

2D and 3D microstructural analysis of the iliotibial tract

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Funding information

Fonds Wetenschappelijk Onderzoek; F.S.R. Fund ("Fonds Spécial pour la Recherche", UCLouvain, Belgium); ARC grant (UCLouvain, Belgium), Grant/Award Number: 18/23-094; Fonds De La Recherche Scientifique - FNRS, Grant/Award Number: 40004991; Fonds Christian Delloye - Fondation Saint-Luc, Belgium

Abstract

The fascial system has gained recognition for its integral role in connecting skin, superficial and deep fasciae, and underlying muscles. However, consensus on its microstructure depending on its topography remains elusive as well as its implications in clinical practices, such as reconstructive surgery and physiotherapy techniques. This study focuses on the iliotibial tract (ITT) implicated in the iliotibial band syndrome. The goal is to describe microstructural characteristics using classical 2D histology and cryogenic contrast-enhanced microcomputed tomography (cryo-CECT) such as the total thickness, number of layers, layer thickness, fibre orientation and tortuosity, according to the specific topography. The total thickness of the ITT varied across topographic regions, with the superior part being on average thicker but non-significantly different from the other regions. The inferior part showed heterogeneity, with the anterior region (AI) being the thinnest and the posterior one (PI) the thickest. The ITT exhibited 1–3 layers, with no significant differences among regions. Most commonly, it consisted of two layers, except for the antero-superior (AS) and antero-middle (AM) regions, which sometimes had only one layer. The posterior regions frequently had 2 or 3 layers, with the PI region having the highest mean (2.7 layers). The intermediate layer was the thickest one, varying from the AI region ($0.368\text{ mm} \pm 0.114$) to the PI region ($0.640\text{ mm} \pm 0.305$). The superficial layer showed regional variability, with the AS region being the thinnest. The deep layer appeared thinner than the superficial one. Fibre orientation analysis indicated that the intermediate layer mainly consisted of oblique longitudinal fibres, orientated downward and forward, while the superficial and deep layers had transversal or oblique transversal fibres. Cryo-CECT 3D observations confirmed these findings, revealing distinct orientations for different layers. Fibre tortuosity exhibited differences based on orientation. Transversal fibres ($>65^\circ$) were significantly less tortuous than longitudinal fibres ($<25^\circ$) and oblique intermediate fibres (25° – 65°), aligning with 3D plot observations. This quantitative study highlights various microstructural characteristics of the ITT, offering insights into its regional variations. The analysis accuracy is increased due to the novel technology of cryo-CECT which emerges as a valuable tool for precise assessment of 3D fibre orientation and tortuosity. These findings contribute to a deeper understanding of the ITT structure, useful in clinical practices, such as reconstructive surgery and physiotherapy, and future research endeavours.

KEYWORDS

2D & 3D histology, fascia lata, fibre orientation, iliotibial band, iliotibial tract, spatial tissue organisation

1 | INTRODUCTION

Whereas the fascial system was not considered by clinicians a few decades ago (Benjamin, 2009; Fede et al., 2018; Stecco et al., 2008, 2011), healthcare givers now recognise the importance of fascial tissues as a system connecting the skin, the superficial and deep fasciae, and the underlying muscles (Benjamin, 2009; Schleip et al., 2019; Stecco et al., 2011).

The iliotibial tract (ITT) or Maissiat's iliotibial band is considered a linking fascia between the gluteal fascia and the fascia lata, below the level of the tensor fasciae latae muscle (Kumka & Bonar, 2012). This lateral thickening of the fascia lata connects the iliac crest to Gerdy's tubercle on the lateral condyle of the tibia (Benjamin, 2009; Fede et al., 2018; Huang et al., 2013; Khaund & Flynn, 2005; Otsuka et al., 2018; Pirri et al., 2021). It is a major insertion for the gluteus maximus muscle (Stecco et al., 2020). No fewer than five distal insertions of the ITT were described, namely the most important insertion being Gerdy's tubercle, followed by the lateral condyle of the femur, the fibular head through the lateral collateral ligament, the linea aspera via the lateral intermuscular septum of the thigh, and the retinaculum of the patella (Flato et al., 2017; Manaster et al., 2008). The ITT is a dense connective tissue whose collagen fibres (Benjamin, 2009; Stecco et al., 2008, 2011) can form different layers with a specific orientation. The proximal part of the ITT is separated into three layers (Huang et al., 2013). The superficial and intermediate ones surround the tensor fasciae latae muscle; the deepest one fuses more distally, coming from the supra-acetabular groove (Flato et al., 2017; Huang et al., 2013). The ITT takes a major part in the hip and knee stabilisation when walking by playing a tendon role for the tensor fasciae latae and gluteus maximus muscles (Benjamin, 2009; Evans, 1979; Otsuka et al., 2018; Stecco et al., 2020).

As with all fasciae, different measurements of the fascia lata were reported depending on the region analysed (Stecco et al., 2011). However, standardized norms are missing, especially for the ITT itself (Pirri et al., 2021). The limited but growing number of studies demonstrates that the measurement of microstructural parameters or the establishment of fascia standards are an emerging research area (Fede et al., 2018). Indeed, the necessity of digging deeper into the comprehension of the fascial microstructure is multiple.

First, the iliotibial band syndrome can cause pain in the iliac tubercle (Flato et al., 2017; Sher et al., 2011), on the greater trochanter (Khoury et al., 2018), or on the lateral condyle of the femur (Benjamin, 2009; Fede et al., 2018; Flato et al., 2017; Khaund & Flynn, 2005; Otsuka et al., 2018; Agridag Ucpinar et al., 2020) in case of irritation or inflammation. This pathology is easily diagnosed but hardly treated. Some physiotherapy techniques use a

deep transverse massage which involves deep, cross-fibre friction applied to the affected area, aiming to break down scar tissue, improve blood flow, and promote healing (Joseph et al., 2012; Stecco et al., 2008). To be effective, the precise orientation of the fibres should then be known by the therapist. Exploring the ITT structure more in detail might also permit to better understand its mechanical role on the lower limb when walking or its associated pathologies (Otsuka et al., 2018; Stecco et al., 2008).

Next, the ITT is largely used in the field of reconstructive surgery. It can be used as a covering flap for duraplasty (Finn et al., 2011), soft tissue defect on the limb (Fox et al., 2014), sealing lung breaches (Molnar et al., 2003), for colpopexy (Bock et al., 2021), as well as for the reconstruction of the acetabular labrum (Philippon et al., 2010), rotator cuff (Matthewson et al., 2020), patellar ligament (Sapino et al., 2019), as interposed material in congenital tarsal coalition resection in adolescents (de Wouters et al., 2014) or even the anterior cruciate ligament (Ferretti et al., 2017). However, the needs for all those reconstructive surgeries are not exactly the same. Sometimes the surgeon might need a thinner flap for tibial coverage or a thicker one to fill a gap. Depending on the number of layers or the fibre orientation, the mechanical characteristics might also be different (Pirri et al., 2021) and could influence the surgical decision on where to harvest the best sample (Stecco et al., 2014).

Since several imaging techniques, like ultrasonography (US) or magnetic resonance imaging (MRI), previously tried to evaluate the microstructure of fasciae with mitigated quality (Fede et al., 2018; Pirri et al., 2021; Agridag Ucpinar et al., 2020), new technologies emerged such as the contrast-enhanced microcomputed tomography (CECT). This technique allows 3D microstructure analysis of biological tissues with limited destruction of the tissue (de Bournonville et al., 2019; Kerckhofs et al., 2018). Since tendons and ligaments have low X-ray attenuation, contrast-enhancing staining agents (CESAs) were developed to enhance discrimination of such tissues (Balint et al., 2016; de Bournonville et al., 2019; Kerckhofs et al., 2018; Sartori et al., 2018; Shearer et al., 2014) but the individual fascicle or fibre visualisation and analysis were still challenging. The newly described cryogenic CECT (cryo-CECT) could allow these microstructural investigations (Maes et al., 2022).

The goal of this paper is to establish quantitative microstructural parameters, specifically for the ITT, using classical 2D histology and cryo-CECT, and to map them according to harvesting regions. Since physiology relies on the close relationship between structure and function (Sieck, 2017; Stecco et al., 2011), this study could allow a deeper understanding of the ITT anatomy, structure, organisation and related function in order to help both the clinical and the research fields. Clinically, this can open the reflexion about the efficacy of physical and manual therapies impacting the fascia, about various deep surgical procedures that require cutting and suturing

fascia, and about the harvesting of fascio-cutaneous flaps for reconstructive surgeries.

