Anisotropy and inhomogeneity of permeability and fibrous network response in the pars intermedia of the human lateral meniscus

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Abstract

Within the human tibiofemoral joint, meniscus plays a key role due to its peculiar time-dependent mechanical characteristics, inhomogeneous structure and compositional features. To better understand the pathophysiological mechanisms underlying this essential component, it is mandatory to analyze in depth the relationship between its structure and the function it performs in the joint. Accordingly, the aim of this study was to evaluate the behaviour of both solid and fluid phases of human meniscus in response to compressive loads, by integrating mechanical assessment and histological analysis. Cubic specimens were harvested from seven knee lateral menisci, specifically from anterior horn, pars intermedia and posterior horn; unconfined compressive tests were then performed according to three main loading directions (i.e., radial, circumferential and vertical). Fibril modulus, matrix modulus and hydraulic permeability of the tissue were thence estimated through a fibril-network-reinforced biphasic model. Tissue porosity and collagen fibers arrangement were assessed through histology for each region and related to the loading directions adopted during mechanical tests. Regional and strain-dependent constitutive parameters were finally proposed for the human lateral meniscus, suggesting an isotropic behavior of both the horns, and a transversely isotropic response of the pars intermedia. Furthermore, the histological findings supported the evidences highlighted by the compressive tests. Indeed, this study provided novel insights concerning the functional behavior of human menisci by

integrating mechanical and histological characterizations and thus highlighting the key role of this component in knee contact mechanics and presenting fundamental information that can be used in the development of tissue-engineered substitutes.

Keywords

Meniscus, anisotropy, inhomogeneity, fibril-network-reinforced biphasic model

1. Introduction

Menisci are wedge-shaped, fibrocartilaginous tissues housed within the knee and located between the femoral condyles and the tibial plateaus [1]. They play a key role in homogeneously transferring 70% to 99% of the axial compressive load acting on the knee during daily life activities [2]. Besides this main mechanical function, menisci also perform further important roles, including secondary joint stabilization [3], lubrication [4], nutrient distribution [5] and proprioception [6]. These multiple functions are promoted by the inhomogeneous structure and composition of the tissue itself [7]. The histological characteristics of human menisci can vary according to the age of the subject and the portion of the tissue considered. In particular, different structural behaviors were noticed between the surface and central regions, whereas compositional differences were observed between both central and horn portions, and meniscal inner and outer areas – the latter presenting different healing capability due to lower blood supply [8][9][10][5][11][12]. Focusing on the collagen fibers network, differences were described between the deeper and superficial part of the tissue, with the fibers oriented mostly along the circumferential direction in the first case, and randomly in the second one. Moreover, changes in the distribution of the cellular populations were highlighted in meniscal tissue; chondrocyte-like round-shaped cells were identified in the inner part, whereas in the outer portion fibroblast-like cells were reported in presence also of fusiform-shaped cells, which could play the role of progenitor cells [13][14][15].

Accordingly, tissue composition and mechanical characteristics should be regionally described. Meniscus behaviour was characterized to be also time-dependent, i.e., viscoelastic; in general, it is widely described by using a biphasic model, consisting of a solid extra-cellular matrix interspersed by a fluid phase [16]. The solid porous matrix consists of collagen types I and II, glycosaminoglycans (GAGs) and elastin [7][17], whereas the fluid phase mainly contains water and free ions [18]. The interaction between these phases produces the characteristic time-dependent behavior of this tissue [2]. To better describe these phenomena, the classic isotropic homogeneous biphasic model evolved in the fibril-network-reinforced biphasic model, by reinforcing the matrix with a network of nonlinear fibrils distributed in the radial, circular and vertical directions [19].
In this perspective, a complete functional assessment of the meniscus should investigate both the mechanical inhomogeneity [20] and anisotropy [16] of the tissue. To the best of the authors' knowledge, detailed mechanical investigations of the human meniscal tissue in compression – considering regional/directional diversity and an appropriate material model – are very limited, so far. Actually, only two studies evaluated the mechanical behavior of human meniscus accounting different regions and directions [16][21]. In particular, Leslie et al. [16] carried out a complete directional assessment, but only meniscal horns were evaluated with no consideration of the fluid phase; moreover, the biomechanical properties of the tissues were altered by storing specimens in formaldehyde before testing them. Chia et al. [21] led a complete regional characterization, but neglecting circumferential direction and, as similarly underlined in the previous work, the contribute of the fluid phase. In their conclusion, both the above studies suggested a plane of isotropy for meniscal tissue, but only considering the elastic modulus.

Therefore, the contribution of the fibrous and fluid components on the local mechanical response of meniscal tissue remains partially unexplored. Such information is crucial to improve the actual knowledge on human tibiofemoral contact mechanics –in both healthy and pathological knees [22][23]; furthermore, reliable advices on meniscal properties can lead to the development of enhanced solutions for meniscal replacement [23]. Moreover, the way we used to model the meniscal mechanical behavior could influence the information we got about the tibiofemoral contact mechanics itself. In the work realized by Kazemi et al., 2014 [24], menisci were modelled, for instance, as fully saturated porous media reinforced with a continuum collagen network, and the tissue anisotropy was determined by considering the primary collagen fibers, oriented in the circumferential direction. The authors reported neither experimental data nor permeability values, specifically concerning the meniscal tissue and, moreover, no specific local model was implemented for the meniscus body and horns. In the same year, Meng et al., 2014 [25] modelled menisci as fibril-reinforced biphasic materials and considered anisotropy, again, with major tensile resistance on the circumferential direction. The authors here presented several material parameters, including permeability values, but they were all derived from scientific literature and not from a dedicated experimental campaign. Even in this work, the dependence of these parameters on the precise tissue region (i.e., body vs horns) was not considered. In this perspective, the knowledge about meniscus mechanical behavior must derive specifically from human specimens, due to the strong differences with the animal source [17][21][26][27].

Accordingly, the aim of this study was to elaborate on the inhomogeneity and anisotropy description of human meniscus through an extensive investigation on the contributes of both solid and fluid phases in response to compressive load. To achieve this purpose, stress-relaxation behaviour of standard-shaped specimens – extracted from anterior horn, pars intermedia and posterior horn of human menisci – was assessed through unconfined compression test realized along three main directions – vertical, radial and circumferential. Mechanical parameters were specifically estimated on the obtained force-displacement curves by using a linear fibril-network-reinforced biphasic model. Furthermore, histological analyses were realized to provide information about meniscal structure and content, thus to integrate the mechanical characterization and support the assessment of any difference among tissue regions and loading directions.

2. Materials and Methods

2.1 Tissue specimens

Human lateral menisci were harvested from twelve patients (age 59-76, mean 66 years) undergoing total knee arthroplasty (TKA, ethical approval EM 249-2018_21/2017/Sper/IOR_EM2, Rizzoli Orthopaedic Institute, Bologna, Italy). Specimens that presented gross signs of degeneration, or which were too small to ensure a correct harvesting of the tissue samples, were preventively discarded. Seven menisci were finally selected for testing. To preserve the structural properties of the tissue, the menisci were wrapped in Ringer's solution-soaked gauze and frozen at -20°C until the experiment [28]. Furthermore, to reliably exclude degenerated specimens, histological evaluation was also performed on an additional meniscal tissue responding the very same inclusion criteria and on a lateral meniscus coming from the musculoskeletal tissue bank of the Institute and matched for age (Supplementary Materials, paragraph SM.1).

