Histological characterization of the human scapholunate ligament

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Abstract

The scapholunate interosseous ligament (SLIL) plays a fundamental role in stabilizing the wrist bones, and its disruption is a frequent cause of wrist arthrosis and disfunction. Traditionally, this structure is considered to be a variety of fibrocartilaginous tissue and consists of three regions: dorsal, membranous and palmar. Despite its functional relevance, the exact composition of the human SLIL is not well understood. In the present work, we have analyzed the human SLIL and control tissues from the human hand using an array of histological, histochemical and immunohistochemical methods to characterize each region of this structure. Results reveal that the SLIL is heterogeneous, and each region can be subdivided in two zones that are histologically different to the other zones. Analysis of collagen and elastic fibers, collagens types I, III and IV, proteoglycans, glycoproteins and versican confirmed that the different regions can be subdivided in two zones that have their own structure and composition. The first part of the dorsal region (zone D1) resembles the control tendons and ligaments, whereas the rest of the SLIL are more similar to the control articular cartilage, especially the first part of the membranous region (zone M1). Cells showing a chondrocyte-like phenotype as determined by S100 were more abundant in M1, whereas the zone containing more CD73-positive stem cells was D2. These results confirm the heterogeneity of the human SLIL and could contribute to explain why certain zones of this structure are more prone to structural damage and why other zones have specific regeneration potential.

INTRODUCTION

The physiological function of the human wrist is strictly dependent on the complex anatomy of the carpal bones and the ligaments providing stability to these bones (Andersson, 2017). The human scapholunate interosseous ligament (SLIL) is a C-shape structure connecting the scaphoid and lunate carpal bones, and it is considered as the primary stabilizer of the scapholunate joint (Johnson et al., 2013). SLIL disruption due to trauma or degeneration is considered the most common cause of carpal instability, which can significantly compromise hand function and lead to wrist arthrosis (Kitay & Wolfe, 2012; Wolff & Wolfe, 2016). The regenerative capability of the human SLIL is very limited, and injuries in this structure cannot heal by themselves and typically require surgical treatment (Mullikin et al., 2020). Although, numerous surgical procedures have been described (Lui et al., 2019; Mullikin et al., 2020), reconstruction of the dorsal component of the SLIL is the usual surgical treatment for SLIL injuries, underestimating the biomechanical role of the membranous and palmar portions of this structure (Naqui et al., 2018).

Despite its key role in carpal physiology, the exact structure and composition of the human SLIL are not fully understood. The gross morphology of the SLIL was originally defined by Berger and cols. (Berger, 1996), who described three major zones in the human SLIL: the dorsal, membranous, and palmar regions. The dorsal region is transversely oriented, whereas the palmar region is oblique, allowing significant relative movement between the two bones (Sokolow & Saffar, 2001). Numerous reports focused on the study of the

SLIL from the anatomical, kinematical and biomechanical standpoints (Kitay & Wolfe, 2012; Rajan & Day, 2015; Wolff & Wolfe, 2016).

Histologically, the human SLIL is thought to be composed of collagen fascicles infiltrated by loosely organized connective tissue, as it is the case of most other intraarticular ligaments, although the SLIL could also share some similarities with human fibrocartilage (Berger, 1996; Sokolow & Saffar, 2001). However, the detailed histological structure of the human SLIL remains to be elucidated.

In the present work, we carried out a comprehensive histological characterization of the human SLIL using an array of histochemical and immunohistochemical methods in order to determine the main extracellular matrix molecules and cells which form part of this ligament.

MATERIALS AND METHODS

Tissue samples and histological analysis

We used six hand specimens obtained from six fresh-frozen cadavers, who provided informed consent to use their organs and tissues for scientific purposes (McHanwell et al., 2008; Riederer et al., 2012). The specimens had no previously documented hand injuries or surgeries. All specimens were thawed at room temperature and dissected in the Human Anatomy Department of the School of Medicine of the University of Granada (Spain). Specifically, the carpal bones were dissected, and the scaphoid and lunate bones joined by the SLIL were carefully extracted and transferred to the Laboratory of Histology of the Medical School of the University of Granada. Then, the SLIL were carefully separated from the carpal bones and fixed in 10% buffered formalin (Panreac Química S.L.U., Barcelona, Spain) for histological analysis. As controls, several structures were also dissected and extracted from the same human hands, including samples of the flexor tendon (FT), carpal ligament (CL), articular cartilage (AC), triangular fibrocartilage (TF) carpal articular capsule (CC) and retinaculum (RT). These tissues were fixed and processed using the same protocols used for the SLIL. Authorization was obtained from the Department of Anatomy of the Medical School of the University of Granada, which approved the study.

Formalin-fixed SLIL and control tissues were dehydrated, cleared in xylene and embedded in paraffin following routine histological methods. 5 μm-thick sections were obtained from each sample, dewaxed in ethanol series, rehydrated and stained with hematoxylin and eosin (HE) (Panreac Química S.L.U.) using standard protocols. Histological images were then obtained using a Pannoramic Desk DW II histological scanner (3DHISTECH, Budapest, Hungary) from controls and from 6 zones of the human SLIL (Figure 1): dorsal region -part 1- (D1), dorsal region -part 2- (D2), membranous region -part 1- (M1), membranous region -part 2- (M2), palmar region -part 1- (P1) and palmar region -part 2- (P2).