2 | METHODS

2.1 | Tissue harvesting and sampling

Human ITT were harvested from deceased donors ($N=5$, four men and one woman; average age = 79 ± 17 years [50–93 years]), received at the Morphology lab of UCLouvain (IRB00008535, Brussels, Belgium). The local ethics committee (Ref 2021-30AOU-356 approved on September 13, 2021) authorized all the experiments.

For classical 2D histology, the ITT was harvested below the level of the tensor fasciae latae muscle extending up to the lateral femoral condyle insertion, following a previously described technique (Manon et al., 2022, 2023, 2024) and then divided into nine regions in order to map and analyse them separately (Figure 1). A specific nomenclature was attributed to each region (PS: postero-superior, MS: medio-superior, AS: antero-superior, PM: postero-middle, MM: medio-middle, AM: antero-middle, PI: postero-inferior, MI: medio-inferior, AI: antero-inferior). Per region, two samples (1×2 cm) were cut ($n=18$ per individual) in order to conduct a systematic analysis. The orientation of the tissue is of uppermost importance to assess the fibre organisation. Into each region, the most superior sample was cut transversally to the main axis of the ITT and the inferior one, longitudinally.

The nine regions allowed to locate and orient the samples in two different directions: the sagittal (postero-, medio-, antero-) and the longitudinal one (superior, middle, and inferior), but the transversal

one was also investigated in order to identify the superficial or the deep side of the ITT. To do so, an ectopic distinct muscle patch was harvested at the same time to be further embedded with each sample to mark the tissue orientation correctly.

For cryo-CECT, random 2 cm^2 samples were harvested from other human ITT ($N=3$ donors, one sample per donor, two men and one woman; average age = 80.6 ± 3.3 years [77–83 years]). A thread served as a landmark to orientate the tissue.

2.2 | Classical 2D histology protocol

A precise protocol was established to keep track of the tissue orientation (Figure 2). The samples were put into cassettes with the muscle patch guide to orientate the tissue in the three planes (Figure 2a). The muscle patch was always positioned on the superior and anterior side of each sample and the superficial side was always facing the operator. All samples contained in their cassettes were fixed in formaldehyde 4% (VWR; 9713.9010) for 48 h before processing for paraffin embedding. The paraffin embedding respected the correct orientation of the samples into their respective moulds (Figure 2b). As previously, the guide was always positioned on the superficial face and the anterior border for transversal samples and on the superficial face and the superior border for longitudinal samples. The anterior border of longitudinal samples as well as the superior part of transversal samples were always facing the cassettes during the embedding phase.

The paraffin embedded blocks were then sliced into $5 \mu\text{m}$ thick sections. These latter were stained with haematoxylin and eosin (H&E) and Masson's trichrome (MTC) before being digitised with a

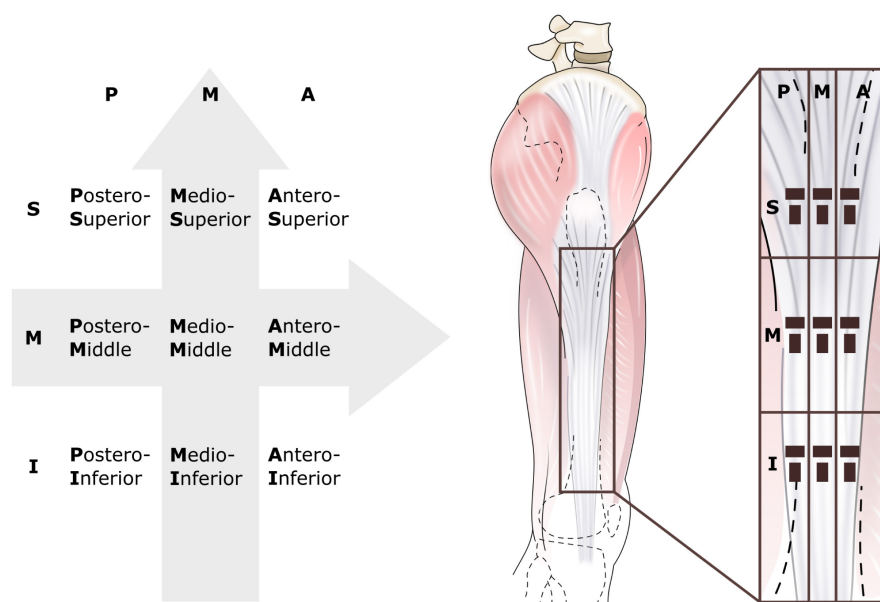


FIGURE 1 Sample harvesting and mapping into nine regions. Each region is called by two capital letters corresponding to the topography in sagittal direction (postero-, medio-, antero-) and longitudinal direction (superior, middle, and inferior). They all contain one sample of 1×2 cm cut transversally and one longitudinally (dark rectangles on the right picture). AI: antero-inferior, AM: antero-middle, AS: antero-superior, MI: medio-inferior, MM: medio-middle, MS: medio-superior, PI: postero-inferior, PM: postero-middle, PS: postero-superior.

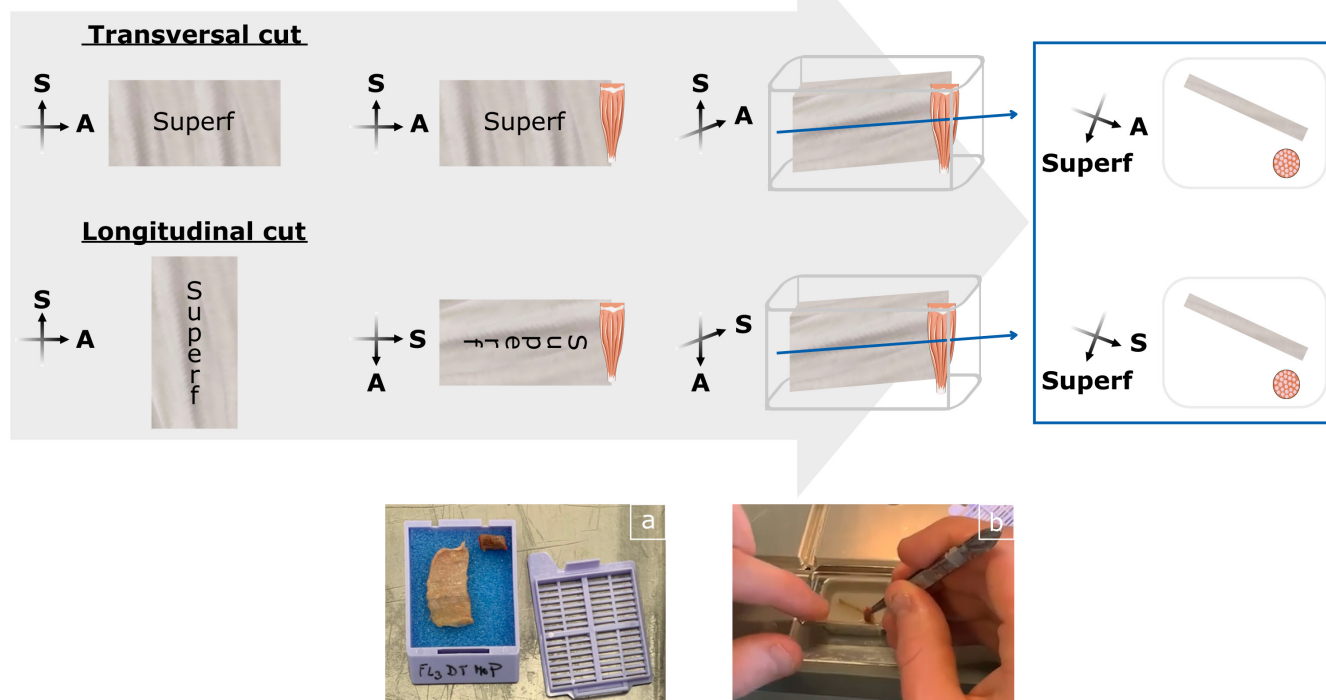


FIGURE 2 Procedure to respect the tissue 3D orientation according to transversal or longitudinal cuts of samples. A muscle guide was associated with each sample to keep a good orientation during the procedure. (a) Correct positioning of the sample and the muscle guide within the cassette. (b) Embedding step respecting the relative position between the sample and the muscle guide. The grey boxes represent the cassettes in which the samples were cut following blue arrows to obtain transversal views through the cassettes shown in the blue rectangle at the right. A: anterior, S: superior, Superf: superficial.

slide scanner (SCN400, Leica Microsystems, Wetzlar, Germany) at a 40-fold magnification and annotated with SlideViewer software (v2.5.0., Budapest, Hungary).

