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RESEARCH ARTICLE

# A comparative study of the epiligament of the medial collateral and the anterior cruciate ligament in the human knee. Immunohistochemical analysis of collagen type I and V and procollagen type III

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ABSTRACT

*Background:* Recent reports in rat models have shown that fibroblasts in the epiligament, an enveloping tissue of the ligament, are not static cells and play an important role during the early ligament healing of isolated grade III injury of the collateral ligaments of the knee. Fibroblasts produce collagen types I, III and V and infiltrate within the ligament body via the endoligament. In addition, similarities have been reported between the structure of the epiligament of the medial collateral ligament and anterior cruciate ligament of the knee in rat and in human. In line with the ascribed role of the epiligament tissue and the synthesis of these collagens and their role in ligament healing, the aim of this study was to determine their presence in the normal epiligament of the aforementioned ligaments in humans, to compare their differential expression and to present a novel hypothesis about the failure of healing of the anterior cruciate ligament in contrast to the medial collateral ligament.
*Materials and methods:* We used samples from the mid-substance of the medial collateral and the anterior cruciate ligament of the knee joint, acquired from 12 fresh knee joints. Routine histological analysis was performed through hematoxylin and eosin stain, Mallory's trichrome stain and Van Gieson's stain. The immunohistochemical analysis was conducted using monoclonal antibodies against collagen type I and V and procollagen type III. The number of cells in the epiligament, endoligament and the ligament tissue was assessed quantitatively through a computerized system for image analysis NIS-Elements Advanced Research and Statistica software.
*Results:* Our observations revealed certain differences in the morphology of the epiligament, as well as variations in the expression of the investigated molecules. Expression of collagen type I was mostly low-positive (1+) in the epiligament and positive (2+) in the ligament tissue of both ligaments. Expression of procollagen type III was mostly positive (2+) in the epiligament and ligament tissue of the medial collateral ligament, low-positive (1+) in the epiligament and negative (0) in ligament tissue of the anterior cruciate ligament. Expression of collagen type V was predominantly low-positive (1+) in the epiligament and negative (0) in the ligament tissue of both ligaments. The immunoreactivity for all three molecules was always higher in the epiligament of the medial collateral ligament than that of the anterior cruciate ligament.
*Conclusions:* The results of our study illustrate for the first time that fibroblasts in the human epiligament are indeed responsible for the synthesis of the main types of collagen participating in the early ligament healing, thus corresponding to previous data of the medial collateral ligament healing in animal models. The differences between the epiligament of the investigated ligaments could add a novel explanation for the failed anterior cruciate ligament healing.



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## 1. Introduction

Ligaments are built of dense regular connective tissue and are hypocellular and hypovascular structures (Frank, 2004; Hsu et al., 2010). Collagen represents around 75% of the dry weight of ligaments, with type I being the predominant type, accounting for nearly 85% of the total collagen found in ligaments (Hauser et al., 2013). The remaining 15% include types III, V, VI, XI, and XIV (Hauser et al., 2013). While collagen type I is responsible for the strength and endurance of ligaments, type III is implicated in ligament repair and its synthesis is significantly increased after grade III injuries to ligaments (Chamberlain et al., 2011; Hauser et al., 2013; Hsu et al., 2010). Collagen type V is also upregulated during ligament healing and has an influence on collagen fibril diameter (Niyibizi et al., 2000), while type XIV plays a role in linear fibril growth (Hsu et al., 2010). Collagen fibers in the ligament are organized in fascicles, enveloped by thin connective tissue sheath, known as the endoligament (Landzhov et al., 2015). The endoligament in turn is connected to a more vascular connective tissue layer, which covers the entire ligament, termed the epiligament (EL) (epi- [Greek – on or upon]; ligament [Latin – *ligare*, to bind]) (Georgiev et al., 2017b).

The EL was first defined as 'any surrounding adherent connective tissue removed simultaneously with the ligament but which was grossly distinguishable from ligament tissue proper' (Bray et al., 1990). Apart from covering the ligament tissue, the EL merges with the periosteum of the bone at the attachment sites of the ligament (Frank, 2004). It has been reported that the EL consists of collagen fibrils with smaller size and different orientation compared to those found in the ligament tissue (Chowdhury et al., 1991; Frank, 2004). Apart from collagen type I, the EL is also rich in types III and V, which are otherwise scarcely represented in the intact ligament tissue and only increase after injury (Chamberlain et al., 2011; Georgiev et al., 2017a). In addition, the EL contains an abundance of blood vessels and is more cellular than the ligament tissue (Frank, 2004). Numerous sensory and proprioceptive nerve elements participate in the formation of an extensive neurovascular network, which courses deeper into the EL (Chowdhury et al., 1991; Frank, 2004). It has been suggested that the EL has a role in the growth and healing of ligaments and controls the water and metabolite influx into the ligament (Chowdhury et al., 1991). A number of studies found that the EL is a donor of fibroblasts and other connective tissue cells, progenitor cells and blood vessels, which migrate towards the body of the ligament via the endoligament and play a key role in the process of ligament repair (Georgiev et al., 2010b, 2015a; Georgiev and Vidinov, 2009a, b, c). Fibroblasts in particular are responsible for the synthesis of various types of collagen molecules, matrix metalloproteinases (MMPs), decorin, fibronectin and fibromodulin which are involved in degradation, as well as proliferation and remodeling of the ligament after injury (Chamberlain et al., 2011; Georgiev et al., 2010a, 2015b, 2016, 2017a; Iliev et al., 2016).