Histochemistry

To evaluate the presence and distribution of relevant components of the extracellular matrix (ECM), all tissue samples were subjected to histochemical methods. To identify the tissue elastic fibers, samples were stained using Verhoeff histochemical staining method (VHF). To reveal the presence of fibrillar collagens, we used picrosirius red (PSR). Non-fibrillar ECM components were evaluated using alcian blue staining (AB) at pH 2.5 for proteoglycans, and periodic acid–Schiff staining (PAS) for glycoproteins. All these methods were performed following previously published standard histochemical methods (Blanco-Elices et al., 2022; V. S. Carriel et al., 2011; García-García et al., 2021; Sánchez-Porras et al., 2023).

Immunohistochemistry

Indirect immunohistochemistry was performed for the specific identification of ECM components and cell phenotype. In relation to the ECM fibers, collagens types I, III and IV were determined using this method. In addition, versican (VSC), a hyaluronan-binding proteoglycan which interacts with other ECM components and contributes to control tissue hydration was also studied (Godoy-Guzmán et al., 2018). To identify specific cell phenotypes, immunohistochemical methods were used for the proteins CD73 (marker of stemness and undifferentiation), S100 (marker of chondrogenic and neural crest lineage) and CD34 a well-known marker

of blood vessels (García-Martínez et al., 2017; Vela-Romera et al., 2019). All immunohistochemical analyses were carried out using routine methods, and were conducted using the same conditions for all samples. Technical details are summarized in Supplementary Table S1.

Quantitative analyses

Results of the histochemical analyses were quantified in each zone of the SLIL and control tissues using the ImageJ software (version 1.53k, National Institute of Health, Bethesda, MD), as previously described (V. Carriel et al., 2014; Ortiz-Arrabal et al., 2021; Rodriguez-Pozo et al., 2020; Ruiz-López et al., 2022). In each image, we randomly selected five 25 μm × 25 μm square areas, and we obtained a numeric value (expressed as intensity units or I.U.) corresponding to the average pixel intensity within each square using the square tool of the software. Results were then subtracted from the blank values and averages and standard deviations were calculated for each group of samples. A total of 30 measurements were obtained for each type of tissue (controls and SLIL zones). Results corresponding to the analysis of cell phenotypes as determined by CD73 and S100 immunohistochemistry were quantified by determining the percentage of positive cells found at in each region of the SLIL. In short, we counted the total number of cells and the number of cells showing positive and negative signal within a 300 μm × 300 μm area randomly selected on each region, and the percentage of positive cells was calculated. The same method was used to quantify the number of blood vessels found at each SLIL region in samples subjected to CD34 immunohistochemistry.

Hierarchical clustering

In order to identify differences and similarities among the different samples analyzed here (SLIL zones and controls) based on their global histochemical and immunohistochemical profiles, we analyzed all samples using hierarchical clustering. With this purpose, average results obtained for all quantitative histochemical and immunohistochemical analyses were analyzed using ClustVis web tool for clustering of multivariate data (Metsalu & Vilo, 2015) (accessible at*https://biit.cs.ut.ee/clustvis/*). A Heatmap dendrogram displaying relative quantitative signals for each marker was generated by the software based on hierarchical clustering analysis using the Euclidean distance and the complete clustering method for both the rows and columns.

Statistical analysis

Results obtained for each SLIL region subjected to each analytical technique were statistically compared with each control tissue. The two regions that we analyzed from each of the zones originally established by Berger were also compared (D1 vs. D2, M1 vs. M2 and P1 vs. P2). First, we evaluated the normality of each distribution using the Shapiro-Wilk test. As none of the distributions fulfilled the criteria of normality, comparison between two specific groups of samples were carried out using the non-parametric test of Mann-Whitney. The comparisons were done with Real Statistics Resource Pack software (Release 7.2) (Dr. Charles Zaiontz, Purdue University, West Lafayette, IN, USA), available at*www.real-statistics.com*. A Bonferroni-adjusted statistical significance p value below 0.001 was set for all double-tailed tests, since multiple comparisons were carried out at the same time.

