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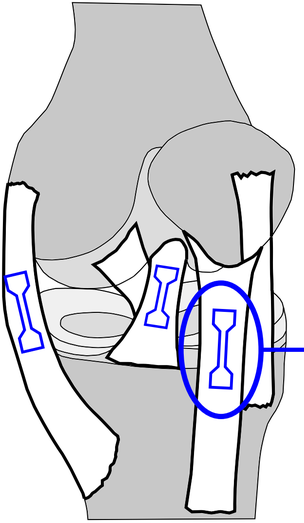

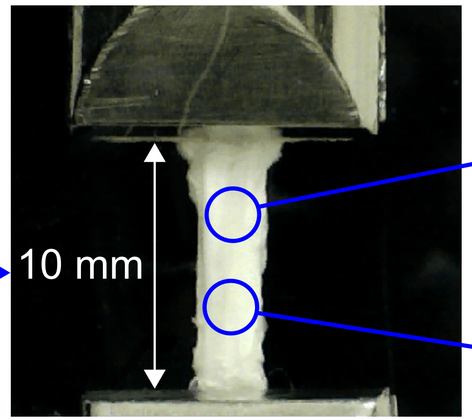


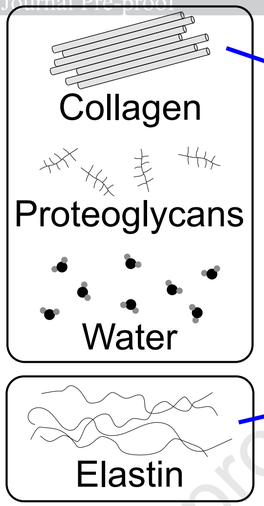


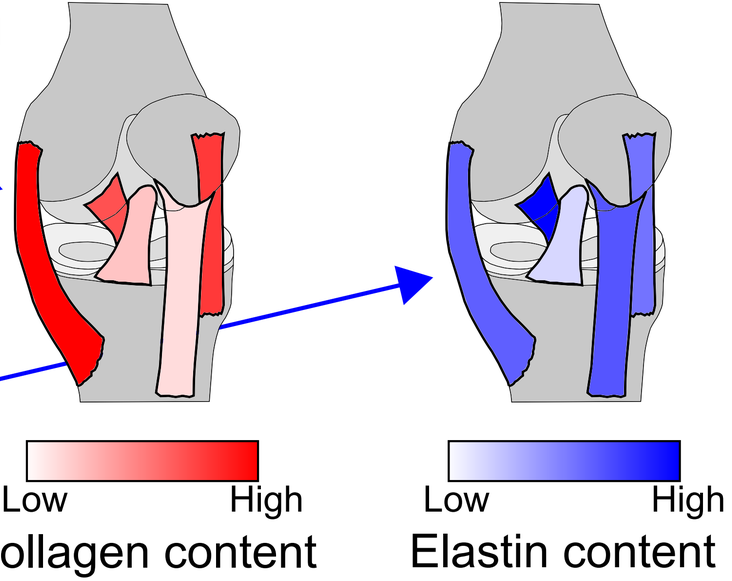


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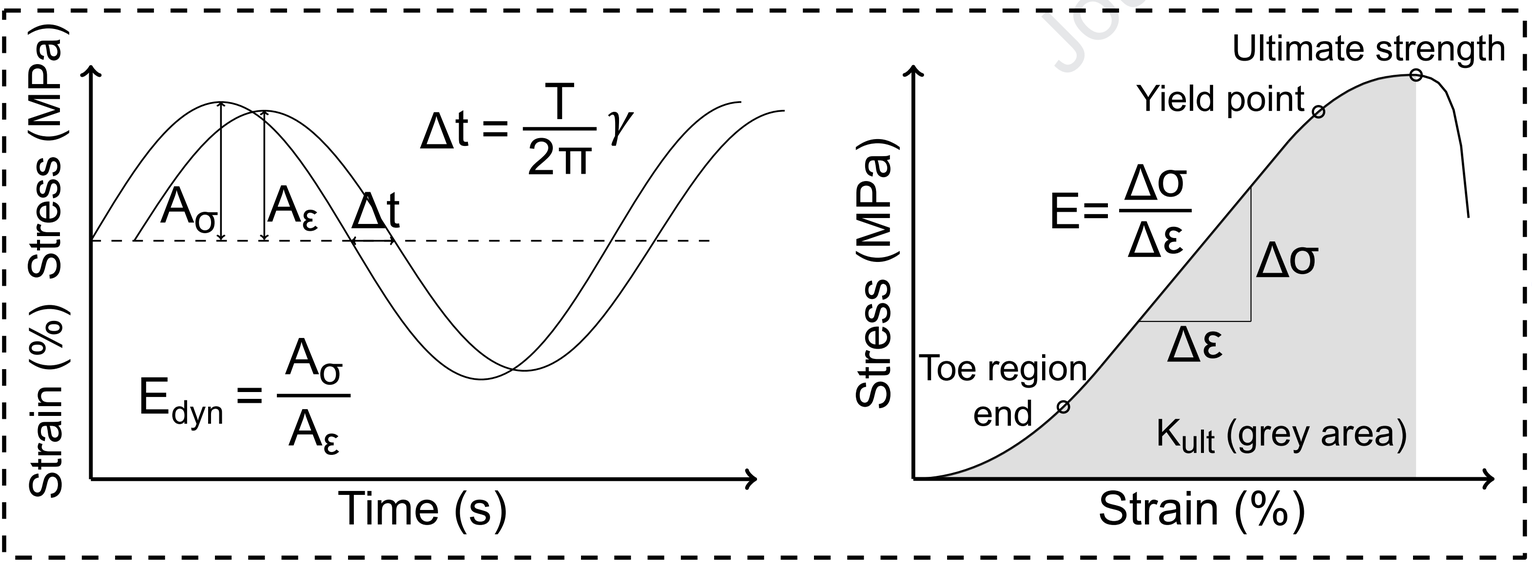


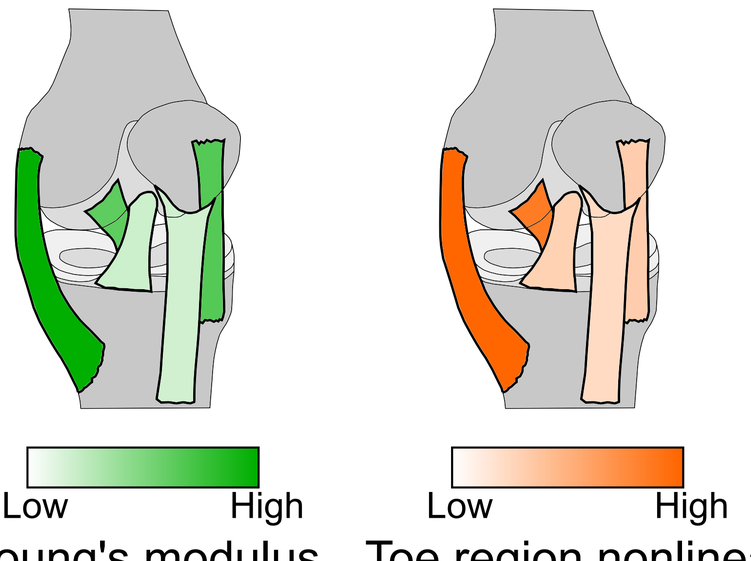

# Comparison of water, hydroxyproline, uronic acid and elastin contents of bovine knee ligaments and patellar tendon and their relationships with biomechanical properties

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*Research paper*

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**Abstract (244 words)**

Mechanical material properties of ligaments originate from their biochemical composition and structural organization. However, it is not yet fully elucidated how biochemical contents vary between knee ligaments and patellar tendon (PT) and how they relate with mechanical properties. The purpose of this study was to compare water, collagen, proteoglycan and elastin contents between bovine knee ligaments and PT and correlate them with tensile material properties.

Hydroxyproline (collagen), uronic acid (proteoglycan) and elastin contents per wet and dry weights were measured using colorimetric biochemical methods for bovine knee ligament and PT samples (n=10 knees). Direct comparison and correlation with multiple linear regression were performed against biomechanical properties measured in our earlier study.

Anterior cruciate ligament (ACL) and PT exhibited lower hydroxyproline content per wet weight compared with other ligaments ($p$<0.05). Cruciate ligaments had higher uronic acid content per dry weight compared with collateral ligaments ($p$<0.05). Posterior cruciate ligament had higher elastin content than ACL ($p$<0.05). Higher hydroxyproline content per wet weight implied higher Young's modulus, strength and toughness. Quantitatively, higher elastin content per wet weight predicted higher toe region nonlinearity and Young's modulus whereas higher uronic acid content per dry weight predicted lower Young's modulus, yield stress and toughness.

Differences between ligaments in biochemical composition highlight differences in their physiological function and loading regimes. As expected, collagen content showed similar trend with stiffness and strength. The predictive role of proteoglycan and elastin contents on the mechanical properties might indicate their important functional role in ligaments.

## 1. Introduction

Ligaments are collagen-rich soft connective tissues that connect bone to bone. In the knee joint, their primary functions are to transmit forces, limit and guide joint translations and rotations, stabilize the joint and serve as mechanical dampers (Birch et al., 2013; Reynolds et al., 2017). They are composed primarily of water, type I collagen, elastin, proteoglycans and fibroblast cells. Cells are estimated to account for a small percentage of total volume (Frank, 2004), but they are important in synthesizing collagen and other extracellular matrix components. Structurally they are organized in an oriented and hierarchical manner, where the ligament divides into fascicles, consisting of fibers, formed from fibrils, eventually comprised of collagen molecules (Ellis and Weiss, 2015).

The mechanics of the whole knee joint is highly affected by the mechanical properties of the four main ligaments, the anterior cruciate ligament (ACL), posterior cruciate ligament (PCL), medial collateral ligament (MCL), lateral collateral ligament (LCL), and patellar tendon (PT). The cruciate ligaments restrict anterior (ACL) and posterior (PCL) tibial translations, and aid in controlling tibial rotations. They mainly act in tension, but may experience localized shear, transverse and compressive loads during locomotion. The collateral ligaments restrict valgus (MCL) and varus (LCL) angulations and control tibial rotations. They must be geometrically adequate to allow for proper flexion-extension movement, but also withstand high tensile loading when resisting valgus and varus angulations. The patellar tendon transmits forces from the quadriceps muscle to tibia, allowing extension of the knee, and in part restricts posterior tibial translation. Due to these different functions in the joint, the ligaments and PT are expected to be adapted to their specific roles and thus exhibit different compositional characteristics.