2.2 Specimen preparation

On test day, menisci were thawed at room temperature and allowed to equilibrate in Ringer's solution. Each meniscus was firstly cleaned from residual non-meniscal connective tissue and then carefully divided into three macro regions – i.e. anterior horn (AH), pars intermedia (PI) and posterior horn (PH) –, characterized by an equal arc length in the circumferential direction [20][26] (**Fig. 1**). From each region, one or two cubic samples – with side dimension in the range of (4-5) mm (measured by a digital caliper, Mitutoyo, Minato-ku, Tokyo, Japan; resolution = 0.01 mm) – were carved (**Fig. 1**). To avoid any possible influence of the superficial layer, cubic meniscal specimens were extracted far from it. In particular, for each meniscal region, a first set of parallel cuts were made along the radial direction, so as to obtain slices of 4-5 mm. Then, by flipping the specimen by 90°, a second set of parallel cuts were made, so as to remove tissue from both the inner and outer areas of the meniscus. Finally, by maintaining the same orientation of the sample, tissue from the top of the wedge-shaped portion was removed. Therefore, specimens were entirely composed by the inner, quantitatively dominant, tissue of the meniscus.

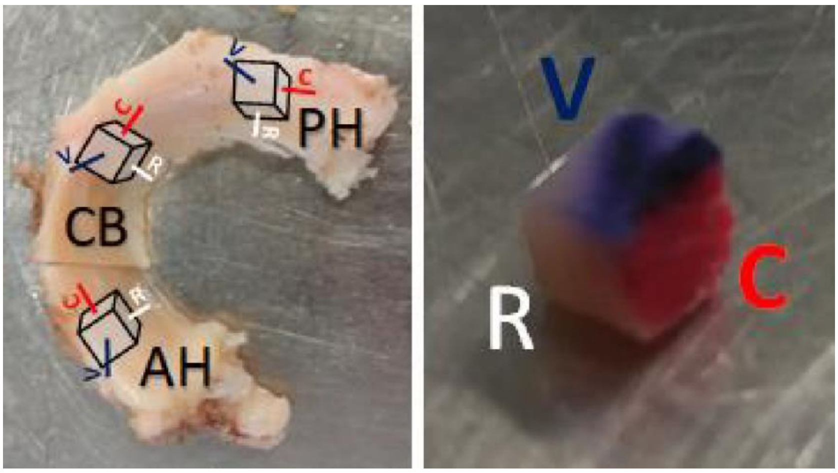


Fig. 1. On the left: anterior horn (AH), pars intermedia (PI) and posterior horn (PH) regions of the meniscus and locations of the cubic samples are clearly visualized. On the right: vertical (V), circumferential (C) and radial (R) directions are identified with different colors (i.e. purple, red and no paint, respectively).

For each region, the most regular specimens were selected for mechanical testing. According to previous literature [29][16] and based on the sample availability, the sample size was set to n = 21 (a sample from each of the three regions that compose each of the seven menisci). On each specimen, radial (-Rad), circumferential (-Circ) and vertical (-Vert) directions were clearly identified and marked (**Fig. 1**). Therefore, each testing session was labelled with region-direction, e.g. PI-Vert; while for the mechanical analysis, direction denotes the loading axis, for the histological assessment it states the corresponding cutting plane. Before testing, specimen dimensions were measured with a digital caliper obtaining a samples size of 4.39 ± 0.27 mm. Before the beginning of the mechanical test, cubic samples were left for 1 hour in Ringer's solution to equilibrate [26].

2.3 Mechanical testing

Unconfined compression test was performed by a multi-axis mechanical tester (Mach-1, Biomomentum Inc., Canada) equipped with a multi-axis load cell (70 N range and 3.5 mN resolution on the vertical axis). The specimen was soaked in Ringer's solution bath within the testing chamber, maintained in position by means of a non-porous metal plate (Φ = 12.5 mm) and then tested following a dedicated procedure [30]. In particular, according to Chia et al. [21], a pre-strain was applied before the unconfined compression protocol, thus to ensure proper contact at the interface between the tissue and the compressive plate, and to avoid any influence of the non-perfect planarity of the sample. Then, the test consisted of five compressive stress relaxation steps with 2% incremental strain and ramp velocity of 0.4% strain/s (relaxation stops when a relaxation rate of 0.05 N/min is reached) [31]. With this approach, meniscal specimens reached a maximum value of strain corresponding to 10%, in agreement with the ranges reported in previous studies [2][32][21][33]. Moreover, the magnitude of strains here applied were close to the ones at which menisci are subjected to during *in vivo* conditions [34][35][36].

After each testing session on a selected direction, specimen was allowed to rest in Ringer's solution at room temperature for 40 minutes (time fixed by a preliminary analysis) to fully recover its original dimensions (i.e. memory loss of the loading history).

To avoid any bias, meniscal regions and directions were tested in random order by considering 63 testing sessions (i.e. 7 menisci x 3 regions x 3 directions).

Finally, the stress relaxation values corresponding to 2% strain increments were fitted by considering a linear fibril-network-reinforced biphasic model, as described in [19]. The testing procedure, as reported by the manufacturer in [37], was here adapted to consider any possible anisotropy of the tissue. Actually, focusing on uniaxial unconfined compression test, the identified model describes the material as a composite, where a homogeneous isotropic biphasic matrix is reinforced by a network of nonlinear fibrils, which are equally distributed along principal directions within the matrix itself and are able to provide stiffness in tension only [19]. In this work, besides inhomogeneity, (i.e., characteristics depend on specific meniscal region), a key element that was introduced is indeed anisotropy. Practically, the biphasic matrix and the fibrils network were not imposed to be isotropic and homogeneously distributed, respectively; their representative mechanical parameters can eventually change with the orientation of the sample in the unconfined compression test, i.e., adapting with respect to the loading direction. The matrix can be mechanically described by considering the drained elastic constants, i.e., Young's modulus E_m (MPa), Poisson's ratio v_m and hydraulic permeability k (10^{-12} m^4/Ns). The fibril network is characterized by the equivalent tensile modulus E_f (MPa). As reported in the general testing procedure [37], sample dimension, strain, and v_m need to be specifically quantified or estimated (v_m = 0.45 [29][38]). Due to the variation that occurs with the increase of strain level, E_f and k were described as

$$E_f = E_{f\_0} * e^{strain * E_{f\_exp}} \qquad (1)$$

and

$$k = k_0 * e^{strain * k_{exp}} \qquad (2)$$

respectively. E_{f_0} and k_0 represents the initial values, whereas E_{f_exp} and k_{exp} – positive and negative, respectively – the exponential coefficients of fibril modulus and permeability.

Regarding the matrix modulus, the value of E_m averaged on five strain steps was used (E_{m_avg}), accordingly to the very same approach used in cartilage characterization [39]. Concerning the exponential coefficients, E_{f_exp}

provides information on how quickly the fibril modulus increases as a function of the strain, whereas the absolute value of k_{exp} – due to its negative sign – represents the attitude of the tissue to be passed through by fluids as the deformation increases. Actually, the values of E_m, E_f and k depend on both meniscal region and loading direction.