Ligament integrity is the key for the proper transmission of forces and facilitation of joint articulation, which is particularly evident in the shoulder and knee joints (Hsu et al., 2010). Knee stability is maintained through the combined activity of both active (neuromuscular) and passive (ligamentous) joint restraints (Kiapour and Murray, 2014). The anterior cruciate ligament (ACL) is a ligamentous structure which arises from the distal femur and attaches for the anterior intercondylar area of the tibia (Nguyen et al., 2014). It is composed of two bundles, termed anteromedial and posterolateral, based on their tibial attachment (Amis, 2012; Sonnery-Cottet et al., 2014). The ACL prevents excessive anterior translation and internal rotation of the tibia with respect to the femur (Kiapour and Murray, 2014; Nguyen et al., 2014). It has been reported that the anteromedial bundle is chiefly responsible for the resistance to anterior drawer, while the posteromedial bundle plays a role in the control of tibial rotational laxity (Amis, 2012). ACL injury is often the cause of recurrent knee instability, which may affect the function of other structures of the knee and lead to meniscal tears and articular cartilage degeneration (Jia et al., 2017). The medial collateral ligament (MCL) is a complex apparatus which acts as the primary static stabilizer of the knee joint against rotation caused by a valgus force (Andrews et al., 2017; Kamawal et al., 2016; Liu et al., 2010). It comprises three components — the superficial MCL (sMCL), the deep MCL (dMCL) and the posterior oblique ligament (POL) (LaPrade et al., 2007; Wijdicks et al., 2010). The sMCL is the largest ligament in the medial aspect of the knee and connects the medial femur and tibia through one femoral and two tibial attachments (Liu et al., 2010; Wijdicks et al., 2010). The dMCL represents the thickened medial portion of the joint capsule and consists of two parts — the meniscofemoral and meniscotibial ligaments (Liu et al., 2010; Wijdicks et al., 2010). The term POL refers to the fibrous extensions from the main common tendon of the semimembranosus, which attach to and reinforce the posteromedial aspect of the joint capsule (LaPrade et al., 2007; Wijdicks et al., 2010). Altogether, the MCL is the most frequently injured ligament of the knee (Andrews et al., 2017; Georgiev et al., 2017a; Wijdicks et al., 2010). Most MCL injuries are isolated and occur predominantly in young athletes as a result of external rotation, valgus loading or a combined force vector in sporting activities such as football, skiing and ice hockey (Wijdicks et al., 2010). MCL injuries are associated with side-to-side instability, especially during cutting or pivoting maneuvers (Wijdicks et al., 2010). Much is known about the different healing potential of the ACL and MCL (Andrews et al., 2017; Kiapour and Murray, 2014; Kim et al., 2013; Vaishya et al., 2015; Wijdicks et al., 2010). However, the forces behind this observation are not entirely clear. The role of the EL as a possible explanation for the difference in healing potential between the two ligaments has not been explored in detail. Herein, we present a comparative study of the structure and organization of the EL of the ACL and MCL. We also analyzed the immunohistochemical expression of collagen type I, procollagen type III and collagen type V and discussed the significance of the difference in their expression within the two ligaments. In addition, a comparative quantitative analysis of the number of cells in the EL, endoligament and ligament tissue was performed. Finally, we explored the importance of the EL for the healing potential of the ACL and MCL.

## 2. Material and methods

### 2.1. Tissue preparation

For the histological and immunohistochemical analysis, we used samples from the mid-substance of the MCL and the ACL of the knee joint, acquired from 12 fresh knee joints The knee joints were obtained from five male and seven female fresh Europid cadavers available at the Department of Anatomy, Histology and Embryology at the Medical University of Sofia. The mean age of the cadavers was 55 (min 49; max 62). No medical history of knee osteoarthritis existed and no scars around the knee joint from previous surgery were detected. The study was approved by the Medical Legal Office, the Local Ethics Committee and the Institutional Review Board (No. 4866). There was no medical or surgical history of previous trauma of the knees of the investigated cadavers.

After skin incision, the underlying subcutaneous tissue was dissected to expose the knee's MCL. The MCL and the external surface of the surrounding EL were precisely dissected and small pieces (1.5 mm in width and 3 mm in length) were immediately fixed in 10% neutral phosphate-buffered formalin solution, prepared under laboratory conditions from 37% formaldehyde solution (Merck Catalogue No. 1040031000, Merck KGga, Darmstadt, Germany) for

24 h and were then dehydrated in increasing concentrations of ethanol (70%, 80%, 95%, 100%) (Merck Catalogue No. 1009835000). After opening the knee joint, samples were also acquired from the midsubstance of ACL and fixed in the previously described way. Ethanol was then removed using cedar oil until samples became translucent. Samples were rinsed in xylene (Merck Catalogue No. 1082984000) and embedded in paraffin (Merck Catalogue No. 1071511000).

### 2.2. Light microscopy

For the routine light microscopy study, sections were cut on a microtome (Leica, Wetzlar, Germany) at a thickness of 5 μm. The obtained paraffin sections were then mounted on the microscope slides. The sections were stained routinely with hematoxylin (Merck Catalogue No. 1051741000) and eosin (Merck Catalogue No. 1170811000), Mallory's trichrome stain and Van Gieson's stain — all according to standard methods.

