Multiscale Poroviscoelastic Compressive Properties of Mouse Supraspinatus Tendons Are Altered in Young and Aged Mice

Citation: Connizzo, Brianne K., and Alan J. Grodzinsky. "Multiscale Poroviscoelastic Compressive Properties of Mouse Supraspinatus Tendons Are Altered in Young and Aged Mice." Journal of Biomechanical Engineering 140, 5 (February 2018): 051002 © 2018 ASME

As Published: http://dx.doi.org/10.1115/1.4038745

Publisher: ASME International

Persistent URL: http://hdl.handle.net/1721.1/117759

Version: Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

Multiscale Poroviscoelastic Compressive Properties of Mouse Supraspinatus Tendons Are Altered in Young and Aged Mice

Brianne K. Connizzo
Department of Biological Engineering,
Massachusetts Institute of Technology,
Cambridge, MA 02139

Alan J. Grodzinsky[1]
Department of Biological Engineering,
Massachusetts Institute of Technology,
Cambridge, MA 02139;
Center for Biomedical Engineering,
Massachusetts Institute of Technology,
Cambridge, MA 02139;
Department of Electrical Engineering
and Computer Science,
Massachusetts Institute of Technology,
Cambridge, MA 02139;
Department of Mechanical Engineering,
Massachusetts Institute of Technology,
Cambridge, MA 02139
e-mail: alg@mit.edu

Rotator cuff disorders are one of the most common causes of shoulder pain and disability in the aging population but, unfortunately, the etiology is still unknown. One factor thought to contribute to the progression of disease is the external compression of the rotator cuff tendons, which can be significantly increased by age-related changes such as muscle weakness and poor posture. The objective of this study was to investigate the baseline compressive response of tendon and determine how this response is altered during maturation and aging. We did this by characterizing the compressive mechanical, viscoelastic, and poroelastic properties of young, mature, and aged mouse supraspinatus tendons using macroscale indentation testing and nanoscale high-frequency AFM-based rheology testing. Using these multiscale techniques, we found that aged tendons were stiffer than their mature counterparts and that both young and aged tendons exhibited increased hydraulic permeability and energy dissipation. We hypothesize that regional and age-related variations in collagen morphology and organization are likely responsible for changes in the multiscale compressive response as these structural parameters may affect fluid flow. Importantly, these results suggest a role for age-related changes in the progression of tendon degeneration, and we hypothesize that decreased ability to resist compressive loading via fluid pressurization may result in damage to the extracellular matrix (ECM) and ultimately tendon degeneration. These studies provide insight into the regional multiscale compressive response of tendons and indicate that altered compressive properties in aging tendons may be a major contributor to overall tendon degeneration. [DOI: 10.1115/1.4038745]

Keywords: tendon, poroviscoelasticity, fluid flow, hydraulic permeability, aging, supraspinatus

1 Introduction

One of the most common causes of shoulder pain and disability in the aging population is disorders of the rotator cuff, which has been reported to affect greater than 50% of the aged population [1]. Unfortunately, one single cause for this degenerative process has been difficult to identify [2–4]. External compression of the rotator cuff tendons, particularly in the most commonly injured supraspinatus tendon, is a factor thought to contribute to tendon degeneration in the clinic [5–7]. Several factors associated with age, such as rotator cuff weakness and poor posture, can cause increased compression of the tendon and may ultimately lead to tendon degeneration, particularly when coupled with repetitive use and normal age-related changes. However, only a few studies have investigated the compressive response of tendons and none of these studies have focused on the rotator cuff tendons [8,9].

Tendons are composed primarily of tenocytes, water, and an extracellular matrix (ECM) comprised predominantly of collagen type I organized in a hierarchical manner, minor collagens, elastin, and proteoglycans with their associated glycosaminoglycan (GAG) chains [10–14]. GAGs have net negative charge, which leads to increased tissue hydration due to osmotic and electrostatic interactions [15–17]. This unique structure dictates tendon's mechanical function, specifically in the supraspinatus tendon, which is one of the most heterogeneous and complex tendons in the human body. Tendon is a compliant, anisotropic material, which exhibits nonlinear biomechanical behavior and several time-dependent properties including *viscoelastic* stress relaxation, hysteresis, and creep [18–20]. Tendons are also thought to exhibit time-dependent *poroelastic* behavior when localized compression causes fluid flow through the tendon's ECM to become the rate-limiting process that determines creep, stress relaxation, and dynamic biomechanical behavior [21,22]. In fact, we recently adapted our high-frequency AFM-based rheology technique to characterize the regional poroelastic behavior of tendon, and found that tendons exhibit a complex dynamic response to compression with both viscoelastic and poroelastic regimes over the large frequency range studied [23].

While it is well known that injury and disease can alter mechanical function, studies on age-related changes in macroscale tendon function have been inconclusive, warranting further investigation. Given that the prevalence of tendon injuries and tendon disease increases with age, it is likely that age-related changes in tendon function are more complicated than that revealed by traditional macroscale mechanical evaluation. Furthermore, dynamic loads are more often reported in vivo and may be more clinically relevant to tendon function, thus necessitating the need for more sophisticated *multiscale* mechanical evaluations. Several recent studies have shown that changes in dynamic responses can occur without changes in macroscale mechanical properties, suggesting

[1]Corresponding author.

Manuscript received August 7, 2017; final manuscript received December 4, 2017; published online February 15, 2018. Assoc. Editor: David Corr.

the importance of changes at the molecular and microscale in early response to loading [9,24–26]. In addition, several studies have found that the dynamic viscoelastic mechanical responses in tension are altered in aged tendons, but it is still unclear how these properties are altered in compression [15,25,27,28].

Therefore, the objective of this study was to measure the multiscale dynamic response of young, mature, and aged mouse supraspinatus tendons to compression, and to utilize the poroelastic response in particular to better understand how fluid pressurization and flow is altered with age. We did this by characterizing the compressive mechanical, viscoelastic, and poroelastic properties of young, mature, and aged tendons at the tissue level using macroscopic compression testing and at the nanoscale using our high-frequency AFM-based rheology method. We hypothesized that aged tendons would have inferior macroscale and nanoscale poroelastic properties.

2 Methods

2.1 Sample Preparation. This study used C57BL/6J male mice at 1 (young, $n = 12$), 4 (mature, $n = 14$), or 18 months (aged, $n = 14$) of age obtained from the Aging Rodent Colony at the National Institute on Aging. The ages were chosen for consistency with previous structure–function studies of maturity and aging in mouse tendon [15,17,27,29,30] as well as due to their mouse-to-human equivalency relationship to clinically relevant ages for age-related rotator cuff injury [31]. While we did not observe any differences between groups, the level of physical activity was not quantified in any of the animal groups and the reduced activity of aged mice should be considered in the future. In addition, body weight was higher in the aged group than the other two groups (young: 22.9 ± 2.1 g, mature: 29.5 ± 1.8 g, aged: 35.7 ± 2.3 g).