RESULTS

1. Histological structure of the human SLIL

Analysis of the human SLIL using HE staining confirmed that this structure was histologically heterogeneous, with several histological differences found at the different zones analyzed in this work (Figure 2). When the dorsal region of the SLIL was analyzed, we first found that the D1 zone consisted of a dense ECM with abundant fibers oriented in different spatial directions and a scarce population of elongated or spindle-shaped cells dispersed across the ECM. Then, D2 showed an ECM containing abundant well aligned and oriented fibers, and an abundant population of spindle-shaped cells displaying similarities with the cells in D1. At the membranous region, we found that M1 contained a dense ECM with no identifiable fibers and rounded cells scattered within the tissue ECM. Interestingly, these cells tended to form groups resembling the human hyaline cartilage isogenic groups, with clusters of 2 to 4 cells surrounded by a clear ECM with a peripheral capsule that was very similar to the structure found in normal human cartilage. In turn, the M2 zone showed

a very dense ECM with fibers apparently oriented in layers with different spatial directions. Cells found in M2 were not abundant and tended to form rows that partially resembled human fibrochondrocytes and were also surrounded by a pericellular capsule. Finally, the palmar region of the SLIL contained abundant fibers and scattered spindle-shaped cells. For P1, we found abundant cells within a dense ECM with properly oriented fibers. However, analysis of the P2 zone revealed the presence of fewer elongated or rounded cells, and the fibers were oriented to different spatial directions.

2. Analysis of the human scapholunate ligament ECM

First, our analysis of elastic fibers as determined by VHF histochemistry showed very low amount of these ECM molecules all samples, with no differences among samples (Figure 3). Elastic fibers were thin and parallel oriented to the longitudinal axis of the abundant collagen network. Quantitative analyses showed significant differences only between the P2 region and FT and RT control tissues and between P1 and FT (Table 1). In contrast, the quantitative analysis of fibrillar collagen fibers identified by PSR histochemical staining revealed that all control tissues and the six regions of the SLIL analyzed here contained high amounts of collagen (Figure 3). Differences were statistically significant for most zones, especially for P1, which showed significantly lower amounts of collagen than the six control tissues (Table 1). Interestingly, comparisons between the two zones distinguished within each region in this study (D1 vs. D2, M1 vs. M2 and P1 vs. P2) were statistically significant for the amount of fibrillar collagen fibers identified by PSR (Table 1).

Then, we quantified three types of collagens by immunohistochemistry, and we found that, in general, control tissues tended to show higher expression of Col-I, Col-III and Col-IV than the SLIL, except for AC (Figure 4 and Table 1). For Col-I, we found that most SLIL regions significantly differed from all CTR tissues, with regions D2, P1 and P2 showing the lowest amounts of this type of collagen. Within the regions, we found that D1 was significantly higher than D2. Regarding Col-III, most SLIL regions expressed low amounts of these fibrillar components, except M2, which was highly positive for this ECM component. Most control tissues tended to express high amounts of type-III collagen, with the only exception of AC. Differences were significant for most comparisons, including the comparisons within each region, but were non-significant for the comparison of AC vs. D2, M1 and P1. For Col-IV, we found that most control tissues and SLIL zones contained very few amounts of this protein, whilst M2 was highly positive. Differences between M2 and all types of control tissues were statistically significant, as well as the differences between M1 and M2 (Table 1).

When the ECM proteoglycans were identified by AB histochemistry (Figure 5 and Table 1), we found that most regions of the SLIL were enriched in these non-fibrillar components of the tissue ECM, especially in the case of the zones M1, M2 and P2. However, the presence of these components was low in most control tissues, with the exception of AC, which was statistically higher than all the other samples (SLIL regions and controls). M1, M2 and P2 were statistically higher than all control tissues, whereas D2 was higher than D1 and P2, than P1. When versican was analyzed, we found that this protein playing an important role in ECM homeostasis was highly positive in D2 and P1, with significant differences with AC, CL and RT in both cases, whereas very low amounts of VRS were found in M1, which statistically differed from all control tissues except RT. Comparisons within the SLIL regions revealed statistical differences between M1 and M2 and between P1 and P2 (Figure 5 and Table 1).

In addition, the analysis of glycoproteins using PAS histochemistry (Figure 5 and Table 1) revealed that the highest expression corresponded again to AC control tissues. For the SLIL zones, M2, P1 and P2 showed the highest glycosaminoglycans content, with statistical differences with all the control tissues. Differences were also statistically significant for the comparison within each SLIL region (P1 vs. P2, M1 vs. M2, and D1 vs. D2).

3. Characterization of the human scapholunate ligament cell populations and blood vessels

In order to determine the phenotype of the cells found at the different regions of the SLIL, we first carried out an immunohistochemical analysis of S100 expression. As shown in Figure 6 and Table 2, our results

show that the zones that were more enriched in S100-positive cells was M1, followed by D2 and M2, whereas the zone with lower content of positive cells was D1. Differences between D1 and the rest of SLIL zones were statistically significant, as well as the differences between P1 and D2 and M1 and between P2 and D2 and M1. Then, we analyzed the expression of the undifferentiation marker CD73, and we found that all regions contained cells showing positive signal for this marker, although the highest number of positive cells corresponded to D2, and the lowest, to P2. Differences between D2 and D1, P1 and P2 were statistically significant (Figure 6 and Table 2).

In order to evaluate the distribution of the blood vessels in the SLIL, we quantified these structures in each zone of the SLIL, after staining each vessel by CD34 immunohistochemistry. As shown in Figure 6 and Table 2, our analysis revealed that blood vessels were abundant in D1, D2, P1 and P2, but were very scarce in M1 and M2. Differences between M1 or M2 and the rest of zones were statistically significant.