### 2.3. Immunohistochemistry

For the immunohistochemical analysis, the sections mounted on gelatin-coated slides were preincubated for one hour in 5% normal goat serum (Vector Laboratories Catalogue No. S-1000, Vector Laboratories, Inc., Burlingame, California, United States) in phosphate-buffered saline (PBS) (Merck Catalogue No. 6505-4 L). After that, incubation with a primary antibody was done for 24 h at room temperature. The following reagents were used: mouse monoclonal anti-collagen type I IgG antibody (Santa Cruz Biotechnology Catalogue No. sc-293182, Santa Cruz Biotechnology, Inc., Heidelberg, Germany); mouse monoclonal anti-procollagen type III IgG antibody (Santa Cruz Biotechnology Catalogue No. sc-166316); mouse monoclonal anti-collagen type V IgG antibody (Santa Cruz Biotechnology Catalogue No. sc-166155); all antibodies were used at concentration 1:500. After rinsing in PBS (Merck Catalogue No. 6505-4 L), incubation in biotinylated goat anti-mouse IgG antibody (Vector Laboratories Catalogue No. BA-9200) at concentration 1:500 for two hours was performed. The sections were washed in PBS (Merck Catalogue No. 6505-4 L) and incubated in avidin-biotin peroxidase complex (Vector Laboratories Catalogue No. PK-6100) for 1 h. This step was followed by rinsing in PBS (Merck Catalogue No. 6505-4 L) and then in 0.05 M Tris–HCl buffer (Sigma Aldrich Catalogue No. T5941, Sigma Aldrich Chemie GmbH, Taufkirchen, Germany), pH 7.6, which preceded incubation in 0.05% 3,3′-diaminobenzidine (DAB) (Sigma Aldrich Catalogue No. D12384) containing 1% hydrogen peroxide ($H_2O_2$) (1:100) for visualization of the reaction. Sections were briefly washed in 0.05 M Tris–HCl buffer (Sigma Aldrich Catalogue No. T5941), pH 7.6. The slides were air-dried for 24 h, then rinsed in distilled water for five minutes, three times, contrastained with hematoxylin (Merck Catalogue No. 1051741000), air-dried again and coverslipped with Entellan (Merck Catalogue No. 1079600500). Eighteen sections were used as controls. All were incubated in the way previously described, but omitting the primary or secondary antibody. All controls were negative.

Photomicrographs of representative fields of the immunohistochemical staining were obtained using Olympus CX 21 microscope fitted with an Olympus C5050Z digital camera (Olympus Optical Co., Ltd, Tokyo, Japan).

### 2.4. Semi-quantitative analysis of the immunohistochemical expression

For semi-quantitative analysis of the expression of collagen type I and V and procollagen type III, we used software ImageJ 1.52a, freely downloaded from the website of the National Institute of Health (NIH) (http://imagej.nih.gov/ij/). The intensity of staining was assessed through the IHC Profiler plugin, freely downloaded from the Sourceforge website (https://sourceforge.net/projects/ihcprofiler/), according to the well-established protocol (Varghese et al., 2014). The IHC Profiler assigned a score to each visual field in a four tier system – high positive (3+), positive (2+), low positive (1+) and negative (0). Five slides were used from each ligament. We analyzed at least ten random visual fields on each slide. The final score was the average of the scores of all visual fields as calculated by the IHC Profiler.

### 2.5. Quantitative analysis of the morphometric parameter number of cells

For the quantitative analysis, we used three randomly selected slides obtained from each paraffin block containing material from the MCL or ACL, respectively (a total of 36 slides from MCL and 36 from ACL). The EL, the endoligament and the ligament tissue were all visualized on each slide. The slides were stained with hematoxylin (Merck Catalogue No. 1051741000) and eosin (Merck Catalogue No. 1170811000) according to standard methods. Quantitative data were obtained with a computerized system for image analysis NIS-Elements Advanced Research, Ver. 2.30 (Nikon CEE GmbH, Vienna, Austria). The regions of interest on each slide were randomly selected from zones without ruptures of the ligament resulting from the laboratory preparation of the samples. These regions of interest were first found on low magnifications (×100, ×200). Next, at magnification ×400, an area which corresponded to surface area of 0.012 $mm^2$ was randomly selected in each of the three studied sub-structures (EL, endoligament, ligament tissue). Five such areas were analyzed in each sub-structure. The nuclei were marked manually with the cursor. Afterwards, the number of cells in each 0.012 $mm^2$ zone was calculated automatically based on the number of nuclei. For more clear representation of the obtained data, the number of cells was then calculated proportionally per surface area of 1 $mm^2$. The obtained data were summarized and analyzed through Microsoft Excel 2010. Statistica software (Dell Software Inc., Round Rock, Texas, United States) was used for the statistical analysis. The quantitative data were evaluated using the Sign test as a nonparametric alternative to the t-test for dependent samples due to the small sample size resulting in a non-normal distribution. The null hypothesis ($H_0$) was rejected, the alternate hypothesis ($H_1$) was accepted and a statistically significant difference was reported in the case of $p < 0.05$.

## 3. Results

### 3.1. Light microscopic observations

The normal structure of the EL of the MCL and ACL in human was quite different from the morphology of the ligament substance (Figs. 1 and 2). The external surface of the EL of the MCL and ACL was comprised of various types of connective tissue cells, including active fibroblasts and non-active fibroblasts (fibrocytes), fat tissue cells and numerous fascicles composed of neuro-vascular units. It appeared that the number of fibroblasts in the EL of the MCL was higher than that in the EL of the ACL; therefore, this morphometric parameter was assessed quantitatively. We also described the presence of a large number of structural randomly organized extracellular collagen fibers, which had uniformly small diameters. The internal surface of the EL of the MCL was in close relation and connected to the medial meniscus. We noted that the EL morphology was quite similar to that of synovium. In contrast to the EL, the ligament tissue was organized in the usual way and was made up

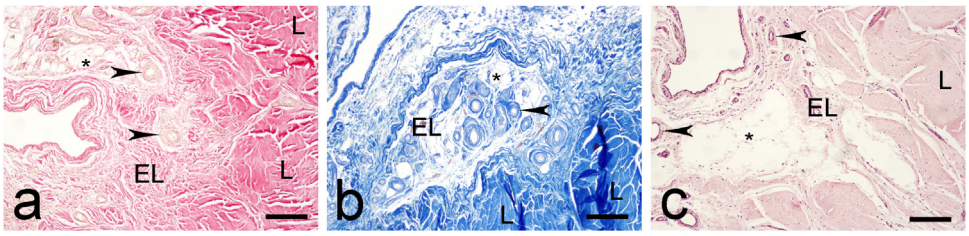


**Fig. 1.** Normal morphology of the epiligament and ligament tissue in the medial collateral ligament in human knee. EL — epiligament; L — ligament tissue; arrows — blood vessels in the EL; asterisks — adipocytes.
a. Hematoxylin and eosin stain. Scale bar — 100 μm.
b. Mallory's trichrome stain. Scale bar — 100 μm.
c. Van Gieson's stain. Scale bar — 200 μm.

