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Histological Analysis of the Structural Composition of Ankle Ligaments

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Abstract

Background: Various ankle ligaments have different structural composition. The aim of this study was to analyze the morphological structure of ankle ligaments to further understand their function in ankle stability.

Methods: One hundred forty ligaments from 10 fresh-frozen cadaver ankle joints were dissected: the calcaneofibular, anterior, and posterior talofibular ligaments; the inferior extensor retinaculum, the talocalcaneal oblique ligament, the canalis tarsi ligament; the deltoid ligament; and the anterior tibiofibular ligament. Hematoxylin-eosin and Elastica van Gieson stains were used for determination of tissue morphology.

Results: Three different morphological compositions were identified: dense, mixed, and interlaced compositions. Densely packed ligaments, characterized by parallel bundles of collagen, were primarily seen in the lateral region, the canalis tarsi, and the anterior tibiofibular ligaments. Ligaments with mixed tight and loose parallel bundles of collagenous connective tissue were mainly found in the inferior extensor retinaculum and talocalcaneal oblique ligament. Densely packed and fiber-rich interlacing collagen was primarily seen in the areas of ligament insertion into bone of the deltoid ligament.

Conclusions: Ligaments of the lateral region, the canalis tarsi, and the anterior tibiofibular ligaments have tightly packed, parallel collagen bundles and thus can resist high tensile forces. The mixed tight and loose, parallel oriented collagenous connective tissue of the inferior extensor retinaculum and the talocalcaneal oblique ligament support the dynamic positioning of the foot on the ground. The interlacing collagen bundles seen at the insertion of the deltoid ligament suggest that these insertion areas are susceptible to tension in a multitude of directions.

Clinical Relevance: The morphology and mechanical properties of ankle ligaments may provide an understanding of their response to the loads to which they are subjected.

Keywords: foot, histology, ligament, morphology

Introduction

Ligaments generally have 3 main functions: (1) to provide mechanical passive stability of joints, thus guiding joints through their normal range of motion during application of tensile or compressive load; (2) viscoelasticity, which helps to preserve joint homeostasis (viscoelastic behavior means that intraligamentous tension decreases if constant ligamentous deformation is applied[5,13,38] and "creep" occurs as a result of elongation under a constant or cyclically repetitive load); and (3) sensory function, where ligaments are recognized as sensory organs, capable of monitoring and supplying afferent kinesthetic and proprioceptive data.[27,28,38] Proprioceptive information transmitted from the mechanoreceptors in ligaments and joint capsule, reacting to changes in joint angle, joint velocity, mechanical distortion, and changes in intra-articular pressure influences the muscular joint stability.[24,29,38]

The ligaments around the ankle joint can be divided into 4 regions: the distal tibiofibular syndesmosis, the lateral region, the deltoid ligament, and the sinus tarsi ligaments.[33] The distal tibiofibular syndesmosis is composed of the anterior (ATiFL), posterior, transversal, and interosseous tibiofibular ligaments and the interosseous membrane.[51] The

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lateral region consists of the calcaneofibular (CFL), anterior (ATFL), and posterior talofibular (PTFL) ligaments. The medial complex includes the deltoid ligament, which can be divided into a superficial layer, containing the tibionavicular (TNL) tibiocalcaneal (TCL) and superficial tibiotalar (STTL) portions, and a deeper layer, consisting of the anterior (ATTL) and posterior (PTTL) tibiotalar portions.[26] The sinus tarsi contains the inferior extensor retinaculum with its lateral (IERL), intermediate (IERI), and medial (IERM) roots; the talocalcaneal oblique ligament (TCOL); and the canalis tarsi ligament (CTL).[36]

Sprains are the most common injury of the ankle joint.[11] Lateral ankle ligament sprains comprise 85% of all ankle sprains while eversion sprains of the deltoid ligament comprise 5% and syndesmosis sprains 10% of these injuries,[31] indicating that the various ankle ligaments have different roles in ankle stability. Furthermore, ligaments are mechano-responsive, which means that they alter their composition and mechanical properties in response to the loads to which they are subjected.[4] Therefore, the aim of our study was to analyze the microscopic and structural composition of the ankle ligaments to further our understanding of their functions in ankle stability.

Materials and Methods

Cadaver Specimens

All protocols in this study were approved by the local ethics committee review board. Ten feet from 5 subjects (2 women and 3 men) with a mean age of 57 ± 20 years (range, 36-86 years) were included in this study. Five left and 5 right feet were analyzed. The cadavers were refrigerated (4°C) pending ligament harvest, and the mean time between death and harvest was 3.6 ± 2.4 days (range, 1 to 7 days). All feet were assessed macroscopically and showed no signs of ligament injury or structural abnormality.

Ligament Specimens

The ATiFL as the anterior part of the distal tibiofibular syndesmosis was resected (Figure 1).[3,14]

The ATFL, PTFL, and CFL were obtained from the lateral region (Figure 2).[14] The ATFL was presented as a single ligament in 7 cases and as a double ligament in 3 cases (Figure 2).

The portions of the deltoid ligament were defined to the description of Pankovich and Shiravam.[26] The TNL, TCL, STTL portions from the superficial layer, the ATTL, and PTTL portions from the deep layer of the deltoid were harvested from the medial region (Figure 3).

Ligaments of the sinus tarsi were defined according to the description of Schmidt.[36] The IERM, IERI, and IERL roots of the inferior extensor retinaculum, the TCOL, and the CTL were resected in the sinus tarsi (Figure 4). The CTL in the sinus tarsi had 2 portions in 8 cases and 1 portion in 2
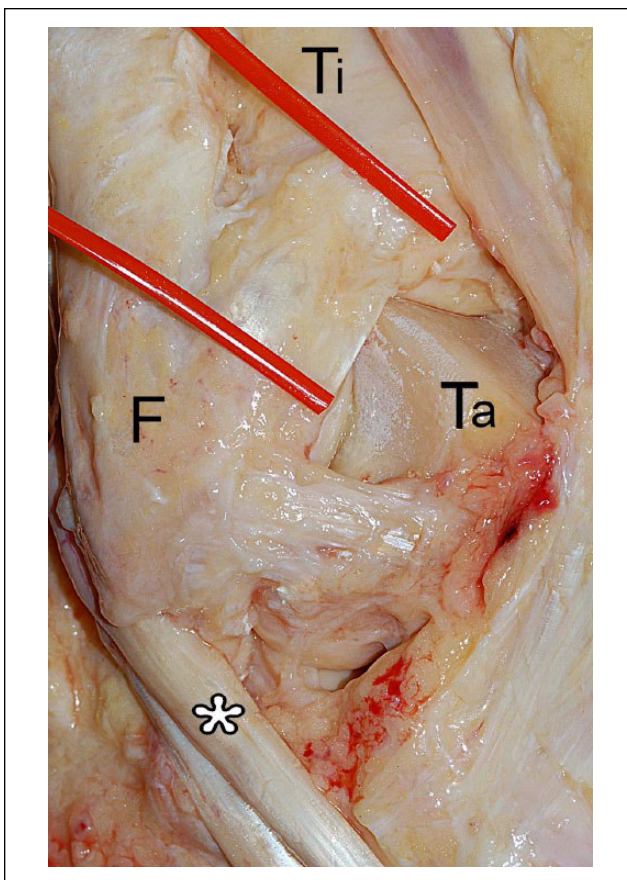

Figure 1. Anterior tibiofibular ligament. A right ankle joint of a human cadaver is seen from the antero-lateral view, where the talus (Ta), the tibia (Ti), the fibula (F), and the peroneal tendons (*) are clearly visible as anatomical key structures. The ATiFL is marked with 2 red soft straps. It descends from the anterior tibial tubercle, so called *tubercule de Tillaux-Chaput* (tubercle of Chaput), to the anterior aspect of the distal fibula, so called *Le Fort's or Wagstaffe tubercle*. ATiFL, anterior tibiofibular ligament. For the color version of this figure, please refer to the online edition.

cases in the sulcus tali (Figure 4). All 140 ligaments were completely dissected from their insertion into bone.