Collagen is the main load-bearing component in ligaments and tendons. Commonly collagen content in connective tissues has been estimated by measuring the hydroxyproline content and assuming that it is present in collagen in a specific proportion based on the amino acid composition of collagen (Brodsky et al., 2008). Proteoglycans in ligaments and tendons attract water, separate and lubricate fibers and facilitate fibril sliding (Berenson et al., 1996; Rigozzi et al., 2013). Proteoglycan content has been commonly estimated by measuring some of its constituents, such as glycosaminoglycans (Amiel et al., 1984; Kharaz et al., 2018; Rigozzi et al., 2013; Rumian et al., 2007; Smith et al., 2014) or uronic acid (Berenson et al., 1996; Danso et al., 2015; Kokkonen et al., 2011; Yoon and Halper, 2005). Elastin has been suggested to provide a restorative effect after loading and influence the stress in the toe-region (Henninger et al., 2013) and was concluded to be the dominant constituent resisting shear and transverse loading in MCL (Henninger et al., 2015).

There is no clear consensus on how collagen content varies between knee ligaments and patellar tendon. Kharaz et al. (2018) found no differences in collagen content (dry weight) between ACL and MCL in canine. Rumian et al. (2007) observed in ovine that cruciate ligaments had the lowest collagen content (dry weight), and collateral ligaments and PT had higher contents, but the differences were not significant. Amiel et al. (1984) discovered a higher collagen content (dry weight) in PT compared with cruciate ligaments and MCL in rabbits. Furthermore, Little et al. (2014) found a higher collagen type I content (dry weight) in human PT compared with ACL, but Suzuki et al. (2008) found no differences in hydroxyproline content (dry weight) between

human PT and ACL. Eleswarapu et al. (2011) observed a higher collagen content in PCL compared with MCL in immature bovine.

Very few studies have investigated the differences in proteoglycan content between knee ligaments and PT. Kharaz et al. (2018) observed ACL to have a higher sulphated glycosaminoglycan (sGAG) content (dry weight) than MCL in canine. Amiel et al. (1984) observed that cruciate ligaments contained significantly more glycosaminoglycans (dry weight) than MCL or PT. Smith et al. (2014) found that ACL had a higher sGAG content than PCL in canine ligaments, whereas Rumian et al. (2007) observed a higher GAG content in PCL compared with all knee ligaments and PT.

Little is known about elastin content of knee ligaments and PT and how the content compares between them. In canine, elastin content (dry weight) was not different between ACL and PCL (Smith et al., 2014), but was higher in ACL compared with MCL (Kharaz et al., 2018). Elastin content was higher in human ACL compared with PT (Little et al., 2014). To our knowledge, there are no studies that have measured the elastin content of all knee ligaments and PT within the same set of knees. Moreover, we found no previous reports on elastin content of LCL.

The structural properties of ligaments and PT, such as the linear region stiffness, are of high importance for joint function (Dhaher et al., 2010; Halonen et al., 2016). The structural properties originate from the combination of material properties, such as Young's modulus, and physical dimensions of the tissue. The material properties, in turn, originate from the biochemical composition and structural organization. The relationships between the composition

and material properties, i.e. the structure-function relationships, provide essential understanding of the tissue function and are useful for example when estimating the effect of degradation of one component on the mechanical function of the tissue, or when developing new constitutive models of ligaments and tendons (Beidokhti et al., 2017; Orozco et al., 2018). Furthermore, understanding structure-function relationships is important when developing clinical imaging modalities for diagnostics (Koff et al., 2014; Naghibi et al., 2019; Novakofski et al., 2016; Torniainen et al., 2019), as imaging reflects the structure and composition of the tissue, which can then be indirectly linked to the functional properties of the joint tissues.

Commonly, the effect of a single biochemical component on the mechanical properties of a certain ligament has been studied. For example, collagen content (dry weight) was found to correlate with Young's modulus and tensile strength in healing rabbit MCL (Frank et al., 1995). Another study reported no correlation between collagen content and Young's modulus in healing rabbit MCL (Woo et al., 1997). Lujan et al. (2007, 2009) reported that GAGs do not affect human MCL quasi-static or viscoelastic behavior. On the other hand, Robinson et al. (2004) analyzed the Young's modulus and tensile strength, and multiple biochemical (chondroitin sulfate/dermatan sulfate content, hydroxyproline content) and structural (mean fibril diameter, fibril diameter deviation, fibril area fraction) parameters of transgenic mouse tail tendon fascicles using a multivariate model. In that multiple linear regression analysis, the mechanical properties were predicted using the biochemical and structural parameters, and they concluded that GAG content was the best predictor of the fascicle mechanical properties. Fascicle properties are, however, missing fascicle-fascicle interactions, present at tissue level observations. To our knowledge, direct comparison or quantitative correlation of biochemical composition and

biomechanical properties has not been previously performed for all the knee ligaments and PT for the same set of healthy skeletally mature knees, with water, collagen, PG and elastin contents considered at the same time.

In our earlier study (Ristaniemi et al., 2018), we observed that MCL had the highest Young's modulus, strain-dependent modulus and toughness, LCL was the most viscous at low-frequency loads and PT was surprisingly soft. We hypothesize that the differences between ligaments observed in the Young's modulus and toughness are mainly explained by the hydroxyproline content (Frank et al., 1995), while differences in the phase difference (viscosity) are primarily explained by water and uronic acid contents (Yin and Elliott, 2004). Additionally, we hypothesize elastin content to be related with the toe region properties (Henninger et al., 2013).

The aims of this study were to 1) compare water, hydroxyproline, uronic acid and elastin contents between skeletally mature bovine knee ligaments and patellar tendon and 2) correlate them with tensile material properties. The biochemical compositions were determined using colorimetric biochemical methods, for the same samples that went through tensile testing to determine the biomechanical properties (Ristaniemi et al., 2018). The results of this study provide important information on the structure-function relationships of bovine knee ligaments and PT.

## 2. Methods

### *2.1 Sample preparation*

The four knee ligaments, ACL, PCL, MCL, LCL, and PT were carefully extracted from 10 skeletally mature bovine stifle joints (age: 14-22 months) obtained from a local abattoir (Figure 1a). Immediately after dissection, the samples were submerged in a phosphate buffered saline (PBS) solution and placed in a freezer (-20 °C) for an average of four months. Prior to the measurements, the samples were thawed at room temperature and dumbbell-shaped tensile testing pieces were cut from the mid-substance of the ligaments (Figure 1b). Two parallel razor blades were used to cut a slice with the desired thickness (approximately 1.8 mm) and a custom punch tool was used to cut the desired width (approximately 2 mm) and shape (length of measurement part approximately 10 mm). Collagen fibers were running along the longitudinal axis of the samples. Mechanical testing was conducted in our previous study (Ristaniemi et al., 2018) (Figure 1c). Immediately after the mechanical testing some interfascicular matrix was often left to create a continuum (no clear rupture to two parts). Therefore, we were able to cut the samples into two approximately equal-sized parts which were stored in a freezer (-20 °C) (Figure 1d). One part was used in the measurements of water, hydroxyproline and uronic acid contents and the other part was used to quantify elastin content. The wet weights of these parts ranged from 7 to 38 mg. Variation in the sample weights was not systematic ($p>0.05$ between ligaments, a linear mixed model) and likely originated from the sample preparation. Prior to the biochemical analyses, the samples were allowed to thaw at room temperature. Ligaments and PTs were tested from one knee at a time to keep storage times the same for all the tissues from the same knee.

180

181 *2.2 Mechanical testing*

182 Detailed description of the mechanical testing can be found in Ristaniemi et al. (2018) and the
183 testing protocol is briefly summarized here. A uniaxial material testing system was used to
184 conduct tensile testing. The zero-load length was determined using a 0.05 MPa tensile stress
185 (Henninger et al., 2013). Preconditioning was performed using 2 % strain for 10 cycles, after
186 which the zero-load length was redetermined. This was repeated five times to stabilize the
187 mechanical behavior (Ebrahimi et al., 2019). A four-step incremental stress-relaxation test was
188 conducted with strains of 2, 4, 6 and 8 % of tissue length with 30 minutes relaxation at each step
189 (results not reported). This was followed by a sinusoidal loading test at 8 % strain with 0.5 %
190 strain amplitude at 0.1, 0.5 and 1 Hz frequencies, with 20 cycles at each frequency. Finally, a
191 quasi-static ultimate tensile test until tissue failure was performed to obtain stress-strain
192 characteristics. The samples were submerged in PBS during the measurements. From these tests,
193 we obtained mechanical properties describing the elastic, viscoelastic and failure tensile material
194 properties, which are summarized in Table 1 and Figure 1f.

195

196 *2.3 Biochemical analyses*

197 Surface of the samples was blot with paper to remove excess PBS, and the wet weight was
198 measured (average of three measurements). The samples were freeze-dried in a lyophilizer for 24
199 hours, and water content was determined from the difference between wet and dry weights
200 (average of three measurements). Then, hydroxyproline and uronic acid contents, indicative of
201 the amount of collagen and proteoglycans, were determined from one part of the samples (Figure
202 1d). We used uronic acid determination, as it may be assumed to reflect PGs in a similar manner

in the studied tissues. The samples were incubated with a 1 mg/ml concentration of papain in 150 mM sodium acetate including 5 mM L-cysteine and 15 mM EDTA at pH of 5.8 and 60 °C for 16 h to digest the proteoglycans (Kokkonen et al., 2011). Next, the samples were boiled for 10 minutes to deactivate the enzyme. The hydroxyproline content was determined as outlined in Brown et al. (2001). First, the papain digested samples were hydrolyzed in 10 M HCl at 108 °C for 16 h. After cooling, the samples were neutralized with 1 M sodium hydroxide. Then, an oxidizing solution containing chloramine-T was added to samples, mixed well and left for 5 minutes. Ehrlich's reagent was added to each sample and mixed by vortex mixer and incubated in a water bath at 60 °C for 45 min. Absorbance was read at 540 nm using a microplate reader. The uronic acid content was measured by following the instructions of Blumenkrantz and Asboe-Hansen (1973). First, the possible salts were removed from the papain digested samples by ethanol-precipitation. Then, sulfuric acid/sodium tetraborate solution was added to the samples that were cooled in crushed ice. The samples were well shaken and heated in a water bath at 100 °C for 5 min. The *m*-hydroxyphenyl reagent was added to the cooled samples and after 5 min incubation absorbances were read at 540 nm. Both the hydroxyproline and uronic acid contents were determined three times and averaged. Finally, the contents were normalized by wet and dry weights.