2.4 Histological evaluation

Histological analyses were performed on two adjacent cubic samples for each identified region, considering three out of the seven harvested specimens; to perform the evaluation on different cutting planes. The agreement between each pair of adjacent cubes was first evaluated in terms of histological parameters, as reported in the Supplementary Materials, paragraph SM.2. Samples were processed for paraffin embedding, taking care to visually maintain the information about the orientation adopted during mechanical test. Histological sections were then stained with Haematoxylin (Sigma-Aldrich, Saint Louis, Missouri, USA)[40] and Eosin (Bio-Optica, Milan, Italy) (H/E) – to evaluate cellularity, cells shape, general tissue morphology and organization – , with Picrosirius Red (Sigma-Aldrich, Saint Louis, Missouri, USA) – to qualitatively assess the collagen fiber arrangement and spatial orientation and collagen content, and with Alcian Blu (Sigma-Aldrich, Saint Louis, Missouri, USA) to assess the proteoglycan content [40]. Details about the methods and the results of the evaluations of the proteoglycan and collagen content are reported in Supplementary Materials, paragraph SM.3.

Images of section stained with H/E were acquired with a digital scanner (Aperio ScanScope Technologies) at different magnifications and analyzed with ImageJ software (NIH) to quantify tissue porosity. For each image, a region of interest (ROI), corresponding to the entire section, was identified. Briefly, after converting images in grayscale level, a method of auto thresholding (i.e., a built-in version of the IsoData algorithm [41]) was applied. A threshold for the identification of the entire ROI was defined (131 -255) as well as those for the identification of the pores (250-255), making the appropriate corrections to avoid bias related to the presence of any artifacts in the histological slides. The area of entire ROI, pores and tissue was measured and the percentage of porosity was then calculated. (**Fig. 2**)

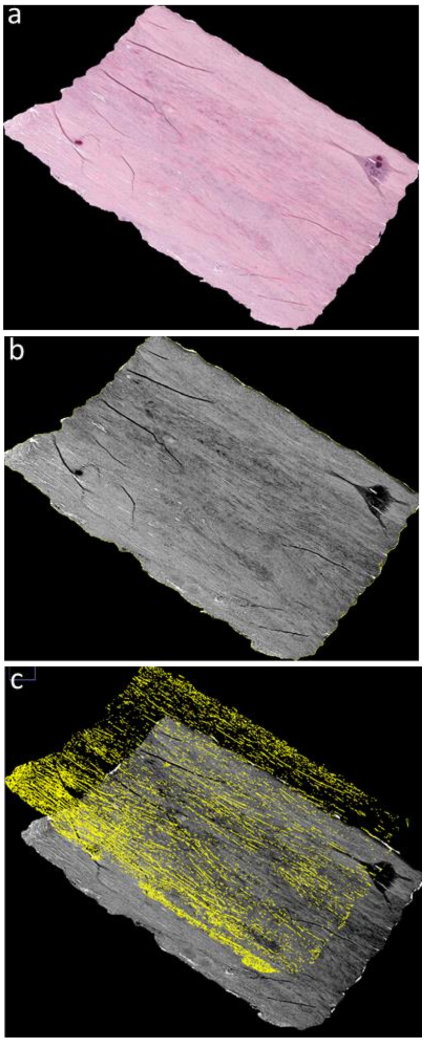


Fig.2. Representative images of the analyses performed with ImageJ Software. a) Identification of the region of interest (ROI), corresponding to the area of the sample evaluated; b) greyscale conversion; c) binarization process to distinguish tissue and pores.

Images of sections stained with Picrosirius Red were acquired with a semi-automatic optical microscope, Olympus BX51 (Olympus Optical, Bochum, Germany) connected to the XC50 Olympus digital camera (Olympus Optical) and the image analysis system CellSense (Olympus Optical). Using a polarized light optical microscope, the birefringence of the collagen structure was visualized. Each image was observed and the spatial orientation of collagen fibers was labeled as “horizontal”, “vertical” or “irregular”, taking as reference for observation the orthogonal plane relative to the load direction of the mechanical test, following what is reported in paragraph 2.2. Finally, the analyses of the local orientation of the collagen fibers were performed by using a dedicated plug-in (OrientationJ), focusing on the “Energy” parameter, where a higher value corresponds to less isotropic and more clearly oriented structures [42].

2.5 Statistical analysis

Statistical analysis was performed in Matlab (MathWorks, Natick, MA). In order to assess the functional inhomogeneity and anisotropy of the meniscal tissue, the parameters resulting by the mechanical analysis (E_{m_avg}, E_{f_0}, E_{f_exp}, k_0, k_{exp}) were pooled both for region and direction and reported as median, minimum and maximum values. For each parameter, statistical difference between groups was assessed by using two-sided Wilcoxon rank sum test with a level of statistical significance $p = 0.05$. Assuming that the constitutive parameters of the solid matrix E_{f_0} and E_{f_exp} were directly related to the amount of fibers within the tissue and that this influenced the hydraulic permeability of the meniscus, correlation between the solid and fluid parameters was computed by using the Spearman's rank correlation coefficients (correlation coefficient *rho*, p-value *p*, see Supplementary Materials paragraph SM.4).

3. Results

3.1 Biomechanical analysis

Solid (E_{m_avg}, E_{f_0}, E_{f_exp}) and fluid (k_0, k_{exp}) phase constitutive parameters (details reported in Supplementary Materials, paragraph SM.5), estimated by fitting the experimental data to a linear fibril-network-reinforced biphasic model (**Fig. 3**) and specified for each region and direction, are presented in **Fig. 4, 5** and **6**.

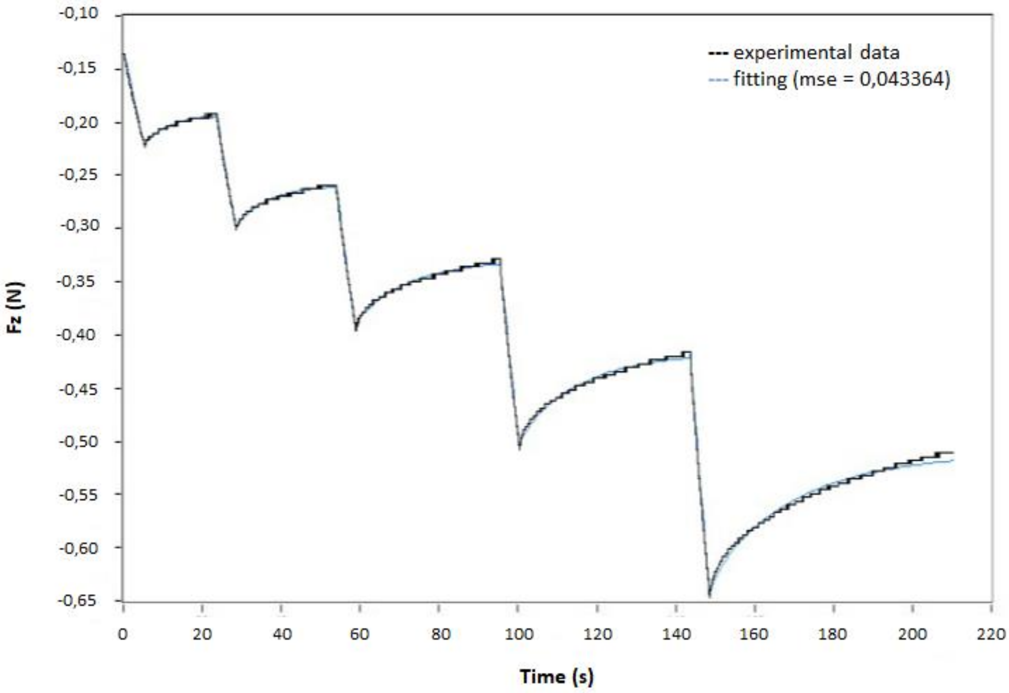


Fig. 3. Result achieved by fitting the experimental value of stress-relaxation by a linear fibril-network-reinforced biphasic model. mse is mean squared error.