Immunohistochemistry

Specimens were immediately fixed in 4% buffered formaldehyde solution (pH = 7.4) for 24 hours at 4°C, decalcified with diaminoethanetetraacetic acid (EDTA), and embedded in paraffin. Sections of 4 μm were cut and mounted on silane-coated slides for conventional staining and immunohistochemistry. Standard immunohistochemical protocol was followed, as previously described in detail.[16,27] The polyclonal antibody against S100 (working dilution 1:500; code Z 0311; DakoCytomation, Glostrup, Denmark) were used, which specifically stains the S100 protein, including Schwann cells of the peripheral nervous system. The monoclonal mouse anti-human 1A4 antibody (sm-actin; working dilution
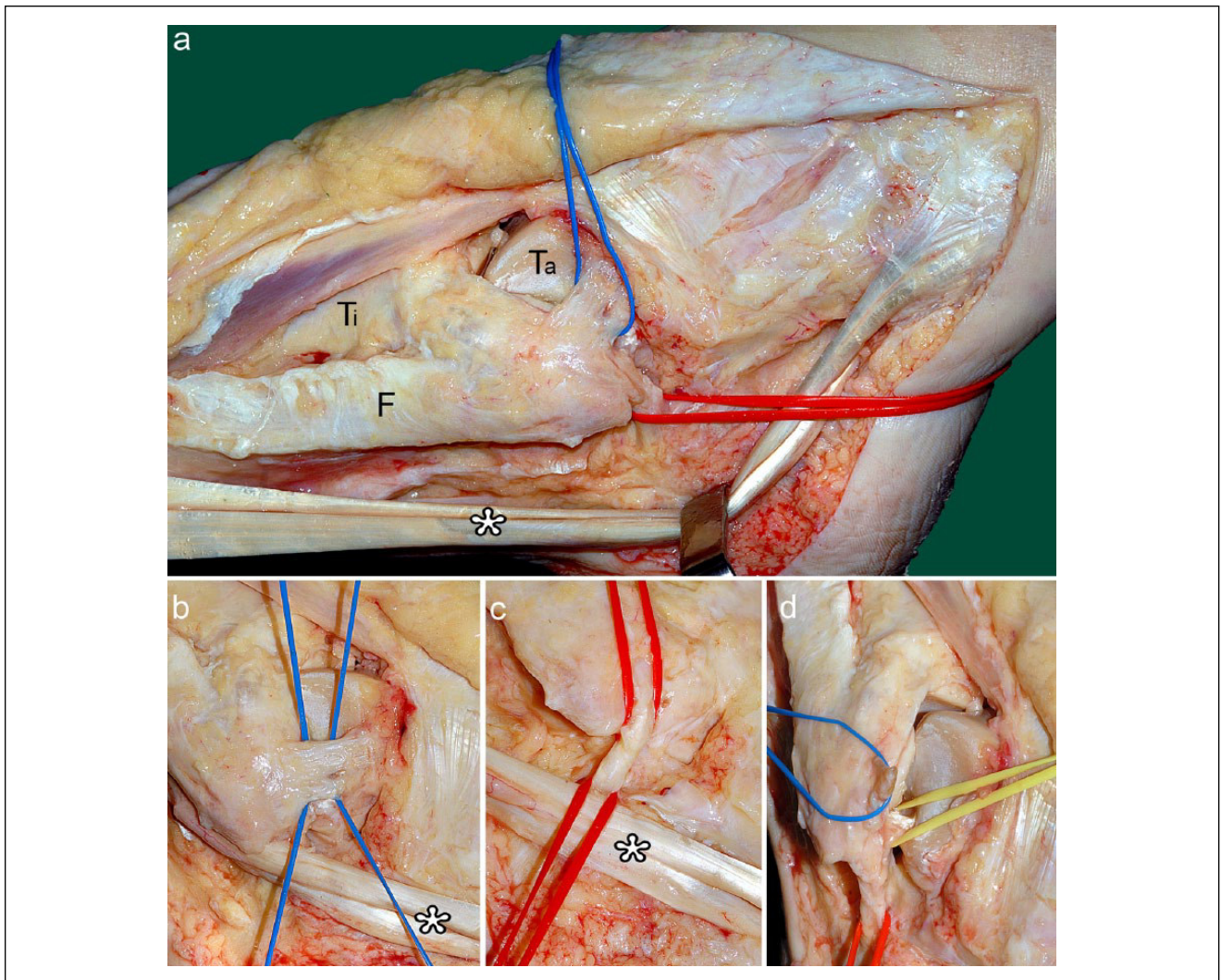
Figure 2. Lateral ligaments. A right ankle joint of human cadaver is considered from the lateral view. The talus (Ta), tibia (Ti), and fibula (F) are identified as main bony structures. The ATFL (blue), divided into 2 parts in this specimen, runs from the anterior edge of the lateral malleolus almost horizontally to the medial neck of the talus (a, b). The red marked CFL passes from the tip of the fibula obliquely dorso-caudal, and medially of the peroneal tendons, over the subtalar joint to its insertion on the calcaneus (c). The short and long peroneal tendons (*) are held away with a hook (a). The PTFL (yellow) is visible from the anterior view after cut of the ATFL (blue) (d). ATFL, anterior talofibular ligament; CFL, calcaneofibular ligament; PTFL, posterior talofibular ligament. For the color version of this figure, please refer to the online edition.

1:750; code M 0851; DakoCytomation, Glostrup, Denmark) labeled smooth muscle cells in blood vessels.

Morphological Analysis

Histological examination of the stained tissue sections was performed using a Leica light microscope (Leitz DMRBE, Wetzlar, Germany) with a Leica camera (Leica DC 300, Leica Microsystems CMS GmbH, Heerbrugg, Switzerland).

Hematoxylin-eosin (H&E) and Elastica van Gieson (EvG) stained slides were used for determination of tissue morphology in the transmission and polarization mode at original magnifications of 25×, 100×, 200×, 400×. Sensory nerve endings were validated in the S100 staining at an original magnification of 400×. Blood vessels were identified by specific staining of sm-actin of the smooth muscle cells in the wall of the vessels.