2.3 | Classical 2D histological analyses

A layer is described by all the fibres that have the same orientation. Four different qualitative orientations were defined: pure longitudinal, pure transversal, oblique longitudinal and oblique transversal. The distinction of layers and their associated fibre orientation were qualitatively assessed on stained sections by two independent examiners. The fibre orientation was indicated for each transversal and longitudinal section as well as each region and was finally defined by the largest headcount in one region.

The quantitative analyses included the total ITT thickness, the number of layers, and the layer thicknesses in each region. They were quantified using the SlideViewer software. In order to establish the total ITT thickness, three measures were taken on one section for each sample. For transversal/longitudinal sections, one measure was taken respectively at 0.5 mm from the antero-/superior extremity, one in the medio-/middle of the samples and one at 0.5 mm from the postero-/inferior extremity. The mean of the three measures was also averaged by the longitudinal and the transversal sections, and the mean of the five donors to best

determine the total average ITT thickness. The number of layers and their respective thickness were assessed by the mean data in longitudinal and transversal sections for each region, followed by the average of the five donors. All quantitative data were expressed as the mean and SDs. Heatmaps were used in order to illustrate quantitative outcomes related to the appropriate region and to compare regions to each other.

2.4 | Cryogenic contrast-enhanced computed tomography and microstructural analysis

All the 2cm² samples were stained with 1:2 hafnium(IV)-substituted Wells-Dawson polyoxometalate (Hf-WD POM, 3.5% [3.5mg Hf-WD POM/mL of phosphate buffered saline solution, 25:1 solution to sample volume ratio]) on a shaker plate for 15 days, following previously described protocol (Maes et al., 2022). The samples were then packed in small plastic bags, snap-frozen by immersion in cold isopentane (−78°C, VWR, 24872.323) for 2 min, and stored at −80°C. Specimens were imaged using a Phoenix NanoTom M (GE Measurement and Control Solutions; Germany) inside an *in-situ* cryo-stage at −40°C (Maes et al., 2022) (parameters in Table 1).

3D fibre analyses were performed using Avizo software (2022.1, ThermoFisher Scientific, France). Successive processing

TABLE 1 Cryo-CECT acquisition and module parameters.

Acquisition parameters		Cylinder correlation parameters		Trace correlation lines parameters	
Voxel size (μm)	3	Cylinder length (μm)	200	Minimum seed correlation	40
Source voltage (kV)	110	Angular sampling	5	Minimum continuation quality	30
Tube current (μA)	114	Mask cylinder radius (μm)	15	Direction coefficient	0.3
Exposure time (ms)	500	Outer cylinder radius (μm)	14	Minimum distance (μm)	28
Number of images	5200	Inner cylinder radius (μm)	0	Minimum length (μm)	200
Average ^a	6			Search cone length (μm)	200
Skip ^a	1			Search cone angle	37
Scan time (min)	317			Minimum step size (μm)	10

^aInformation about those parameters were previously given in the literature (Maes et al., 2022).

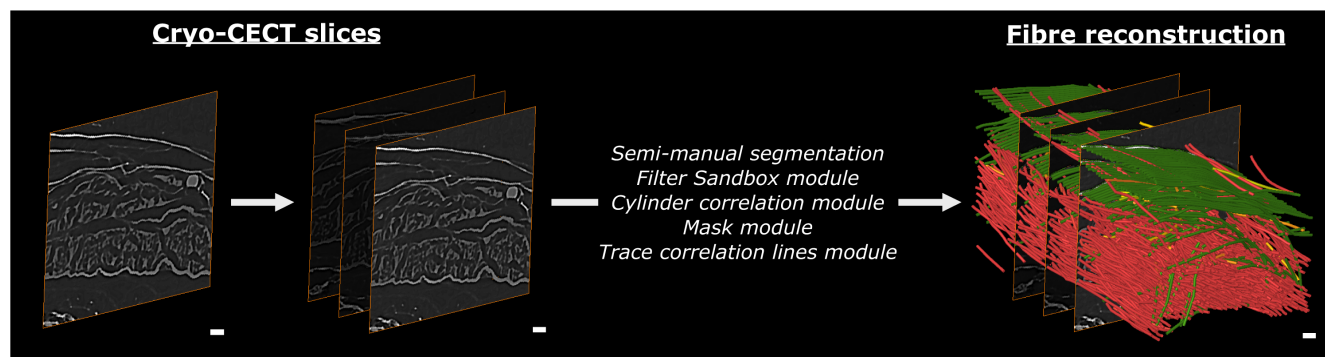


FIGURE 3 Cryogenic contrast-enhanced computed tomography (cryo-CECT) of the iliotibial tract from slices to fibre 3D reconstruction using different modules from Avizo software (2022.1, ThermoFisher Scientific, France). Scale bars: 100 μm .

modules were applied following the *XFiber protocol* previously described (Blanc & Westerberger, 2017, Figure 3). First, *Extract Subvolume* module permitted to analyse exactly the same volume ($0.9 \times 0.9 \times 1.8 \text{ mm}$) in each sample, centred on a random ITT area. The main axis was orientated following the main limb axis. The fibres were segmented and extracted using *semi-manual segmentation tools* (*magic wand*, *brush*, ...) and the *Filter Sandbox* module thanks to their grey values. The fibre analysis included *Cylinder correlation* module, *Mask* module, and *Trace Correlation Lines* module. All the detailed acquisitions and module parameters are resumed in Table 1.

A *Spatial Graph View* module allowed to reconstruct the 3D organisation of the fibres following the theta orientation (θ angle formed with the Z-axis, aligned with the main limb axis, and ranging from 0° [longitudinal fibres] to 90° [transversal fibres]). A *Plot 3D orientation* module plotted and schematised the density of different fibre orientations.

The *Spatial Graph Statistics* module quantified the tortuosity of the reconstructed fibres. Tortuosity is independent of fibre orientation angles, pertains to the microscopic intrinsic waviness of an individual fibre, and is defined by the ratio of curved length on the chord length of a fibre (ratio starting from 1 = no tortuosity at all, and increasing progressively with the tortuosity).

2.5 | Statistical analysis

All statistics were made using GraphPad Prism software (v.8.0.1, San Diego, CA; United States). Independent quantitative values were first analysed by descriptive analyses, boxplots, and histograms to evaluate their distribution. Regarding orientation data highlighting two main almost orthogonal orientations, the fibres were divided into three groups according to the θ angle (Group 1: $<25^\circ$, group 2: between 25° and 65° , group 3: $>65^\circ$) in order to discriminate the tortuosity.

The normal distribution of all quantitative data was evaluated by QQ plots and Shapiro-Wilk tests. Since no distribution was normal, only nonparametric Kruskal-Wallis tests were used, with a Dunn's correction for multiple comparisons. Data are expressed as the mean $\pm$ SD. The threshold for significance is set at 0.05.