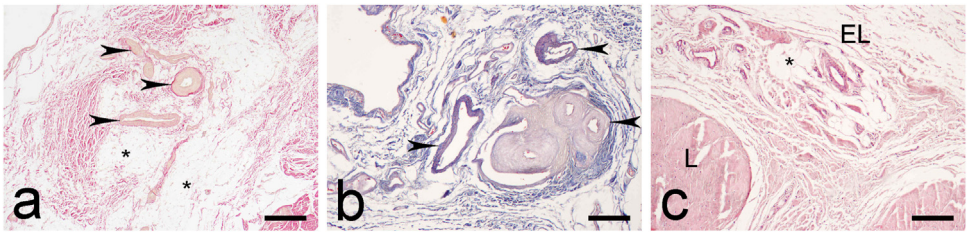


**Fig. 2.** Normal morphology of the epiligament and ligament tissue in the anterior cruciate ligament in human knee. EL — epiligament; L — ligament tissue; arrows — blood vessels in the EL; asterisks — adipocytes.
a. Hematoxylin and eosin stain. Scale bar — 200 μm.
b. Mallory's trichrome stain. Scale bar — 100 μm.
c. Van Gieson's stain. Scale bar — 100 μm.

of collagen fibers with parallel orientation which formed fascicles. Each fascicle appeared hypocellular and the cells were interspersed between the collagen fibers. There were also chaotically orientated small groups of collagen fibers. The ligament tissue contained very few blood vessels and nerve elements.

### *3.2. Expression of collagen type I and V and procollagen type III in the EL of the MCL and ACL*

In the EL, immunostaining for collagen type I was observed predominantly in the tunica media of the blood vessels (Figs. 3a–c

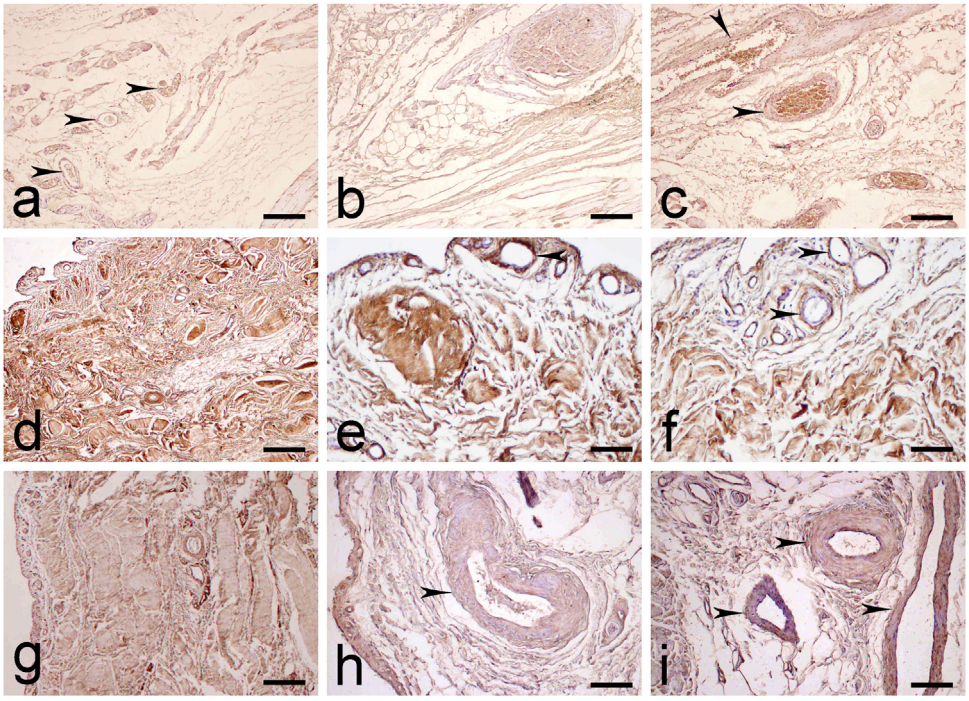


**Fig. 3.** Immunohistochemical expression of collagen type I and V and procollagen type III in the epiligament of the medial collateral ligament in human knee. Arrowheads — immunoreactivity in the adventitia and tunica media of blood vessels.
a–c. Immunohistochemical expression of collagen type I. Scale bar — 100 μm.
d–f. Immunohistochemical expression of procollagen type III. Scale bar — d: 200 μm; e, f: 50 μm.
g–i. Immunohistochemical expression of collagen type V. Scale bar — g: 200 μm; h, i: 50 μm.