The complete ligamentous section was evaluated for determination of the structural composition on 5 slides in the H&E and EvG staining, respectively. In principle, 2 general structural compositions of ligaments were found according to the arrangement of the collagen bundles into interlaced or parallel collagen. The latter one could be further divided into densely packed and fiber-rich collagen or mixed tight and loose collagenous connective tissue.[23]
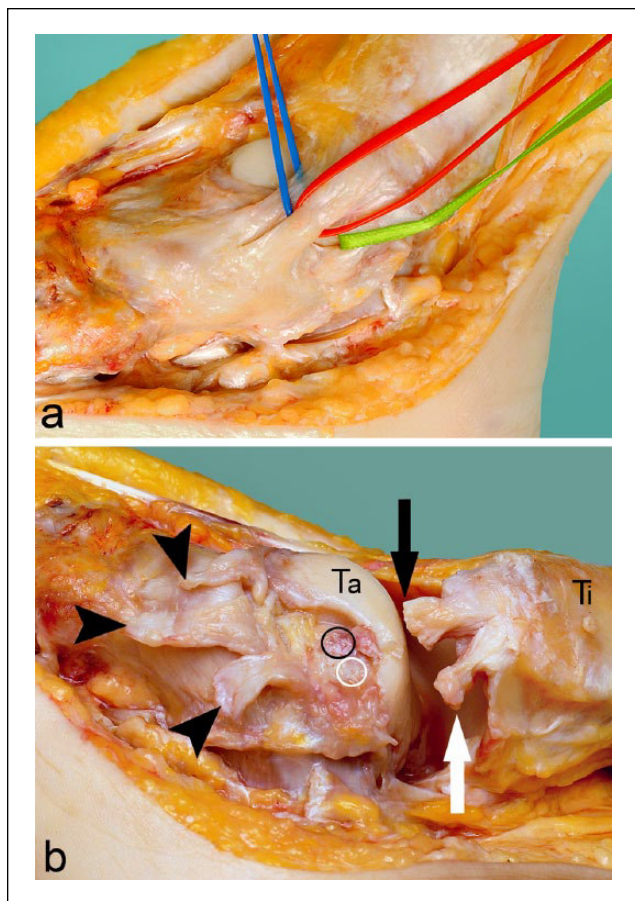
Figure 3. Deltoid ligaments. The superficial layer of the deltoid ligament is demonstrated in a right ankle of a human cadaver foot. The superficial layer consists of the TNL (blue), the TCL (red), and the STTL (green) (a). The deep layer of the deltoid ligament with the ATTL (black arrow) and the PTTL (white arrow) is seen after cutting of the superficial layer (arrow heads) at the bony tibial insertion (Ti). The insertion area of the ATTL is marked with a black circle and of the PTTL with a white circle at the medial talus (Ta) (b). TNL, tibionavicular ligament; TCL, tibiocalcaneal ligament; STTL, superficial tibiotalar ligament; ATTL, anterior tibiotalar ligament; PTTL, posterior tibiotalar ligament. For the color version of this figure, please refer to the online edition.

Statistical Analysis

Absolute values were used for descriptive statistics throughout the article. The structural compositions of ligaments between the 4 anatomical complexes as well as the inter-ligamentous structural composition in each anatomical complex were examined. Statistical analysis was performed with contingency tables using Fisher's exact test. The level of 2-sided significance was considered high with $P \leq .05$.

Results

Three different morphological compositions were identified in the ankle ligaments (Figure 5): dense, mixed, and interlaced

compositions. The distribution of these 3 structural compositions between the anatomical ligamentous regions was statistically significant ($P < .0001$).

Densely packed ligaments, characterized by parallel bundles of collagen, were primarily the ligaments of the lateral region, the CTL, STTL, and the ATiFL (Figures 5, 6).

Second, ligaments with mixed tight and loose, parallel bundles of collagenous connective tissue were mainly found in the IERL, IERI, IERM, and TCOL (Figures 5, 7).

Lastly, densely packed and fiber-rich interlacing collagen with round-shaped fibroblasts was especially seen in the areas of ligament insertion into bone of the deltoid ligament as the third type (Figures 5, 8). In this morphological type, the densely interlaced collagen arrangement at the ligamentous insertion was changed into densely packed parallel collagen bundles in the central part of the different portions of the deltoid ligament, where the polarized mode showed a homogenous view similar to the ligaments of the lateral region. The collagen fibers at the insertion often displayed a multidirectional arrangement, whereas an inhomogeneous multicolored view was seen in the polarized mode (Figure 8). Some of the ligaments, particularly the ligaments with mixed tight and loose parallel bundles of collagenous connective tissue displayed an undulating homogenous polarization (Figure 7) that decreased in amplitude when progressing from the central to the peripheral regions of the ligament (Figure 7). The structural composition between the ligaments of each region was not statistical significant.

Furthermore, all ligaments had a typical structural composition, consisting of collagen bundles in the central area of the ligament, surrounded by an external epiligamentous region, which appeared macroscopically as a collagen layer enveloping the ligament in its entirety. It extended to the periosteum and microscopically had a multidirectional fiber arrangement (Figure 9). Particularly ligaments with mixed tight and loose, parallel bundles of collagenous connective tissue were divided into collagen fascicles through interstitial septa of connective tissue (Figure 10). These interstitial septa as well as the epiligamentous region contained sensory nerve endings and blood vessels (Figures 9, 10).

Discussion

Understanding the different types of ligament compositions helps to assign biomechanical functions of the individual ligaments in joint stability. The results of this study suggest that 3 different structural configurations of the ligaments stabilizing the ankle joint may be reflected in different kinetic and kinematic properties.[2,8,12,30,37,42]