3 | RESULTS

3.1 | ITT thickness

The total ITT thickness varied from one region to another (Figure 4). The superior part of the ITT was thicker ($\text{PS} = 0.653 \text{ mm} \pm 0.173$, $\text{MS} = 0.654 \text{ mm} \pm 0.160$, $\text{AS} = 0.690 \text{ mm} \pm 0.245$) than others

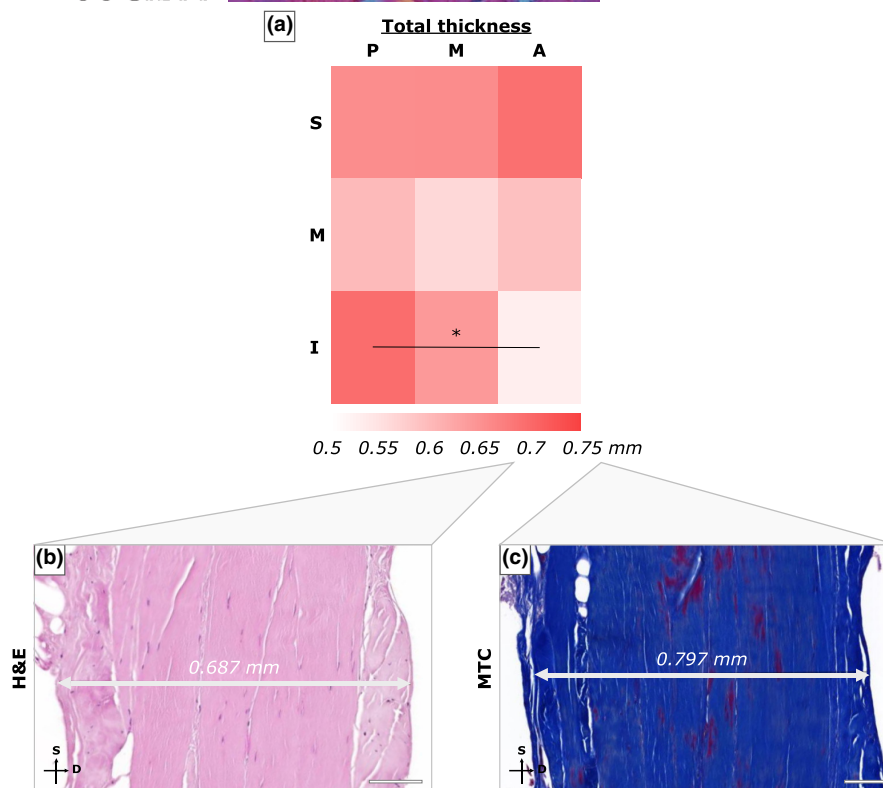


FIGURE 4 Total thickness depends on the region. (a) Heatmap of iliotibial tract total thickness (mm). Data represent multiple consecutive averages of three measurements, two section orientations, and five donors. Horizontal = P: postero-, M: medio-, A: antero-, Vertical = S: superior, M: middle, I: inferior. * $p < 0.05$. (b) Longitudinal cut of a sample from the PS region stained with H&E. (c) Longitudinal cut of a sample from the MS region stained with MTC. H&E: haematoxylin and eosin, MTC: Masson's trichrome; S: superior, D: deep. White scale bar: 100 μm.

(MP = $0.597 \text{ mm} \pm 0.158$, MM = $0.560 \text{ mm} \pm 0.173$, AM = $0.590 \text{ mm} \pm 0.247$, MI = $0.638 \text{ mm} \pm 0.227$) although not significantly, and its thickness was the most uniform. On the contrary, the inferior part was the most heterogeneous with the thinnest anterior region (AI = $0.527 \text{ mm} \pm 0.182$) and the thickest posterior one (PI = $0.693 \text{ mm} \pm 0.206$), which were statistically different ($p = 0.016$).

3.2 | Number of layers

The heatmap illustrated the average number of layers per region (Figure 5a) and the bubble chart displayed their prevalence (Figure 5b). The number of layers varied from one to a maximum of three but was never significantly different among regions. In most cases, the ITT was composed of two layers (Figure 5c,e). The AS ($1.9 \text{ layers} \pm 1.0$) and the AM ($1.8 \text{ layers} \pm 0.7$) regions were the only ones that sometimes contained only one layer. The posterior parts of the ITT are more frequently composed of two or three layers (Figure 5d,f) with the maximum mean for the PI region ($2.7 \text{ layers} \pm 0.5$). However, the most constant region was the PM one with two constant layers in 80% of cases.

3.3 | Layer thickness

After being measured on histological sections (Figure 6a,b), the different heatmaps showed the thickness depending on each layer and each region (Figure 6c). The predominant layer is obviously the intermediate one with thicknesses ranging from $0.368 \text{ mm} \pm 0.114$ in the AI region to $0.640 \text{ mm} \pm 0.305$ in the PI one. Regarding the superficial layer, it was the thinnest in the AS region ($0.053 \text{ mm} \pm 0.034$) while the intermediate layer was one of the thickest in this same region ($0.606 \text{ mm} \pm 0.319$). The rest of the superficial layer was homogeneous varying from $0.110 \text{ mm} \pm 0.029$ in the AI region to $0.154 \text{ mm} \pm 0.103$ in the PS one. The deep layer looked thinner than the superficial one with thicknesses comprised between $0.058 \text{ mm} \pm 0.048$ for the MM region and $0.201 \text{ mm} \pm 0.124$ for the MS region.

The boxplots indicated the data dispersion and homogeneity depending on the region (Figure 7). In the superior regions, the intermediate layer is the most variable while the superficial one is the most constant. The middle regions had the most predictable uniform thickness, whether for superficial, intermediate, or deep layer values (no significant differences, $p > 0.05$). The most variable region in the ITT is the inferior part with a high fluctuation in the intermediate

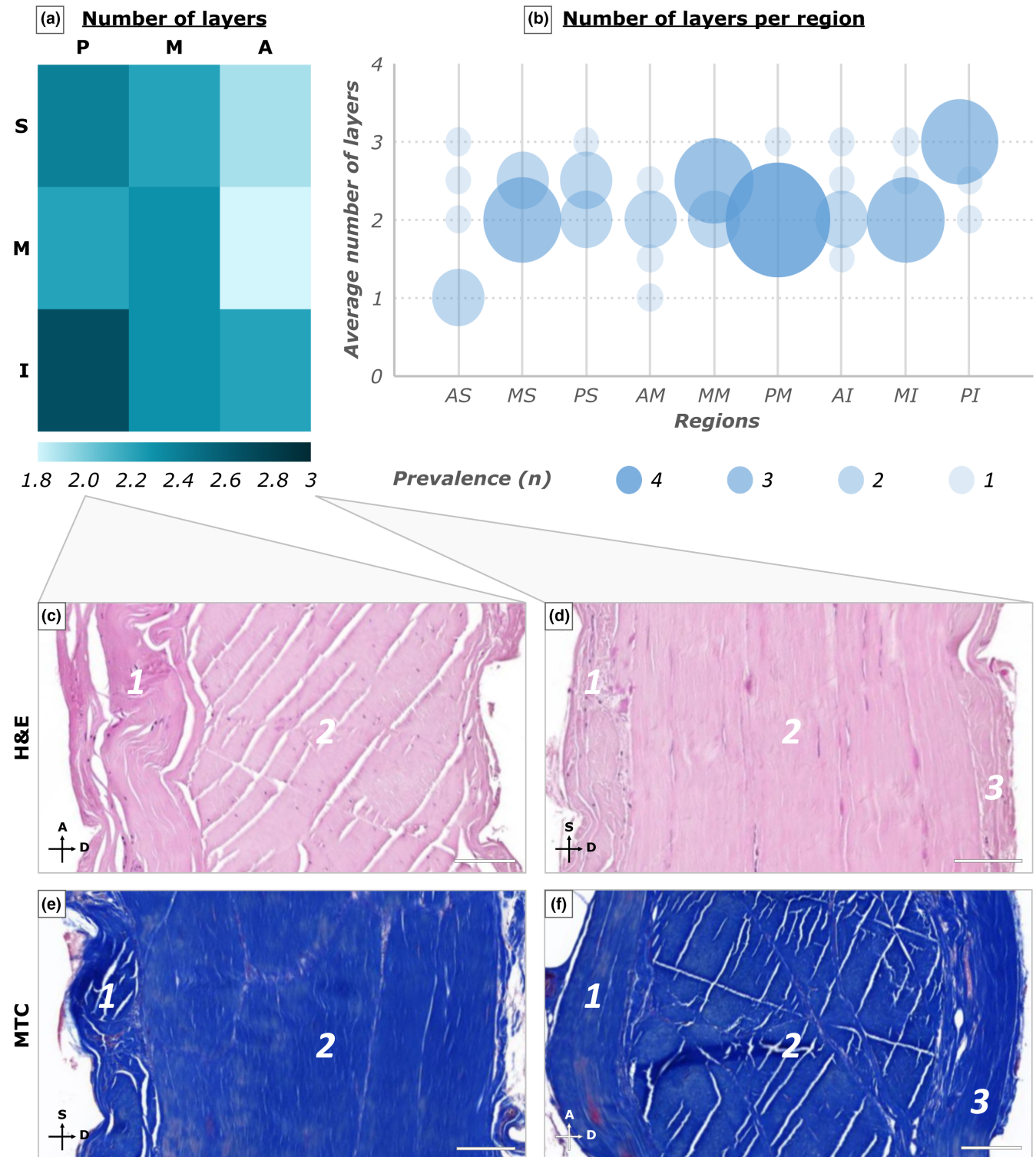


FIGURE 5 Number of layers according to the region, illustrated by (a) the heatmap of the iliotibial tract, (b) the bubble chart, and (c–f) histology. Horizontal = P: postero-, M: medio-, A: antero-; Vertical = S: superior, M: middle, I: inferior. The bubble chart allows us to identify the number of layers per region associated with their respective prevalence illustrated by the width of the bubble. (c) Transversal cut of a sample from the AI region stained with H&E. (d) Longitudinal cut of a sample from the MI region stained with H&E. (e) Longitudinal cut of a sample from the AI region stained with MTC. (f) Transversal cut of a sample from the PI region stained with MTC. H&E: haematoxylin and eosin, MTC: Masson's trichrome, A: anterior, S: superior, D: deep. White scale bar: 100 μ m.