Elastin content was quantified from the other part of the samples (Figure 1d) using Fastin Elastin Assay (Biocolor Ltd., Carrickfergus, County Antrim, United Kingdom) following manufacturer's instructions (Biocolor Ltd., 2011). Briefly, the samples were incubated with 0.25 M oxalic acid for one hour at 98°C. To extract all elastin, this step was repeated three times, which was estimated to be sufficient in our pilot measurements. Elastin was precipitated from

two aliquots of pooled extracts, then allowed to react with dye for 90 minutes and dissociated. The solution was placed in a microwell plate, absorbance was read at 513 nm, and the elastin content was determined with a standard curve. The elastin content was normalized by wet and dry weights.

*2.4 Statistical analyses*

Statistical comparisons of biochemical composition between ligament types were made with SPSS Statistics 25.0.0.1 (SPSS Inc., IBM Company, Armonk, NY, USA) using a linear mixed model (McCulloch et al., 2008), which takes into account that the samples originate from the same set of knees. Each biochemical parameter was analyzed with the ligament type (ACL, LCL, MCL, PCL, and PT) as a fixed variable with knee-specific random intercepts. The data in the figures is presented as mean ± standard deviation.

To investigate the relationships between the biochemical composition and mechanical properties, first differences between ligaments in the mechanical properties were compared against differences in their biochemical contents, similar to previous studies (Danso et al., 2017; Mäkelä et al., 2014; Mommersteeg et al., 1994). Then, the relationships were examined using a multiple linear regression. Separate regression models were formulated for each mechanical property, where the uronic acid content, hydroxyproline content, elastin content and ligament type were used as predictors. Two separate analyses were performed, one with contents normalized by wet weight and the other with contents normalized by dry weight. Water content was omitted as a predictor due to strong correlations with other constituents (Table 2). Uninformative predictors were removed from each model using a backward elimination based on the Akaike Information

Criterion. Regression analyses were performed in R (R Core Team, 2013). For reference, in supplementary material we present similar multiple linear regression analysis, but with water content, dry weight contents and ligament type as predictors.

## 3. Results

The biochemical and biomechanical data related to this study have been deposited to Mendeley Data repository. ACL had significantly higher water content than MCL or LCL ($p<0.01$) (Figure 2b). PT had higher water content than MCL ($p<0.05$) (Figure 2b). The hydroxyproline content per wet weight in ACL and PT was significantly lower compared with LCL ($p<0.05$), MCL ($p<0.01$) and PCL ($p<0.05$) (Figure 2c). The hydroxyproline content per dry weight was significantly lower in PT compared with all ligaments ($p<0.01$) (Figure 2c). The uronic acid content per wet weight was significantly higher in PCL compared with LCL and MCL ($p<0.05$) (Figure 2d). The uronic acid content per dry weight was significantly higher in ACL and PCL compared with LCL and MCL ($p<0.05$) (Figure 2d). The elastin content per wet weight was significantly lower in ACL compared with MCL, PCL and PT ($p<0.05$) (Figure 2e). The elastin content per dry weight was significantly higher in PCL compared with ACL and LCL ($p<0.05$) (Figure 2e). The water content correlated negatively with the hydroxyproline content per wet weight ($r=-0.91$, $p<0.01$, Table 2) and positively with the uronic acid content per dry weight ($r=0.62$, $p<0.01$, Table 2).

The differences observed between the ligaments in the Young's modulus, yield stress, toughness at yield, ultimate strength and toughness at failure were observed similarly in the water and hydroxyproline (wet weight) contents (Figures 2b, 2c, 2e). Additionally, the difference in the

toughness at yield was observed also in the hydroxyproline content per dry weight (Table 4). Quantitatively, the hydroxyproline content (wet weight) was found to predict the phase difference at 0.1 Hz, toe region strain and coefficient *D* describing the toe region (all $p<0.05$) (Table 3). The elastin content (wet weight) predicted the dynamic moduli ($p<0.01$), strain dependency coefficients *A* ($p<0.01$) and *F* ($p<0.05$) and Young's modulus ($p<0.01$). In the wet weight analysis, the ligament type was observed to predict the phase difference at 0.1 and 0.5 Hz (both $p<0.05$). In the dry weight analysis, the uronic acid content predicted the dynamic moduli, strain dependency coefficient *A*, Young's modulus, yield stress and toughness at yield (all $p<0.05$), while the ligament type predicted the phase differences at 0.1 and 0.5 Hz, dynamic moduli and strain dependency coefficients *A* and *F* (all $p<0.05$) (Table 4).

## 4. Discussion

In this study, we compared the water, hydroxyproline, uronic acid and elastin contents between bovine knee ligaments and PT. In addition, we studied the relationships between the biochemical contents and the tensile material properties. The main results were that ACL and PT had the lowest hydroxyproline contents, collateral ligaments exhibited the lowest uronic acid contents, while PCL had the highest elastin content. Differences between the ligaments in the Young's modulus, yield and ultimate strengths corresponded with differences in the hydroxyproline content (wet weight). The hydroxyproline and elastin contents (wet weight) predicted the toe region properties, and the uronic acid content (dry weight) predicted the Young's modulus, yield stress and toughness at yield. This study brings novel information of the structure-function relationships of bovine knee joint ligaments and PT.

*4.1 Comparison of biochemical composition between ligaments and PT*

The water contents measured in this study (ligament means 72.0–77.6 %) were higher than those reported by Rumian et al. (2007) for ovine knee ligaments, where the mean values ranged from 53.1% in PT to 67.7% in PCL. In their study, PT had significantly lower water content compared with other ligaments, which is not in agreement with the current study as PT had higher water content compared to MCL and LCL, and similar to ACL and PCL. The differences may arise from different species, low number of samples in their study, or wet weight measurements, as our samples were in PBS prior to the measurement, whereas their samples were thawed in air with no further hydration. PBS has been reported to cause swelling in tendons (Safa et al., 2017; Screen et al., 2006), and thus water contents measured here could be higher than in vivo. Additionally, the water content was measured from ruptured samples, which may have caused the tissue to imbibe additional water. These effects are however similar for all samples, enabling comparisons within this study.

Differences observed between the ligament types in the hydroxyproline content (wet weight) indicate that collateral ligaments require more densely packed collagen for normal physiological function. Collateral ligaments must be geometrically of adequate size and shape, while maintaining the force transmit capability to control for varus and valgus rotations. Interestingly, ACL and PCL were significantly different from each other, which might indicate a different physiological loading or anatomical restriction. A similar result to our study was observed by Mommersteeg et al. (1994) where hydroxyproline density was lower in ACL compared with LCL, MCL and PCL. In a study by Eleswarapu et al. (2011), the collagen content was

317 significantly lower in ACL and PCL compared with MCL, LCL and PT, but they used immature
318 bovine joints in which ligaments are still developing.
319
320 With respect to dry weight, the hydroxyproline content was significantly lower in PT than in
321 other ligaments, suggesting that in PT there is more of some other constituent not measured in
322 this study. The other ligaments had a similar, almost constant, hydroxyproline content (dry
323 weight). This is in line with the results of Kharaz et al. (2018) where the collagen content (dry
324 weight) showed no differences between ACL and MCL in canine. Our results contradict earlier
325 findings by Rumian et al. (2007) in ovine where cruciate ligaments had the lowest collagen
326 content (dry weight), and collateral ligaments and PT had higher collagen contents, though
327 differences were not statistically significant. Furthermore, Amiel et al. (1984) observed a higher
328 collagen content (dry weight) in PT compared with cruciate ligaments and MCL in rabbits and
329 similarly Little et al. (2014) found higher collagen type I content (dry weight) in human PT
330 compared with ACL. Suzuki et al. (2008) did not observe differences in the hydroxyproline
331 content (dry weight) between ACL and PT in humans and Eleswarapu et al. (2011) found no
332 differences between PT and all other ligaments in immature bovine. The collagen contents (dry
333 weight) in ligaments thus appear to be dependent on the species and maturation.
334
335 The uronic acid content, indicative of the amount of PGs, both per wet and dry weight, showed a
336 clear difference between cruciate and collateral ligaments. This highlights the different
337 functional requirements of cruciate and collateral ligaments, as higher PG content facilitates
338 fibril sliding (Rigozzi et al., 2013), which might be needed more in cruciate ligaments due to the
339 complex loading environment. The uronic acid content (dry weight) correlated positively with

the water content, which may be an indication of its ability to attract water, suggested also for other tissues (Poole, 1986; Vogel and Koob, 1989; Yoon and Halper, 2005). The results are in line with Kharaz et al. (2018) where ACL was observed to have a higher sGAG content than MCL. Moreover, our results are similar to Amiel et al. (1984) where cruciate ligaments contained significantly more glycosaminoglycans (dry weight) than MCL or PT. However, Smith et al. (2014) found that ACL had a higher sGAG content than PCL in canine ligaments. Again, these differences between studies may be related to different species.

Surprisingly, elastin content per wet weight was lowest in ACL, though ACL might require more resistance to shear loading, which has been suggested to be governed by elastin (Henninger et al., 2015). PCL had the highest elastin content, suggesting PCL to experience more shear and transverse loads compared to ACL. PT had elastin content comparable to PCL, implying bovine PT to require high restorative capability due to large strain range in normal operation. Our results contradict earlier findings in canine where ACL and PCL had no difference in elastin contents (Smith et al., 2014), ACL had higher elastin content than MCL (Kharaz et al., 2018), or in humans where ACL had higher elastin content than PT (Little et al., 2014).