4. Hierarchical classification of the human scapholunate ligament

Once we quantified eight relevant ECM components in SLIL and control tissues, we classified the different samples based on their global profile for these ECM components using hierarchical clustering. As shown in Figure 7, our results showed two main groups of samples. In the first branch, we found five out of the six control tissues analyzed here (TF, CC, CL, FT, and RT), along with the SLIL zone D1, although the control tissues and D1 formed independent classification subbranches. In the second branch, however, we found five out of the six SLIL zones (D2, M1, M2, P1 and P2) clustering together with AC control tissues. Interestingly, AC clustered together with M1, with both types of tissues forming an independent classification subbranch.

DISCUSSION

Despite its crucial role as a major stabilizer of the wrist bone, the exact structure of the human SLIL is not properly understood. For this reason, in the present study, we evaluated the human SLIL using an array of histological, histochemical and immunohistochemical methods, trying to shed light on the specific composition of each zone of the SLIL. In the first place, we analyzed each SLIL region after establishing two subregions in each of the classically defined regions (dorsal, membranous and palmar) (Berger, 1996; Berger et al., 1991). This subdivision of each region was done to define the histological characteristics of each zone of the ligament with higher precision. In fact, the original classification was made based on the gross aspect of the SLIL, what may not coincide with the histological features of each zone of the SLIL.

The results of our histological analysis revealed that the SLIL was heterogeneous, and each subregion had specific histological characteristics. First, our histological analysis using HE staining confirmed that the dorsal region of the SLIL consisted of a dense ECM with dispersed elongated cells that partially resembles a human ligament, as other authors already demonstrated (Berger, 1996; Berger et al., 1991; Berger & Blair, 1984; Sokolow & Saffar, 2001). However, the three-dimensional disposition of the fibers and the cell distribution were different in both subregions of this area (D1 and D2), which supports the idea that the dorsal area consisted of two distinct zones. Then, the analysis of the membranous region revealed the presence of a dense tissue containing cells resembling human chondrocytes that were surrounded by a well-defined pericellular matrix and a capsule, as it is the case of the human cartilage. Although the similitude of the membranous region with human fibrocartilage was previously preconized (Berger, 1996; Berger et al., 1991; Berger & Blair, 1984), we found that the ECM was more dense in M2 than in M1, and both the cells and the ECM in M1 were more similar to hyaline chondrocytes than to fibrochondrocytes, supporting again the possibility that both subregions could be histologically different. Finally, our characterization of the palmar region revealed a structure containing abundant fibers and scattered spindle-shaped cells, as previously described (Berger, 1996; Berger et al., 1991; Berger & Blair, 1984). Again, differences in fiber orientation and cell content were detected between P1 and P2, suggesting again that this region could be heterogeneous.

To confirm our hypothesis that each region could be heterogeneous, we analyzed the ECM composition at each subregion. The ECM plays a key role in controlling the physical properties of human tissues, supporting compressive and extension forces and providing resilience and shock absorption capacity, especially during

continuous biomechanical stress (Hoffmann et al., 2019). For this reason, understanding of the ECM configuration at each subregion of the SLIL could contribute to understand the pathomechanics of the human carpus (Wolff & Wolfe, 2016). In this milieu, we first analyzed the presence of ECM fibers in the SLIL and compared the results with control tissues. Regarding elastic fibers, we found that these components were very scarcely present in the SLIL and in control tissues. Although elastic fibers have been found in high amounts at certain ligaments such as the nuchal ligament or the ligamentum flavum, its presence is location-specific, and many ligaments have very low amounts of these fibers (Hill et al., 2020). Despite the low amount of elastic fiber found within the SLIL, it is probably that these fibers, which run parallel to the collagen networks, contribute to the resistance of this complex structure to tensile or shear stress (Henninger et al., 2019).

Then, we analyzed the presence and distribution of collagen fibers. Collagen is the main fibrillar component of the ECM and plays a crucial role in controlling tissue stiffness and resistance to tensile forces, as these fibers can store elastic energy by stretching the flexible regions of the fibrils in their triple-helix tridimensional structure (Silver et al., 2003, 2021). Collagen fibers are typically very abundant in mature tendons and ligaments (Barros et al., 2002) and in other mechanically demanding areas, where they are the main responsible for mechanotransduction of incoming forces. As expected, we found that all SLIL regions and control tissues contained high amounts of collagen fibers, with some differences among samples. Among controls, the higher concentration of collagen was found in AC, which is known to contain high amounts of these fibers (Bloebaum et al., 2021). Regarding the SLIL, the highest contents of collagen fibers were found in D1 and M1, whereas the lowest amounts of collagen corresponded to both subregions of the palmar region (P1 and P2). These results are in agreement with previous biomechanical studies suggesting that the palmar region could be mechanically weaker than the dorsal region (Kakar et al., 2019), and could contribute to understand why most SLIL lesions begin with a disruption of the palmar region of this structure (Andersson & Garcia-Elias, 2013). Interestingly, differences between both subregions of each region (D1 vs. D2, M1 vs. M2 and P1 vs. P2) were statistically significant, suggesting again that each region could be heterogeneous. Strikingly, our analysis of specific types of collagens also revealed differences among areas, with the highest contents of collagens types I, III and IV corresponding to the M2 subregion, with significant differences between both subregions of each region for some collagen types. Specifically, M1 was significantly different to M2 for collagens III and IV. The fact that M2 showed the highest contents of the three types of collagens is consistent with the possibility that M2 could be more similar to a hyaline cartilage than a fibrous cartilage, since it has been demonstrated that hyaline cartilage contains high amounts of collagens I, III and IV (Alcaide-Ruggiero et al., 2021).