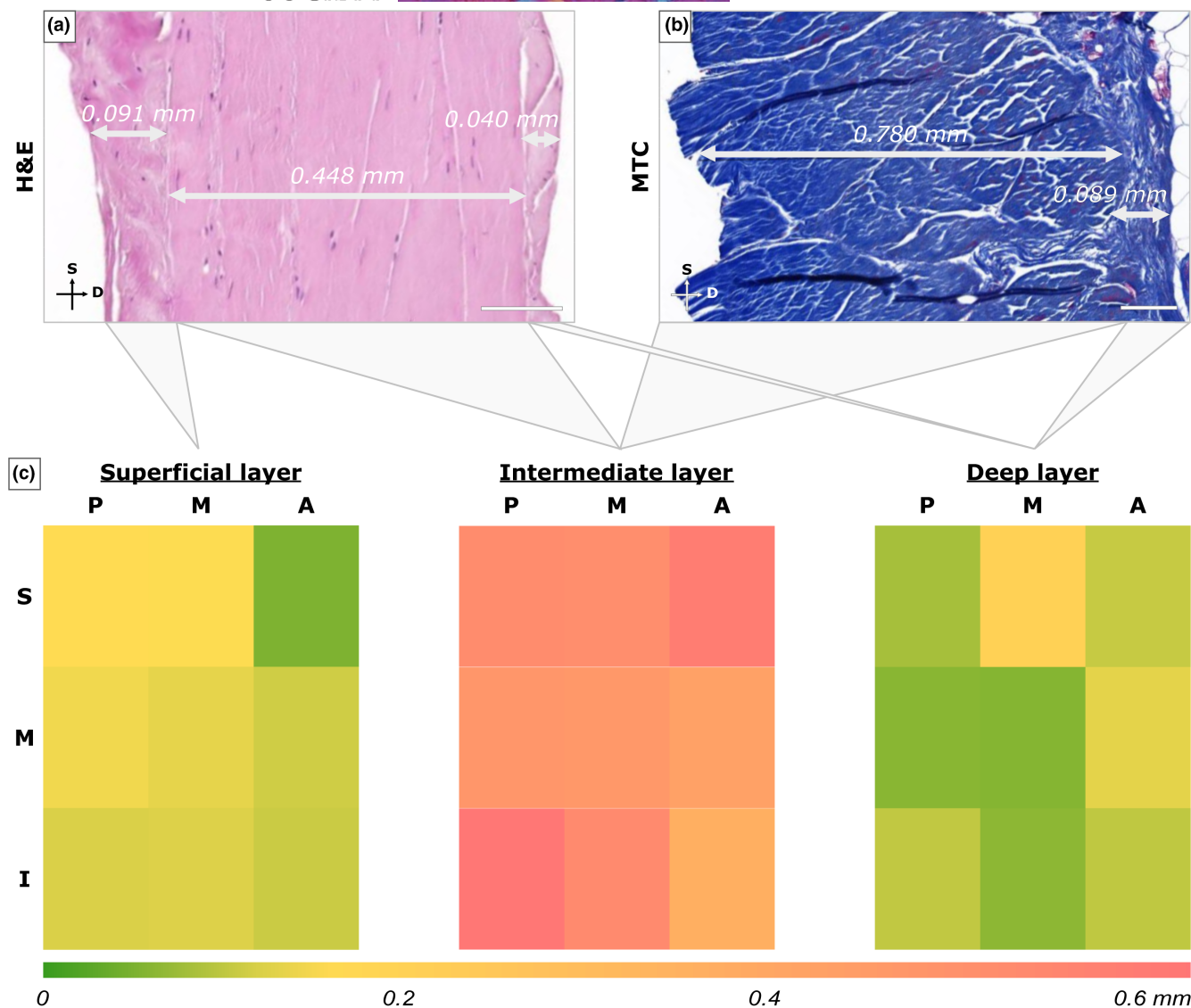


FIGURE 6 Layer thickness depending on the regions. (a) Longitudinal cut of a sample from the PS region stained with H&E. (b) Longitudinal cut of a sample from the PI region stained with MTC. D; deep; H&E, haematoxylin and eosin; MTC, Masson's trichrome; S, superior. White scale bar: 100 μ m. (c) Heatmap of each layer thickness according to the nine regions. Data represent multiple consecutive averages of three measurements; two section orientations; and five donors. Horizontal = P: postero-; M: medio-; A: antero-; Vertical = S: superior; M: middle; I: inferior.

layer thickness. The maximal thickness found among the whole ITT is in the PI region (1.2 mm).

3.4 | Fibre orientation

The main fibre orientation is represented for each layer of each region in the Figure 8a. The intermediate layer is mostly composed of oblique longitudinal fibres (Figure 8c,g) while the superficial and the deep layers are mostly composed of transversal (Figure 8b,f) or oblique transversal fibres (Figure 8d,h). The deep layer is made of more strict transversal fibres than the superficial one.

Cryo-CECT also allowed to examine the fibre orientation in full 3D (Figure 9). Among the three subvolumes analysed, one has only one layer (ITT 3), one has two main layers (ITT 1) and the last one has three layers (ITT 2). This 3D representation also shows that the main thick intermediate layer is composed of longitudinal fibres (red) while the thin superficial and deep layers are mainly composed of transversal fibres (green). The 3D plots schematise the θ angle between the fibres. When a minimal of two layers are present, two main orientations emerge, both in a sagittal plane, and with an interlayer angle from 80.11° to 84.78°. The height of the bars shows that green transversal fibres are more numerous for one same single orientation. This means that they are more parallel to each other

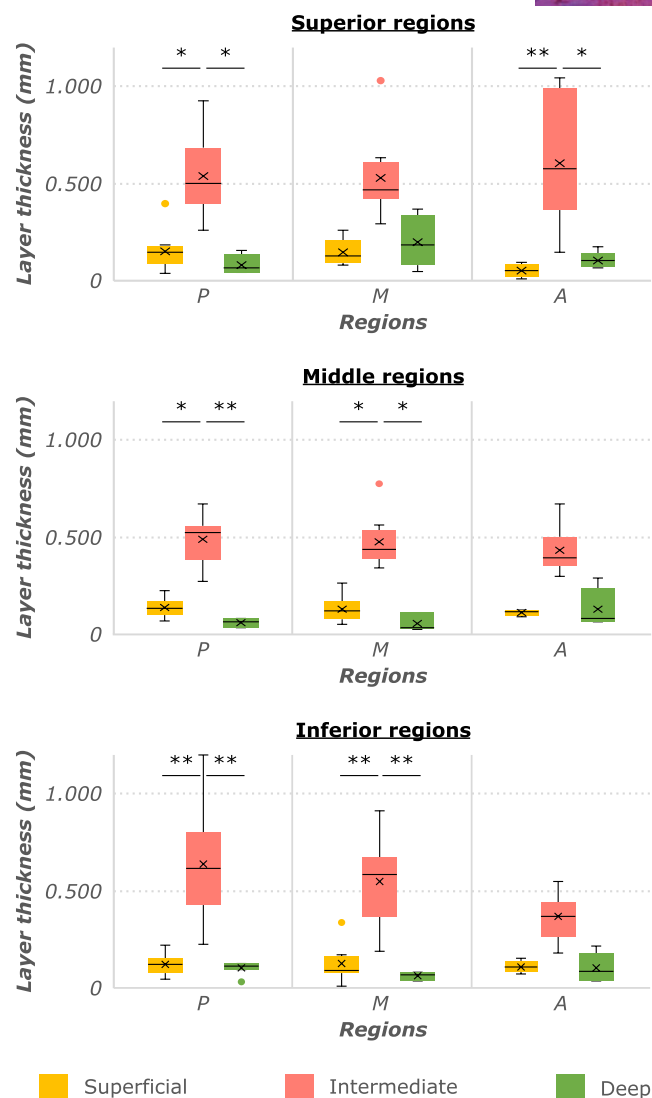


FIGURE 7 Boxplots of the layer thickness depending on respective regions and each layer. P: postero-, M: medio-, A: antero-, x: mean. * $p < 0.05$, ** $p < 0.01$.

than the red longitudinal fibres that are more spread around an axis almost perpendicular to the latter.