Supraspinatus tendon and muscle along with the humerus were dissected free from the shoulder, as described previously [32,33]. Excess soft tissue was gently removed from the tendons, but the bony insertion and myotendinous junction were kept intact. Samples were then fixed to either a metal disk for macroscale compression testing (Fig. 1) or a custom stage for wide bandwidth rheological testing (Fig. 2) using cyanoacrylate glue. All samples were kept hydrated with phosphate-buffered saline throughout experiments and tested only on the articular surface of the tendon.

2.2 Macroscale Compression Testing. Verhoeff's stain lines were applied to the tissue at 1 mm increments (Fig. 1(*a*)), to define the insertion site (0–1 mm) and the midsubstance (1–2 mm). Tendons were then subjected to a dynamic compression protocol using a custom-built rounded 0.5 mm diameter indenter. The protocol began with an initial compressive preload to 0.025 g with a 300 s hold (Fig. 1(*b*)). The tissue was then subjected to consecutive ramp-and-hold indentations to 2%, 4%, 8%, 12%, 16%, 20%, and 24% cumulative compressive strain at 0.1% strain/second followed by 1000 s of relaxation. Our maximum cumulative compression value was chosen based on previous studies in mouse tendon [8], as well as unpublished pilot studies indicating that this maximum provided multiple points within the linear region of the equilibrium stress–strain response in order to calculate equilibrium modulus. Following the 4% step and subsequent relaxation, a 2% dynamic strain-amplitude sinusoidal frequency sweep spanning from 0.001–2.0 Hz was applied. We then calculated the compressive equilibrium modulus (the slope of a linear fit to the equilibrium stress–strain points) as well as the magnitude $|E^*|$ (stress amplitude divided by the strain amplitude) and phase angle δ (between the measured stress and the applied dynamic strain) of the dynamic modulus (Fig. 1(*b*)). A higher phase angle would represent more energy dissipation, while a lower phase angle would represent more elastic storage. Two-way analyses of variance with effects for age, location, and the interaction between age and location were then used to analyze the data statistically, followed by Bonferroni-corrected post hoc comparisons. Significance was defined as $p < 0.025$ (solid bar) and a trend defined as $p < 0.05$ (dashed bar or hash (#) symbol).

2.3 High-Frequency AFM-Based Rheology. For regional nanoscale evaluation, indentations were performed at fourteen regions along the length of the tendon from bone to muscle (Fig. 2(*a*)). The first region was defined as the closest indentation site to the humeral head, typically less than 100 μm from the bone, and this was done consistently for all samples. Samples were then indented every 50 μm for the first 200 μm and then every 100 μm until the junction with muscle. Using this procedure, there was variation in the number of regions between samples, but there was no relationship between the number of regions and any particular age group. Given the variance in the data, we estimated that approximately seven samples were necessary for sufficient power for statistical comparisons and therefore, we only considered regions where there were at least seven samples in each group, which were the first 14 regions in the present study. Indentations at each region were performed in the middle of the tissue (away from the glued edges) at nine locations within each region. Built-in top-view optics and translational stage were used to visualize positions of locations.

We indented supraspinatus samples on the articular surface of the tendon with ~25 μm diameter polystyrene colloidal probe tips attached to tipless cantilevers having nominal spring constant $K \sim 7.4$ N/m (Fig. 2(*b*)). Data were obtained over a wide frequency range (1 Hz to 10 kHz) using our custom rheology system

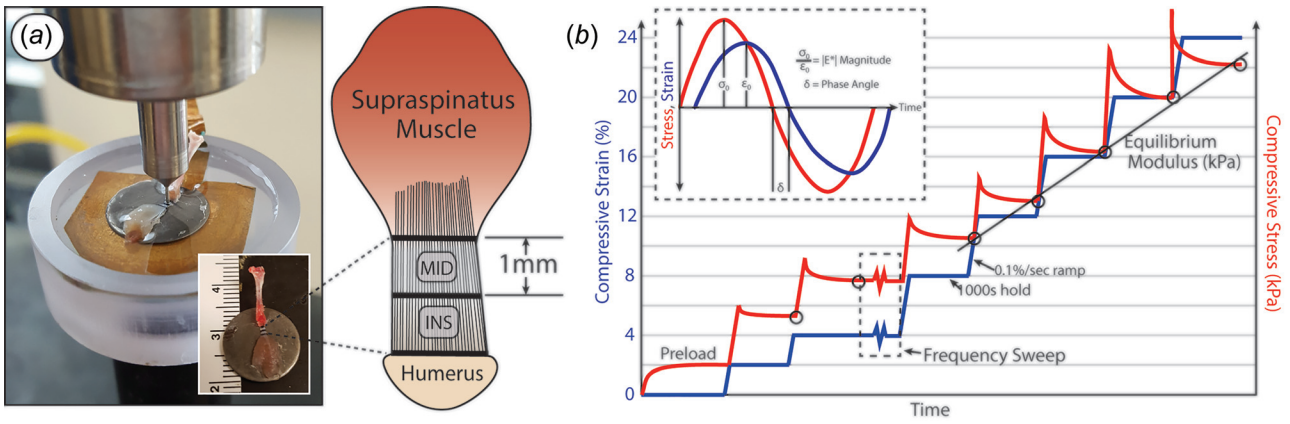

Fig. 1 (*a*) Testing setup for macroscopic compressive indentation of mouse supraspinatus tendon. Inset image shows dimensions of prepared tendon, and the expanded schematic depicts regional definition. (*b*) Indentation testing protocol, including preload, sequential compressive strain ramp-and-hold indentations, and dynamic frequency sweep.