Densely Packed Parallel Ligaments

The ligaments of the lateral region were found to mainly have a tight fiber-rich, parallel, densely packed collagen fiber composition, which is in accordance with the literature.[21] An almost
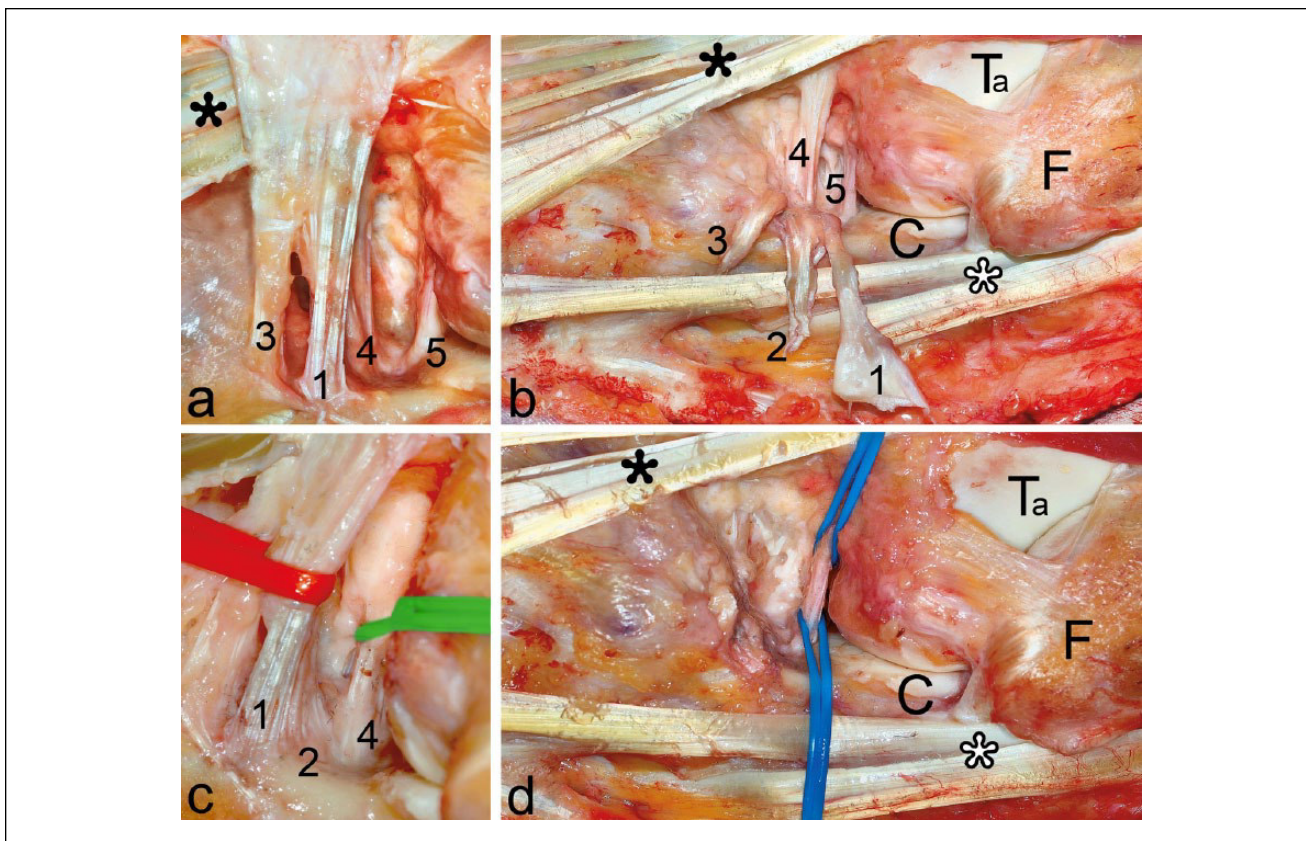
Figure 4. Sinus tarsi ligaments. Lateral view of the sinus tarsi ligaments in a human cadaver specimen with the following visible bony structures: talus (Ta), calcaneus (C), and fibula (F). The sinus tarsi contains the IERL (1), the IERI (2), the IERM (3), the TCOL (4), and the CTL (5). The IERL (1), which runs over the tendons of the long digital extensor muscles and the peroneal tertius muscle (black star), the IERM (3), the TCOL (4), and the CTL (5) are visible in the overview (a). The strong TCOL (4) and the CTL (5) in the inner part of the sinus tarsi are clearly discernible after cutting of the 3 roots of the IER (1, 2, 3) at the talar insertion (b). The IERI (2) can be clearly differentiated after cutting the IERL (1) at the dorsum of the foot because both ligaments have the same calcaneal insertion point (marked red in c), whereas the IERI (2) lies medial of the IERL (1) and runs underneath the tendons of the long digital extensor muscles and the peroneal tertius muscle to the sulcus tali. The IERM (3) lies medial of the IERI and extends into the IERI (b). The IERL and IERI (red marked) could be clearly differentiated from the TCOL (4, green marked) (c). Inset "d" shows the blue marked CTL after resection of the 3 roots of the IER and the TCOL. Furthermore, the tendons of the short and long peroneal muscles (white star in b and d) blend behind the lateral malleolus at the latero-caudal edge of the sinus tarsi entrance in distal direction. IER, inferior extensor retinaculum; IERL, inferior extensor retinaculum, lateral root; IERI, inferior extensor retinaculum, intermediate root; IERM, inferior extensor retinaculum, medial root; TCOL, talocalcaneal oblique ligament; CTL, canalis tarsi ligament. For the color version of this figure, please refer to the online edition.

parallel array of fibrils in ligaments and tendons produces an ideal physical adaptation for tensile-force transmission.[9] The fibular ligaments thus appear to be particularly well adapted to the substantial loads acting on the lateral malleolus.[22,43] Although the following ligaments are located in different regions at the ankle joint, the deep CTL of the sinus tarsi, the ATiFL of the syndesmosis, and the STTL of the deltoid ligament were similarly found to consist of a densely packed collagenous structure, as a morphological expression of the distinct static and mechanical functions of these ligaments.

Previous studies showed that the ATFL and CFL have a significant role in talocrural stability.[7,18] Increased internal rotation and rotational instability in the transverse plane is present along with instability in anterior translation after ATFL sectioning.[32] Furthermore, a recent immunohistochemical study revealed the presence of fibrocartilage at the lateral talar insertion where the ATFL wraps around the lateral talar articular cartilage, which is regarded as an adaptation to resisting compression.[21] However, the ATFL is the weakest of the 3 lateral ankle ligaments, having the least elastic transformation properties.[2,35] Surprisingly, no significant differences in the structural composition between the ATFL, CFL, and PTFL have been found in this study, which could not explain from a histological point of view why the ATFL is more prone to injury in comparison with the CFL and PTFL.[52] The ATFL is a flat, quadrilateral
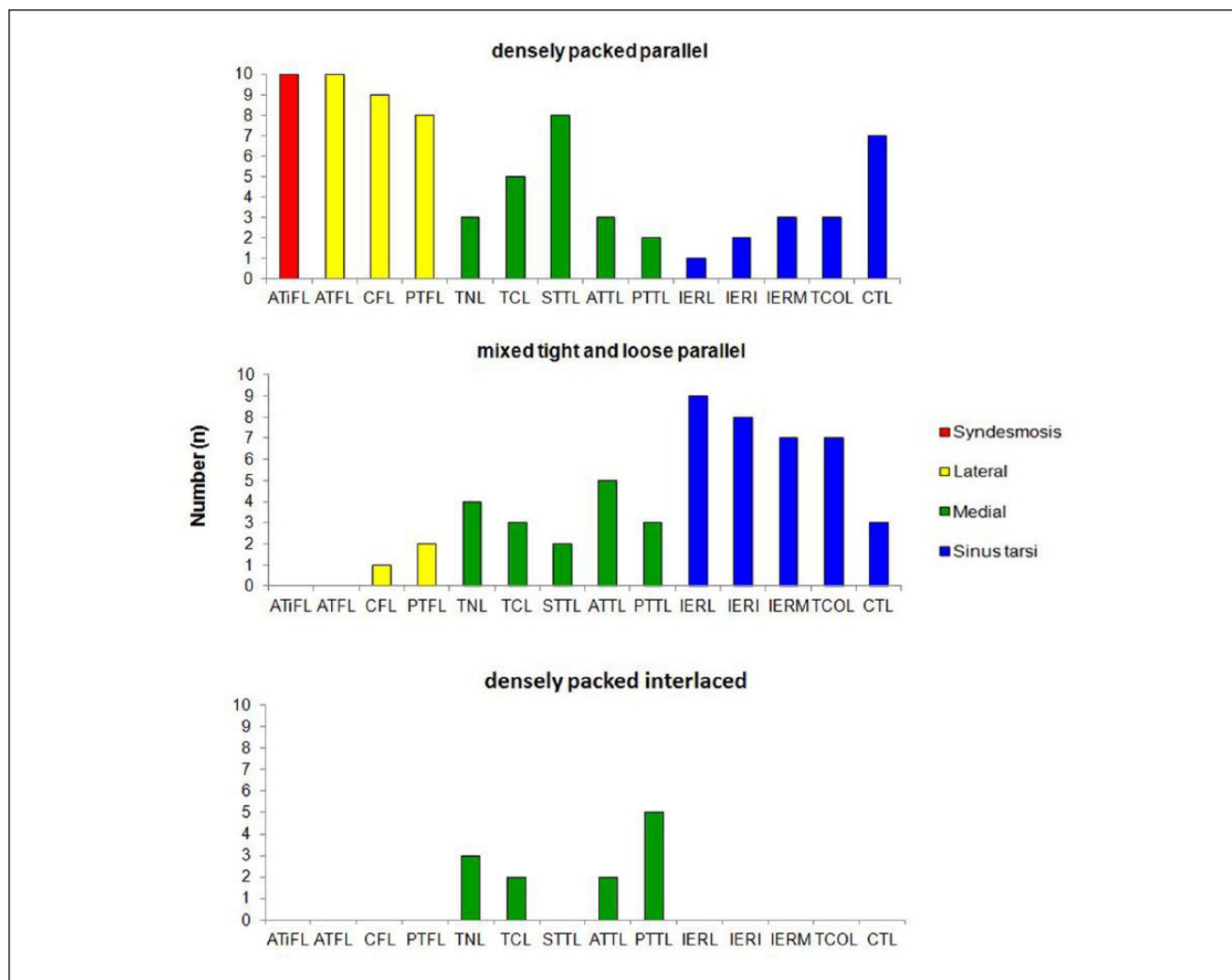
Figure 5. Tissue morphology. The appearance of the 3 typical arrangements of the collagen fibers in the analyzed ligaments is demonstrated. Ligaments of the lateral region, the CTL, STTL, and the ATiFL consist predominantly of parallel fiber-rich collagen with densely packed collagenous tissue. Meanwhile the 3 roots of the IER and the TCOL of the sinus tarsi most often have parallel arranged collagenous fibers with mixed tight and loose connective tissue. The insertion regions of the different portions of the deltoid ligaments especially have densely packed, fiber rich, interlacing collagen bundles. ATiFL, anterior tibiofibular ligament; CTL, canalis tarsi ligament; IER, inferior extensor retinaculum; TCOL, talocalcaneum oblique ligament.