*4.2 Relationship between biochemical composition and mechanical properties*

Differences between ligaments in the Young's modulus, yield stress, toughness at yield, ultimate strength and toughness at failure were observed also in the water and hydroxyproline (wet weight) contents. In particular, MCL was the stiffest and had the highest collagen (hydroxyproline) content and lowest water fraction. The water content is also emphasized by its strong negative correlation with the hydroxyproline content (wet weight). On the other hand,

LCL was the most viscous at low-frequency loads and viscosity in tension seems not to be controlled by water content, as we earlier speculated (Ristaniemi et al., 2018), but possibly by collagen content due to the viscoelasticity of the collagen fibers. This is supported also by positive correlation of hydroxyproline content (wet weight) with phase difference at 0.1 Hz loading (Table 3). Collagen viscoelasticity is a well-documented property and has been implemented in several computational models of collagenous soft tissues (Gupta et al., 2010; Kazemi and Li, 2014; Li et al., 2005; Shen et al., 2011; Svensson et al., 2012, 2010). The results imply that hydroxyproline content with respect to wet weight, rather than dry weight, is an important determinant of the ligament mechanical function. These findings are in agreement with an earlier study by Mommersteeg et al. (1994) where the variations of the hydroxyproline density corresponded to variations in the Young's modulus in ACL, PCL and MCL obtained from other studies. It must be noted, however, that all the differences in the biochemical constituents between ligaments were not reflected by significant differences in the mechanical properties. This might indicate that some other properties, such as collagen architecture and crimping, could have a role in controlling certain mechanical properties.

Quantitatively, the negative correlation between the hydroxyproline (wet weight) and the toe region strain might indicate that when there is more collagen, the relative proportion for the component suggested to be responsible for restoring the collagen crimp (elastin (Eekhoff et al., 2017; Godinho et al., 2017; Smith et al., 2011; Thorpe et al., 2015)) is lower and thus the collagen is less crimped and strains are lower. This effect was also observed in the coefficient *D*, describing the toe region nonlinearity, with a positive correlation with the hydroxyproline content. Additionally, elastin content correlated positively with toe region nonlinearity

(parameters $A$ and $F$), supporting the observation that elastin affects stress especially in the toe region (Henninger et al., 2013). Elastin may be the main load bearing component in tension when collagen fibers are crimped and do not support tensile loads. Though elastin is small in quantity relative to collagen, it was observed to positively correlate with dynamic moduli and Young's modulus. This contradicts Henninger et al. (2013) where elastin digestion did not affect Young's modulus of porcine MCL, but is in line with Millesi et al. (1995), where elastin digestion reduced Young's modulus of human palmaris longus tendons. Our result suggests that elastin may serve as a load-bearing component together with collagen or influence load transfer within ligaments and tendons.

With respect to dry weight, interestingly, the uronic acid content per dry weight was a significant predictor for the dynamic moduli, strain dependent modulus, Young's modulus, yield stress and toughness at yield, with lower content associated with higher values in the mechanical properties. This observation is in line with Robinson et al. (2004) where low GAG content (dry weight) was the best predictor of high modulus and strength in mouse tail tendon fascicles. The GAGs were, however, observed not to affect the quasi-static or viscoelastic behavior of human MCL (Lujan et al., 2007, 2009) or human PT (Svensson et al., 2011). Therefore, the observed correlation might not imply causality, meaning that the uronic acid content might be related with or regulate other properties, which may cause the observed behavior. These other properties could include fibril diameters (Robinson et al., 2004), separation of collagen fibrils (fibril assembly) (Berenson et al., 1996), lubrication or fibril sliding (Berenson et al., 1996; Rigozzi et al., 2013). Though facilitated fibril sliding due to GAGs could lead to higher tissue-level strains, we did not observe a positive relationship between uronic acid content and strains. Collagen per

dry weight was expected to predict the Young's modulus and strength (Frank et al., 1995). However, with respect to dry weight, the hydroxyproline or elastin contents were not significant predictors of any mechanical parameter.

Interestingly, the ligament type was a significant predictor for many mechanical properties. In those properties, some ligaments were clearly different from the others, such as higher phase difference in LCL at 0.1 and 0.5 Hz or higher dynamic moduli of MCL. The ligament type as a predictor suggests that these differences were not thoroughly explained by hydroxyproline, uronic acid or elastin contents. There may be other matters, such as ligament structure, which affect the mechanical properties and are different between ligaments.

*4.3 Limitations*

For complete description of the structure-function relationships in ligaments, also other factors than those measured here may affect the ligament biomechanics. Those are, for instance, collagen crosslinks (Ng et al., 1996), elastic fibers (fibrillin I and II), crimp angle, crimp length (Spiesz et al., 2018), collagen fibril diameter (Hart et al., 1992; Rumian et al., 2007) and collagen fibril orientation dispersion (Stender et al., 2018). Statistically, we did not investigate the interaction effects of the hydroxyproline, uronic acid and elastin contents on the mechanical properties due to high number of samples required for such analyses. This could be a subject of future investigations.

We used bovine stifle joints due to easy availability and best resemblance to the human knee joint in ligament anatomy compared to other species (Proffen et al., 2012). In addition, the

biochemical composition of bovine ligaments and PT is similar to human tissues, as reported collagen contents (dry weight) for human are up to 80 % (Hanada et al., 2014; Samiric et al., 2009; Suzuki et al., 2008; Woo et al., 2005), and for bovine 71-87 % (Eleswarapu et al., 2011), which are in agreement with the current study (83-98 %), assuming 14 % of collagen to be hydroxyproline (Birch et al., 1998). Furthermore, when investigating structure-function relationships, the relations can be assumed to be independent of species. The differences observed between ligaments may not be, however, directly translatable to human knees as typical range of motion and joint dimensions are different.

The tensile testing samples were cut with fascicles running along the longitudinal direction, and positioning of the cutting tool was made by hand based on visual cues. This may have resulted in some edge fibers to be cut. However, we estimate the effect of the sample preparation on the results to be small, as the sample encompasses several intact fascicles. To minimize the error, all the samples were cut by the same person using the same tool. Moreover, this type of sample preparation has been common with ligaments and tendons (Bonifasi-Lista et al., 2005; Chokhandre et al., 2015; Eleswarapu et al., 2011; Henninger et al., 2013; Lujan et al., 2007; Quapp and Weiss, 1998; Stabile et al., 2004).

Hydroxyproline was used to indicate the amount of collagen by assuming that the level of proline hydroxylation in ligaments and PT is similar (Birch et al., 2013). However, the literature on this topic is limited and minor differences in the level of proline hydroxylation may exist. One might argue that storage of the ligament samples in the freezer may have caused alterations to the structure and mechanical properties. However, we believe freezing effect to be small, as no

differences were found earlier in the mechanical properties and midsubstance PG content of rat Achilles tendons between fresh and 9-month frozen groups (Quirk et al., 2018).

The water content was omitted as a predictor from the multiple linear regression analysis due to correlations with other contents (Table 2). Therefore, the importance of water could not be clearly established. For instance, water could facilitate fibril sliding at the toe region, as it predicted toe region strain and coefficients A and D, describing the toe region (see supplementary material).

*4.4 Conclusion*

In conclusion, bovine LCL, MCL and PCL exhibited the highest hydroxyproline contents, the cruciate ligaments had the highest uronic acid contents and PCL had the highest elastin content. By investigating the differences between ligaments, higher hydroxyproline content, particularly that in MCL, implied higher Young's modulus, higher yield and failure stresses and toughness. Quantitatively elastin content was related to toe region nonlinearity and Young's modulus. Uronic acid content predicted the Young's modulus, yield stress and toughness, but it is likely not the cause, but rather relates to other quantities affecting these properties. The results of this study bring novel information on the structure-function relationships of bovine knee ligaments and PT and are important when estimating the effect of structural changes on tissue and joint function, when developing new constitutive models of ligaments and PT, and when developing different imaging methods for diagnostics.

## Conflict of interest statement

Authors declare no conflicts of interest.

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716 **Table 1.** Summary of the mechanical properties measured in our earlier study (Ristaniemi et al.,
717 2018) and the biochemical results of the current study with their descriptions. The data from
718 different ligaments and patellar tendon is pooled.