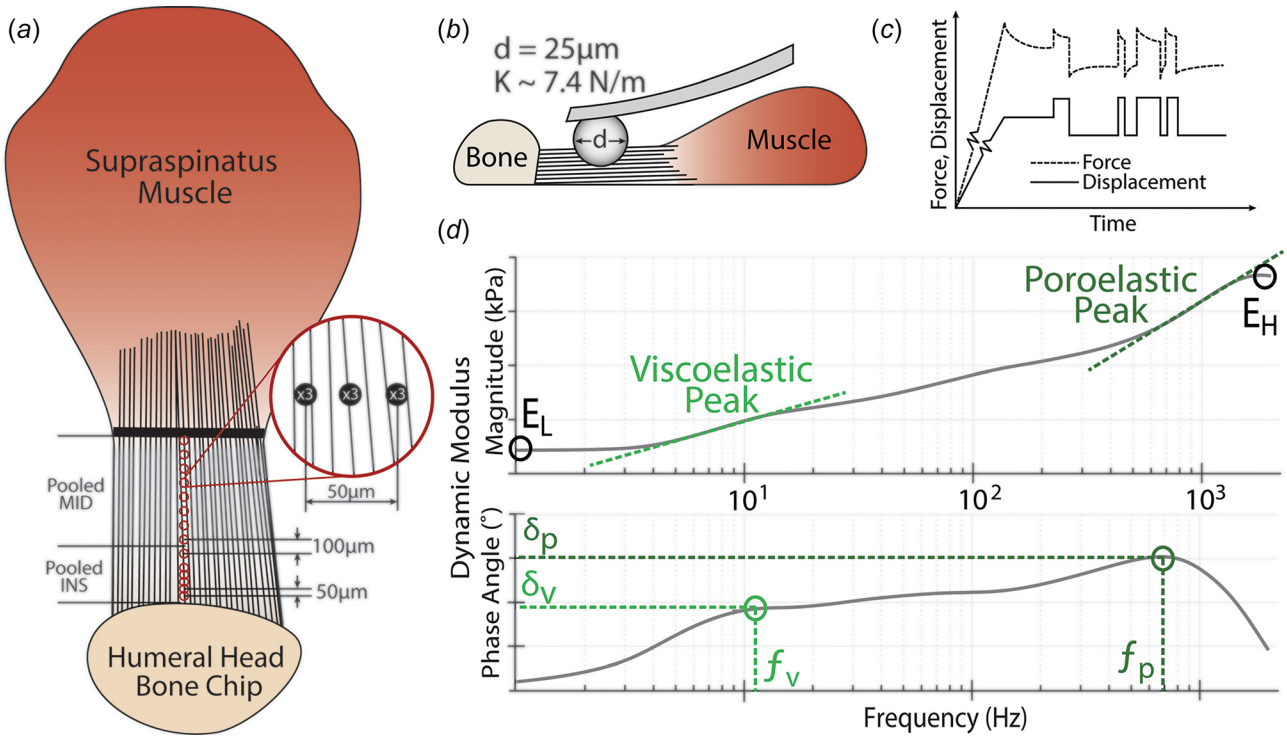

Fig. 2 (*a*) **Depiction of mouse supraspinatus tendon nanoindentation protocol, where the circles represent indentation regions. Nine indentations were performed within each region; solid lines mark the distance between indentation regions and the areas defined for pooled regional comparisons. (*b*) Depiction of indentation of mouse supraspinatus tendon using a probe tip having diameter (*d*) and AFM cantilever spring constant (*k*) (note: AFM cantilever is not to scale) (*c*) nanoindentation displacement–force protocol for each indentation consisted of an initial load-controlled ramp-and-hold pre-indentation of ~3.5 μm followed by random binary sequence displacements of 8–12 nm amplitude. (*d*) Representative schematic of the magnitude and phase angle versus frequency for tendon, indicating the definitions of low and high frequency moduli values (E_L and E_H) as well as the angle and frequency of viscoelastic and (δ_v, f_v) and poroelastic poroelastic peaks (δ_p, f_p) (panels (*b*)–(*d*) adapted from Ref. [23]).**

coupled to the MFP-3D AFM (Asylum Res, CA), as described [34–36]. This system allows us to measure how the response of the tissue varies with loading rate, which is critical toward understanding injury mechanisms including those involving very high impact rate. In addition, the poroelastic (but not the viscoelastic) phase response of soft tissues to compressive deformation scales with the square of the characteristic length associated with the fluid-flow response to deformation (see below) and thus, AFM-based indentation at high frequencies can reveal biomechanical behavior very relevant to normal tissue loading of larger tendons by larger indenters (such as the subacromial bursa in the human rotator cuff).

The indentation loading profile (Fig. 2(*c*)) consisted of an initial load-controlled ramp-and-hold pre-indentation of approximately ~3.5 μm followed by superimposed random binary sequence displacements of 8–12 nm, applied using previously established methods [23,34,35]. The contact between the indenter probe and the tissue was maintained for the duration of experiments. While we are indenting with microscale-sized probe tips, our study utilizes applied indentation displacements that are truly nanoscale (only ~8–12 nm). As a result, our measurements interrogate tissue compressive strains that are nanoscale regarding depth dependence, and the corresponding nanoscale fluid flow profiles associated with these compressive strains; hence our use of "nanoscale" [34–37]. This is an important distinction and should be not confused with studies using nanoscale-sized probes. A discrete Fourier transform was used to obtain the fundamental frequency component of the force and displacement signals and we then obtained the magnitude and phase of the dynamic complex modulus from the measured force and the applied displacement as we have described extensively previously [34,35].

Tendons typically exhibit a dynamic phase response having two characteristic peaks in the frequency range measured here, but only the 2nd peak, which occurs at higher frequencies, is associated with fluid–solid poroelastic energy dissipation [23]. The poroelastic peak in the phase response (Fig. 2(*d*)) occurs at the frequency, $f_p = (kE_L/d^2)$, where k is the hydraulic permeability, E_L is the low frequency modulus (Fig. 2(*d*)), and γ is a constant associated with probe tip geometry. The probe size used in this study (25 μm) insures that the poroelastic phase peak is higher than the viscoelastic peak (e.g., see Fig. 2(*d*), [23], for the properties of these tendons), but lower than the resonance frequency of the piezo testing system. From the measured dynamic modulus at low and high frequency limits, we quantified the equilibrium (low-frequency, E_L) and instantaneous (high-frequency, E_H) moduli, the self-stiffening ratio (ratio of high to low frequency moduli [38]), and the angle and frequency of the peak viscoelastic (δ_v, f_v) and poroelastic (δ_p, f_p) phase angles (Fig. 2(*d*)). For pooled comparisons, the insertion site was defined as the first 300 μm from the first indentation and the midsubstance was defined as the next 400–1100 μm. Pooled comparisons were made using two-way analyses of variance, and with Bonferroni corrected post hoc *t*-tests where applicable ($p < 0.025$ significant (solid bar); $p < 0.05$ trend (dashed bar)).

3 Results

In macroscale compressive testing, the equilibrium modulus was increased in the aged tendons compared to the young and mature tendons at both the insertion site and the midsubstance (Fig. 3(*a*)). The magnitude of the dynamic modulus was increased in the aged tendons compared to the mature tendons at all