ligament, which is incorporated in the joint capsule, whereas the CFL is a strong cord-like or flat oval ligament, and the strong PTFL has a trapezoidal contour.[47]

The CTL is an important stabilizer of the subtalar joint,[32] stabilizing it against drawer forces applied to the calcaneus from lateral to medial.[20,45] After isolated dissection of the CTL, dorsiflexion of the talocalcaneal joint increased to 43%.[19] In contrast, an isolated dissection of the TCOL resulted only in an increase of 7% in dorsiflexion of the talocalcaneal joint.[19] Combined sectioning of the ATFL and CTL can induce anterolateral rotatory instability of the ankle joint under conditions of axial loading.[46]

The integrity of the distal tibiofibular syndesmosis is fundamental for an adequate function and stability of the ankle joint.[17] The ATiFL is an important stabilizer of the distal tibiofibular syndesmosis,[44] contributing with 35% to the stability of the ankle joint.[25] In addition to holding the fibula tight to the tibia, this ligament prevents excessive movement of the fibula and external rotation of the talus.[10,34] This biomechanical function is histologically reflected by a densely packed parallel collagen fiber orientation.

Mixed Tight and Loose Parallel Ligaments

In contrast, the ligaments at the entrance of the sinus tarsi, namely, the IERL, IERI, IERM, and TCOL, showed mixed tight and loose parallel bundles of collagenous connective tissue, suggesting that these ligaments are not exposed to
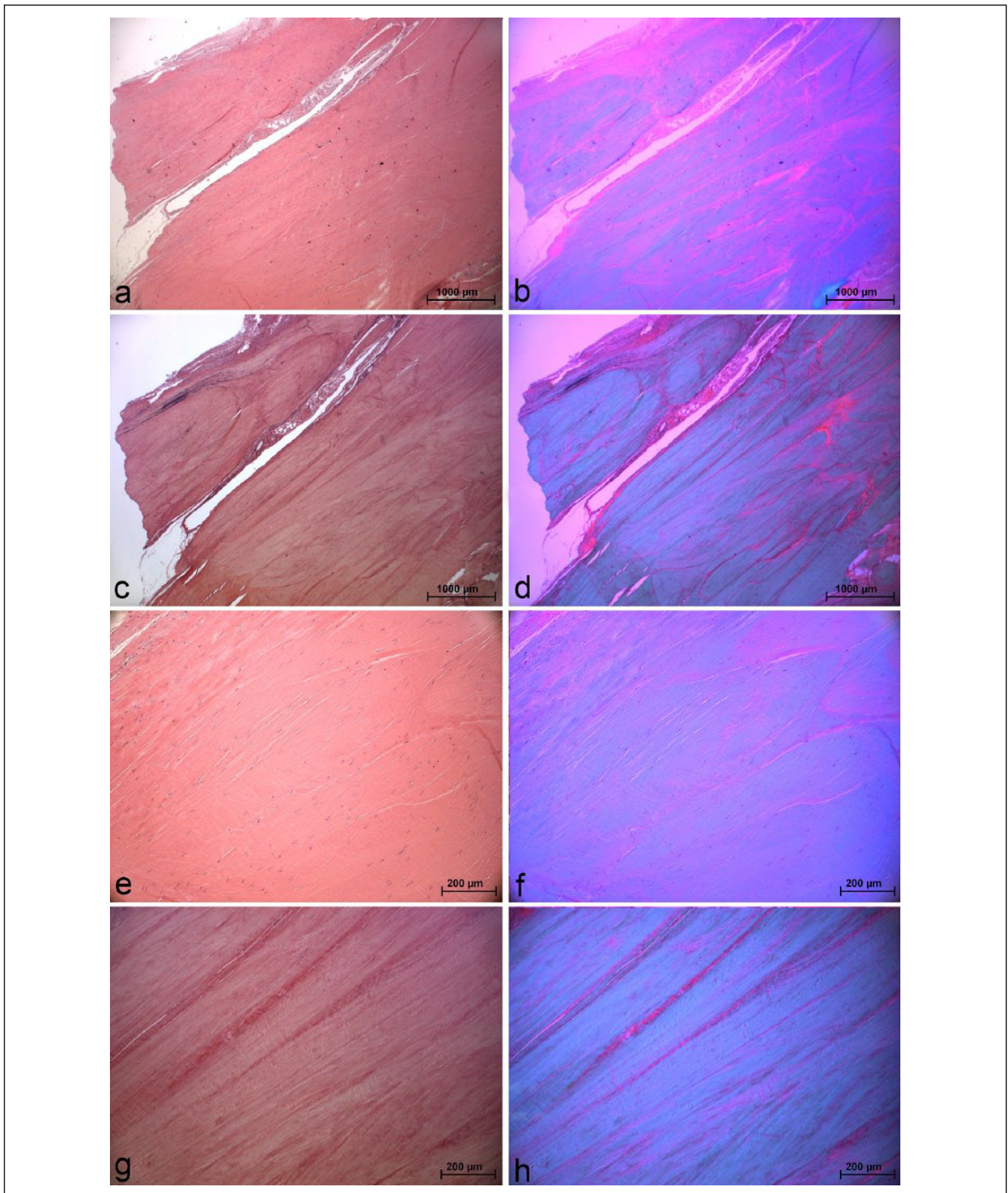