| Property | Symbol | n | Mean | STD | Unit | Description |
|---|---|---|---|---|---|---|
| Phase difference (0.1 Hz) | $\gamma_{0.1\ Hz}$ | 50 | 4.1 | 1.1 | deg | Stress-strain phase difference at 0.1 Hz sinusoidal loading |
| Phase difference (0.5 Hz) | $\gamma_{0.5\ Hz}$ | 50 | 4.8 | 1.2 | deg | Stress-strain phase difference at 0.5 Hz |
| Phase difference (1 Hz) | $\gamma_{1\ Hz}$ | 50 | 5.6 | 1.6 | deg | Stress-strain phase difference at 1 Hz |
| Dynamic modulus (0.1 Hz) | $E_{dyn\ 0.1\ Hz}$ | 50 | 192.8 | 117.9 | MPa | Stress-strain amplitude ratio at 0.1 Hz sinusoidal loading |
| Dynamic modulus (0.5 Hz) | $E_{dyn\ 0.5\ Hz}$ | 50 | 198.5 | 121.8 | MPa | Stress-strain amplitude ratio at 0.5 Hz |
| Dynamic modulus (1 Hz) | $E_{dyn\ 1\ Hz}$ | 50 | 200.5 | 122.8 | MPa | Stress-strain amplitude ratio at 1 Hz |
| Toe region strain | $\varepsilon_{toe}$ | 49 | 7.8 | 3.4 | % | Strain at the end of the toe region |
| Toe region stress | $\sigma_{toe}$ | 49 | 7.0 | 3.7 | MPa | Stress at the end of the toe region |
| Strain dependency coefficient, $\frac{1}{2}$ strain dependent modulus | $A$ | 49 | 1213.6 | 733.1 | MPa | Coefficients describing the toe region $\sigma = A\varepsilon^2 + B\varepsilon + C$ |
| Initial modulus | $B$ | 49 | 1.4 | 14.6 | MPa | |
| Coefficient | $C$ | 49 | 0.2 | 0.1 | MPa | |
| Coefficient | $D$ | 48 | 1.9 | 3.0 | MPa | Coefficients describing the toe region $\sigma = D(e^{F\varepsilon} - 1)$ |
| Strain dependency coefficient | $F$ | 48 | 24.4 | 9.4 | - | |
| Young's modulus | $E$ | 49 | 255.6 | 107.1 | MPa | Young's modulus of the linear region |
| Yield strain | $\varepsilon_{yield}$ | 41 | 19.5 | 3.5 | % | Strain at tissue yield |
| Yield stress | $\sigma_{yield}$ | 41 | 32.5 | 14.6 | MPa | Stress at tissue yield |
| Toughness at yield | $K_{yield}$ | 41 | 2.6 | 1.3 | mJ/mm$^3$ | Energy density at yield |
| Ultimate strain | $\varepsilon_{ult}$ | 39 | 22.8 | 3.9 | % | Strain at failure |
| Ultimate strength | $\sigma_{ult}$ | 39 | 35.4 | 17.1 | MPa | Stress at failure |
| Toughness at failure | $K_{ult}$ | 39 | 3.8 | 2.0 | mJ/mm$^3$ | Energy density at failure |
| Water content | - | 50 | 74.7 | 5.6 | % | Water mass fraction |
| Hydroxyproline (wet weight) | - | 50 | 32.5 | 7.5 | µg/mg | Hydroxyproline content per wet weight |
| Hydroxyproline (dry weight) | - | 50 | 128.8 | 11.6 | µg/mg | Hydroxyproline content per dry weight |
| Uronic acid (wet weight) | - | 50 | 0.46 | 0.14 | µg/mg | Uronic acid content per wet weight |
| Uronic acid (dry weight) | - | 50 | 1.94 | 0.77 | µg/mg | Uronic acid content per dry weight |
| Elastin content (wet weight) | - | 50 | 11.7 | 3.3 | µg/mg | Elastin content per wet weight |
| Elastin content (dry weight) | - | 50 | 45.5 | 12.8 | µg/mg | Elastin content per dry weight |

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721 **Table 2.** Pearson correlation coefficients between biochemical constituents with two-tailed
722 significance indicated by * ($p$<0.05) and ** ($p$<0.01).

| | Water content | Hydroxyproline (wet weight) | Hydroxyproline (dry weight) | Uronic acid (wet weight) | Uronic acid (dry weight) | Elastin (wet weight) |
|---|---|---|---|---|---|---|
| Hydroxyproline (wet weight) | -0.91** | | | | | |
| Hydroxyproline (dry weight) | 0.069 | 0.34* | | | | |
| Uronic acid (wet weight) | 0.060 | -0.0059 | 0.071 | | | |
| Uronic acid (dry weight) | 0.62** | -0.56** | 0.0039 | 0.79** | | |
| Elastin (wet weight) | -0.30* | 0.31* | 0.095 | -0.010 | -0.23 | |
| Elastin (dry weight) | 0.26 | -0.21 | 0.069 | 0.13 | 0.22 | 0.78** |

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**Table 3.** Results of the relationships between the biochemical composition normalized by wet weight and the mechanical properties. A black arrow indicates that the differences between ligaments in a mechanical property were observed also in the biochemical content, and the direction indicates the nature of the differences (↗ higher value in the biochemical content associated with higher value in the mechanical property; ↘ higher value in the biochemical content associated with lower value in the mechanical property). The quantitative correlation results show a number if the biochemical constituent is an informative predictor according to Akaike Information Criterion. The number indicates how much the mechanical property will change, in its units, if the corresponding biochemical content increases by one standard deviation. Ligament type is indicated by "Yes" if it is an informative predictor and adjusted $R^2$ values are shown for each regression model. Statistical significance of predictors is indicated by dark grey background ($p$<0.05).

| | | | | Quantitative correlation: wet weights | | | | |
|---|---|---|---|---|---|---|---|---|
| Mechanical property | Symbol | Unit | Water content (for investigation of differences only) | Hydroxyproline (wet weight) | Uronic acid (wet weight) | Elastin (wet weight) | Type | $R^2_{adj}$ |
| Phase difference (0.1 Hz) | $\gamma_{0.1\ Hz}$ | deg | | 0.3 ± 0.2 ($p$<0.05) | -0.2 ± 0.1 | -0.2 ± 0.1 | Yes ($p$<0.05) | 0.26 |
| Phase difference (0.5 Hz) | $\gamma_{0.5\ Hz}$ | deg | | | -0.2 ± 0.2 | | Yes ($p$<0.05) | 0.15 |
| Phase difference (1 Hz) | $\gamma_{1\ Hz}$ | deg | | | -0.3 ± 0.2 | | | 0.02 |
| Dynamic modulus (0.1 Hz) | $E_{dyn\ 0.1\ Hz}$ | MPa | | | | 45.4 ± 15.3 ($p$<0.01) | Yes | 0.29 |
| Dynamic modulus (0.5 Hz) | $E_{dyn\ 0.5\ Hz}$ | MPa | | | | 47.1 ± 15.8 ($p$<0.01) | Yes | 0.29 |
| Dynamic modulus (1 Hz) | $E_{dyn\ 1\ Hz}$ | MPa | | | | 47.2 ± 16.0 ($p$<0.01) | Yes | 0.29 |
| Toe region strain | $\varepsilon_{toe}$ | % | | -1.0 ± 0.5 ($p$<0.05) | | | | 0.06 |
| Toe region stress | $\sigma_{toe}$ | MPa | | | | | | 0.00 |
| Strain dependency coefficient, $\frac{1}{2}$ strain dependent modulus | $A$ | MPa | | | | 299.0 ± 92.1 ($p$<0.01) | Yes | 0.32 |
| Initial modulus | $B$ | MPa | | | | -3.1 ± 2.0 | | 0.03 |
| Coefficient | $C$ | MPa | | | -0.03 ± 0.02 | | | 0.03 |
| Coefficient | $D$ | MPa | | 1.3 ± 0.4 ($p$<0.01) | | | | 0.19 |
| Strain dependency coefficient | $F$ | - | | | | 2.9 ± 1.3 ($p$<0.05) | Yes | 0.18 |
| Young's modulus | $E$ | MPa | ↘ | ↗ | | 40.5 ± 14.8 ($p$<0.01) | Yes | 0.20 |
| Yield strain | $\varepsilon_{yield}$ | % | | | -1.0 ± 0.6 | | | 0.04 |
| Yield stress | $\sigma_{yield}$ | MPa | ↘ | ↗ | | 4.6 ± 2.4 | | 0.06 |
| Toughness at yield | $K_{yield}$ | mJ/ mm$^3$ | ↘ | ↗ | | 0.3 ± 0.2 | | 0.03 |
| Ultimate strain | $\varepsilon_{ult}$ | % | | | | | | 0.00 |
| Ultimate strength | $\sigma_{ult}$ | MPa | ↘ | ↗ | | 5.2 ± 2.9 | | 0.06 |
| Toughness at failure | $K_{ult}$ | mJ/ mm$^3$ | ↘ | ↗ | | 0.7 ± 0.3 | | 0.07 |

**Table 4.** Results of the relationships between the biochemical composition normalized by dry weight and the mechanical properties. A black arrow indicates that the differences between ligaments in a mechanical property were observed also in the biochemical content, and the direction indicates the nature of the differences (↗ higher value in the biochemical content associated with higher value in the mechanical property). The quantitative correlation results show a number if the biochemical constituent is an informative predictor according to Akaike Information Criterion. The number indicates how much the mechanical property will change, in its units, if the corresponding biochemical content increases by one standard deviation. Ligament type is indicated by "Yes" if it is an informative predictor and adjusted $R^2$ values are shown for each regression model. Statistical significance of predictors is indicated by dark grey background ($p$<0.05).