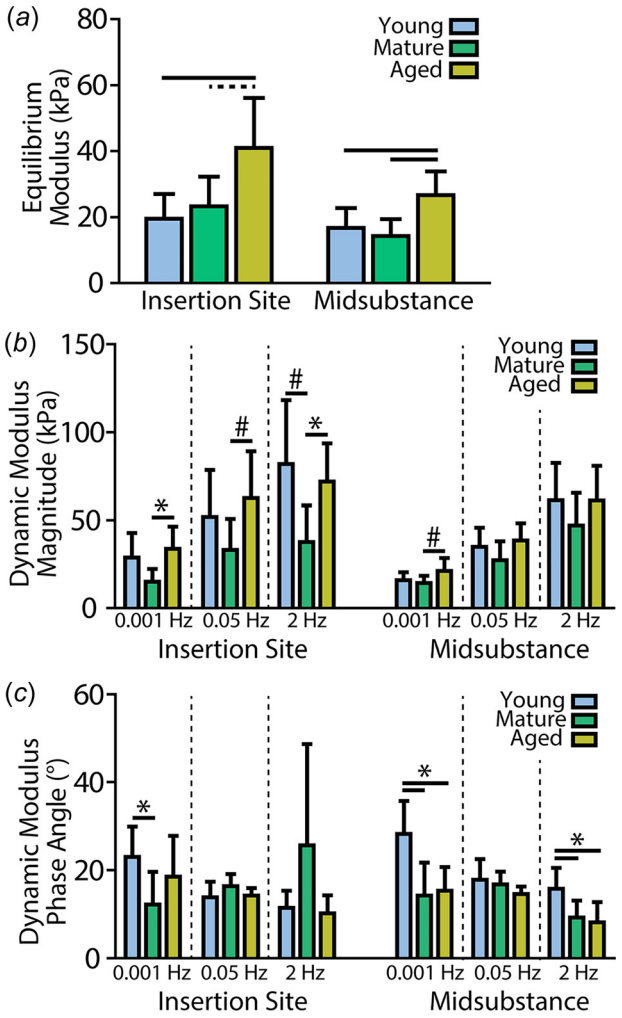

Fig. 3 In macroscale indentation testing, (*a*) equilibrium modulus was significantly higher in the aged tendons compared to both the young and mature tendons. (*b*) Similarly, the magnitude of the dynamic modulus was higher in aged tendons compared to mature tendons, particularly at the insertion site and the lowest frequency of the midsubstance. The young tendons were not significantly different from the aged group at any frequency in either regions, but were higher compared to the mature group at the highest frequency. (*c*) Finally, the phase angle of the dynamic modulus was higher in the young group compared to the mature group at the lowest frequency of the insertion site and compared to mature and aged tendons at low and high frequencies in the midsubstance. Data are presented as mean ±95% confidence interval and statistical significance is denoted by a solid line or star (*) symbol, while trends are denoted by a dashed line or hash (#) symbol.

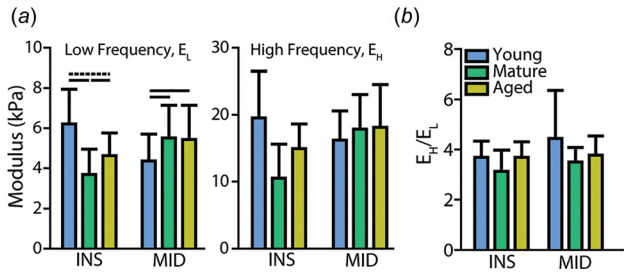

Fig. 4 In nanoindentation testing: ((*a*), left) the low frequency, or equilibrium, modulus was significantly higher in the young and aged groups at the insertion site and in the mature and aged groups compared to the young group at the midsubstance. No significant differences were found in the ((*a*), right) high frequency, or instantaneous, modulus or the (*b*) self-stiffening ratio. Data are presented as mean ±95% confidence interval and statistical significance between groups is denoted by a solid line, while trends are denoted by a dashed line.

frequencies at the insertion site and at the lowest frequency at the midsubstance (Fig. 3(*b*)). The young tendons also had increased dynamic modulus magnitude when compared to the mature tendons at the insertion site, which reached significance at the highest frequency. The phase angle was increased in the young tendons compared to the mature and aged tendons at both low and high frequencies at the midsubstance, as well as compared to the mature tendons at the lowest frequency in the insertion site. No other significant differences were found in either region (Fig. 3(*c*)).

At the nanoscale, the magnitude of the low frequency modulus (E_L, Fig. 4(*a*)) was higher in the young and aged tendons compared to the mature tendons at the insertion site and in the aged and mature tendons compared to the young tendons at the midsubstance. There were no significant differences at either region in the high frequency modulus (E_H, Fig. 4(*a*)). Similarly, self-stiffening ratio (E_H/E_L) was no different between the three ages at either the insertion site or the midsubstance (Fig. 4(*b*)). Interestingly, poroelastic properties were significantly altered in the young and aged tendons compared to the mature tendons. The frequency of the poroelastic peak phase angle (f_p, depicted in Fig. 2(*d*)) was slightly lower in the aged tendons than the mature tendons at the insertion site (Fig. 5). At the midsubstance, the frequency of this poroelastic peak was higher in both young and aged tendons when compared to mature tendons. The phase angle at the peak was also higher in the young and aged tendons when compared to the mature tendons at both regions. The frequency of the viscoelastic peak phase angle (f_v, depicted in Fig. 2(*d*)) was slightly but significantly higher in the aged and young tendons when compared to the mature tendons at the midsubstance as well (Fig. 6). However, there were no significant differences in the phase angle of the viscoelastic peak between groups in either region.

Direct comparisons of parameter values between the midsubstance and the insertion site were made to detect regional differences within each age group (Fig. 7). The midsubstance of the mature tendons, when compared to the insertion site, had higher indentation modulus, higher low and high frequency moduli, higher viscoelastic and poroelastic peak phase angles, higher viscoelastic and lower poroelastic peak frequencies. Regional variation was lost in aged tendons, with no significant differences between insertion site and midsubstance in almost all parameters. In the young tendons, regional variation was lost in the poroelastic peak phase angle and frequency. Furthermore, the indentation modulus, as well as the low and high frequency moduli, was greater at the insertion site than the midsubstance, opposite to the mature tendons. However, there were no differences in regional variation in either of the viscoelastic peak parameters between groups.

4 Discussion

In this study, the first to measure the regional compressive properties of mouse supraspinatus tendons, the equilibrium compressive modulus was found to be lower than the tensile modulus as expected and similar to other studies of tendon in compression [8]. Aged tendons were found to be stiffer than mature tendons in both equilibrium and dynamic loading conditions at the tissue level and at the nanoscale, contrary to our original hypothesis. As the supraspinatus is a major joint stabilizer, this stiffening could be associated with general joint stiffening or could suggest an accumulation of GAG; ongoing studies are investigating this possibility. Interestingly, self-stiffening with increased frequency of

