to a higher risk of strain injury.[25,26] While not validated thoroughly yet, it is supportive to this idea that the most frequently injured muscles, the hamstrings, generally contain a high proportion of type II compared with type I fibers.[26] The finding in the current study of a smaller area of force transmission between muscle and tendon in type II compared with type I fibers in the hamstring muscle semitendinosus introduces an additional weak element that may be important to explain the increased risk of strain injury in muscles with a large proportion of type II fibers. It is suggested from animal studies that the foldings of the muscle membrane at the MTJ have a high degree of plasticity and are able to increase in size following repeated loading. Based on this, targeting the type II fibers in a prevention program may increase the interface area of the MTJ and be advantageous to prevent strain injuries.

However, not all force is transmitted linear through the MTJ. An unknown amount is also distributed laterally and does not load the MTJ.[39] This lateral force transmission pathway is relatively undescribed but important in the understanding of the load exerted on the MTJ. Differences in the ability to transmit force laterally between fiber types have not been documented but any such difference would significantly affect the susceptibility of the given fiber type towards strain injury.

Two previous studies have compared the area of the MTJ between fiber types in animals, but with conflicting results.[27,38] A stereological approach was used to estimate the area of the MTJ on TEM sections in chicken, and muscles consisting of type II fibers were found to have larger area compared with the type I dominant muscles. However, the opposite was demonstrated in muscles from snake by the same method. An explanation of the conflicting results can be that the relative proportion of MTJ interface areas between type I and II fibers may depend on species.

In most of the previous studies, the surface of the MTJ is expressed relative to size or diameter of the fiber, as theoretically, a larger fiber would be expected to have a larger interface area at the MTJ. However, in the current study, it was observed that the size of the MTJ was very much determined by morphology, as some MTJs had very short foldings and others had long thread-like foldings (Figure 6). Fiber diameter was chosen as normalization since it best resembles what has been used in previous studies.[16–20] However, whether cross-sectional area, fiber circumference or maybe the volume of the tip of the muscle fiber is better suited for the normalization could be an interesting aspect in future studies. In the current study, the interface area of type I fibers was still significantly larger than type II fibers when the measurements were expressed relative to fiber diameter.

TEM-images from human MTJ's have shown that the thickness (width) of the smallest foldings ranges between 220 and 300 nm (Figure 7). The current confocal setup has a calculated resolution of 200 nm, which is probably enough to visualize the morphology of the foldings. To the best of our knowledge, it is not possible to measure the same fibers using both confocal and