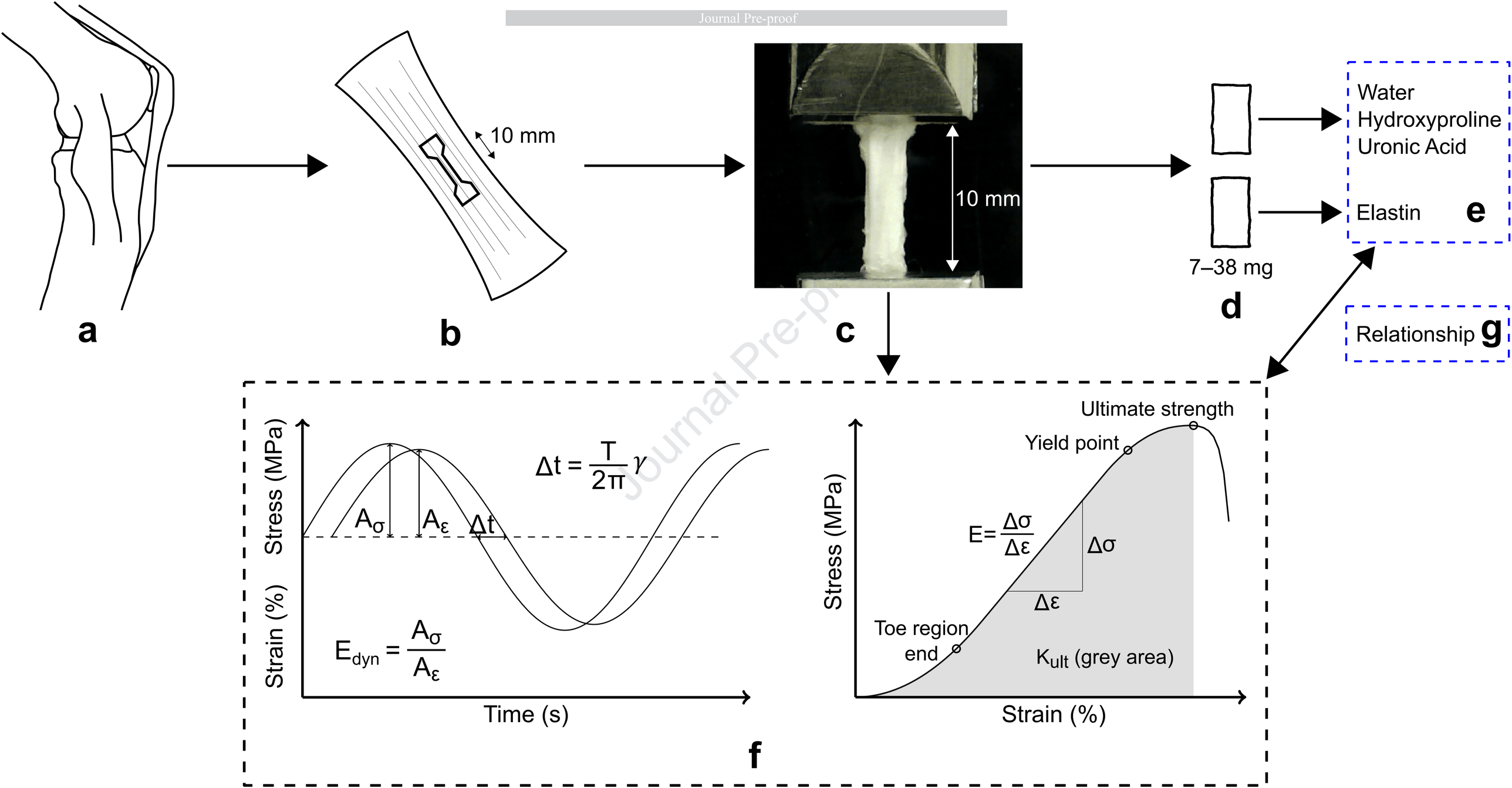
| | | | Quantitative correlation: dry weights | | | | |
|---|---|---|---|---|---|---|---|
| Mechanical property | Symbol | Unit | Hydroxyproline (dry weight) | Uronic acid (dry weight) | Elastin (dry weight) | Type | $R^2_{adj}$ |
| Phase difference (0.1 Hz) | $\gamma_{0.1\ Hz}$ | deg | 0.2 ± 0.2 | -0.2 ± 0.1 | -0.2 ± 0.1 | Yes ($p$<0.05) | 0.25 |
| Phase difference (0.5 Hz) | $\gamma_{0.5\ Hz}$ | deg | | | | Yes ($p$<0.05) | 0.13 |
| Phase difference (1 Hz) | $\gamma_{1\ Hz}$ | deg | | | | | 0.00 |
| Dynamic modulus (0.1 Hz) | $E_{dyn\ 0.1\ Hz}$ | MPa | -35.2 ± 18.1 | -36.2 ± 16.1 ($p$<0.05) | 24.3 ± 16.0 | Yes ($p$<0.05) | 0.27 |
| Dynamic modulus (0.5 Hz) | $E_{dyn\ 0.5\ Hz}$ | MPa | -36.2 ± 18.7 | -37.3 ± 16.7 ($p$<0.05) | 25.1 ± 16.6 | Yes ($p$<0.05) | 0.27 |
| Dynamic modulus (1 Hz) | $E_{dyn\ 1\ Hz}$ | MPa | -36.5 ± 18.9 | -37.8 ± 16.8 ($p$<0.05) | 24.9 ± 16.8 | Yes ($p$<0.05) | 0.26 |
| Toe region strain | $\varepsilon_{toe}$ | % | -0.9 ± 0.5 | | | | 0.04 |
| Toe region stress | $\sigma_{toe}$ | MPa | -0.8 ± 0.5 | -0.9 ± 0.5 | | | 0.06 |
| Strain dependency coefficient, $\frac{1}{2}$ strain dependent modulus | $A$ | MPa | -189.2 ± 117.4 | -212.8 ± 98.9 ($p$<0.05) | 195.7 ± 98.4 | Yes ($p$<0.05) | 0.27 |
| Initial modulus | $B$ | MPa | | | -3.4 ± 2.0 | | 0.04 |
| Coefficient | $C$ | MPa | | -0.03 ± 0.02 | | | 0.02 |
| Coefficient | $D$ | MPa | 0.7 ± 0.4 | | | | 0.03 |
| Strain dependency coefficient | $F$ | - | -2.7 ± 1.6 | | 2.5 ± 1.3 | Yes ($p$<0.05) | 0.17 |
| Young's modulus | $E$ | MPa | -33.1 ± 18.3 | -35.5 ± 15.4 ($p$<0.05) | 24.0 ± 15.3 | Yes | 0.19 |
| Yield strain | $\varepsilon_{yield}$ | % | | | | | 0.00 |
| Yield stress | $\sigma_{yield}$ | MPa | | -5.1 ± 2.2 ($p$<0.05) | | | 0.10 |
| Toughness at yield | $K_{yield}$ | mJ/ mm$^3$ | ↗ | -0.4 ± 0.2 ($p$<0.05) | | | 0.09 |
| Ultimate strain | $\varepsilon_{ult}$ | % | -1.2 ± 0.7 | | | | 0.04 |
| Ultimate strength | $\sigma_{ult}$ | MPa | | -5.0 ± 2.7 | | | 0.06 |
| Toughness at failure | $K_{ult}$ | mJ/ mm$^3$ | | | | | 0.00 |

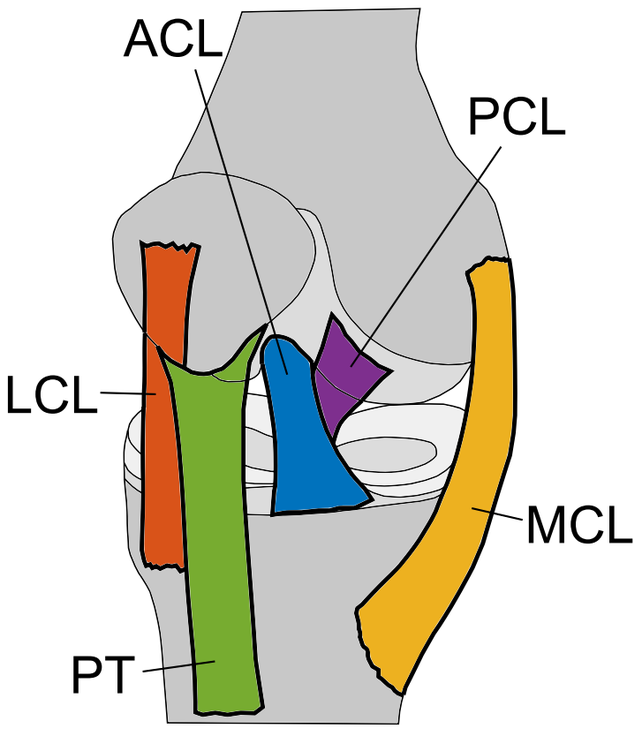
**Figure captions**

**Figure 1.** Ligaments were carefully dissected from bovine knee joints (a) and dumbbell-shaped tensile test samples were cut from the ligament mid-substance (b). Ligaments were subjected to tensile testing (c) to determine the mechanical material properties, of which certain are highlighted (f). After the mechanical testing, the samples were cut to two equal-sized pieces (d), which were used to determine the biochemical composition (e). Relationship was investigated between the mechanical properties and biochemical composition (g).

**Figure 2.** The knee joint ligaments are highlighted in (a). The water content is shown in (b) as mean (± SD). Subfigures (c), (d) and (e) show the mean (± SD) hydroxyproline, uronic acid and elastin contents, expressed in µg/mg, per wet and dry weights. Subfigures (f) and (g) show the Young's modulus, phase difference at 0.5 Hz loading frequency, strain dependent modulus and yield stress of the same samples as mean (± SD) (Ristaniemi et al., 2018). Significant differences are indicated with * ($p$<0.05) and ** ($p$<0.01).

**Figure 3.** Summary of the results of this study, highlighting PCL (purple), MCL (yellow) and ACL (blue). The collateral ligaments exhibited the highest collagen contents, and the cruciate ligaments had the highest proteoglycan contents, while PCL had the highest elastin content. All ligaments pooled, high collagen content was associated with low toe-region strain, high Young's modulus, high yield and failure stresses and high toughness. High proteoglycan content was associated with low Young's modulus, low yield stress and low toughness at yield. High elastin content implied higher toe region nonlinearity and Young's modulus.



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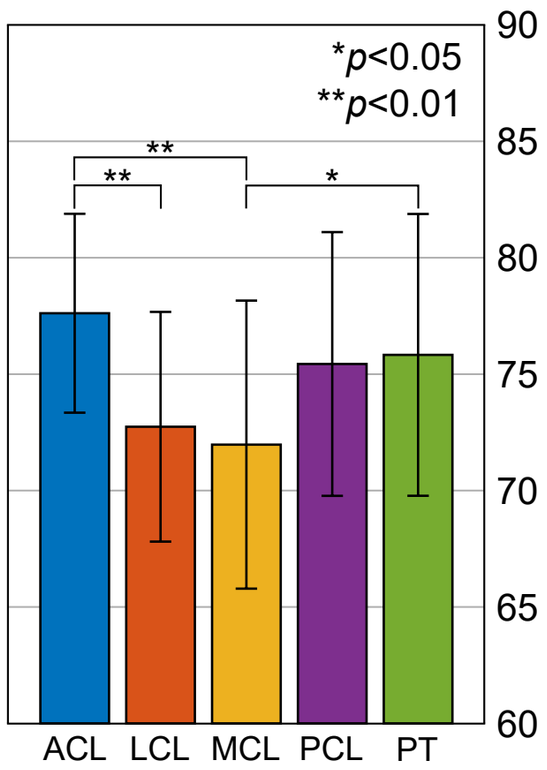