Figure 6. Densely packed parallel collagen tissue. The ligament type of fiber-rich parallel organized collagen and densely packed collagen tissue is demonstrated in an ATFL in the H&E staining (a, b, e, f) and EvG staining (c, d, g, h) in the transmission (a, c, e, g) and polarization mode (b, d, f, h). The collagen is so densely packed that there are hardly interstitial septa for this collagen type. This likely gives the ligament its tensile strength. A homogeneous ligamentous structure without undulating course of the collagen fibers is seen in the polarization mode due to the densely packed parallel fiber arrangement (b, d, f, h). Elastic fibers are not seen (c, g). Original magnification ×25 (a, b, c, d), original magnification ×100 (e, f, g, h). ATFL, anterior talofibular ligament.
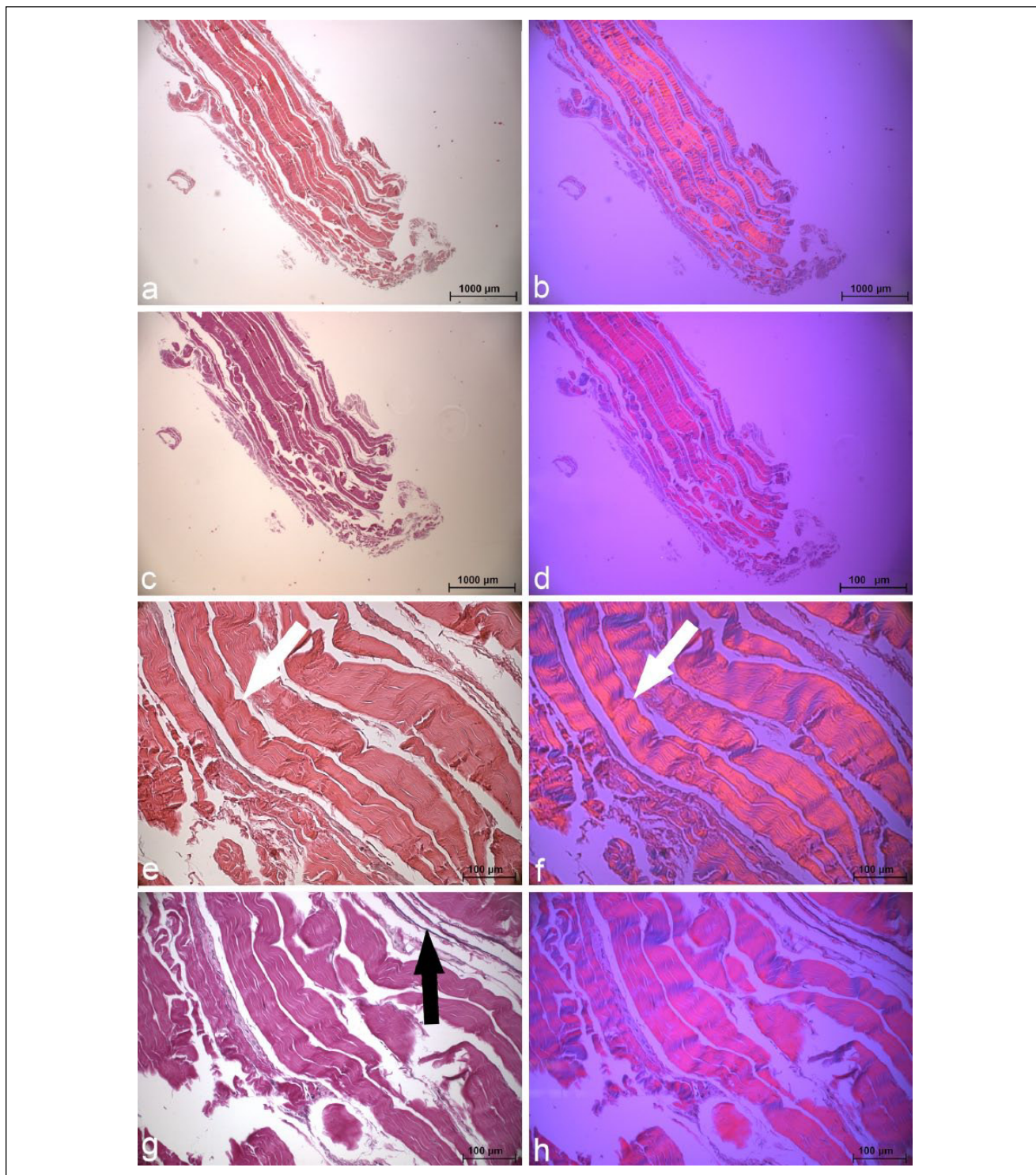
Figure 7. Mixed densely packed and loose collagen tissue. An IERI as a characteristic example for the parallel fiber arrangement of mixed densely packed and loose collagen is shown in the H&E (a, b, e, f) and the EvG staining (c, d, g, h) in the transmission (a, c, e, g) as well as in the polarization mode (b, d, f, h). The ligament structure is homogeneous in the polarization mode due to the parallel collagen fiber arrangement (b, d, f, h). The pronounced undulating structure of the single collagen bundles is a morphological aspect of the elongation ability (arrows in e, f). Furthermore, elastic fibers can be seen in the loose interstitial connective tissue (arrow in g). The undulating ligamentous structure, the loose interstitial connective tissue, and the elastic fibers give the ligament a certain flexibility to elongate without immediately rupture. Original magnification ×25 (a, b, c, d), original magnification ×200 (e, f, g, h). IERI, inferior extensor retinaculum, intermediate root.
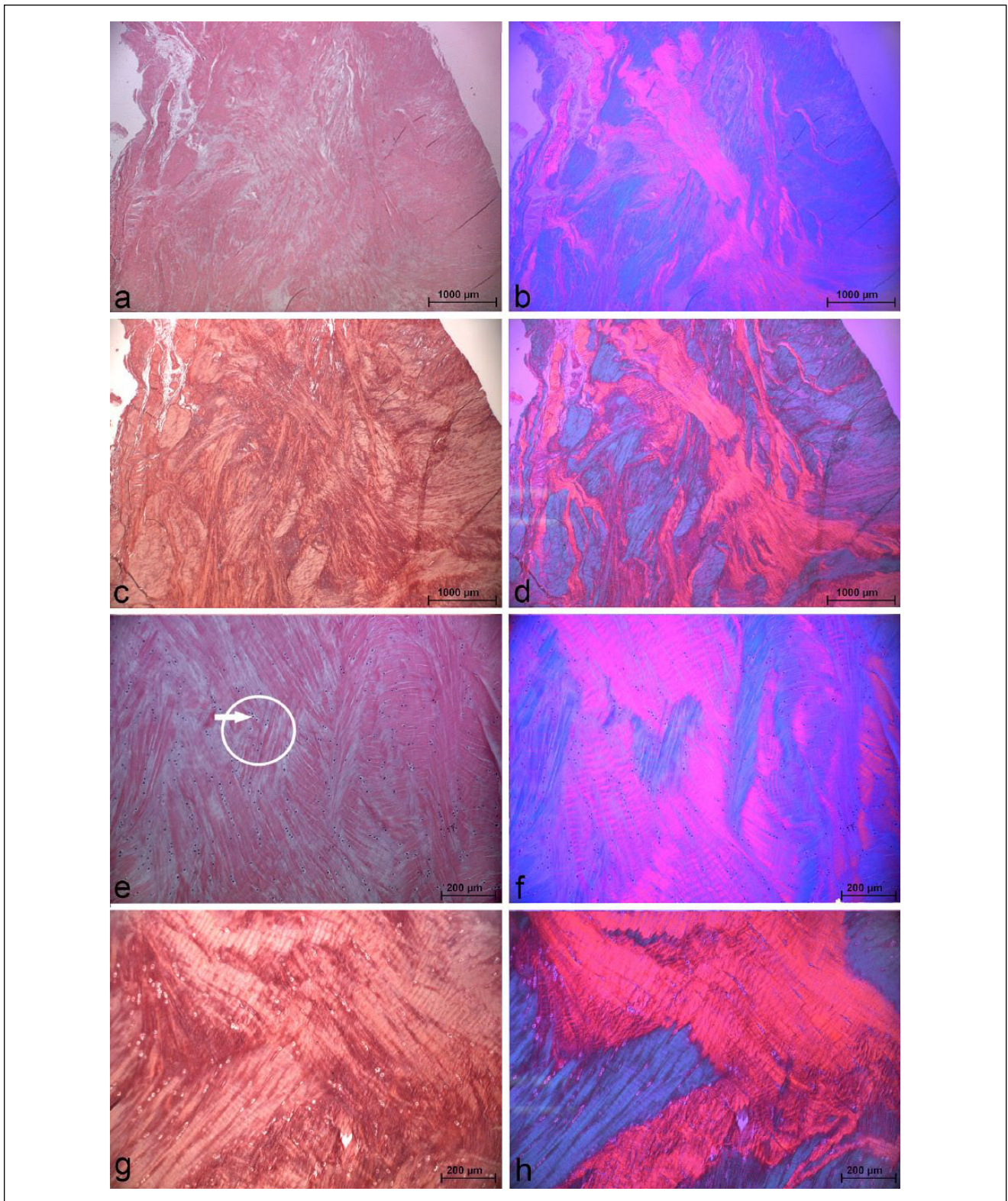