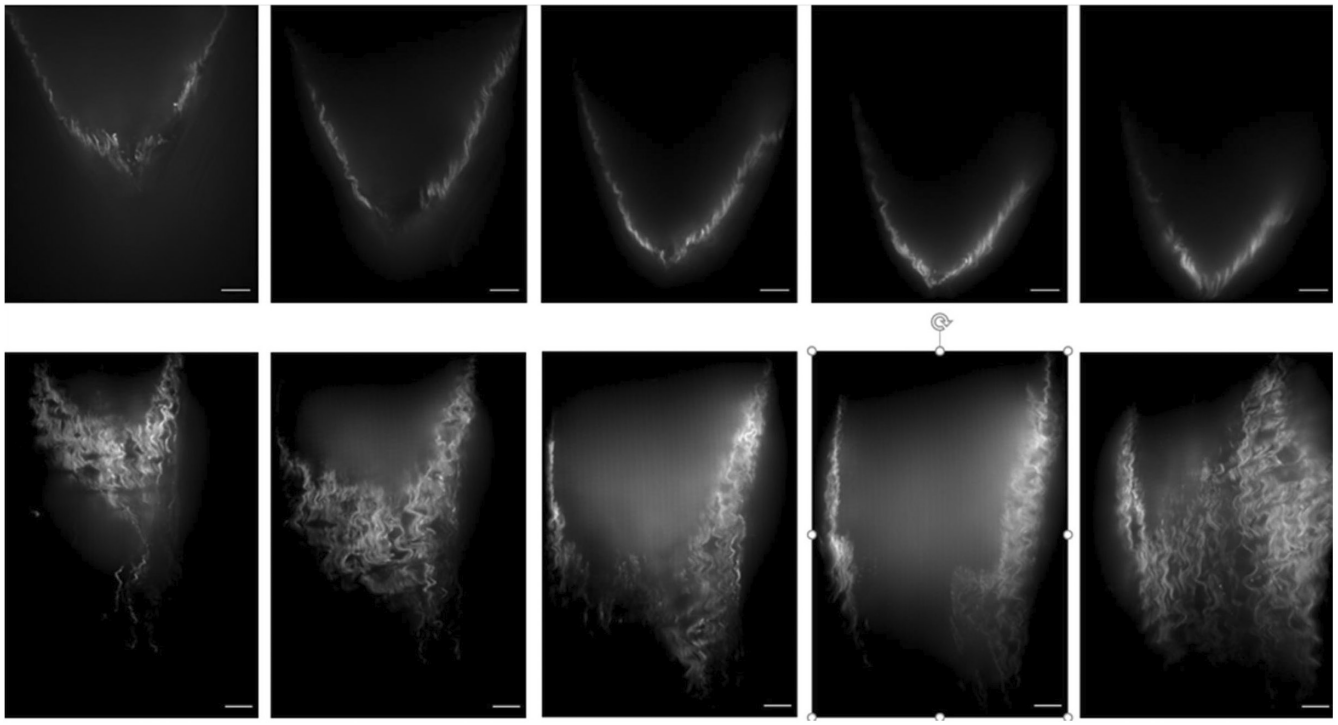

FIGURE 6 Representative sections of two type II fibers, illustrating that the size of the fiber is not necessarily determining the interface area of the MTJ. The upper row shows 5 images randomly picked from the z-stack of the whole fiber with a total surface area of 20 692 μm^2 and a fiber diameter of 80 μm. The bottom row shows 5 images from a fiber with a diameter of 42 μm but with an MTJ interface area of 76 759 μm^2. Scale bars are 10 μm

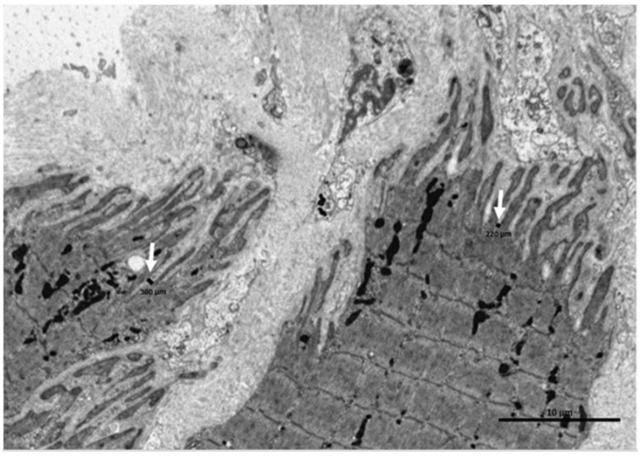

FIGURE 7 Electron microscope image of human MTJ. The width of the base of the foldings (indicated by white arrow) is 220–300 nm. Scale bar 10 μm. Image acquired by Andreas Billa Knudsen, made at the Centre for Integrated Microscopy (CFIM) at the Panum Institute in Copenhagen, Denmark, used by permission

electron microscopy, and therefore, we were not able to validate the confocal measurements in the current study. In theory, smaller details below 200 nm are not detected in the current setup. If the distribution of thin foldings (<200 nm) and thicker foldings is independent of fiber type the threshold will not affect the results, but if type II fibers have many thin foldings compared with type I fibers, the surface area of type I fibers is underestimated. However, such a difference in thickness of foldings has not been reported in previous literature using TEM.[27,28] Finer details are better visualized by TEM-images that have a resolution of around 1 nm. The advantage of the confocal method used in the current study compared with TEM is that whole fibers can be measured in large quantity and that muscle fiber type can easily be identified. That it is important to measure a large number of fibers is documented by the remarkable variation in the size of the interface area among fibers of same type from same individual. In the most extreme cases, a 9-fold difference was seen between the smallest and largest fiber from the same subject.

No significant difference between genders were seen in the normalized size of interface area of the MTJ. However, due to the low number of female participants ($n = 3$), a potential gender difference cannot be excluded and could be of interest for future studies.

In conclusion, this study introduces a novel method to analyze the entire interface area of the MTJ from human muscle fibers, allowing analysis of a large number of fibers. The interface area between muscle and tendon was higher for type I fibers compared with type

II, but there was a large variation in this area between muscle fibers, even in the same individual. Although future studies need to confirm this, it suggests that type II fibers might be more susceptible to strain injury due to a smaller MTJ interface area, and that interventions aiming to prevent strain injury may focus on strengthening of these fibers.

4.1 | Perspective

By the new method to measure the size of the MTJ interface area in whole muscle fibers, it is possible to include many fibers in studies of the MTJ. This is valuable, as the high variance in MTJ interface area shows that quantitative results by electron microscopy of small sections may not be representative and must be interpreted with caution and cannot necessarily be extrapolated to be valid for whole fibers.

In future studies of the MTJ, it will be relevant to distinguish between fiber types, as it seems to influence the proportions of the MTJ. Whether it is possible to change the MTJ interface area through exercise as suggested from electron microscope studies in animals is a subject for further study. Likewise, it is relevant to study if training aimed at strengthening type II fibers leads to a more even MTJ interface area in type I and II fibers, and if this adaptation of type II fibers is accompanied by a clinical relevant effect on risk of strain injury.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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