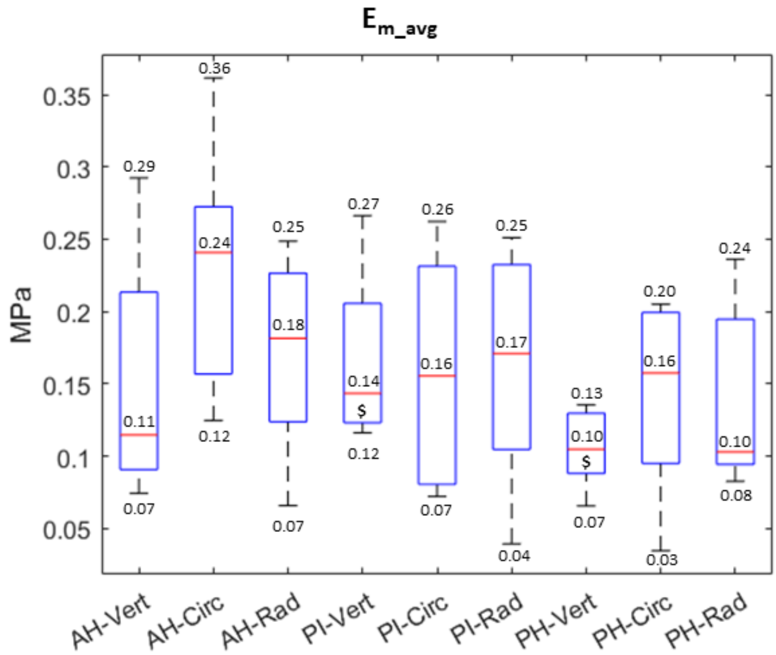


Fig. 4. Boxplot (median, minimum and maximum values) of matrix modulus (E_{m_avg}) depending on region (AH, PI, PH) and direction (Vert, Circ, Rad), determined from unconfined compression of meniscal cuboid

specimens (n = 7 for all groups). The $ symbol represents significant differences (using two-sided Wilcoxon rank sum test) between regions or directions ($p < 0.05$).

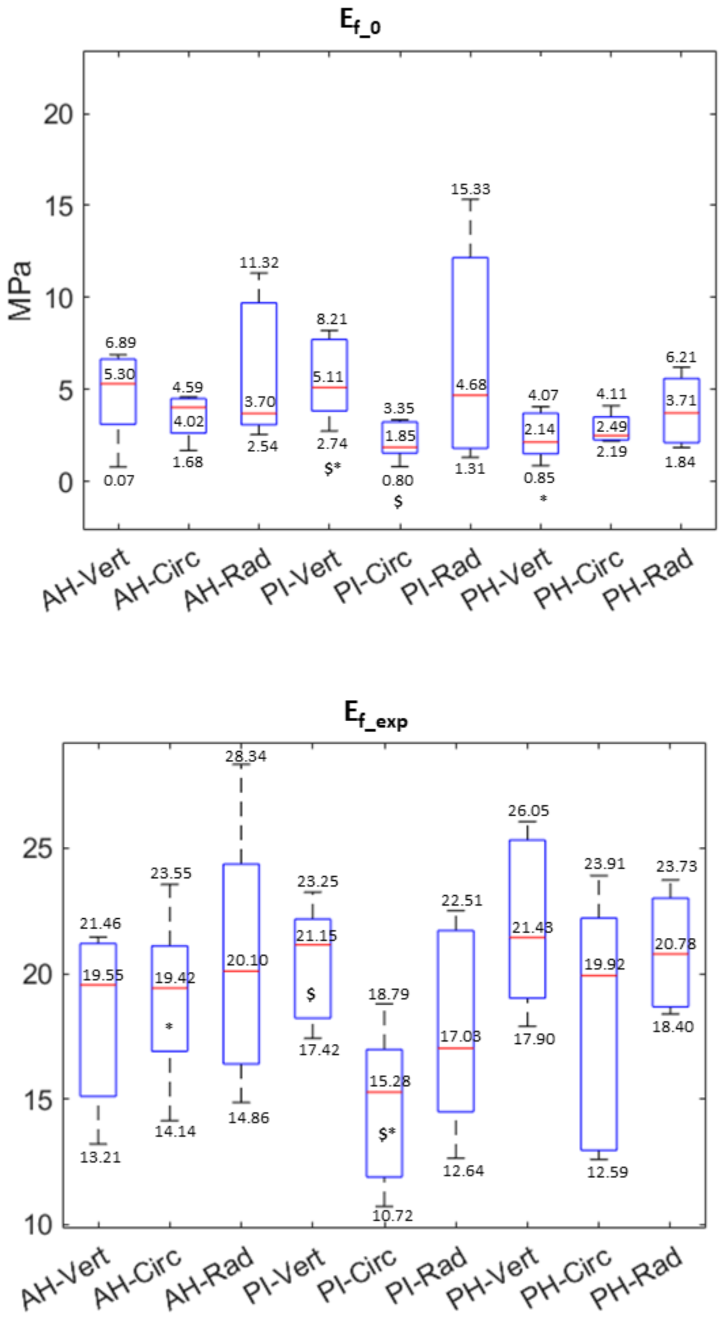
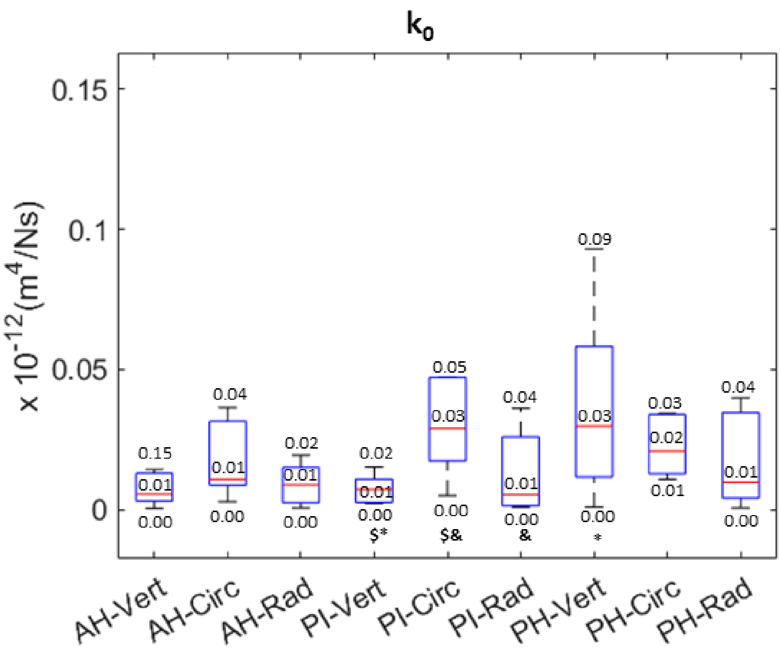