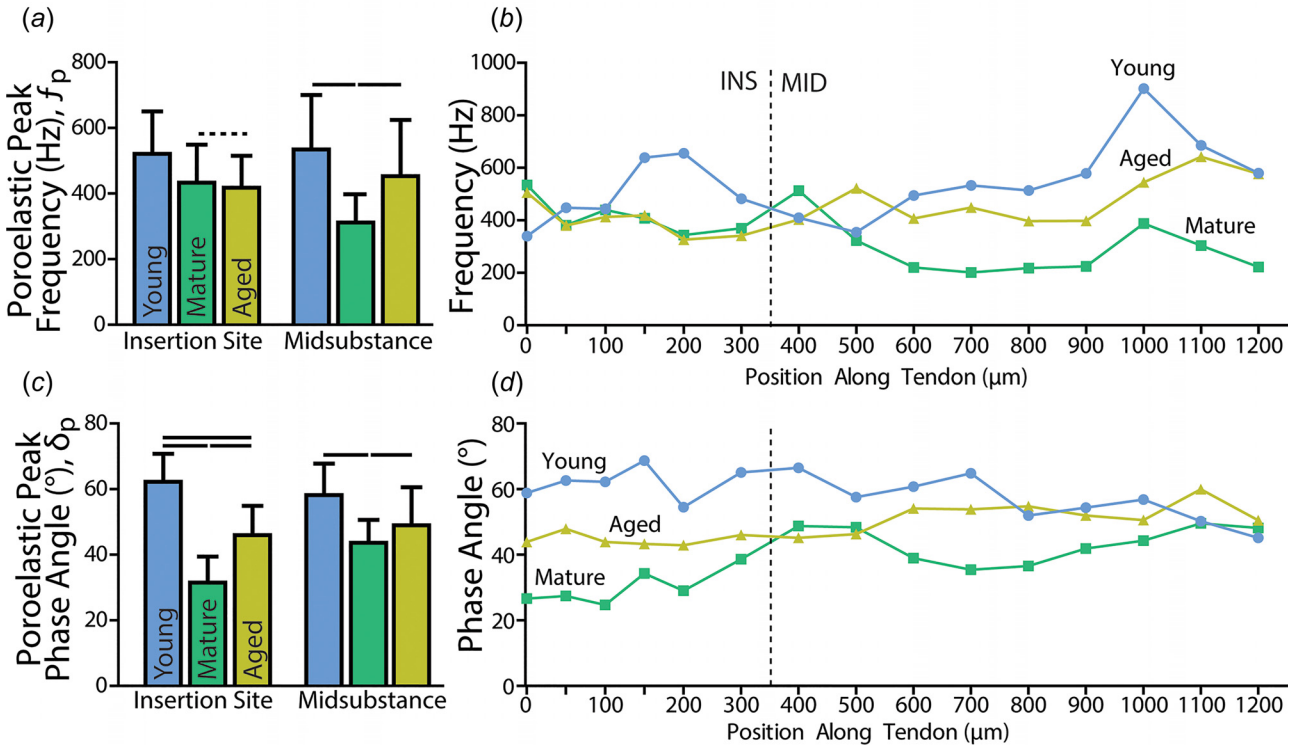

Fig. 5 Poroelastic properties were significantly altered in young and aged tendons when compared to the mature group (pooled comparisons ((*a*) and (*c*)), entire regional dataset ((*b*) and (*d*))). Specifically, the peak frequency ((*a*) and (*b*)) was higher in young and aged tendons at the midsubstance, and the peak phase angle (*c*) and (*d*) was higher in both groups in both regions of the tendon. Pooled data are presented as mean ±95% confidence interval, while entire dataset is presented as a single mean at each region. Statistical significance between groups is denoted by a solid line, while trends are denoted by a dashed line.

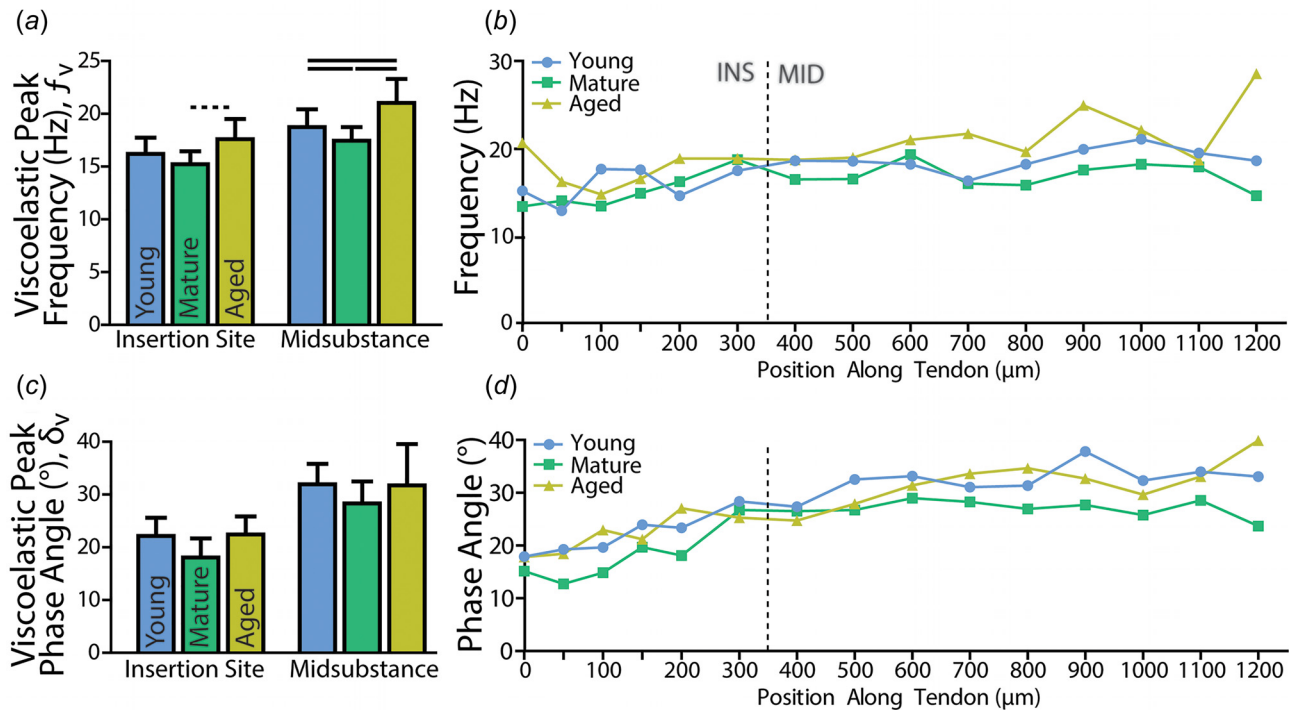

Fig. 6 Viscoelastic properties were altered only slightly with maturation and aging (pooled comparisons ((*a*) and (*c*)), entire regional dataset ((*b*) and (*d*))), with increased peak frequency (*a*) and (*b*) in young and aged tendons at the midsubstance. No differences were found in viscoelastic peak phase angle in either region of the tendon (*c*) and (*d*). Pooled data are presented as mean ±95% confidence interval, while entire dataset is presented as a single mean at each region. Statistical significance between groups is denoted by a solid line, while trends are denoted by a dashed line.