3.5 | Fibre tortuosity

While the distribution of θ orientation values follows a U-shape with highest prevalences at the extreme values (data not shown), the fibre tortuosity does not follow the same distribution. These data follow a decreasing exponential prevalence starting from the ratio of 1, meaning no tortuosity at all, with the curved length equal to the chord length. The transversal fibres ($>65^\circ$) were significantly less tortuous (1.019 ± 0.047) than longitudinal fibres ($<25^\circ$, 1.024 ± 0.027) and oblique intermediate fibres (25° – 65° , 1.039 ± 0.070 , $p < 0.0001$). These observations are in line with the 3D fibre reconstructions.

4 | DISCUSSION

This study demonstrated specific differences in fibre organisation between anatomical regions of the ITT, i.e. a higher thickness of the intermediate layer, especially in the superior and the postero-inferior regions, a higher number of layers of the PI region, and an almost perpendicular orientation of 2 consecutive layers.

4.1 | Total thickness

The superior part of the ITT is the thickest and the most homogeneous part, where the different layers of fascia converge after enveloping the tensor fasciae latae muscle. Its posterior region is reinforced by tendinous fibres coming from the gluteal aponeurosis covering the gluteus maximus (Huang et al., 2013), confirmed by our outcomes. As previously mentioned (Pirri et al., 2021; Stecco et al., 2008, 2015), the fascial thickness tends to decrease in the middle region and to increase again distally around the knee. In our study, the thickest region is the PI one, which corresponds to the gathering of fibres before insertion on Gerdy's tubercle. However, the comparison with the literature is not easy, since different anatomical regions into the whole fascia lata were screened (Fede et al., 2018; Pirri et al., 2021) but never specific regions into the ITT itself, nor the different layers insofar as this is possible. The thickness is also often evaluated precisely at the level of the lateral condyle *in vivo* (Goh et al., 2003; Gyaran et al., 2011; Kim & Yoon, 2012; Agridag Ucpinar et al., 2020); which is one of the thickest zones where fibres gather towards Gerdy's tubercle.

While the average total thickness of a fascia is around 1 mm (Stecco et al., 2011), different tools permit to measure it. Using the US, the ITT thickness was measured from $1.1 \text{ mm} \pm 0.2$ to $1.95 \text{ mm} \pm 0.3$ (Goh et al., 2003; Gyaran et al., 2011; Kim & Yoon, 2012; Pirri et al., 2021; Wang et al., 2008), which is almost double our data. *Ex vivo* tissue processing for classical 2D histology is known to induce some fibre shrinkage and therefore to diminish the data compared to *in vivo* US (Pirri et al., 2021) or MRI measurements (Agridag Ucpinar et al., 2020). Those authors were not able to highlight the relationship between the thickness and the age, sex, weight, or side dominance (Gyaran et al., 2011). Their population was also younger (18–68 years) than our old one (50–93 years). Additionally, the MRI is not so accessible, and US is operator dependent. The intra- and inter-operator reliability can vary substantially (Fede et al., 2018). The probe position may influence the outcomes because of tissue anisotropy and dynamic variation of the measures. In living patients, the interstitial space between fascial layers can vary and may affect the measured thickness. Factors such as hydration status, tissue health, and even the dynamic nature of tissues during movement might influence the appearance and measurements obtained via imaging techniques like US. As a general example, the deep fascia sometimes seems adherent to the superficial one, thus leading to wrong measurements of both fasciae (Fede et al., 2018). The US and MRI are very useful to participate in the diagnosis and the treatment follow-up as non-invasive techniques (Fede et al., 2018; Agridag

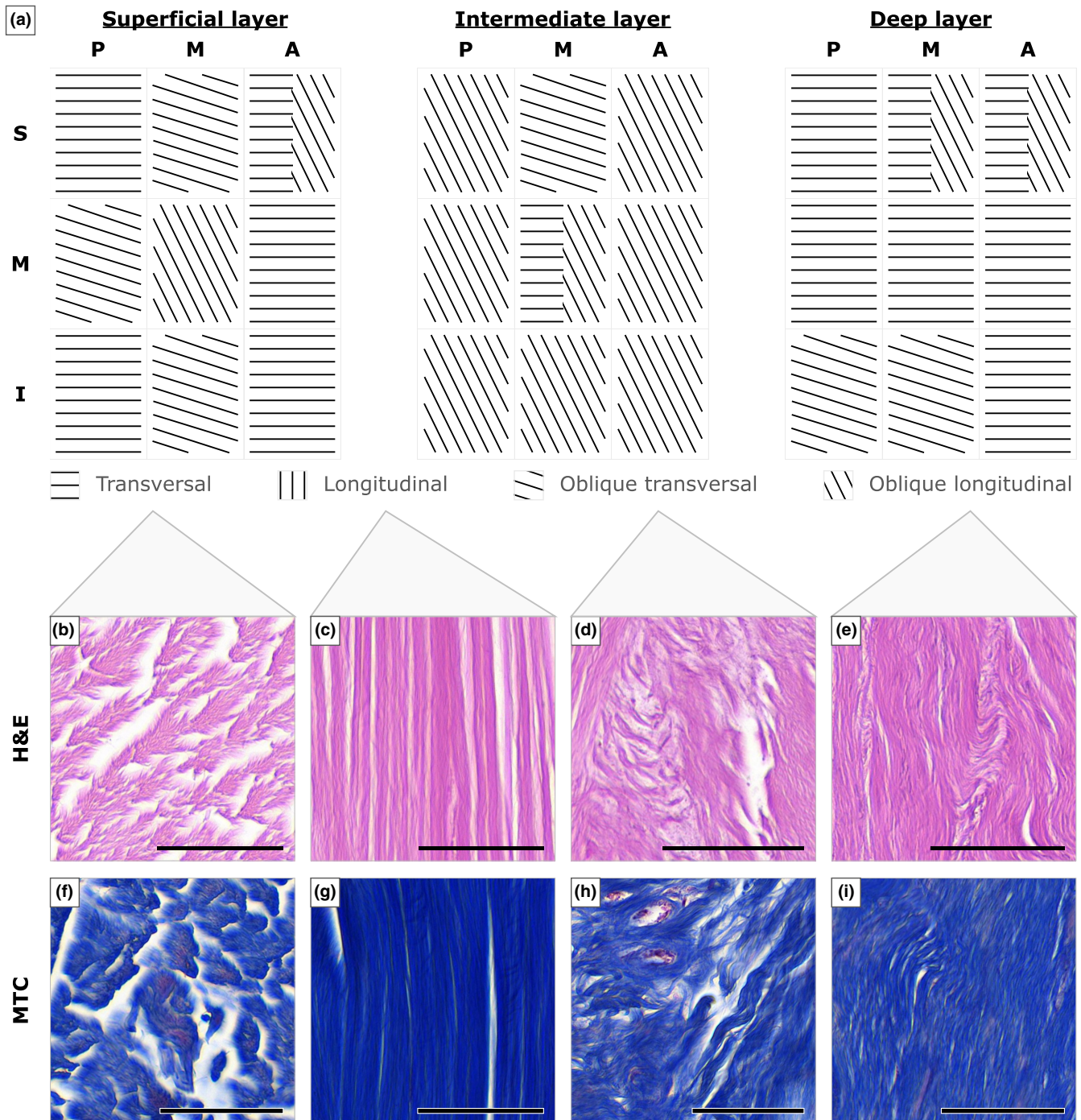


FIGURE 8 Fibre orientation analyses using histology. (a) Fibre orientation map according to each region from each layer in the ITT samples analysed with classical histology. Horizontal=P: postero-, M: medio-, A: antero-, Vertical=S: superior, M: middle, I: inferior. (b) Transversal cut of a sample from the PI region stained with H&E. (c) Longitudinal cut of a sample from the PI region stained with H&E. (d) Longitudinal cut of a sample from the PI region stained with H&E. (e) Longitudinal cut of a sample from the AI region stained with H&E. (f) Longitudinal cut of a sample from the PI region stained with MTC. (g) Longitudinal cut of a sample from the MM region stained with MTC. (h) Longitudinal cut of a sample from the PI region stained with MTC. (i) Longitudinal cut of a sample from the PM region stained with MTC. H&E: Haematoxylin and eosin, MTC: Masson's trichrome. Black scale bar: 50µm.