Fig. 5. Boxplots (median, minimum and maximum values) of initial (E_{f_0}) and exponential coefficient (E_{f_exp}) of the fibril modulus depending on region (AH, PI, PH) and direction (Vert, Circ, Rad), determined from unconfined compression of meniscal cuboid specimens (n = 7 for all groups). The $, * symbols represent significant differences (using two-sided Wilcoxon rank sum test) between regions or directions ($p < 0.05$).



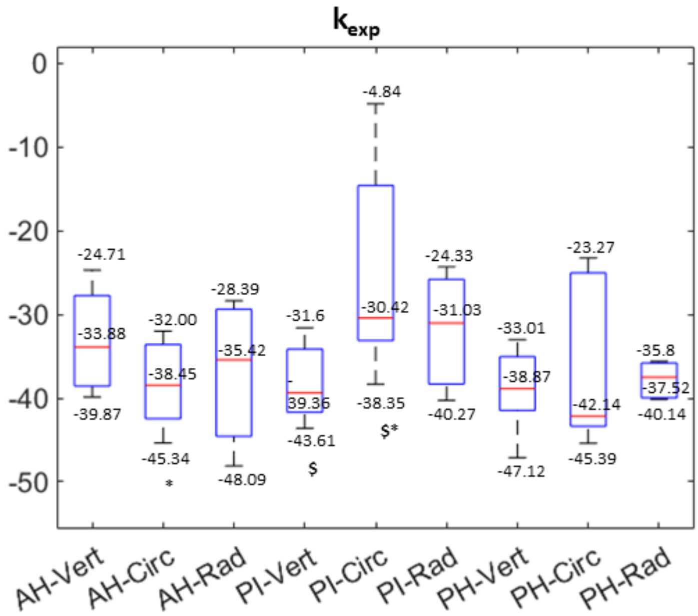


Fig. 6. Boxplots (median, minimum and maximum values) of initial (k_0) and exponential coefficient (k_{exp}) of the hydraulic permeability depending on region (AH, PI, PH) and direction (Vert, Circ, Rad), determined from unconfined compression of meniscal cuboid specimens (n = 7 for all groups). The $, *, & symbols represent significant differences (using two-sided Wilcoxon rank sum test) between regions or directions ($p < 0.05$).

Regarding the comparison between directions, the following statistical differences were found: vertical vs. circular direction in PI for E_{f_0} (Δ = 3.26 MPa, p = 0.0175), E_{f_exp} (Δ = 5.87, p = 0.0023), k_0 (Δ = 0.02 10^{-12} m^4/Ns, p = 0.0111) and k_{exp} (Δ = 8.94, p = 0.0111); circular vs. radial in PI for k_0 (Δ = 0.02 10^{-12} m^4/Ns, p = 0.0428).

Regarding the comparison between regions, the following statistical differences were found: PI vs PH in the vertical direction for E_{m_avg} (Δ = 0.4 MPa, p = 0.049), E_{f_0} (Δ = 2.97 MPa, p = 0.049) and k_0 (Δ = -0.02 10^{-12} m^4/Ns, p = 0.049); AH vs PI in the circumferential direction for E_{f_exp} (Δ = 4.14, p = 0.0262), and k_{exp} (Δ = 8.03, p = 0.0111).

Statistically significant inverse correlations ($p < 0.05$, $rho < 0$, see Supplementary Materials paragraph SM.4) were found between the exponential coefficients E_{f_exp} and k_{exp}, for each direction within all the investigated regions, and between E_{f_0} and k_0 for each direction within all the investigated regions, confirming that permeability is influenced also by the fiber network.

3.2 Histological analysis

Normal cellularity was observed in most of the histological samples, with some areas presenting hyper cellularity. In several cases, collagen bundles appeared well organized, presenting fusiform-shaped meniscal cells aligned among bundles; on the other hand, in some samples, collagen network was quite unorganized and homogeneous, presenting round-shaped cells and reporting an expected intra-donors variability (**Fig. 7**).

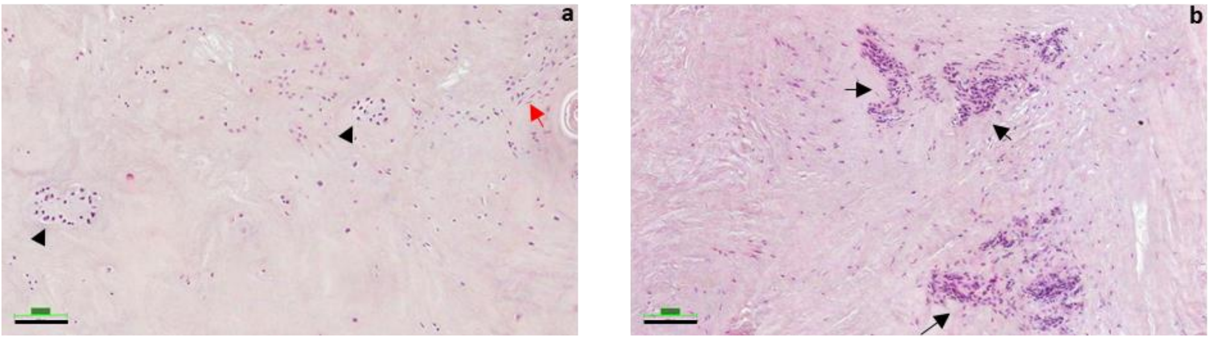


Fig. 7. Histological pictures of analyzed menisci, taking as reference for the observation the plane orthogonal to the load direction, as identified for the mechanical tests as reported in paragraph 2.2. Presence of round-shaped fibro-chondrocytes (a- black arrows) and fibroblast-like cells (a- red arrows), with zone of hyper cellularity (b). Hematoxylin/Eosin staining, 20x magnification. Scale bar length 200 um.

The evaluation of porosity in histological samples with cuts orthogonal to mechanical directions is shown in **Figure 8**.