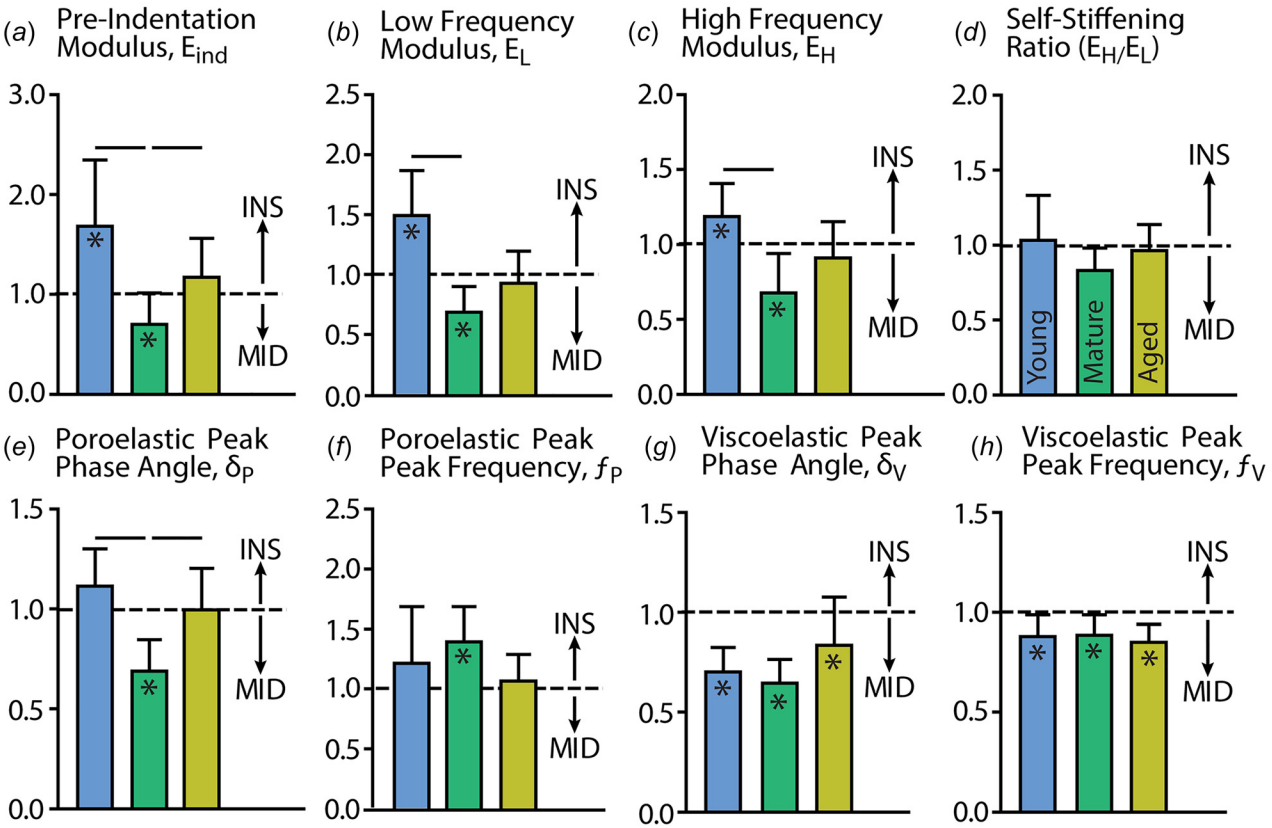

Fig. 7 Regional variation in each material parameter is shown here, where each bar represents the ratio of insertion to midsubstance properties; a value less than one indicates higher properties at the midsubstance, and a value greater than one indicates higher properties at the insertion site. A star (*) within the bar indicates a statistical significance between the insertion and midsubstance, while solid bars indicate significant differences between age groups. Many parameters were regionally different in the mature tendons (middle bars), but this regional heterogeneity was lost in the aged tendons (right bars), particularly in moduli (*a*)–(*c*) and poroelastic properties (*e*) and (*f*). Young tendons showed a loss of regional differences in poroelastic parameters (*e*) and (*f*) and a complete reversal of where higher properties appeared in the moduli values (*a*)–(*c*). Regional variations in viscoelastic parameters appeared to be constant across all age groups (*g*) and (*h*). Data are presented as mean ±95% confidence interval.

loading over the frequency range studied was not different between the three age groups, which would be expected with alterations in GAG content or with aging, as in cartilage [34,35]. However, there currently is not a consensus on alterations in GAG or proteoglycan content with aging [15,39–41]. We do know that GAGs and proteoglycans are important for the dynamic tissue response in tendon, and thus they likely contribute in compression as well. Differences between groups in the magnitude of the dynamic modulus (Figs. 3(*b*) and 4(*a*)), however, were present throughout the frequency range studied, suggesting that the mode by which mature tendon is able to self-stiffen in compression is likely similar in young and aged tendons even if the aged tendons were overall stiffer.

A number of regional differences in the nanoscale compressive properties were present between the insertion site and midsubstance of the mature tendons. We originally hypothesized that the insertion site of the mature tendon would be stiffer than the midsubstance at both hierarchical scales, as the insertion site of the supraspinatus is thought to experience higher compressive loads due to its anatomical positioning around the humeral head and because of the increased glycosaminoglycans in the fibrocartilage of the insertion site. Interestingly, while we did not see a dramatic difference in either equilibrium or dynamic properties at the macroscale, our nanoscale experiments demonstrated that midsubstance was significantly stiffer than the insertion site (Figs. 3, 4, and 7). It has been well established that bulk tissue properties and nanoscale properties can vary dramatically because of the dynamic responses occurring at multiple hierarchical scales, and we hypothesize that this may be the case in our study. Furthermore, we posit that specialization for the regional response to tensile loading is related to regional response to compressive loading [24,33]. Collagen fibrils at the insertion site must be flexible to both compress when wrapped around the humeral head during loading and re-organize during tensile loading via collagen realignment and crimp. Similarly, fibrils in the midsubstance are primarily required to slide against one another during tensile loading and provide strength and durability against levels of stress past the toe region of the stress–strain response.

In addition, the insertion site of the mature tendons also had higher poroelastic peak frequency and lower energy dissipation than that of the midsubstance, confirming again our previous results [23]. Based on our previous studies using finite element and analytical modeling [34–36], there is a direct relationship between the frequency of the poroelastic peak in the phase response, f_p, and the hydraulic permeability of the tissue, $f_p = (kE_L/d^2)$, where k is the hydraulic permeability, E_L is the low frequency modulus (Fig. 2(*d*)), and γ is a constant associated with probe tip geometry. Therefore, we infer that hydraulic permeability is also increased at the insertion site compared to the midsubstance. This combination may play specific roles in tendon homeostasis, allowing for nutrient influx to the cells of the three transitional tissues present while also maintaining fluid pressurization when this region is subjected to compressive and shear loading during shoulder motion. Interestingly, this regional heterogeneity, which we also suspect may be responsible in part for directed fluid flow along the length of the tendon, was lost in the

young and aged tendons. This is not only likely due to regional alterations in structure and composition, as we will discuss below, but also alludes to the possibility of different maturation and aging processes in the two regions of the tendon [25,27].

Furthermore, young and aged tendons both had higher energy dissipation and hydraulic permeability than the mature tendons, as demonstrated by the changes in poroelastic peak phase angle and frequency, respectively, (e.g., see Fig. 5). Unlike cartilage, in which the many GAGs of the large proteoglycan aggrecan are known to restrict fluid flow and thereby regulate matrix poroelasticity [35,38], we hypothesize that collagen organization is a more likely candidate to govern the poroelastic response of normal mature tendons. In tendons, the resident small leucine-rich proteoglycans are less abundant overall and each have only one or a few GAG chains; therefore, compared to the packed collagen architecture, this much lower GAG density would offer far less resistance to fluid flow associated with local compression-induced hydrostatic pressure gradients [23]. Although mice are considered to be skeletally and sexually mature at 28 days following birth, fibril diameter size, density, and organization are well known to fluctuate dramatically throughout life. Following fibrillogenesis, which is completed around 1 month of age in the mouse, tendon fibril diameters shift modestly toward a population with higher fibril diameter as the mouse ages [15,17,42], and therefore fibril diameters are smallest at the young tendons and largest at the aged tendons. However, organization and density of collagen fibrils have been shown to decreased in both young and aged tendons [15,17,43], which could be responsible for the increased hydraulic permeability in both groups as found here (Fig. 5). We hypothesize that fibril spacing mat also be a major determinant of intrinsic hydraulic permeability, as several modeling studies have shown that fluid preferentially flows in the direction of the collagen fibrils rather than across them toward the outside of the tendon [21,44,45]. However, there are many other likely candidates for involvement in the poroelastic response of tendon, including cross-linking of the collagen fibrils and the presence of elastic fibers, which both appear to impact multiscale tendon mechanics [46–50].