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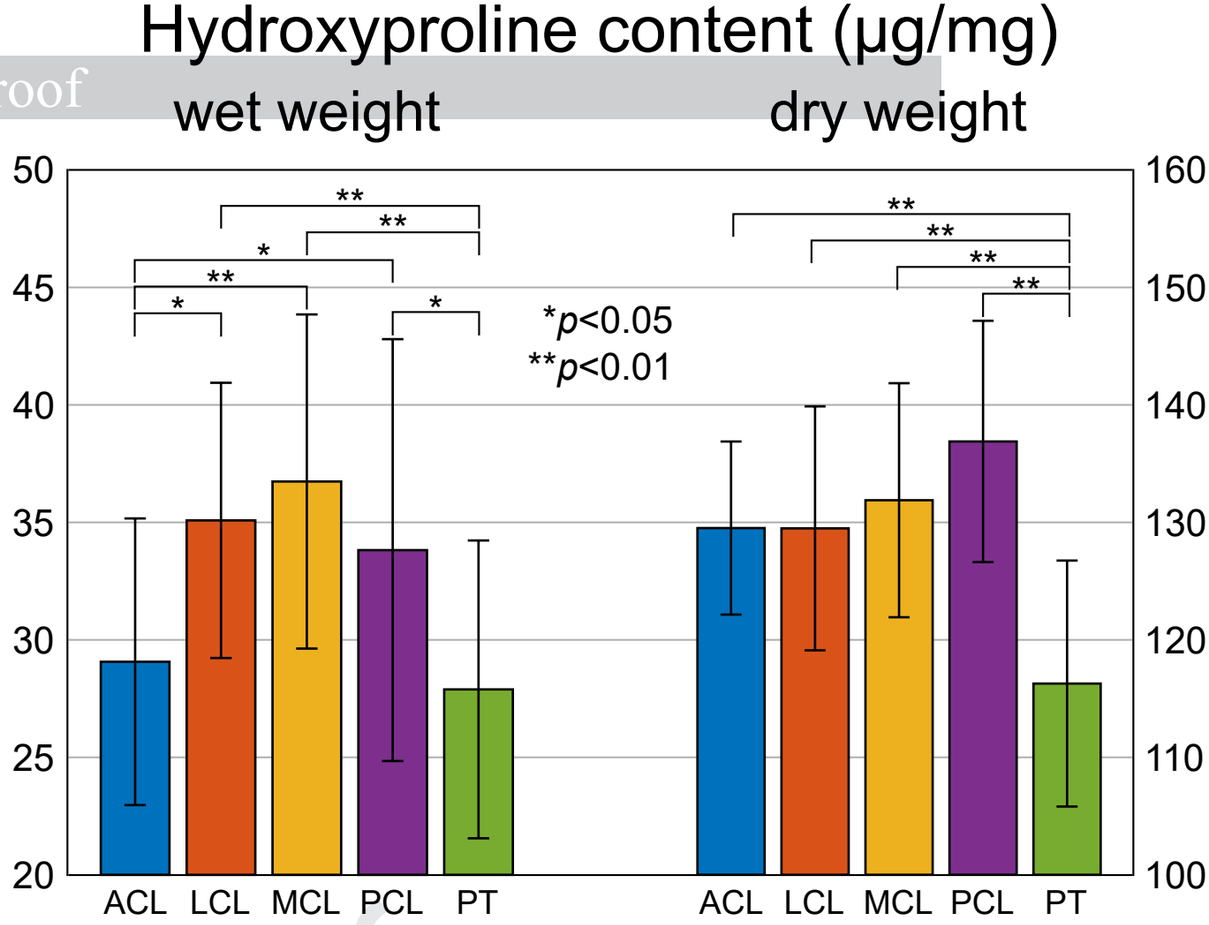


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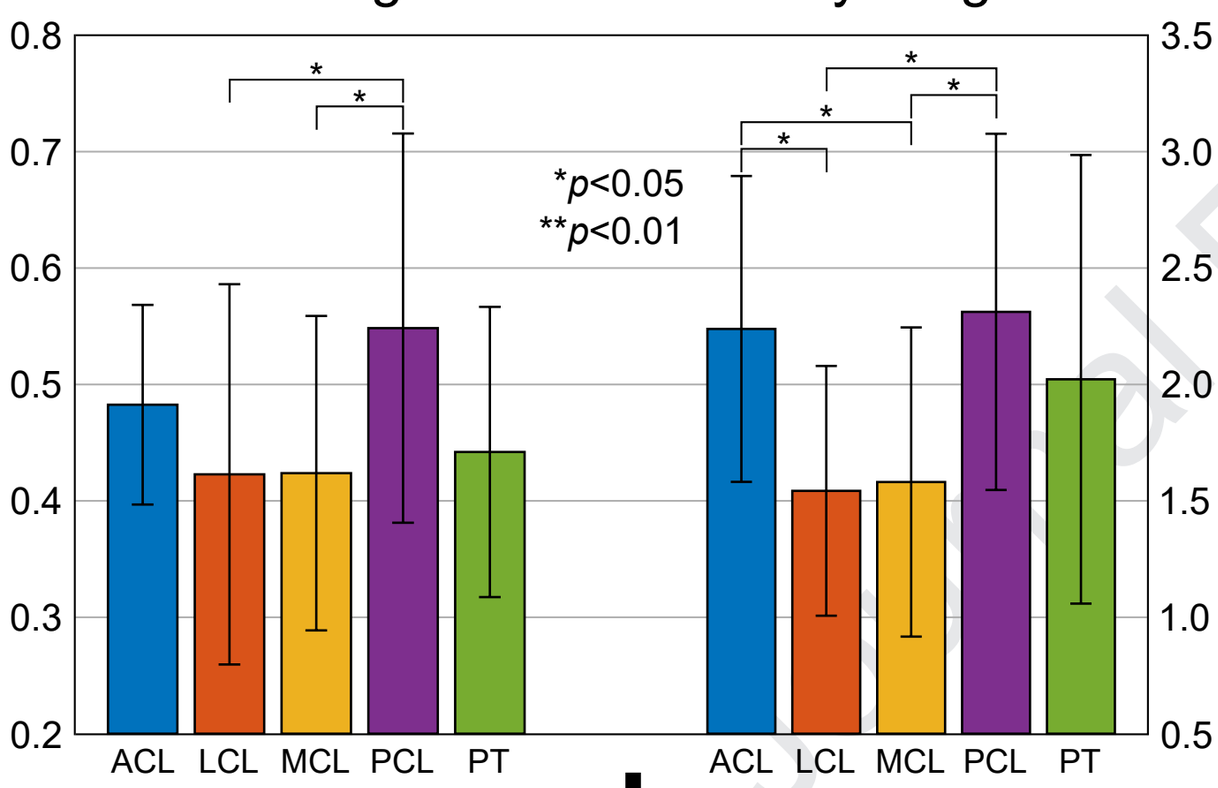


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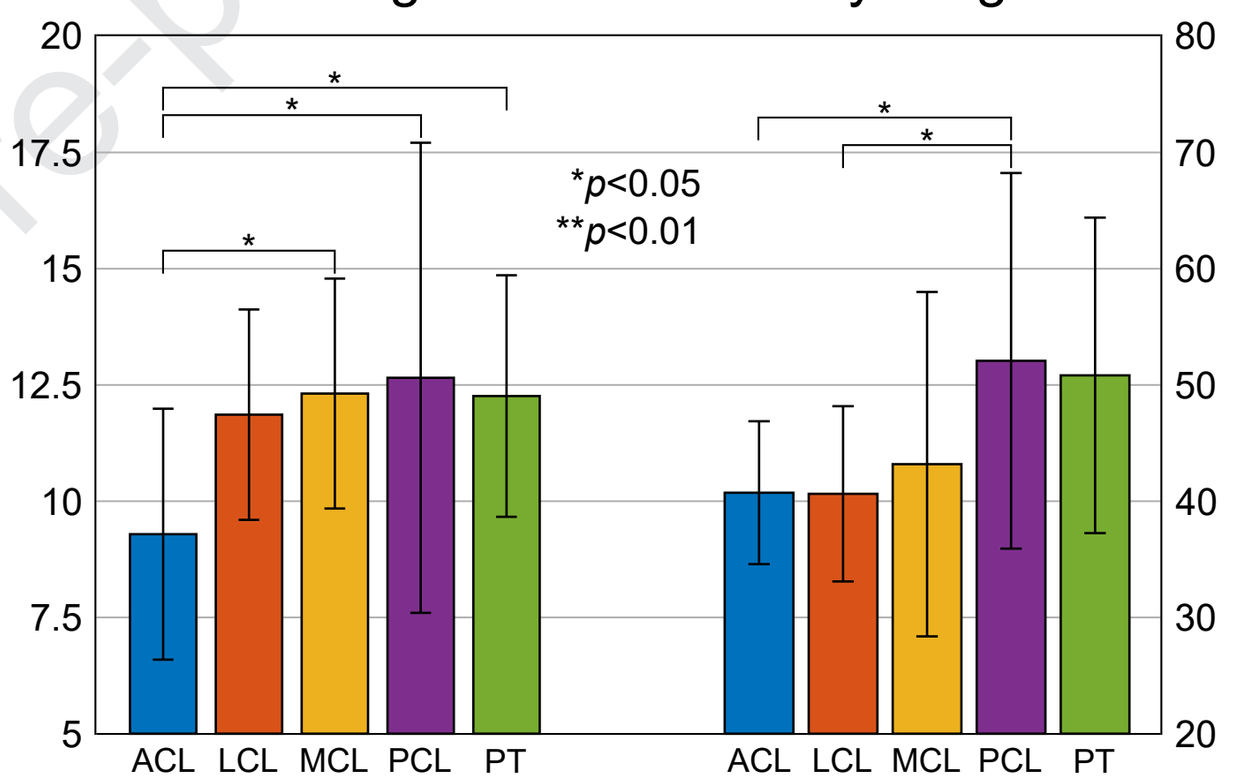