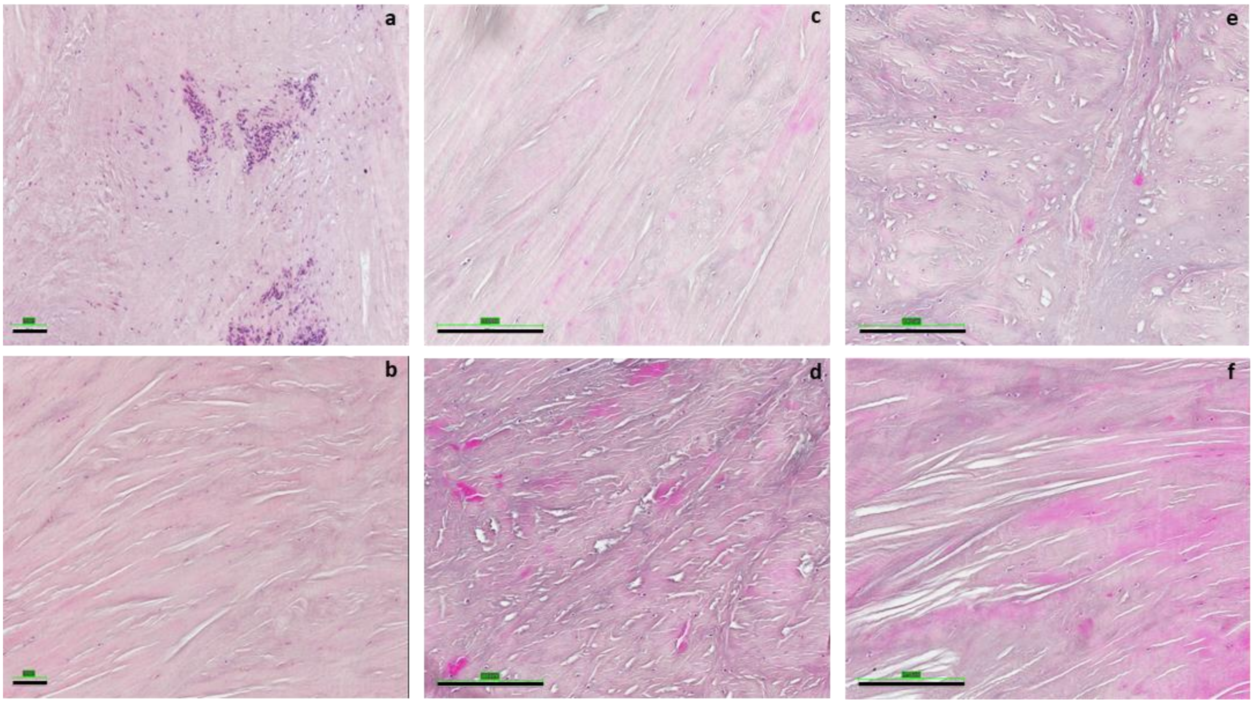


Fig.8. Comparison panel of the specimen porosity, expressed as percentage. a) PI- Circ (2.1% ± 0.6) *vs* b) PI-Vert (-3.1% ± 0.8); c) PI- Circ (4.4% ± 1.3) *vs* d) PI- Rad (4.9% ± 0.6); c) PI- Circ + d) Rad (9.3% ± 0.4) *vs* e) PH-Circ_ + f) Rad (9.9% ± 0.6). Hematoxylin/Eosin staining, 20x magnification. Scale bar length 200 um.

Staining with Picrosirius Red under the polarized light, in general, well highlighted the meniscal fibers. Distribution of the fibers appeared quite homogenous and fibers bundles appeared well organized, with spatial orientation clearly defined in horizontal or vertical direction; in few cases an irregular fibers network was observed. The comparison between samples, considering different regions and planes, highlighted differences in fibers orientation as shown in **Figure 9**.

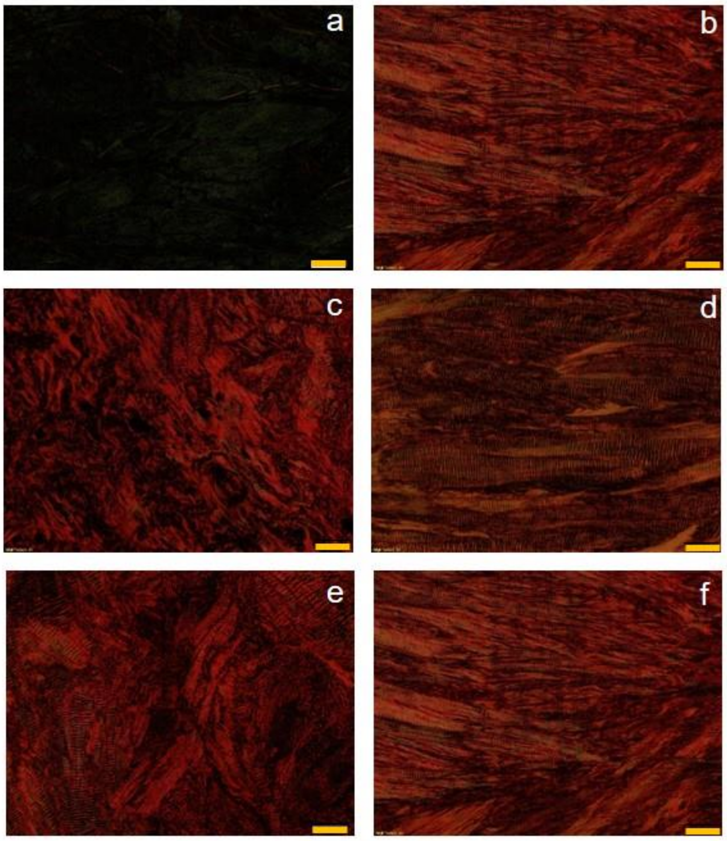


Fig. 9. Comparison panel of the fiber orientation. (a) AH- Circ+Vert *vs* (b) PI- Vert+Rad; (c) PI- Circ+Rad *vs* (d) PH- Rad+Circ; (e) PI- Circ+Rad *vs* (f) PI- Vert+Rad. Picrosirius Red staining, 8x magnification, polarized light. Scale bar length 100 um.

“Energy” values showed to be lower in PI Circ+Rad (69±20) and AH Circ+Vert (65.3±21) in comparison to the other samples PI Vert+Rad (118.5±9.6), PH Rad+Circ (123.8±29.8), PI Circ+Rad (87.6±12).

4. Discussion

This study reported, for the first time, region- and strain-dependent parameters considering a fibril-reinforced biphasic model fitted on the characteristics of the human lateral meniscus. The performed analysis highlighted that this tissue specifically resulted to be isotropic in the horns and transversely isotropic (i.e., isotropy plane formed by the radial and vertical directions) in the pars intermedia.

Meniscus degeneration represents a very frequent and widespread condition, presenting very different etiological characteristics and an observed major incidence in the elderly population (> 70 years, with an estimated prevalence > 50% between 70 and 90 years of age). Considering possible treatments of meniscal lesions, in the last decade preserving surgery has been preferred to meniscal resection. However, meniscectomy still remains the most used approach in severe cases, despite the high risk of early development of osteoarthritis and the need for arthroplasty. In this perspective, a deeper comprehension of structural and mechanical characteristics of menisci is mandatory to optimize both surgical treatments and the design of tissue-engineered meniscal substitutes [43].