Figure 8. Densely packed interlaced collagen tissue. A TCL with densely packed and fiber rich collagen with interlacing collagen arrangement at the insertion area is represented in the H&E (a, b, e, f) and the EvG staining (c, d, g, h) in the transmission (a, c, e, g) and polarization mode (b, d, f, h). The polarization mode shows the interlacing ligamentous structure well by the color change (b, d, f, h). The fibroblasts have a roundish chondroid shape appearing (circle with an arrow in e). No elastic fibers could be found in that ligament area (c, g). Original magnification ×25 (a, b, c, d), original magnification ×100 (e, f, g, h). TCL, tibiocalcaneal ligament.
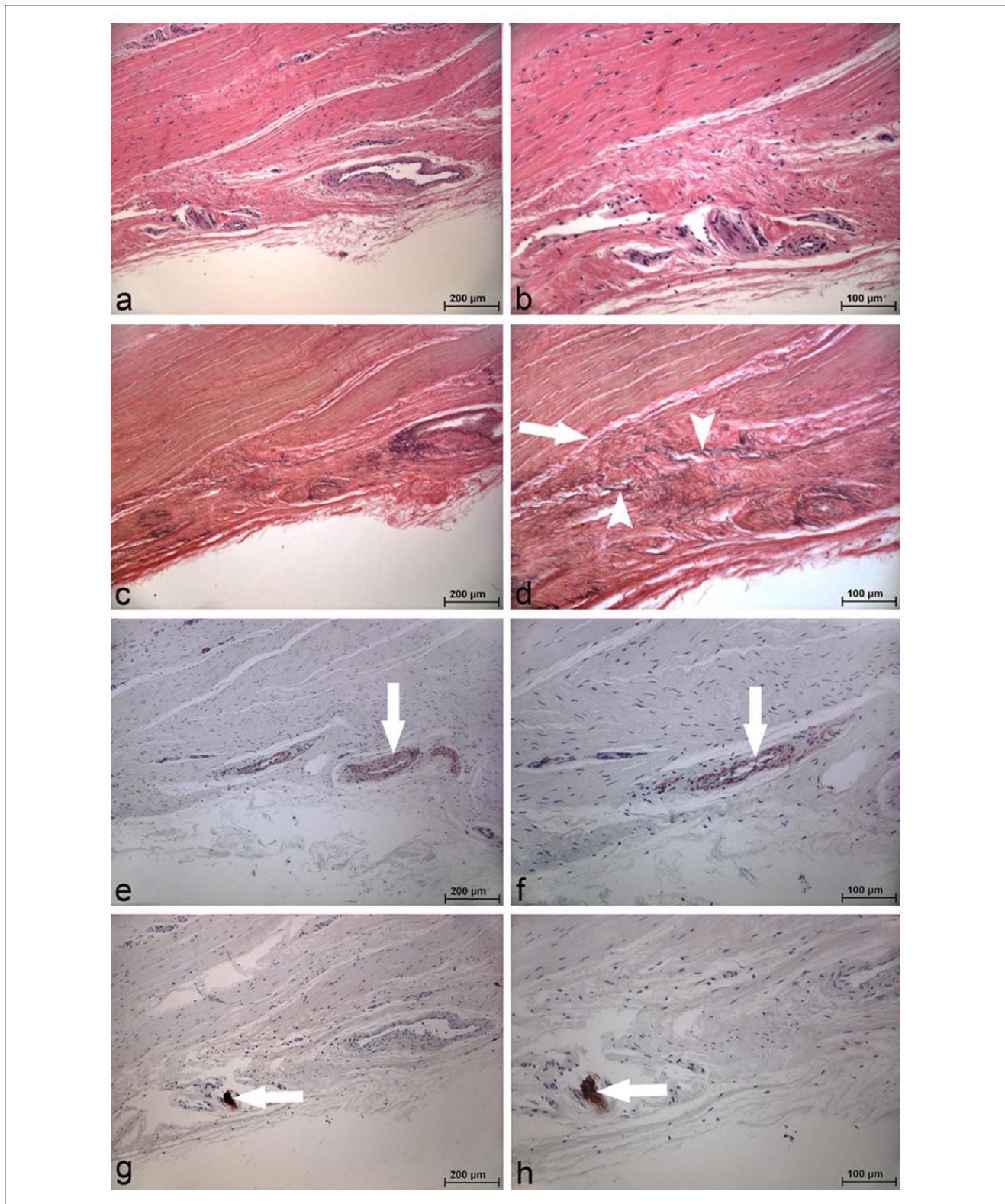
Figure 9. Epiligament. The epiligamentous region of a TNL is shown in the H&E (a, b) and the EvG staining (c, d) as well as with immunoreactivity of sm-actin (e, f) and S100 (g, h). The epiligament can be clearly distinguished by the loosened collagen well from the tight parallel collagen fiber orientation of the ligament (arrow in d). It contains abundant black stained elastic fibers (c, arrow heads in d), blood vessels (arrow in e and f), as well as free nerve endings (arrow in g and h). Original magnification ×100 (a, c, e, g), original magnification ×200 (b, d, f, h). TNL, tibionavicular ligament.
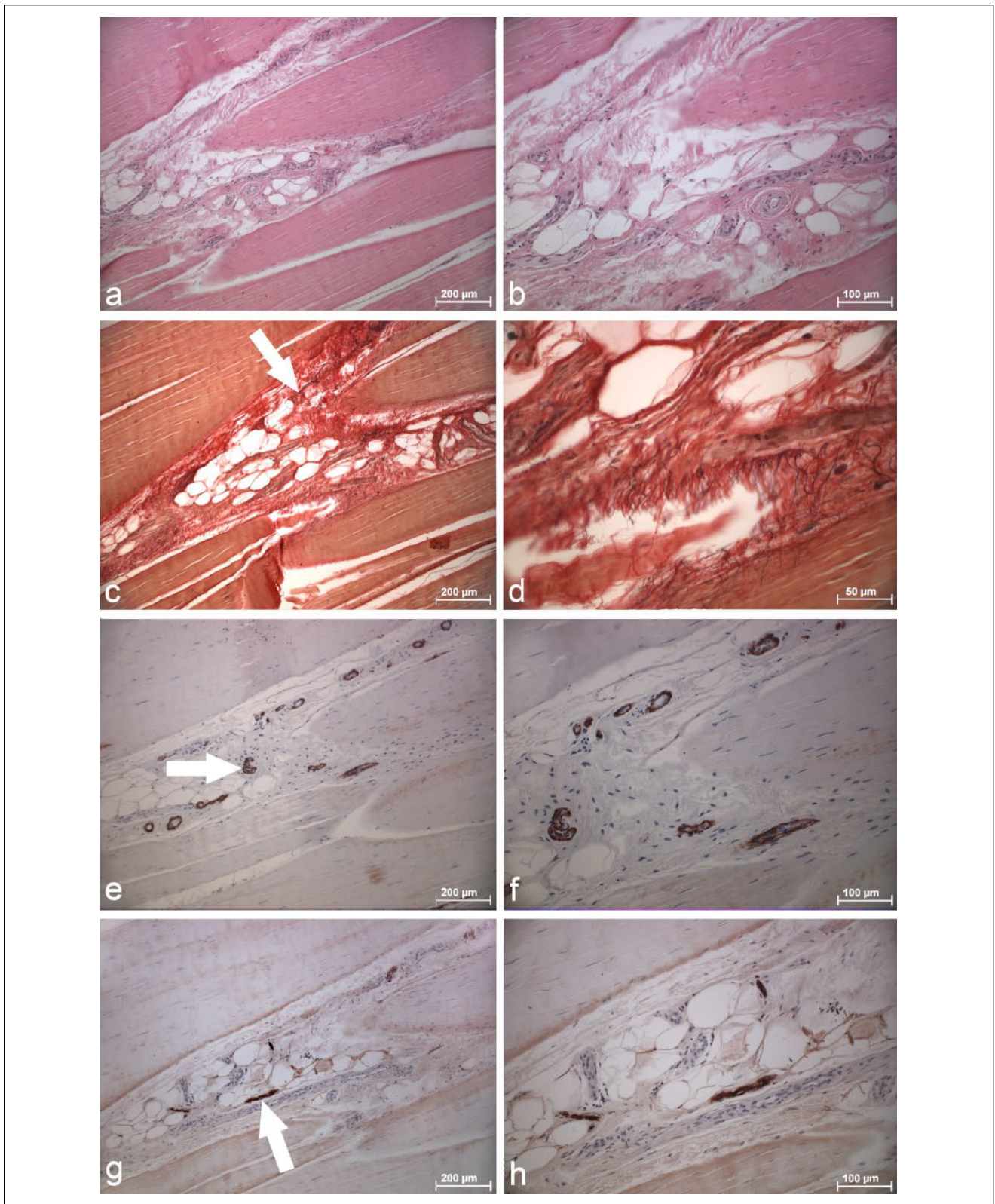
Figure 10. Interstitium of ligaments. The interstitial area of an IERL with parallel fiber arrangement of mixed densely packed and loose collagen is shown in the H&E (a, b) and EvG staining (c, d), with immunoreactivity of sm-actin (e, f) and S100 (g, h). The interstitium of ligaments also contains abundant elastic fibers (arrow in c; d), blood vessels (arrow in e; f), free nerve endings (arrow in g; h). Original magnification ×100 (a, c, e, g), original magnification ×200 (b, f, h), original magnification ×400 (d). IERL, inferior extensor retinaculum, lateral root.