The overall purpose of and the downstream biological consequences of increased hydraulic permeability unfortunately are unknown. We hypothesize that increased hydraulic permeability in young tendons may allow for increased communication between tenocytes along the length of the tendon via growth factors and signaling proteins, thus contributing to the maturation and appropriate cellular programming for assembly of the growing tendon structure. Alternatively, increased permeability in aged tendons could mean a decreased ability to resist compressive loading via fluid pressurization. This could result in direct loading of the collagen fibers and cells, particularly during high impact loading, promoting unknown downstream biological consequences. Tendinopathy samples have been reported to a contain cartilage-like phenotype, with rounded cells and GAG accumulation, that could be an effect of increased compressive damage [5–7,50,51] but this is also currently unknown. We must also consider the altered signaling response of aging tenocytes [51–54], which could further exacerbate tissue damage, leading to the development and/or progression of tendon damage. Finally, an increase in fluid flow or permeability could also indicate an increased ability of cytokines and/or growth factors to reach cells in the interior of the tendon, thereby potentially spreading any metabolic response from one tendon to the rest of the rotator cuff (cartilage, joint capsule, tendons, etc.) more easily. In the case of inflammation or matrix degradation, this would quickly devolve from single tendon injury to a multitissue joint degeneration as has been reported in several studies. However, this is beyond the scope of this study and future studies should investigate these potential mechanisms.

While this study was the first to directly investigate the multiscale compressive properties of mouse supraspinatus tendons, there are a number of limitations and considerations that should be addressed in future studies. First, indenting and measuring regions very close to the bone proved to be difficult due to the geometry of the supraspinatus tendon wrapping around the humeral head. Future studies should focus on determining a better method to probe closer to and possibly within the transition from tendon through fibrocartilage into bone as this region remains a major interest in tendon research. In addition, these studies only investigate the articular surface of the tissue. Since the bursal side is in direct contact with the subacromial bursa and therefore may be subjected to additional shear and compressive forces, future studies should investigate properties on the bursal surface of the tendon. This study is particularly focused on maturation and aging and as such used only postnatal mouse tendons. However, given the role of proteoglycans and GAGs in the developmental regulation of fibril assembly as well as the growing fibril structure, investigating poroelasticity during postnatal development could provide insight into the relative contributions of various structural elements to the overall tissue response. Finally, our study draws conclusions on tendon fluid flow, yet there is no determination yet of flow direction. This should be confirmed in follow-up studies using computational modeling and, potentially, the incorporation of strain-generated potential measurements that directly confirm flow direction [55], to understand directionality and mechanics of fluid flow.

Overall, we found that many of the properties governing fluid flow and energy dissipation are altered during maturation and aging. We hypothesize that this could be a contributing factor to the development of age-related tendinopathy. However, several questions still remain and should be answered in follow-up studies. While we hypothesize and provide evidence that fibril organization and morphology are likely the cause of age-related alteration in tendon hydraulic permeability, direct evidence is still needed. Altering the composition and structure of tendon in mouse models is challenging due to importance of collagen type I in almost every major organ system, but we believe we may be able to gain insight in this respect through the use of targeted transgenics. Nevertheless, these studies provide insight into the regional compressive response of and indicate that altered dynamic compressive properties and energy dissipation in aging tendons may be a major contributor to overall tendon degeneration.

Acknowledgment

The authors thank Dr. Eliot Frank and Han-Hwa Hung for their expertise and assistance.

Funding Data

- Division of Civil, Mechanical and Manufacturing Innovation (Grant No. CMMI-1536233).
- National Institute on Aging (Grant No. F32-AG052284).

References

[1] Buckwalter, J. A., Heckman, J. D., Petrie, D. P., and AOA, 2003, "An AOA Critical Issue: Aging of the North American Population: New Challenges for Orthopaedics," J. Bone Jt. Surg. Am., **85A**(4), pp. 748–758.

[2] Millar, N. L., Murrell, G. A. C., and McInnes, I. B., 2017, "Inflammatory Mechanisms in Tendinopathy—Towards Translation," Nat. Rev. Rheumatol., **13**(2), pp. 110–122.

[3] Soslowsky, L. J., Thomopoulos, S., Esmail, A., Flanagan, C. L., Iannotti, J. P., Williamson, J. D., and Carpenter, J. E., 2002, "Rotator Cuff Tendinosis in an Animal Model: Role of Extrinsic and Overuse Factors," Ann. Biomed. Eng., **30**(8), pp. 1057–1063.

[4] Thomopoulos, S., Parks, W. C., Rifkin, D. B., and Derwin, K. A., 2015, "Mechanisms of Tendon Injury and Repair," J. Orthop. Res., **33**(6), pp. 832–839.

[5] Attia, M., Scott, A., Duchesnay, A., Carpentier, G., Soslowsky, L. J., Huynh, M. B., Van Kuppevelt, T. H., Gossard, C., Courty, J., Tassoni, M.-C., and Martelly, I., 2012, "Alterations of Overused Supraspinatus Tendon: A Possible Role of Glycosaminoglycans and HARP/Pleiotrophin in Early Tendon Pathology," J. Orthop. Res., **30**(1), pp. 61–71.