The mechanical behaviour of human menisci was commonly investigated through indentation and confined compression tests, both allowing the estimation of the constitutive parameters of the solid and fluid phases [2][27][44][32]. Hydraulic permeability of meniscal tissue assumes values in the range of (10^{-15} - 10^{-14}) m^4/N sec for confined compression [45][27] and in the range of (10^{-17} - 10^{-15}) m^4/N sec for indentation [32][46]. The small value of this parameter is indicative of the high flow resistance of the extracellular matrix, which is fundamental in determining biomechanics and function of this tissue [47]. The high variability in reported hydraulic permeability values is likely due to several differences in the used measurement techniques, tested specimens and constitutive models [47]. To the best of the authors' knowledge, unconfined compression test coupled with the presented linear fibril-network-reinforced biphasic model has never been used to obtain hydraulic permeability of human menisci. In the present study, this parameter was estimated to have values in the order of 10^{-14} m^4/N sec, close to the higher values of the range reported above.

Concerning meniscus solid phase, the application of the linear fibril-network-reinforced biphasic model to the unconfined compression experimental data allowed to estimate both the fibrillar (E_f) and the non-fibrillar (E_m) modulus of the tissue. These parameters, generally achievable through indentation test, reported values in the order of (10^{-2} - 10^{1}) MPa and 10^{-2} MPa, respectively [32][46][48]. Accordingly, the solid phase parameters here assessed - for the first time through unconfined compression - highlighted a greater fibrillar contribution (10^{1} MPa) with respect to the non-fibrillar matrix (10^{-2} MPa). This finding was expected by considering the composition of human meniscus, where the collagen fibers are the main constituent of the solid part and are fundamental in the compressive response of the tissue [7][49]. Moreover, proteoglycan content, as investigated, did not show significant differences with respect to portions and orientations, supporting the fact that non-fibrillar modulus was less affected by tissue region and loading direction.

The analysis of the relationship between the estimated parameters and the applied deformation reported values that were comparable to the information available in literature [47][50][51]. Especially at high levels of deformation, the non-linear behaviour of hydraulic permeability plays a key role in the physiological functioning of load-bearing tissues, by preventing a rapid and excessive fluid exudation during loading [47]. The non-linear response of meniscal fibril modulus was reported to be typical of fibrils stress-strain behaviour [46][47]. Finally, non-fibrillar matrix modulus showed to be independent on strain, as previously highlighted [19].

In addition to magnitude and strain-dependence of the estimated parameters, the present study allowed to find significant differences between directions (i.e., anisotropy) and regions (i.e., inhomogeneity) in terms of mechanical response. Both these aspects are discussed in the following paragraphs.

4.1 Anisotropy of lateral human menisci

The pars intermedia (PI) of the lateral meniscus highlighted significant differences in the response to compressive stress along the different directions.

By analyzing the differences between vertical and circumferential direction of the PI, the lower E_{f_0} and E_{f_exp} values along circumferential axis can be likely due to the collagen fibers distribution and organization. The hypothesis is confirmed by the observations of collagen fibers network under polarized light. In fact, fibers appeared to be differently oriented, presenting an horizontal pattern *vs* an irregular orientation **(Fig.9, a *vs* b).** As suggested by Li et al., compressing the menisci along a specific direction generates tensile stresses on fibers oriented perpendicularly to the load itself [52]. Accordingly, a compression along the vertical axis stresses the fibers that present circumferential and radial orientation; for the same reason, if the load direction is circumferential, stresses in both vertically and radially oriented fibers are generated. Therefore, differences between the fibers with circumferential and vertical orientation should be sought. As reported by Li et al. [51] and Aspden et al. [53], collagen fibers in human menisci tend to be oriented circumferentially in the bulk tissue and radially in the surface region; on the other hand, no vertically oriented fibers are detected. Therefore, the higher values of E_{f_0} and E_{f_exp} in the vertical direction of the PI are ascribable to a greater number of fibers, that are radially and circumferentially oriented and which are tensioned during the compressive loading, with respect to those fibers that are able to respond if compressed in the circumferential direction, i.e., the only radially oriented ones. This hypothesis - in addition to the inverse correlations between fibril modulus and hydraulic permeability - can also explain the differences found between PI vertical and circumferential directions in terms of fluid phase parameters. In particular, PI showed a statistically lower k_0 and a higher absolute value of k_{exp} (with $k_{exp} < 0$) in the vertical direction compared to the circumferential one. This finding suggests a more difficult flow of the fluid in that specific direction - i.e. vertical one -, probably due to a denser fibers network formed on the plane specifically formed by fibers in circumferential and radial directions. This behaviour was also evident when comparing the histological values of porosity of PI, which result to be higher in the sagittal plane with respect to the transverse one (**Fig.8, a *vs* b**).

By analyzing the differences in k_0 between the radial and circumferential axes of the PI, a higher value in the latter was supported by a lower - although not presenting a statistically significant difference - initial value of the fibril modulus (E_{f_0}); this evidence is also in agreement with the inverse correlations found between fibril modulus (E_{f_0}) and hydraulic permeability (k_0). Even in this case, there is an agreement between the mechanical results and the tissue porosity, since the percentage of porous measured is lower in the sagittal plane of the PI samples with respect to the coronal one **(Fig. 8, c *vs* d).**

Due to the statistical differences found between circumferential and vertical/radial direction - both in terms of solid (E_{f_0} and E_{f_exp} for vertical) and fluid phase (k_0 for radial, k_0 and k_{exp} for vertical) constitutive parameters - the use of a transversely isotropic constitutive model for the PI is definitely suggested. The presence of an isotropic plane - defined by the radial and vertical directions - in the compressive response of the PI can be explained by the forces acting on menisci during everyday activity [7]. In particular, due to their wedge shape, the axial tibio-femoral forces decompose in a vertical component - countered by the upward force of the tibia - and in an horizontal component - exerted radially and directed outward in each meniscus [7]. As a result, the response of the meniscal tissue located in the PI differs along circumferential direction compared to the vertical and radial ones, which bear most of contact forces within the tibio-femoral joint.

Literature confirms the use of a plane of isotropy, formed by radial and vertical directions, in describing the mechanical behaviour of human menisci [16][21]. However, the evidence reported concerned only the elastic/compressive modulus [16][21]. Moreover, Chia et al. [21] pooled together the results obtained on samples deriving from different regions of the meniscus (AH, PI and PH) and therefore they did not specifically report anything about the anisotropy of each specific location. At present, a full investigation focused on the hydraulic permeability of human meniscus under different loading directions has never been reported, neither considering unconfined compression nor different loading configurations (e.g. indentation, tensile or confined compression tests).

4.2 Inhomogeneity of lateral human menisci

Regarding inhomogeneity, the obtained results suggest different constitutive parameters for PI and AH/PH; statistical differences were indeed found between PI and PH along the vertical direction and between PI and AH along the circumferential direction, both in terms of solid (E_{f_0} and E_{m_avg} in the first case, E_{f_exp} in the second one) and fluid phase (k_0 in the first case, k_{exp} in the second one).

Concerning the differences between PI and PH in the vertical direction, data suggested that the latter is generally more permeable - i.e. higher k_0 highlighted by PH compared to PI -, due to its lower amount of solid content, i.e. lower E_{f_0} and E_{m_avg}.

By analyzing the regional differences between PI and AH in the circumferential direction, a higher absolute k_{exp} value for AH highlighted its lower permeability; this was also supported by the higher values of E_{f_0}, E_{f_exp} and E_{m_avg} showed along circumferential axis, if compared to PI parameters. Definitely, the pars intermedia of the lateral meniscus is more permeable than the horns.