mechanical loads as extreme as the lateral ligaments. These findings are in accordance with Viladot et al,[48] who described mixed tight and loose collagenous connective tissue for the IERL, IERI, IERM, and TCOL but a tight and dense parallel aligned collagen fibers for the CTL. IER is suggested to have a modest effect on the mechanical stability of the ankle but play an important role in proprioception.[40,48] This is supported by a recent immunohistochemical study, which showed that the IERL has significantly more free nerve endings than the CTL.[28] Functionally, the IER serves as a pulley for the extensor tendons, which demands a more pliable configuration.[33] According to Stephens and Sammarco[41] the IER plays a significant role in subtalar joint stability in neutral and dorsiflexion positions. Another biomechanical cadaver study suggested that the IER is also important for the inversion and eversion of the foot.[49]

Densely Packed Interlaced Ligaments

The ligaments of the medial region often have at their insertion area densely packed fiber-rich and interlaced collagen bundles. The densely packed interlaced collagen bundles visualized at these insertion areas are susceptible to tension in a multitude of directions, and they serve to anchor the ligament to its bony insertion, allowing the ligaments adapt to the bony surface like a "stocking."[23]

Epiligament and Interstitium of Ligaments

The interstitial septa, containing loose collagenous connective tissue, sensory nerve endings, and elastic fibers, as well as a pattern of decreasing undulating or crimp collagen alignment from center to periphery, supports the function of the IER and TCOL in the dynamic adaptation of the foot to the ground.[39,50] Crimp plays a biomechanical role, as it is related to the loading state of ligaments. Increased loading will cause certain areas of the ligament to uncrimp, thereby allowing the ligament to elongate without sustaining damage.[1]

The multidirectional collagen fiber orientation of the epiligament indicates that these fibers must resist pressure and tension in various directions and can be correlated to the relative mobility of the epiligament and its role as a sliding surface to adjacent tissues.[6] The epiligamentous vascular network has an important nutritional function for the ligament.[6] Due to the high vascular density and the surrounding loose connective tissue, it can be assumed that the epiligament is also crucial for obtaining a special hydrostatic environment in the ligament.[6] Furthermore, the epiligament protects the collagen fibers as well as the ligamentous neurovascular bundles.[6] Hagert et al[15] observed less sensory nerve endings in wrist ligaments with densely packed collagen due to their minimally existing interstitial connective tissue and small epiligamentous regions, as compared to wrist ligaments with mixed dense and loose collagenous connective tissue and a broad epiligament. These observations could not have been confirmed in the present study on the innervation patterns of the ankle ligaments.[27] The ligaments of the lateral region, the ATiFL, and CTL consisted of densely packed parallel collagen fibers, whereas the IER and TCOL had a mixed dense and loose parallel collagenous composition. No statistically significant differences were found for the number of Ruffini endings, Pacini corpuscles, or Golgi-like endings between the ATiFL, the ligaments of lateral and sinus tarsi regions.[27] In addition, when analyzing the mechanoreceptors between the anatomical regions, significantly more free nerve endings have been counted in the ligaments of the lateral and medial regions in comparison to the sinus tarsi ligaments,[27] despite the fact that the IER and TCOL have parallel arranged collagenous fibers with mixed tight and loose connective tissue as well as interstitital septa containing sensory nerve endings and blood vessels. It can be assumed that the wrist ligaments in general are smaller than ankle ligaments, so that existing differences may appear with starker contrast. The ankle ligaments have variable collagenous appearance, which enhance our understanding of their innate biomechanical functions in ankle stability. No correlation could be made between ligament composition and degree of innervation, as the former is variable but the latter more uniform in disposition.

Conclusion

In conclusion, the 3 different morphological compositions correlate with the functions of the various ankle ligaments in joint stability. Ligaments of the lateral region, the CTL and ATiFL were composed of densely packed parallel collagen bundles, which is important to resist high tensile forces. Mixed tight and loose, parallel oriented collagenous connective tissue was found in the IER and TCOL, which enables a dynamic adaptation during foot movement. The areas of ligament insertion into bone of the deltoid ligament were characterized by densely packed interlaced collagen arrangement, by which tensile strength in different directions is achieved. All ligaments were surrounded by an epiligamentous region, which has nutritional functions containing nerve endings and blood vessels.

Declaration of Conflicting Interests

The authors declare no potential conflicts of interest with respect to the research, and/ or authorship of this article.

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