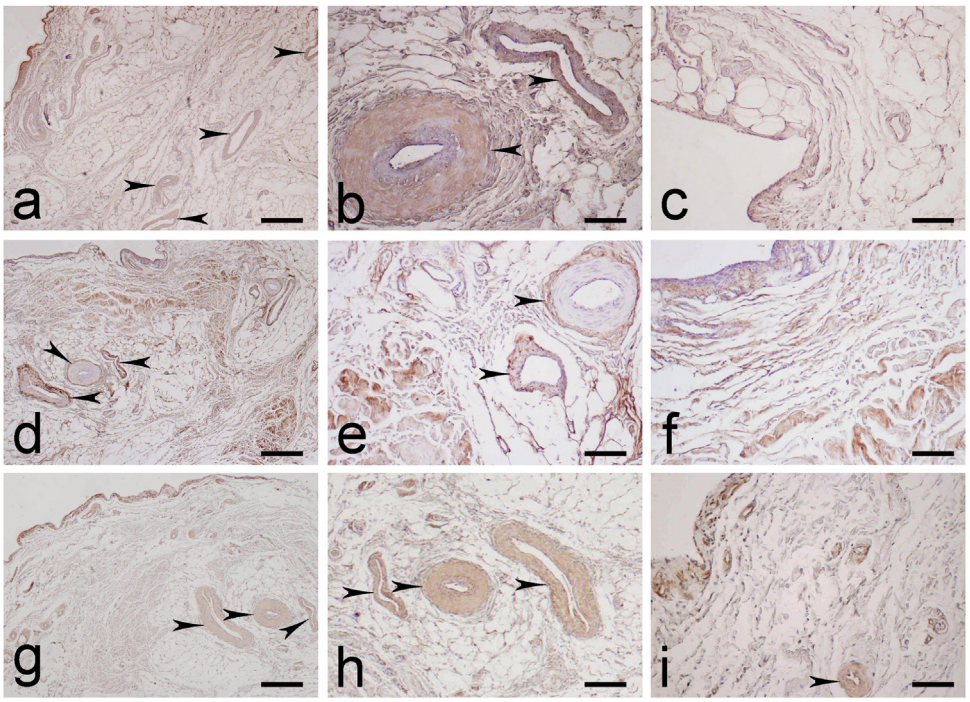


**Fig. 4.** Immunohistochemical expression of collagen type I and V and procollagen type III in the epiligament of the anterior cruciate ligament in human knee. Arrowheads — immunoreactivity in the adventitia and tunica media of blood vessels.
a–c. Immunohistochemical expression of collagen type I. Scale bar — a: 200 μm; b, c: 50 μm.
d–f. Immunohistochemical expression of procollagen type III. Scale bar — d: 200 μm; e, f: 50 μm.
g–i. Immunohistochemical expression of collagen type V. Scale bar — g: 200 μm; h, i: 50 μm.

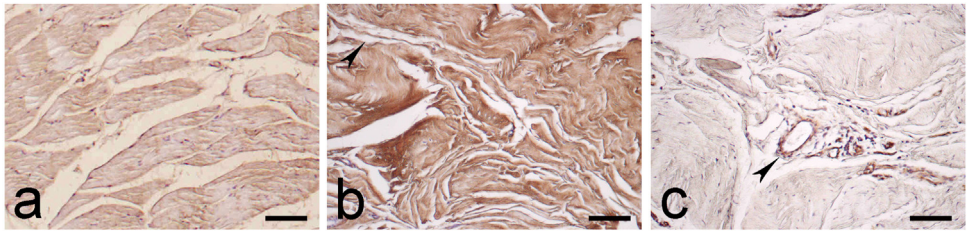


**Fig. 5.** Immunohistochemical expression of collagen type I and V and procollagen type III in the ligament tissue of the medial collateral ligament in human knee. Arrowheads — sheets of epiligament tissue spreading throughout the ligament tissue, thus forming the endoligament.
a. Immunohistochemical expression of collagen type I. Scale bar — 50 μm.
b. Immunohistochemical expression of procollagen type III. Scale bar — 50 μm.
c. Immunohistochemical expression of collagen type V. Scale bar — 50 μm.

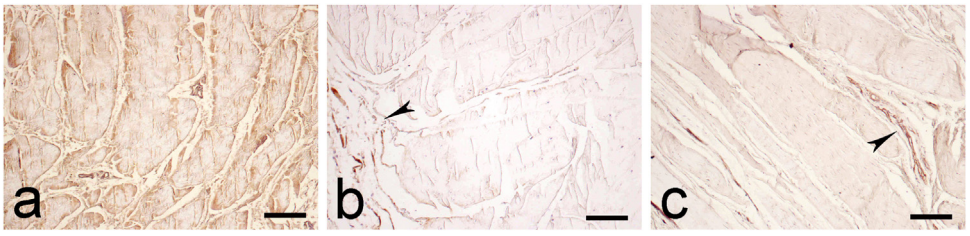


**Fig. 6.** Immunohistochemical expression of collagen type I and V and procollagen type III in the ligament tissue of the anterior cruciate ligament in human knee. Arrowheads — sheets of epiligament tissue spreading throughout the ligament tissue, thus forming the endoligament.
a. Immunohistochemical expression of collagen type I. Scale bar — 100 μm.
b. Immunohistochemical expression of procollagen type III. Scale bar — 200 μm.
c. Immunohistochemical expression of collagen type V. Scale bar — 100 μm.

and 4a–c). In the ligament tissue of both the MCL and the ACL, immunoreactivity was expressed ubiquitously and appeared moderate (Figs. 5a and 6a). The immunohistochemical reaction for procollagen type III was detected in the adventitia of blood vessels and on the periphery of adipocytes in the EL and was much stronger in the EL of the MCL (Figs. 3d–f and 4d–f). In the ligament tissue of the MCL, expression again appeared strong and ubiquitously distributed (Fig. 5b). In contrast, in the ligament tissue of the ACL, immunoreactivity appeared very low to absent (Fig. 6b). Collagen type V was localized mostly in the tunica media of the blood vessels

**Table 1**
Semi-quantitative analysis of the immunohistochemical expression of collagen type I and V and procollagen type III in the epiligament, endoligament and ligament tissue of the anterior cruciate and medial collateral ligament. The percentage for each score represents the percentage of visual fields that the IHC Profiler assigned this score to.