In addition, we assessed the presence of relevant non-fibrillar components of the ECM in the tissues analyzed in this work. Non-fibrillar ECM components are fundamental molecules able to control the biomechanical properties of human tissues by regulating the tissue response to external mechanical forces (Ghadie et al., 2021). Specifically, proteoglycans and glycosaminoglycans mediate collagen fibrils alignment and regulate water content of the ECM, which in turn, is able to control tissue stiffness (Müller et al., 2004; Smith & Melrose, 2015). Our results showed that the human SLIL was enriched in these components, and proteoglycans and glycoproteins tended to be more abundant in SLIL than in control tissues, with the exception of AC, which showed the highest contents of all tissues. Interestingly, the membranous and palmar regions displayed the most intense signal for both types of components, although the levels of AC were not reached. When versican was analyzed, we found that the lowest expression of this proteoglycan corresponded to AC, M1, M2 and P2. Again, significant differences were found between both subregions of the dorsal, membranous and palmar regions for all non-fibrillar components, except for some specific comparisons.

In order to furtherly characterize each zone, we analyzed the phenotype of cells found at each area using S100 immunohistochemistry, a typical cartilage-linked marker that is especially expressed by hyaline chondrocytes (Yammani, 2012). The finding that cells were positive at the M1 area followed by M2, along with the ECM structure showing the typical capsule and ECM arrangement of the human cartilage, confirms the cartilaginous nature of these zones. Furthermore, the analysis of blood vessels showed very few vessels at M1 and M2, as it is the case of cartilage. In general, these results, support the idea that the membranous region

could indeed be composed by a variety of cartilage tissue, and that M1 could be more similar to hyaline cartilage than M2.

Finally, we aimed to assess the presence of stem cells at each zone of the SLIL in order to identify those areas that could have higher self-regeneration potential. Our results suggest that the SLIL in general contains low amounts of stem cells, what is in agreement with previous reports questioning the intrinsic healing capability of the human SLIL (Minami et al., 2003). However, the fact that subregion D2 could be more enriched in stem cells than other areas may imply that the dorsal area has more intrinsic regenerative potential, what could be related to the presence of stronger mechanical forces affecting the dorsal region of the SLIL, as compared with other regions (Kakar et al., 2019).

Altogether, these results support our hypothesis that each region of the human SLIL could be structurally heterogeneous. In fact, a global analysis of the ECM evaluation results using hierarchical clustering confirmed the existence of differences within the SLIL. Remarkably, the D1 zone was structurally more similar to ligaments and tendons used as controls than to other areas of the SLIL, although the control tissue showing the lowest distance with D1 was a fibrocartilage (TF). Thus, our results suggest that the rest of SLIL zones shared ECM composition similarities with hyaline cartilage (AC), with M1 showing the closest distance with AC. Although the SLIL is generally considered to be histologically related to a human fibrocartilage (Berger, 1996; Sokolow & Saffar, 2001), our comprehensive analysis suggests that this structure could be more close to an articular cartilage, with high heterogeneity among zones.

The present study has several limitations. First, results should be confirmed in a larger cohort of human hand donors. Second, biomechanical studies should be carried out in the future to correlate our histological results with biomechanical data of stiffness and elasticity of each specific zone of the SLIL. Finally, the study could be complemented with the implant of different biomaterials or bioartificial tissues generated by tissue engineering to determine the potential usefulness of this approach to repair the human SLIL, as previously reported for other wrist ligaments (Lui et al., 2021).

To our knowledge, this is the first study that characterized the SLIL after stablishing a subclassification of the classical SLIL regions. Overall, our results support the idea that each subregion has a definite structure and composition not only at a morphological level, but also when the expression of relevant tissue components were analyzed. These results could be clinically relevant, as they could contribute to explain why most disruptions of the human SLIL typically commence at the palmar region, and support the surgical approaches based on a reinforcement of this region. In addition, the higher similitude of the SLIL with a cartilage-like tissue contributes to understand the poor regenerative properties of the human SLIL, especially in the areas devoid of blood vessels. However, the heterogeneity of this structure supports the idea that certain zones, such as D2, could have more intrinsic regeneration potential derived from the presence of stem cells. In consequence, lesions affecting the zones with lower regeneration potential, especially M1 and M2, should be treated with the use of tissue grafts containing mesenchymal stem cells or other approaches based on tissue engineering and regenerative medicine, as suggested for the human knee meniscus (Kwon et al., 2019). In addition, our results suggest that surgical repair of injuries affecting the membranous region should preferentially be repaired using cartilage grafts that can be obtained from articular surfaces, meniscus grafts or other similar anatomical regions, as described for the knee joint repair (Ashton Tan et al., 2022). However, surgical treatment of the dorsal region of the SLIL could be favored by the use of grafts whose histological structure is more similar to this region, such as the human ligaments and tendons. Future works carried out *in vivo* should confirm or not this statement.