Ucpinar et al., 2020) but consequently, not the best tools to establish thickness measurements. In addition, measurements taken from different sources lead to inaccurate and non comparable data (Pirri et al., 2021). For classical 2D histology, another point to consider is the impact of the cutting orientation on histological slices. If the

sample is not strictly perpendicular to the cutting plane, the thickness might be falsely increased. Anatomical dissection remains the historical reference, but classical 2D histology can still help to discern microscopic structures such as layer and fibre orientation. Knowing that, our results closely approximate the anatomically measured ITT

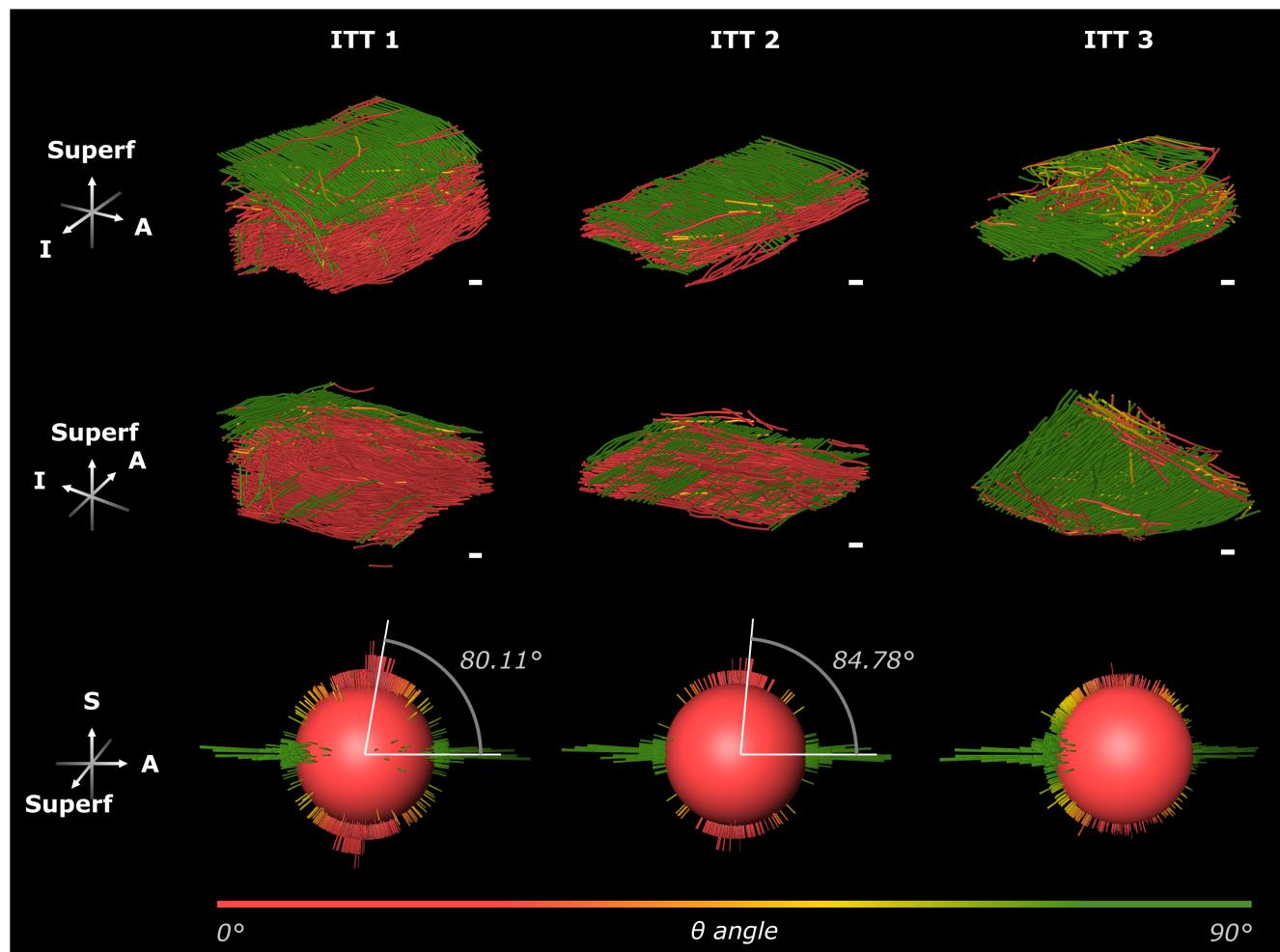


FIGURE 9 Fibre orientation analyses using the cryo-CECT. 3D fibre reconstruction of ITT subvolumes coming from three different donors (ITT 1, ITT 2, ITT 3), and 3D plots schematising the θ orientation of fibres. The fibre colour is associated with the θ angle (red 0° =longitudinal fibres – green 90° =transversal fibres). The height of the bars expresses the fibre count. White scale bar: $100\mu\text{m}$. Colormap scale bar: θ angle (orientation) of the fibres. ITT: iliotibial tract, θ : theta angle. Superf: superficial, A: anterior, S: superior, I: inferior.

thickness (0.8–0.9 mm) obtained using callipers on cadaveric specimens (Bonaldi et al., 2023; Otsuka et al., 2018).

4.2 | Number of layers and layer thickness

All these observations bring more details and knowledge than the single mean layer thickness of about $115\text{--}277\mu\text{m}$ described in the literature (Benetazzo et al., 2011; Stecco et al., 2011).

The ITT is a fusion of three collagen tissue layers including superficial, intermediate and deep layers (Huang et al., 2013), each one separated by a light stratum of loose connective tissue (Fede et al., 2018; Pirri et al., 2021; Stecco et al., 2008). This organisation is essential for the inter-layer and tissue sliding (Stecco et al., 2008, 2011). However, no study to our knowledge linked the number of layers with the specific regions of the ITT. Our results showed that the mean number of layers is above 2 in most of the regions, excluding the AS and the AM ones. If only the longitudinal orientation is considered, the inferior level is the only one that always contains

an average of 2 layers, regardless of the region in the sagittal plane. This could reflect a more complex organisation of the distal ITT. This difference could be related to the complexity of the ligamentous and tendinous network around the knee and the Gerdy's tubercle (Pirri et al., 2021; Stecco et al., 2015). The PI region is the area with the highest average number of layers, the most frequent three layers, as well as the largest total thickness. On the contrary, the AS region is also a thick region but generally counts only one layer. This observation can corroborate the fusion of the thin superficial and deep layers with the intermediate one. The high thickness of these two regions is mostly explained by the thickness of the mean intermediate layer. Indeed, when AS region contains seldom one superficial layer, the latter is the thinnest one. In addition, the AI region with the thinnest intermediate layer corresponds to the thinnest total thickness, and the PI region with the thickest intermediate layer corresponds to the thickest total thickness. The high thickness fluctuation of the inferior part of the ITT also matches the fluctuation of the intermediate layer thickness. In all the regions, this intermediate layer was always the thickest, and in the middle when three layers existed. When only two

layers existed, this thick layer was more often found on the deep side than on the superficial side. Contrariwise, when three layers existed, the deep one was usually thinner than the superficial one. Actually, the fascia has the ability to adapt to changes in muscular volume in a horizontal direction, which could explain the thinner thickness in the middle of the deep layer (Stecco et al., 2014).

4.3 | Fibre orientation

The variability of fibre orientation was previously established, depending on the harvested site of the fascia lata (Otsuka et al., 2018; Stecco et al., 2008). However, the different regions in the ITT itself were not discriminated. While linking fasciae are described as dense unidirectional collagen tissue (Kumka & Bonar, 2012), our study demonstrated that different layers of a single linking fascia such as the ITT can be composed of parallel ordered collagen fibres with several different orientations. The majority of fibres of superficial and deep layers are transversal or oblique transversal while they are oblique longitudinal for the intermediate layer. The interlayer angle approached an orthogonal plane, described in the literature from 75° to 80° (Benetazzo et al., 2011; Bonaldi et al., 2023).