| Type of collagen/procollagen | ACL | | MCL | |
|---|---|---|---|---|
| | EL | L + EndoL | EL | L + EndoL |
| Collagen type I | Positive (2+) (11%)<br>Low-positive (1+) (50%)<br>Negative (0) (39%) | Positive (2+) (54%)<br>Low-positive (1+) (31%)<br>Negative (0) (15%) | Positive (2+) (13%)<br>Low-positive (1+) (65%)<br>Negative (0) (22%) | Positive (2+) (61%)<br>Low-positive (1+) (29%)<br>Negative (0) (10%) |
| Procollagen type III | Positive (2+) (38%)<br>Low-positive (1+) (52%)<br>Negative (0) (10%) | Low-positive (1+) (23%)<br>Negative (0) (77%) | High-positive (3+) (31%)<br>Positive (2+) (45%)<br>Low-positive (1+) (17%)<br>Negative (0) (7%) | High-positive (3+) (33%)<br>Positive (2+) (48%)<br>Low-positive (1+) (15%)<br>Negative (0) (4%) |
| Collagen type V | Positive (2+) (27%)<br>Low-positive (1+) (49%)<br>Negative (0) (24%) | Low-positive (1+) (25%)<br>Negative (0) (75%) | Positive (2+) (34%)<br>Low-positive (1+) (46%)<br>Negative (0) (20%) | Low-positive (1+) (29%)<br>Negative (0) (71%) |

ACL — anterior cruciate ligament; MCL — medial collateral ligament; EL — epiligament; L — ligament tissue; EndoL — endoligament.

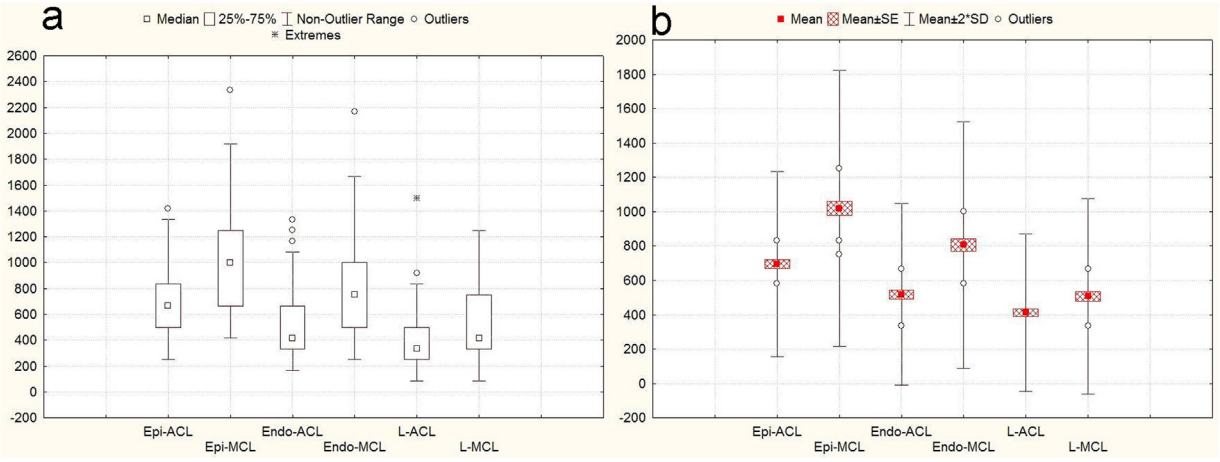


**Fig. 7.** Graphical representation of the descriptive statistics of the quantitative data for the number of cells in the anterior cruciate and medial collateral ligament. Epi — epiligament; Endo — endoligament; L — ligament tissue; MCL — medial collateral ligament; ACL — anterior cruciate ligament.
a. Box and whisker plots showing the median (square), surrounded by a 'box', the vertical edge of which is the interval between the lower and upper quartile [25%–75%]. 'Whiskers' originating from this 'box' represent the non-outlier range. Circles — outliers; asterisks — extremes.
b. Box and whisker plots showing the mean (red square), surrounded by a 'box', the vertical edge of which is the interval [Mean-SE, Mean + SE]. 'Whiskers' originating from this 'box' represent the 2-sigma confidence interval of the mean, i.e. [Mean-2*SD, Mean+2*SD]. Circles — outliers; SE — standard error of mean; SD — standard deviation.

and in the superficial layer of the EL in both ligaments (Figs. 3g–i and 4g–i). In the ligament tissue, its expression appeared very low to absent (Figs. 5c and 6c). Since the intensity of the immunohistochemical reaction varied between the EL and ligament tissue of the MCL and ACL for the studied types of collagen and procollagen type III, it was calculated semi-quantitatively using the IHC Profiler. Results are summarized in Table 1.

### 3.3. Quantitative analysis

The comparative quantitative analysis of the parameter number of cells in the MCL and the ACL revealed certain differences between the mean and median values obtained in the EL of the two ligaments (Fig. 7; Table 2). The mean number of cells in the EL of the MCL was approximately 32% higher than that in the EL of the ACL; the median – approximately 33% higher. Perhaps it is interesting to mention that this difference was even higher in the endoligament, where the endoligament of the MCL contained approximately 36% more cells than that of the ACL; the median was 44% higher. As for the ligament tissue, we only noted a difference in the mean values of slightly less than 19% and 20% in the medians – again a higher number of cells being reported in the MCL.

The statistical significance of the difference in the number of cells in each of the three substructures of the two ligaments was evaluated with the Sign test. Comparing the medians in the EL of the

**Table 2**
Numerical representation of the descriptive statistics of the quantitative data for the number of cells in the anterior cruciate and medial collateral ligament.

| | Epi-ACL | Epi-MCL | Endo-ACL | Endo-MCL | L-ACL | L-MCL |
|---|---|---|---|---|---|---|
| MEAN | 694.4 | 1019.4 | 518.5 | 806.4 | 412.5 | 507.6 |
| MEDIAN | 666.7 | 1000.0 | 416.7 | 750.0 | 333.3 | 416.7 |
| SD | 268.6 | 400.9 | 263.9 | 358.9 | 229.7 | 284.2 |
| MIN | 250.0 | 416.7 | 166.7 | 250.0 | 83.3 | 83.3 |
| MAX | 1416.7 | 2333.3 | 1333.3 | 2166.7 | 1500.0 | 1250.0 |
| 25th% | 500.0 | 666.7 | 333.3 | 500.0 | 250.0 | 333.3 |
| 75th% | 833.3 | 1250.0 | 666.7 | 1000.0 | 500.0 | 750.0 |

ACL — anterior cruciate ligament; MCL — medial collateral ligament; Epi — epiligament; Endo — endoligament; L — ligament tissue; SD — standard deviation.