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TABLES

	VHF	**PSR**	**COL-I**	**COL-III**	**COL-IV**	**AB**	**PAS**
FT	31.63±3.46	174.29±7.94	83.71±8.29	87.45±24.08	47.97±18.26	34.05±5.91	56.4±1.72
CL	33.03±13.53	204.42±13.66	96.09±14.42	92.87±15.14	64.47±22.68	38.8±3.22	72.19±4.87
AC	38.23±26.8	217.87±8.02	95.9±27.36	48.65±5.39	31.03±6.32	144.46±4.49	119.6±6.51
TF	33.43±21.4	190.05±13.78	69.26±9.06	102.32±11.56	46.03±22.97	51.48±9.74	76.75±9.84
CC	32.4±16.72	183.48±17	70.06±11.2	99.5±16.87	52.83±20.22	49.48±6.17	71.2±14.16
RT	28.37±10.2	187.44±27.94	90.28±19.36	130.66±23.94	52.67±29.31	30.51±2.51	45.74±11.93
D1	30.4±4.41	189.94±5.08	61.43±8.19	56.91±6.56	45.47±30.47	48.77±2.37	69.85±7.88
D2	31.23±7.16	177.89±6.79	48.58±7.32	48.97±6.22	59.7±20.79	66.6±22.08	80.43±6.82
M1	31.37±16.56	189.23±8.14	56.27±16.45	46.65±7.03	46.93±21.24	80.94±8.2	84.12±7.02
M2	30.97±18.63	178.29±6.92	70.09±7.53	68.54±10.22	129.87±60.51	88.9±13.99	97.4±6.28
P1	33.67±18.51	145.15±18.13	45.2±5.54	45.9±4.41	61.6±28.95	54.67±6.94	101.96±9.86
P2	35.13±15.57	179.75±8.27	43.82±6.93	56.06±6.11	49.13±27.4	111.37±11.02	92.72±5.09
AC vs. D1	0.0026	<0.0001*	<0.0001*	<0.0001*	0.0162	<0.0001*	<0.0001*
CC vs. D1	0.0551	0.1194	0.0005	<0.0001*	0.2751	0.2132	0.7747
CL vs. D1	0.0012	<0.0001*	<0.0001*	<0.0001*	0.0083	<0.0001*	0.0965
FT vs. D1	0.1304	<0.0001*	<0.0001*	<0.0001*	0.7016	<0.0001*	<0.0001*
RT vs. D1	0.2478	0.3280	<0.0001*	<0.0001*	0.3548	<0.0001*	<0.0001*
TF vs. D1	0.0015	0.9007	0.0005	<0.0001*	0.9355	0.7412	0.0030
AC vs. D2	0.0023	<0.0001*	<0.0001*	0.9357	<0.0001*	<0.0001*	<0.0001*
CC vs. D2	0.0906	0.0747	<0.0001*	<0.0001*	0.1999	0.0055	0.0142
CL vs. D2	0.0058	<0.0001*	<0.0001*	<0.0001*	0.3997	<0.0001*	<0.0001*