In both the comparisons, the histological quantification of porosity in the specimens highlighted a trend in the percentage of pores that supports the mechanical evidences **(Fig.8, c+d *vs* e+f)**. Mechanical results are supported also by the different orientation of the network fibers, as shown in **Fig. 9 (c *vs* d; e *vs* f)**.

Literature provides contrasting evidence regarding the dependence of the human menisci constitutive parameters on the analyzed region [16][44][32][48]. Leslie et al. did not find any difference between different portions of both medial and lateral human menisci [16]. Danso et al. found differences both in solid (i.e., the strain-dependent fibril network modulus) and fluid phase (i.e., the initial and strain-dependent permeability coefficient) between middle and anterior/posterior regions of human menisci [32][46][48]. Statistically significant differences in the region-dependent mechanical response of meniscal tissue were also reported by Ala-Myllymaki et al. [48], particularly in terms of strain-dependent fibril network modulus and hydraulic permeability. In these studies, indentation prevailed as characterizing approach; nevertheless, only the vertical direction was assessed. Despite some studies partially investigated the variability in human menisci depending on the region both in terms of composition - e.g. glycosaminoglycan, proteoglycan and collagen content, collagen orientation angle etc. - and mechanical properties [16][44][32][46][54][33][55], a comprehensive evaluation of both the solid and fluid phase regional-dependence was still missing, especially when considering the unconfined compression test. In this perspective, the differences found between body and horns can be mainly ascribable to the variation in tissue composition and structure. The collagen fibers arrangement of meniscal tissue is ideal for transferring a vertical compressive load into circumferential “hoop” stresses; therefore, it is quite reasonable to suggest that a gradient is present in the variation of the fibers orientation, not only depending on depth but also on region, from the more radially directed in the pars intermedia to the more circumferentially oriented in the horns [55]. The analysis here performed reported a homogeneity in the collagen content among the samples, thus suggesting that the orientation of the fiber network could be the main discriminating factor.

Finally, some caveats must be highlighted.

Freezing the samples appeared to have not influenced the histological examination, despite the process can damage the cells in the tissue. In mechanical characterization, freezing the samples without previous fixation of the meniscal tissue represents indeed a common protocol described in literature; reported information seem to support that a short-term storage does not affect the mechanical properties and morphological characteristics of the tissue [56][57][58].

The small sample size (i.e., 7 samples), represents indeed one of the major limitations of this study. A proper power analysis was not performed, mainly due to the lack of consistent information present in literature about the analyzed parameters, practical considerations regarding tissue harvesting and the samples numerosity highlighted in several studies with very similar approaches [16][29]. Due to this limitation, we are well aware that it is quite difficult to generalize our findings; however, we were able to highlight a clear dependency of tissue permeability – the most dispersed parameter – from loading direction that is one of the most relevant conclusions coming from this investigation.

Regarding the adopted mechanical test, compression seems to be not perfectly coherent with meniscal function, since the physiological joint loading conditions are reported to elicit a tensional stress on the radial and circumferential direction of the menisci due to their attachments [59]. However, the primary aim of the unconfined compression test is not to replicate physiological stress, but to allow the estimation of several parameters describing material mechanical behaviour [37]. Unconfined compression characterization represents, at the same time, the simplest and still most spread mechanical test for soft tissues involved in load-bearing human joints, such as cartilage and menisci [60]. In opposition to confined approach, where cylinder specimens are needed, unconfined compression can be applied to simple cubic specimens allowing, under appropriate conditions, to evaluate the influence of loading direction, i.e. anisotropy, on the mechanical behaviour of the same tissue portion [16]. Moreover, adopting specific loading protocol and material models, unconfined compression test can be adopted to reveal the contributions of both the solid and fluid phases on tissue mechanics [37]. Actually, not confining the sample during compression, we were able to highlight the fibres tension resisting to bulging and the permeability on the lateral side. In this way, from a rather “artificial” testing condition, it is however possible to provide useful information for designing tissue-engineered materials that could mimic meniscus mechanical behaviour.

Swelling should be taken into account when testing specimens excised from soft tissues, in particular whenever considering menisci and cartilage [61][62]. Of course, the lack of a systematic evaluation of this phenomenon represents a limitation of the study. Although we did not quantify the effect of the swelling nor its impact on the estimated mechanical parameters, we are quite confident that the differences highlighted among stressing directions and meniscal regions were not highly affected by this process, since the very same protocol was applied for all the experimental steps - i.e. thawing, dissection, testing and equilibrating phase, realized after each compressive session.

In this study, the Poisson’s ratio was set at 0.45. In order to develop a sample-specific model, this ratio should have been measured by using specific approaches, such as - for instance - imaging solutions applied to the lateral deformation of each specimen achieved during compression. This represents indeed a proper limitation, but such measurements were not possible in this work due to the implemented setup, which was specifically designed to achieve the primary goals of this study. However, in order to make our findings more reliable, we investigated the effects of the Poisson’s ratio on the estimated mechanical parameters (see supplementary file

“Poisson”). These preliminary analyses highlighted that changes in the Poisson’s ratio did not affect the initial values of E_f and k, neither their evolution with respect to strain, and not even the value of E_m. Therefore, a “wrong” definition of the Poisson’s ratio may indeed have introduced an error in the estimated values of the reported mechanical parameters, but this did not heavily affect the possibility of comparing the outcomes obtained from different regions and directions.

Regarding the histological assessment, since it is inherently a 2D evaluation, it could have been affected by the position of the slices. Other methodologies, more suitable to characterize 3D structure, such as Scanning Electron Micrography or microtomography, should be used as well, although they present other intrinsic limitations (e.g. use of contrast agent for soft tissue). At present, a microtomography procedure specific for the characterization of human menisci has been employed, able to highlight the tissue structure, without altering it. The first obtained preliminary information about porosity are in agreement with histological findings (see Supplementary materials, paragraph SM.6).

Finally, despite the acquired information relates to an adult population undergoing TKA – thus affected at least by aging effects, considering a mean age of 66 years – gross and histological evaluations confirmed that all the analyzed specimens did not present significant evidence of degenerative processes (see Supplementary materials, paragraph SM.1), as previously reported for the macroscopic behaviour of the matrix with respect to “healthy” joints [17].

Despite these limitations, the here reported evidences can represent one further step in understanding the physiological biomechanics of human menisci, and help in establishing updated requirements for the development of tissue-engineered meniscal implants.

5. Conclusion

In this study, the anisotropy and inhomogeneity of human lateral menisci were systematically investigated. Accordingly, region- and strain-dependent parameters for a fibril-reinforced biphasic model of the lateral meniscus were proposed, being specifically isotropic in the horns and transversely isotropic (i.e., an isotropy plane formed by radial and vertical directions) in the body. This study gave a functional assessment of meniscal tissue, endorsing the correspondence between mechanical behaviour and tissue structure, and thus providing further insights for the constitutive analysis of menisci. This in-depth evaluation permits an advancement in the modelling approaches related to the tibio-femoral contact mechanics, specifically addressing both clinical diagnosis and prognosis, and supporting the design and development of novel meniscal replacement solutions.

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