MCL and ACL yielded a $p = 0.000...$ ($p < 0.05$), the null hypothesis ($H_0$) was rejected, the alternate hypothesis ($H_1$) was accepted and statistically significant difference was reported. The comparison of the values in the endoligament yielded a $p = 0.000004$ ($p < 0.05$), $H_0$ was rejected, $H_1$ was accepted and statistically significant difference was reported. When we compared values in the ligament tissue, we obtained $p = 0.113846$ ($p > 0.05$), $H_0$ was accepted, $H_1$ was rejected and the reported difference between the median of the number of cells in the ligament tissue of the MCL and ACL was considered not to be statistically significant.

## 4. Discussion

In 1990, Bray et al. provided the first description of the EL of the MCL in their work 'Fine vascular anatomy of adult rabbit knee ligaments' (Bray et al., 1990). Later, Chowdhury et al. (1991) also examined the external surface of the EL of the MCL in rabbits and described two types of cells – spinous-shaped adipocytes and fibroblasts. The latter participate in the formation of the ligament scar tissue and are chiefly responsible for the synthesis of collagen fibers. More recently, Georgiev and Vidinov (2009c), and Georgiev et al. (2010b) described in detail the histological appearance and ultrastructural characteristics of the EL under normal conditions and in the setting of experimental ligament injury. They reported that the external aspect of the EL consisted of fibroblasts, fibrocytes, adipocytes, neurovascular bundles, and a multitude of multidirectional collagen fibers. Due to their ultrastructural appearance, Georgiev et al. (2010a, b, 2019) concluded that fibroblasts in the EL may be involved in the differentiation, phagocytosis and synthesis of collagen, which may define their possible role in the regeneration of the injured ligament.

Later, Georgiev et al. (2017a) extended their investigations on the morphology of the EL of the MCL in human knee. The authors made a detailed description of the normal morphology of the EL and demonstrated differences between the structure of the EL and that of the ligament tissue. Their electron microscopic analysis revealed the specific ultrastructural characteristics of the various types of cells in the EL and supported the hypothesis that fibroblasts in particular, together with the abundant blood vessels, are essential for the nutrition and normal function of the MCL, as well as its healing after injury (Georgiev et al., 2017a).

Ninety percent of knee ligament injuries involve the MCL or the ACL. In contrast to MCL, the ACL has a poor healing ability (Andrews et al., 2017; Kiapour and Murray, 2014; Wijdicks et al., 2010). So far, multiple explanations for this phenomenon have been suggested. These include different ultrastructural characteristics of the connective tissue cells in the MCL and ACL (Lyon et al., 1991); variations in the proliferative potential of fibroblasts (Amiel et al., 1995; Yoshida and Fujii, 1999); higher levels of nitric oxide produced by the ACL which inhibit collagen and proteoglycan synthesis (Cao et al., 2000); a superior capacity of the MCL to increase its blood supply after injury through angiogenesis and increased blood flow (Bray et al., 2003); distinctive, ligament-specific properties of stem cells (Furumatsu et al., 2010; Zhang et al., 2011); differential expression of MMP-2, -9 and -13 (Georgiev et al., 2018; Nishikawa et al., 2018). The unsatisfactory healing of the ACL may also be due to the failure of cells and blood vessels to bridge the gap between the ruptured ends of the ACL in an adequate way, as well as the lack of the wound-site to fill within the intra-articular environment (Chen, 2009). The intra-articular location of the ACL means that it is exposed to the synovial fluid which has been shown to inhibit ACL fibroblasts (Andrish and Holmes, 1979). Furthermore, plasmin circulating in the synovial fluid breaks down the fibrin clot, thereby slowing down healing (Vavken and Murray, 2011).

We herein suggest that some of the additional possible reasons for the discrepancy in healing potential may be: 1) lower number of cells; 2) lower expression of collagen type I, III and V. To confirm our hypothesis, we investigated the EL of the MCL and ACL qualitatively, semi-quantitatively and quantitatively. Our results showed that: 1) the number of connective tissue cells, namely fibroblasts, was higher in the EL of the MCL compared to the ACL and this difference was statistically significant; 2) the fibroblasts in the EL of both ligaments were not static cells but produced collagen type I and V and procollagen type III; 3) expression of collagen type I was predominantly positive (2+) in the ligament tissue of both ligaments but remained mostly low-positive (1+) in the EL; 4) procollagen type III was expressed in the EL of both ligaments, however expression was stronger in the EL of the MCL; 5) procollagen type III was also expressed in the ligament tissue of the MCL, with immunoreactivity being predominantly positive (2+) (48%), while in the ligament tissue of the ACL, it was predominantly negative (0) (77%); 6) expression of collagen type V was predominantly low-positive (1+) in the EL of both ligaments and remained mostly negative (0) in the ligament tissue.