	VHF	PSR	COL-I	COL-III	COL-IV	AB	PAS
FT vs. D2	0.6228	0.0462	<0.0001*	<0.0001*	0.0238	<0.0001*	<0.0001*
RT vs. D2	0.0415	0.0043	<0.0001*	<0.0001*	0.2887	<0.0001*	<0.0001*
TF vs. D2	0.0027	0.0009	<0.0001*	<0.0001*	0.0189	0.0105	0.1727
AC vs. M1	0.0105	<0.0001*	<0.0001*	0.1922	0.0004	<0.0001*	<0.0001*
CC vs. M1	0.4492	0.1462	0.0035	<0.0001*	0.2751	<0.0001*	0.0001*
CL vs. M1	0.4946	<0.0001*	<0.0001*	<0.0001*	0.0031	<0.0001*	<0.0001*
FT vs. M1	0.0029	<0.0001*	<0.0001*	<0.0001*	0.8406	<0.0001*	<0.0001*
RT vs. M1	0.0096	0.2665	<0.0001*	<0.0001*	0.3896	<0.0001*	<0.0001*
TF vs. M1	0.0371	0.6228	0.0032	<0.0001*	0.8753	<0.0001*	0.0037
AC vs. M2	0.0332	<0.0001*	0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
CC vs. M2	0.7973	0.0878	0.9007	<0.0001*	<0.0001*	<0.0001*	<0.0001*
CL vs. M2	0.9240	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
FT vs. M2	0.0430	0.0654	<0.0001*	0.0009	<0.0001*	<0.0001*	<0.0001*
RT vs. M2	0.0263	0.0052	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
TF vs. M2	0.1304	0.0004	0.6125	<0.0001*	<0.0001*	<0.0001*	<0.0001*
AC vs. P1	0.1547	<0.0001*	<0.0001*	0.0183	<0.0001*	<0.0001*	<0.0001*
CC vs. P1	0.5039	<0.0001*	<0.0001*	<0.0001*	0.1798	0.0063	<0.0001*
CL vs. P1	0.0654	<0.0001*	<0.0001*	<0.0001*	0.6711	<0.0001*	<0.0001*
FT vs. P1	0.0002	<0.0001*	<0.0001*	<0.0001*	0.0340	<0.0001*	<0.0001*
RT vs. P1	0.0019	<0.0001*	<0.0001*	<0.0001*	0.2398	<0.0001*	<0.0001*
TF vs. P1	0.4232	<0.0001*	<0.0001*	<0.0001*	0.0248	0.1547	<0.0001*
AC vs. P2	0.1504	<0.0001*	<0.0001*	<0.0001*	0.0013	<0.0001*	<0.0001*
CC vs. P2	0.5229	0.2478	<0.0001*	<0.0001*	0.5543	<0.0001*	<0.0001*
CL vs. P2	0.0191	<0.0001*	<0.0001*	<0.0001*	0.0217	<0.0001*	<0.0001*
FT vs. P2	<0.0001*	0.0052	<0.0001*	<0.0001*	0.8469	<0.0001*	<0.0001*
RT vs. P2	0.0001*	0.0110	<0.0001*	<0.0001*	0.6314	<0.0001*	<0.0001*
TF vs. P2	0.2665	0.0063	<0.0001*	<0.0001*	0.6367	<0.0001*	<0.0001*
D1 vs. D2	0.0878	<0.0001*	<0.0001*	<0.0001*	0.0395	0.0003	<0.0001*
M1 vs. M2	0.6333	<0.0001*	0.0063	<0.0001*	<0.0001*	0.0797	<0.0001*
P1 vs. P2	0.5619	<0.0001*	0.1727	<0.0001*	0.0920	<0.0001*	0.0001*

Table 1. Quantitative analysis of extracellular matrix (ECM) components in the human scapholunate ligament (SLIL) zones and control tissues. For each histochemical and immunohistochemical method, average intensity units (I.U.) ± standard deviation values are shown for each sample. Statistical p values correspond to the comparison of each SLIL zone vs. each control tissue and between both zones of each region using the Mann-Whitney test. Statistically significant values are labeled with asterisks (*). FT: Flexor tendon, CL: Carpal ligament, AC: Articular cartilage, TF: Triangular fibrocartilage, CC: Carpal articular capsule, RT: Retinaculum, D1: dorsal region (part 1), D2: dorsal region (part 2), M1: membranous region (part 1), M2: membranous region (part 2), P1: palmar region (part 1), P2: palmar region (part 2). VHF: Verhoeff histochemistry for elastic fibers, PSR: picrosirius red histochemistry for collagen fibers, COL-I, COL-III and COL-IV: immunohistochemistry for collagens types I, III and IV, respectively, AB: alcian blue histochemistry for proteoglycans, PAS: periodic acid–Schiff histochemistry for glycosaminoglycans, VSC: versican immunohistochemistry.

	S100	CD73	VESSELS
D1	0.4±0.55	10.2±1.92	19±6.16
D2	11.6±4.39	24.4±8.26	16.6±5.18
M1	13.6±4.93	14.2±4.49	1±1
M2	8±2.92	13.6±5.98	1.6±2.07

	S100	CD73	VESSELS
P1	5.8±1.92	11.2±3.27	17.2±2.59
P2	6.8±1.92	9.8±1.92	17±2.24
D1 vs. D2	<0.0001*	0.0003*	0.4813
D1 vs. M1	<0.0001*	0.0232	<0.0001*
D1 vs. M2	<0.0001*	0.2475	<0.0001*
D1 vs. P1	<0.0001*	0.6842	0.4813
D1 vs. P2	<0.0001*	0.7959	0.4813
D2 vs. M1	0.2475	0.0052	<0.0001*
D2 vs. M2	0.0524	0.0089	<0.0001*
D2 vs. P1	<0.0001*	0.0007*	0.4813
D2 vs. P2	0.0007*	0.0003*	0.3930
M1 vs. M2	0.0089	0.6842	0.7959
M1 vs. P1	<0.0001*	0.0753	<0.0001*
M1 vs. P2	<0.0001*	0.0147	<0.0001*
M2 vs. P1	0.1431	0.5787	<0.0001*
M2 vs. P2	0.4813	0.1431	<0.0001*
P1 vs. P2	0.2475	0.4813	0.7959