The transversal deep layer surrounds the thigh muscles and can be confounded with the epimysial fascia (Stecco et al., 2008). The superficial layer contains the superficial fibres of the gluteus maximus that are oblique transversal in the superior region, converging downward and forward. Concerning the inferior region, fibrous expansions coming from the ITT form an arc covering the patella, especially for the lateral retinaculum (Flato et al., 2017; Kaplan, 1957; Merican & Amis, 2008). This could explain the predominance of arciform or transversal fibre orientation in the superficial and deep layers, respectively, in this region (Merican & Amis, 2008).

Structure is associated with function (Benjamin, 2009; Otsuka et al., 2018; Ottani et al., 2001). The transversal and circumferential layers could be involved in muscle containment such as an exoskeleton protecting underlying tissues and organs, and in proprioception (Benjamin, 2009; Stecco et al., 2008, 2011). This description was proposed for compression fasciae that play a role in compartment pressure, its venous return, and muscular force distribution (Benjamin, 2009; Kumka & Bonar, 2012; Stecco et al., 2011, 2014). Otherwise, this specification is well known to be harmful during excessive pressure in compartment syndrome of lower limbs (Stecco et al., 2014). Interestingly, the transversal stiffness and Young's modulus were statistically the lowest for the ITT compared to the rest of the fascia lata (Otsuka et al., 2018). Fasciae are known as anisotropic (Bonaldi et al., 2023; Otsuka et al., 2018). While the majority of fibres are orientated oblique longitudinally in the intermediate layer, the stiffness and tensile forces are higher in an oblique vertical direction (Bonaldi et al., 2023; Manon et al., 2022; Otsuka et al., 2018; Stecco et al., 2014). In surgical application, this is potentially important, as a graft required to withstand stresses over an extended length must be cut along this longitudinal axis obliquely downwards and forwards, rather than strictly longitudinally, to prevent shear forces that could

cause graft rupture. This layer is implicated into the main function of the ITT, namely the force transmission through the lower limb and walk stability (Otsuka et al., 2018; Ottani et al., 2001; Stecco et al., 2008, 2011, 2014). In this study, histological measurements were taken without tension, thus not replicating weight-bearing force transmission conditions. Tension might potentially influence fibre tortuosity and layer thickness, while rigor mortis could also slightly affect fibre organization. Further studies are thus warranted to investigate the precise relationship between tissue structure and organization under tension transmission conditions. A structure modification, such as an adhesion point or cicatricial scars in a fascia after trauma or surgery, can alter the function, *i.e.* tensile forces lines leading to changes in muscle glide (Stecco et al., 2011). The presence and function of fasciocytes in between the layer could also influence fascia gliding and might cause subsequent myofascial pain (Stecco et al., 2018). A stiffer ITT could also increase mechanical loading to the knee leading to osteoarthritis (Yagi et al., 2023). Moreover some surgical techniques involve a structure change to improve the function, such as an ITT release in ITT syndrome (Benjamin, 2009; Inoue et al., 2018). In the opposite, the function can also drive the structure. For example, the stiffness and the thickness of the ITT can be modified according to postural positions or repeated dynamic friction, sometimes leading to ITT syndrome (Tateuchi et al., 2016). The thickness of the ITT is then related to the pain in conditions such as patellofemoral pain (de Albuquerque et al., 2022), lateral knee pain (Agridag Ucpinar et al., 2020), or greater trochanteric pain syndrome (Khoury et al., 2018). Donor characteristics such as weight, height, or femur length could also be considered in relation to the structure and function of the ITT.

4.4 | New techniques

For less-destructive evaluation of the microstructure of the ITT, clinical CT is not the imaging of choice for soft tissue, and US and MRI have too low spatial resolution (Dejea et al., 2020; Fede et al., 2018). Both imaging techniques are not efficient enough to discriminate each single fibres and analyse their characteristics. For research purposes or to establish precise norms, cryo-CECT could be a good compromise to associate spatial resolution and fibre visualisation (Maes et al., 2022). Its precision is sufficient to characterize fascicles and individual fibres, without shrinking the tissue. Freezing can create microcrystals of ice that can spread the fibres slightly apart, which must be considered when analysing the microstructure, even if snap-freezing reduces this phenomenon. Even if this technique cannot be applied *in vivo*, it allows more quantitative measurements than possible with previous techniques. For example, the 3D fibre orientation and the tortuosity can be quantified. Even if all ratios were very close to 1 (no tortuosity), this study showed that the longitudinal fibres, mainly localised in the intermediate layer, and oblique fibres are significantly more tortuous than transversal fibres of the deep/superficial layers. This newly developed technology is doubtless to be considered for further assessment on more numerous samples.

5 | CONCLUSION

Given the interdependence of function and structure, establishing precise standards is imperative for a comprehensive understanding of tissue dynamics. Advanced techniques for assessing the morphological characteristics of the fascia not only reveal its functional significance but also hold promise for refining therapeutic strategies. This understanding could pave the way for the creation or adaptation of more effective therapeutic interventions such as in the treatment of ITT syndrome, in order to enhance the coordination of fascial function and contractions of underlying muscles, and to harvest tissue where the structure and the function are the most adapted to the surgical transplant site.

While our study offers valuable insights, it is important to note that further studies on larger sample size and with younger persons are needed to determine strict norms. Looking ahead, future research could explore the implications of our findings for conditions like ITT syndrome and optimize the judicious use of an ITT graft in reconstructive procedures.

AUTHOR CONTRIBUTIONS

All authors agree to be accountable for the content of the work. Julie Manon: Concept/design, Acquisition of data, Data analysis/interpretation, Drafting of the manuscript. Laurie Gallant: Acquisition of data, Data analysis/interpretation. Paul Gerard: Acquisition of data, Data analysis/interpretation. Lies Fieve: Acquisition of data, Critical revision of the manuscript. Pierre Schneidewind: Acquisition of data, Data analysis. Grzegorz Pyka: Concept/design, Supervision. Greet Kerckhofs: Supervision, Critical revision of the manuscript. Benoît Lengele: Supervision, Critical revision of the manuscript. Olivier Cornu: Funding acquisition, Supervision, Critical revision of the manuscript. Catherine Behets: Concept/design, Supervision, Data interpretation, Critical revision of the manuscript.

ACKNOWLEDGEMENTS

Figure 2 was partly generated using templates provided by Servier Medical Art (<http://smart.servier.com/> downloaded on the 14 July 2022), licensed under a Creative Commons Attribution 3.0 unported license (<https://creativecommons.org/licenses/by/3.0/>, accessed on the 14 July 2022).

The authors would like to deeply thank the people who have been involved in this work, either directly or indirectly, or who have made it possible to carry it out, including Christine de Ville de Goyet, Eléonore Gillerot, Delia Hoffmann, Caroline Bouzin, Michèle De Beukelaer, Aurélie Daumerie, Marc de Bournonville and all members of the 2IP platform (UCLouvain, Brussels, Belgium), Bernard Caelen, Nicolas Charles-Pirlot, Michelle Cougnon and Walter Hudders. The X-ray microCT images were generated at UCLouvain (supported by the ConstrastTeam, headed by Greet Kerckhofs). Last but not least, the authors would explicitly thank the donors of the cadavers used in this study, as well as their families. This study was made possible thanks to the generous contributions from the Fonds Christian Delloye, for which the authors express their heartfelt gratitude.

Additionally, support was provided by the F.S.R. Fund ("Fonds Spécial pour la Recherche", UCLouvain, Belgium) and F.N.R.S. Aspirant Fund awarded to Julie Manon ("Fonds National de la Recherche Scientifique", Application ID 40004991, Belgium). Benoît Lengele, Greet Kerckhofs and Grzegorz Pyka acknowledge the support from an ARC grant (ARC 18/23-094, UCLouvain, Belgium) and the SBO project of the Research Foundation Flanders (FWO, grant S007219N), respectively.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

All data analysed during the study are included in this published article. The complete original datasets generated during the current study are available from the corresponding author on reasonable request.

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How to cite this article: Manon, J., Gallant, L., Gérard, P., Fievé, L., Schneidewind, P., Pyka, G. et al. (2024) 2D and 3D microstructural analysis of the iliotibial tract. *Journal of Anatomy*, 00, 1–15. Available from: <https://doi.org/10.1111/joa.14125>