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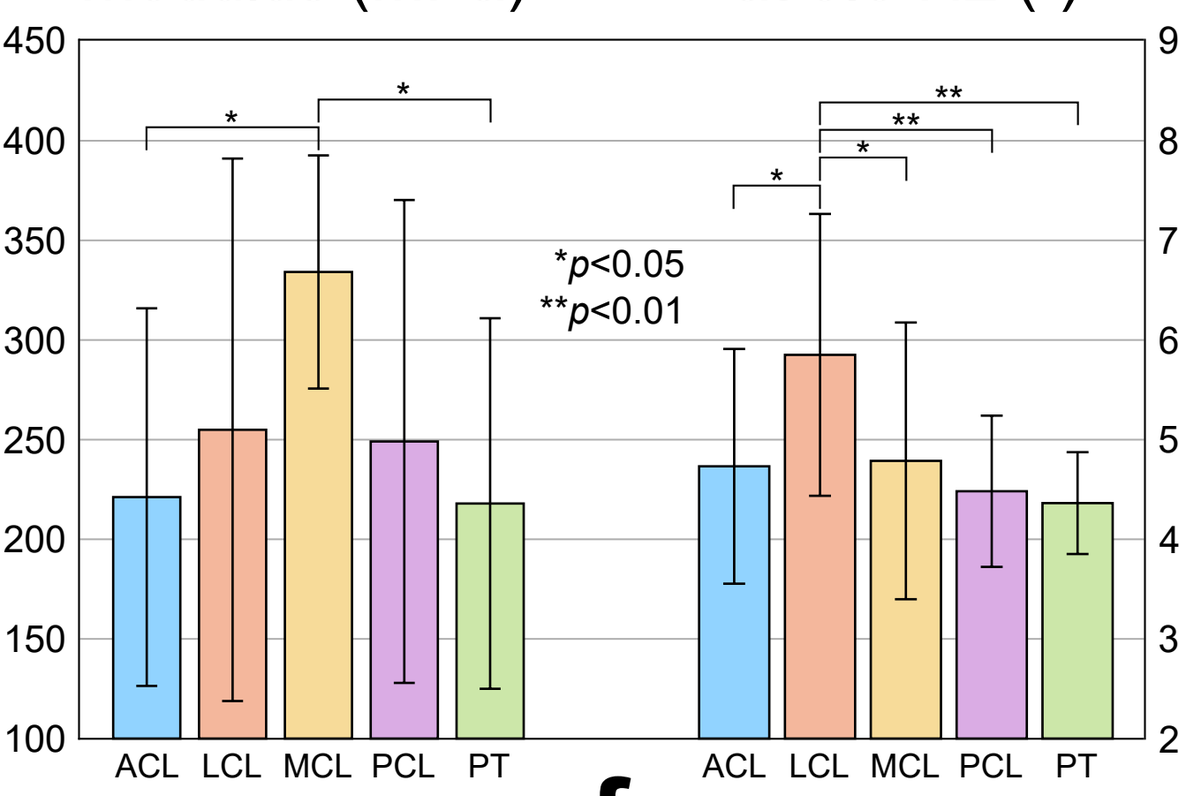


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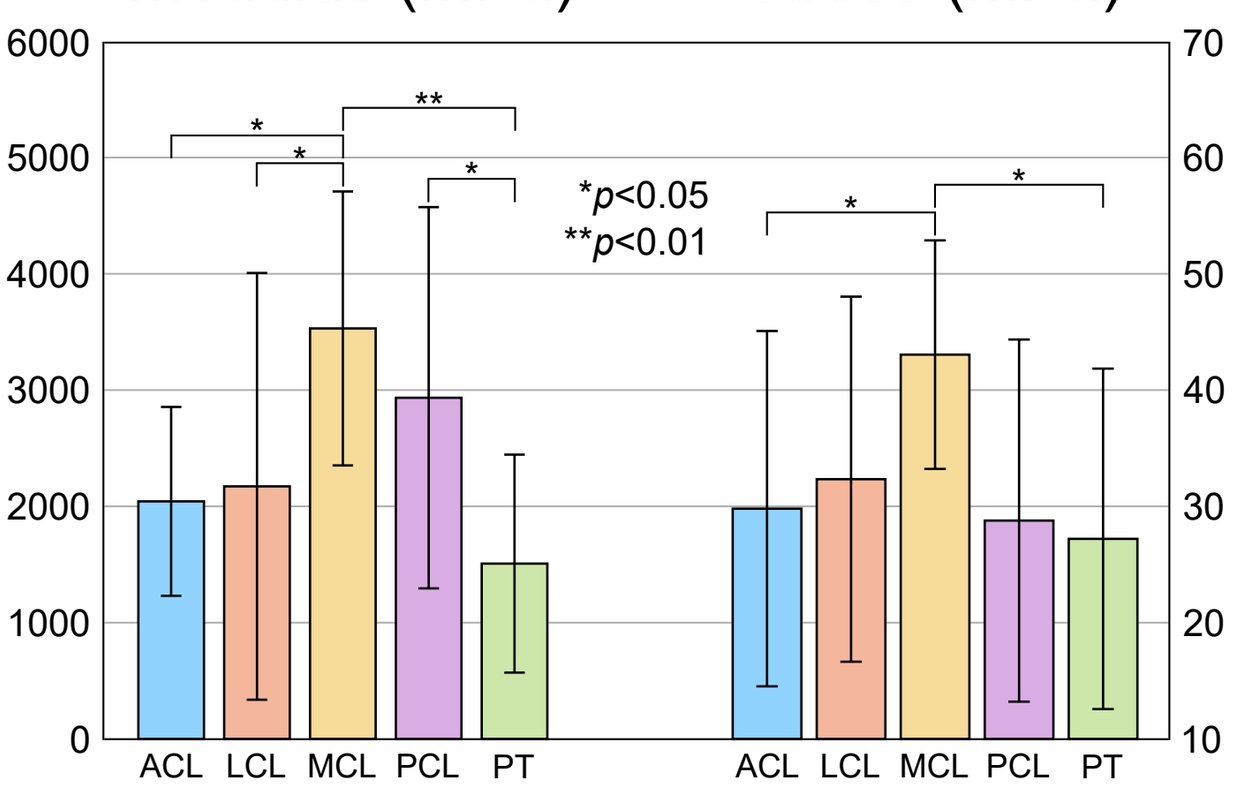


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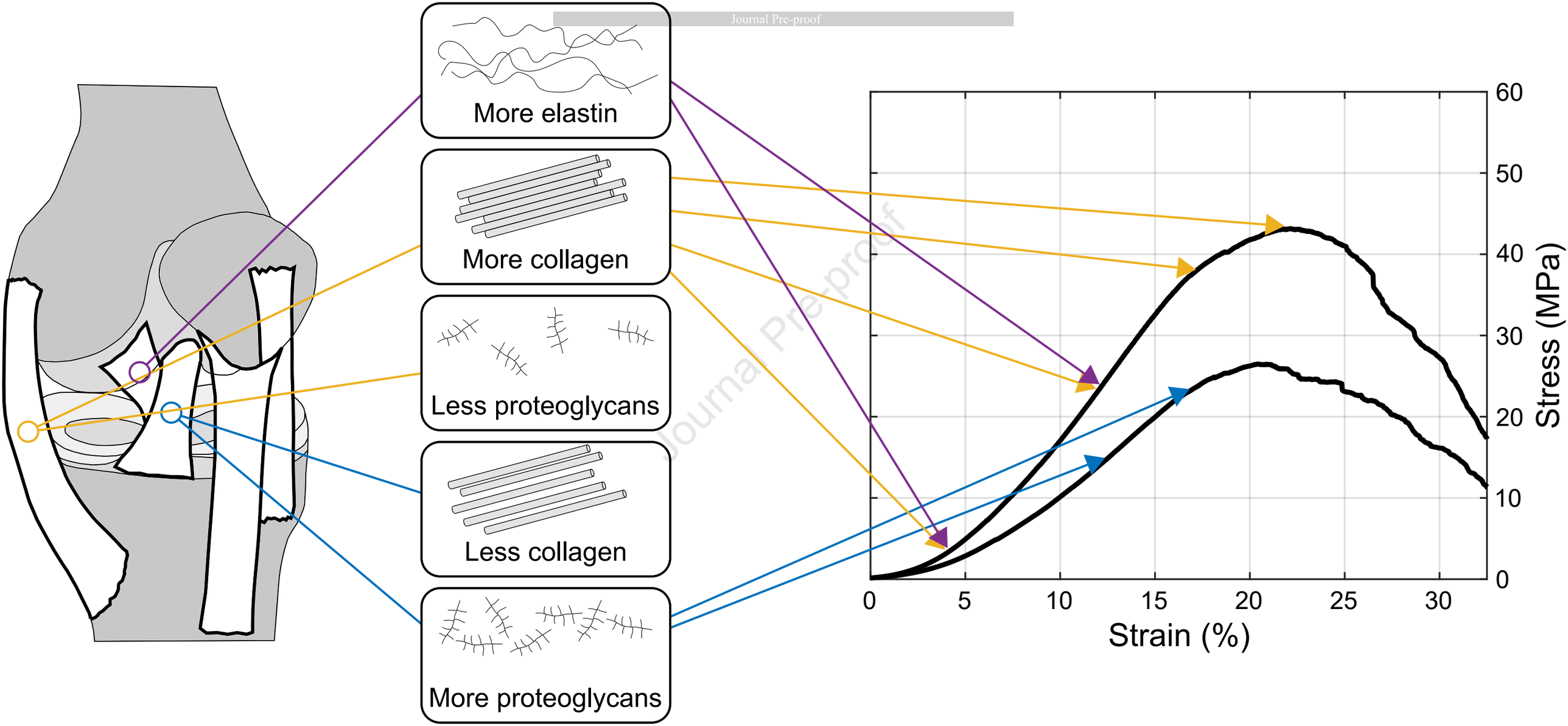

- Medial collateral ligament exhibited highest hydroxyproline content
- Cruciate ligaments had highest uronic acid content
- Posterior cruciate ligament exhibited highest elastin content
- High elastin content predicted high toe region nonlinearity and Young's modulus
- High uronic acid content predicted low Young's modulus, yield stress and toughness

**Author Statement**

**A. Ristaniemi:** Writing - Original Draft, Investigation, Visualization, Formal analysis, Conceptualization; **J. Torniainen:** Methodology, Formal analysis, Writing - Review & Editing; **L. Stenroth:** Conceptualization, Visualization, Writing - Review & Editing; **M.A.J. Finnilä:** Methodology, Writing - Review & Editing; **T. Paakkonen:** Methodology, Investigation, Writing - Review & Editing; **J. Töyräs:** Conceptualization, Supervision, Writing - Review & Editing; **R.K. Korhonen:** Conceptualization, Resources, Supervision, Visualization, Writing - Review & Editing

**Conflict of Interest Statement**

Authors declare no conflicts of interest.

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