Table 2. Quantitative analysis of cell phenotype and blood vessel quantification in the human scapholunate ligament (SLIL) zones. For each analysis method, the average number of positive cells or the average number of blood vessels per unit of area ± standard deviation is shown for each sample. Statistical p values correspond to the comparison of each SLIL region vs. the rest of the regions using the Mann-Whitney test. Statistically significant values are labeled with asterisks (*). FT: Flexor tendon, CL: Carpal ligament, AC: Articular cartilage, TF: Triangular fibrocartilage, CC: Carpal articular capsule, RT: Retinaculum, D1: dorsal region (part 1), D2: dorsal region (part 2), M1: membranous region (part 1), M2: membranous region (part 2), P1: palmar region (part 1), P2: palmar region (part 2). S100: immunohistochemistry for the chondrocyte marker S100, CD73: immunohistochemistry for the stem cell marker CD73, Vessels: quantification of blood vessels showing positive immunohistochemical signal for CD34.

FIGURE LEGENDS

Figure 1. Macroscopic images of the human scapholunate interosseous ligament (SLIL) analyzed in this work. The left image shows the lunate bone (L) attached to the scaphoid bone (S) by the SLIL. The image to the right shows the different zones within each region of the SLIL: D1: dorsal region (part 1), D2: dorsal region (part 2), M1: membranous region (part 1), M2: membranous region (part 2), P1: palmar region (part 1), P2: palmar region (part 2). The asterisk corresponds to the radio-scapho-lunate ligament.

Figure 2. Histological analysis of the human scapholunate ligament (SLIL) stained with hematoxylin-eosin staining showing the 6 zones analyzed in the present work. Illustrative high-augmentation images are also shown for each zone. D1: dorsal region (part 1); D2: dorsal region (part 2); M1: membranous region (part 1); M2: membranous region (part 2); P1: palmar region (part 1); P2: palmar region (part 2). Scale bars: 100μm. The histogram to the right corresponds to the quantification of the number of cells per unit of area in each zone of the SLIL.

Figure 3. Analysis of elastic and collagen fibers in the human scapholunate ligament (SLIL) and control tissues using Verhoeff (VHF) and picrosirius red (PSR) histochemistry, respectively. Histograms represent the results of the staining signal quantification for each analysis method. FT: Flexor tendon, CL: Carpal ligament, AC: Articular cartilage, TF: Triangular fibrocartilage, CC: Carpal articular capsule, RT: Retinaculum, D1: dorsal region (part 1), D2: dorsal region (part 2), M1: membranous region (part 1), M2: membranous region (part 2), P1: palmar region (part 1), P2: palmar region (part 2). Scale bars: 100μm.

Figure 4. Analysis of collagen types I, III and IV in the human scapholunate ligament (SLIL) and control tissues as determined by immunohistochemistry. Histograms represent the results of the staining signal quantification for each analysis technique. FT: Flexor tendon, CL: Carpal ligament, AC: Articular cartilage, TF: Triangular fibrocartilage, CC: Carpal articular capsule, RT: Retinaculum, D1: dorsal region (part 1), D2: dorsal region (part 2), M1: membranous region (part 1), M2: membranous region (part 2), P1: palmar region (part 1), P2: palmar region (part 2). Scale bars: 100μm.

Figure 5. Analysis of proteoglycans and glycoproteins in the human scapholunate ligament (SLIL) and control tissues as determined by alcian blue (AB) and Periodic acid–Schiff (PAS) histochemistry and versican (VSC) immunohistochemistry. Histograms represent the results of the staining signal quantification for each analysis technique. FT: Flexor tendon, CL: Carpal ligament, AC: Articular cartilage, TF: Triangular fibrocartilage, CC: Carpal articular capsule, RT: Retinaculum, D1: dorsal region (part 1), D2: dorsal region (part 2), M1: membranous region (part 1), M2: membranous region (part 2), P1: palmar region (part 1), P2: palmar region (part 2). Scale bars: 100μm.

Figure 6. Analysis of expression of the cell markers S100 and CD73 and quantification of blood vessels in the different regions of the human scapholunate ligament (SLIL). Histograms represent the results of the quantitative analysis as number of positive cells or number of blood vessels per unit of area. FT: Flexor tendon, CL: Carpal ligament, AC: Articular cartilage, TF: Triangular fibrocartilage, CC: Carpal articular capsule, RT: Retinaculum, D1: dorsal region (part 1), D2: dorsal region (part 2), M1: membranous region (part 1), M2: membranous region (part 2), P1: palmar region (part 1), P2: palmar region (part 2). Scale bars: 100μm.

Figure 7. Hierarchical clustering analysis of the tissues analyzed in this work (controls and human scapholunate ligament -SLIL- zones) based on the quantitative analysis of ECM composition. FT: Flexor tendon, CL: Carpal ligament, AC: Articular cartilage, TF: Triangular fibrocartilage, CC: Carpal articular capsule, RT: Retinaculum, D1: dorsal region (part 1), D2: dorsal region (part 2), M1: membranous region (part 1), M2: membranous region (part 2), P1: palmar region (part 1), P2: palmar region (part 2). Tissues with high relative signal for each analysis method are represented in red, whereas tissues with the lowest signal appear in blue.