Collagen is the most abundant solid constituent of the extracellular matrix forming the major tensile-bearing component of soft connective tissues (Frank et al., 1999; Georgiev et al., 2010a, 2015a, b; Wan et al., 2013). Collagen type I is the main type of collagen found in ligaments. It is responsible for the ligament tensile strength (Georgiev et al., 2010a, 2015a, b; Woo et al., 2006). Type III and V are the other major components of ligaments (Georgiev et al., 2010a, 2015a, b; Wan et al., 2013). Their synthesis is upregulared after injury to levels higher than those in mature ligaments and as we have stated previously in a rat model of MCL injury, they are expressed mainly in the EL tissue (Georgiev et al., 2010a, 2015a, b). Niyibizi et al. (2000), Hsu et al. (2010) and Georgiev et al. (2010b) have all reported that collagen type III is essential for proper ligament healing. These authors established that in the early phases of ligament healing, production of collagen type III was higher than that of collagen type I and returned to normal levels by week 52 of healing. Thus, the higher expression of procollagen type III, the precursor molecule from which collagen type III is synthesized, in the EL of the MCL may point to a higher 'readiness' of the ligament to respond to injury by initiating a proper repair process. Furthermore, this may be another factor contributing to the better healing potential of the MCL as opposed to the ACL. According to Yang et al. (1999) collagen type III is also essential for the genesis of the tissue matrix, fetal tissue matrix, and scar formation. These authors hypothesized that the ability of collagen type III to crosslink by disulfide bridges may contribute to its favorable deposition in sites of tissue regeneration. Amiel et al. (1986) also found that collagen type III was increased during the ligamentization after tendon graft.

Collagen type V is a member of the fibrillar subfamily of collagens. It is involved in the organization and regulation of collagen type I fibril diameters (Breuls et al., 2009; Georgiev et al., 2010a, 2015a, b; Liu et al., 1995). Collagen type V may affect cell morphology, growth kinetics, protein synthesis and migration during tissue development, the process of inflammation and wound repair (Breuls et al., 2009; Georgiev et al., 2010a, 2015a, b; Liu et al., 1995). Georgiev et al. (2010a, b, 2015b) observed a low-positive immunohistochemical reaction for collagen type V on the $8^{th}$ and $16^{th}$ day after grade III injury of the collateral ligaments of the knee in a rat model and similar to normal reaction on the $30^{th}$ day. According to Breuls et al. (2009) collagen type V may play an important regulatory role in collagen fibril initiation, in the modeling and remodeling of the extracellular matrix. In addition, Andresen et al. (2000) suggested that the increased levels of this collagen can modify the stiffness of the extracellular matrix by changing the fibril organization.

Based on the fact that the EL is the major source of cells and blood vessels that ensure early MCL healing and the current results on the differences between MCL and ACL EL morphology, we present a confirmation of our hypothesis about the failure of ACL healing. In the healthy knee, the cells in the EL of ACL are lower in number compared to MCL, and therefore cannot ensure adequate healing capacity. Moreover, the expression of collagen type I (responsible for the ligament tensile strength), procollagen type III (essential for proper ligament healing) and collagen V (organize and regulate collagen type I fibril diameters) from fibroblasts are also lower in the EL tissue of the ACL as opposed to the MCL. All aforementioned variations of EL morphology and differences in fibroblast activity in healthy knee, especially the expression of procollagen type III, provide additional explanation about the failure of ACL healing after trauma.

Limitations of the current study existed and should be outlined. Usually, the age of the cadavers used for scientific purposes may compromise results owing to age-related alterations in the morphology of the examined samples. In particular, knee osteoarthritis (gonarthrosis), a typical age-related disease, may result in joint instability, thereby leading to MCL bursitis, ACL rupture, increased ligament stiffness and others (Fishkin et al., 2002; Hill et al., 2005; Nur et al., 2018). In the present study, samples were obtained from fresh cadavers with a mean age of 55 years with no previous history of gonarthrosis or trauma. Thus, we attempted to eliminate the impact of aging on the obtained results. The assessment of differences in the structure of the EL of the MCL and the ACL on histological slides stained routinely with hematoxylin and eosin was of somewhat descriptive nature, which represented another limitation. We attempted to eliminate researcher bias by conducting a quantitative analysis of the number of connective tissue cells in each of the three sub-structures of the ligament – EL, endoligament and ligament tissue and comparing the obtained values between the MCL and ACL. We used non-parametrical statistics, namely the Sign test, due to the small sample size. While this test yields plausible results, it is associated with more general applicability and may thus lack the power of alternative parametric tests. Visual quantification of immunohistochemical images is also associated with significant inter- and intra-observer variation. We attempted to resolve this issue by using the IHC Profiler plugin for the ImageJ software, which eliminates inter-observer visual perception bias. In fact, 88.6% of scoring determined by the IHC Profiler coincides with blinded manual scoring determined by trained pathologists ($P < 0.0001$, CI = 95%) (Varghese et al., 2014).

## 5. Conclusions

The results of our study confirm that: 1) the EL of the MCL and the ACL is a quite different structure from the ligament tissue; 2) the EL of these ligaments is hypercellular compared with the ligament tissue; 3) cells found in the EL include fibroblasts, fibrocytes and adipocytes and are accompanied by the presence of neurovascular bundles; 4) fibroblasts in the EL are not static cells and produce different types of collagens; 5) sheets of EL tissue extend towards the ligament tissue, thus forming the endoligament; 6) collagen fibers in the EL have uniformly small diameters and are organized in bundles with different orientations; 7) the number of blood vessels in the EL is relatively abundant and higher than that in the ligament tissue. Furthermore, herein we have reported for the first time in human knees that: 1) the number of connective tissue cells in the EL of the ACL is lower than that in the EL of the MCL, and the difference is statistically significant; 2) expression of collagen type I and V and procollagen type III is higher in the EL of the MCL than in the EL of the ACL; 3) procollagen type III is also expressed in the ligament tissue of the MCL under physiological conditions. Therefore, the expression of these collagens in line with their ascribed roles, as well as the structural differences between the EL of the ACL and the MCL, could provide yet another explanation for the difference in the healing potential of these ligaments.

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