[6] Thornton, G. M., Shao, X., Chung, M., Sciore, P., Boorman, R. S., Hart, D. A., and Lo, I. K. Y., 2010, "Changes in Mechanical Loading Lead to Tendon-specific Alterations in MMP and TIMP Expression: Influence of Stress Deprivation and Intermittent Cyclic Hydrostatic Compression on Rat Supraspinatus and Achilles Tendons," Br. J. Sports Med., **44**(10), pp. 698–703.
[7] Thornton, G. M., and Hart, D. A., 2011, "The Interface of Mechanical Loading and Biological Variables as They Pertain to the Development of Tendinosis," J. Musculoskeletal Neuronal Interact., **11**(2), pp. 94–105.
[8] Dourte, L. M., Pathmanathan, L., Mienaltowski, M. J., Jawad, A. F., Birk, D. E., and Soslowsky, L. J., 2013, "Mechanical, Compositional, and Structural Properties of the Mouse Patellar Tendon With Changes in Biglycan Gene Expression," J. Orthop. Res., **31**(9), pp. 1430–1437.
[9] Fang, F., and Lake, S. P., 2015, "Multiscale Strain Analysis of Tendon Subjected to Shear and Compression Demonstrates Strain Attenuation, Fiber Sliding, and Reorganization," J. Orthop. Res., **33**(11), pp. 1704–1712.
[10] Birk, D. E., Zycband, E. I., Woodruff, S., Winkelmann, D. A., and Trelstad, R. L., 1997, "Collagen Fibrillogenesis In Situ: Fibril Segments Become Long Fibrils as the Developing Tendon Matures," Dev. Dyn., **208**(3), pp. 291–298.
[11] Birk, D. E., Nurminskaya, M. V., and Zycband, E. I., 1995, "Collagen Fibrillogenesis In Situ: Fibril Segments Undergo Post-Depositional Modifications Resulting in Linear and Lateral Growth During Matrix Development," Dev. Dyn., **202**(3), pp. 229–243.
[12] Cribb, A. M., and Scott, J. E., 1995, "Tendon Response to Tensile Stress: An Ultrastructural Investigation of Collagen: Proteoglycan Interactions in Stressed Tendon," J. Anat., **187**(Pt. 2), pp. 423–428.
[13] Mow, V. C., and Huiskes, R., 2005, *Basic Orthopaedic Biomechanics & Mechano-Biology*, Lippincott Williams & Wilkins, Philadelphia, PA.
[14] Scott, J. E., Orford, C. R., and Hughes, E. W., 1981, "Proteoglycan-Collagen Arrangements in Developing Rat Tail Tendon. An Electron Microscopical and Biochemical Investigation," Biochem. J., **195**(3), pp. 573–581.
[15] Dunkman, A. A., Buckley, M. R., Mienaltowski, M. J., Adams, S. M., Thomas, S. J., Satchell, L., Kumar, A., Pathmanathan, L., Beason, D. P., Iozzo, R. V., Birk, D. E., and Soslowsky, L. J., 2013, "Decorin Expression Is Important for Age-Related Changes in Tendon Structure and Mechanical Properties," Matrix Biol., **32**(1), pp. 3–13.
[16] Nimer, E., Schneiderman, R., and Maroudas, A., 2003, "Diffusion and Partition of Solutes in Cartilage Under Static Load," Biophys. Chem., **106**(2), pp. 125–146.
[17] Zhang, G., Ezura, Y., Chervoneva, I., Robinson, P. S., Beason, D. P., Carine, E. T., Soslowsky, L. J., Iozzo, R. V., and Birk, D. E., 2006, "Decorin Regulates Assembly of Collagen Fibrils and Acquisition of Biomechanical Properties During Tendon Development," J. Cell. Biochem., **98**(6), pp. 1436–1449.
[18] Connizzo, B. K., Yannascoli, S. M., and Soslowsky, L. J., 2013, "Structure-Function Relationships of Postnatal Tendon Development: A Parallel to Healing," Matrix Biol., **32**(2), pp. 106–116.
[19] Weiss, J. A., and Maakestad, B. J., 2006, "Permeability of Human Medial Collateral Ligament in Compression Transverse to the Collagen Fiber Direction," J. Biomech., **39**(2), pp. 276–283.
[20] Woo, S. L., Debski, R. E., Zeminski, J., Abramowitch, S. D., Saw, S. S., and Fenwick, J. A., 2000, "Injury and Repair of Ligaments and Tendons," Annu. Rev. Biomed. Eng., **2**, pp. 83–118.
[21] Ahmadzadeh, H., Freedman, B. R., Connizzo, B. K., Soslowsky, L. J., and Shenoy, V. B., 2015, "Micromechanical Poroelastic Finite Element and Shear-Lag Models of Tendon Predict Large Strain Dependent Poisson's Ratios and Fluid Expulsion Under Tensile Loading," Acta Biomater., **22**, pp. 83–91.
[22] Atkinson, T. S., Haut, R. C., and Altiero, N. J., 1997, "A Poroelastic Model That Predicts Some Phenomenological Responses of Ligaments and Tendons," ASME J. Biomech. Eng., **119**(4), pp. 400–405.
[23] Connizzo, B. K., and Grodzinsky, A. J., 2017, "Tendon Exhibits Complex Poroelastic Behavior at the Nanoscale as Revealed by High-Frequency AFM-Based Rheology," J. Biomech., **54**, pp. 11–18.
[24] Connizzo, B. K., Adams, S. M., Adams, T. H., Jawad, A. F., Birk, D. E., and Soslowsky, L. J., 2016, "Multiscale Regression Modeling in Mouse Supraspinatus Tendons Reveals That Dynamic Processes Act as Mediators in Structure-Function Relationships," J. Biomech., **49**(9), pp. 1649–1657.
[25] Miller, K. S., Connizzo, B. K., Feeney, E., and Soslowsky, L. J., 2012, "Characterizing Local Collagen Fiber Re-Alignment and Crimp Behavior Throughout Mechanical Testing in a Mature Mouse Supraspinatus Tendon Model," J. Biomech., **45**(12), pp. 2061–2065.
[26] Szczesny, S. E., and Elliott, D. M., 2014, "Interfibrillar Shear Stress Is the Loading Mechanism of Collagen Fibrils in Tendon," Acta Biomater., **10**(6), pp. 2582–2590.
[27] Connizzo, B. K., Sarver, J. J., Birk, D. E., Soslowsky, L. J., and Iozzo, R. V., 2013, "Effect of Age and Proteoglycan Deficiency on Collagen Fiber Re-Alignment and Mechanical Properties in Mouse Supraspinatus Tendon," ASME J. Biomech. Eng., **135**(2), p. 021019.
[28] Fessel, G., and Snedeker, J. G., 2009, "Evidence Against Proteoglycan Mediated Collagen Fibril Load Transmission and Dynamic Viscoelasticity in Tendon," Matrix Biol., **28**(8), pp. 503–510.
[29] Miller, K. S., Connizzo, B. K., Feeney, E., Tucker, J. J., and Soslowsky, L. J., 2012, "Examining Differences in Local Collagen Fiber Crimp Frequency Throughout Mechanical Testing in a Developmental Mouse Supraspinatus Tendon Model," ASME J. Biomech. Eng., **134**(4), p. 041004.
[30] Ansorge, H. L., Adams, S., Jawad, A. F., Birk, D. E., and Soslowsky, L. J., 2012, "Mechanical Property Changes During Neonatal Development and Healing Using a Multiple Regression Model," J. Biomech., **45**(7), pp. 1288–1292.
[31] Fox, J., Barthold, S., Davisson, M., Newcomer, C., Quimby, F., and Smith, A., eds., 2017, *The Mouse in Biomedical Research*, 2nd ed., Vol. 4, Academic Press, Burlington, MA.
[32] Connizzo, B. K., Han, L., Birk, D. E., and Soslowsky, L. J., 2016, "Collagen V-Heterozygous and -Null Supraspinatus Tendons Exhibit Altered Dynamic Mechanical Behaviour at Multiple Hierarchical Scales," Interface Focus, **6**(1), p. 20150043.
[33] Connizzo, B. K., Sarver, J. J., Han, L., and Soslowsky, L. J., 2014, "In Situ Fibril Stretch and Sliding Is Location-Dependent in Mouse Supraspinatus Tendons," J. Biomech., **47**(16), pp. 3794–3798.
[34] Azadi, M., Nia, H. T., Gauci, S. J., Ortiz, C., Fosang, A. J., and Grodzinsky, A. J., 2016, "Wide Bandwidth Nanomechanical Assessment of Murine Cartilage Reveals Protection of Aggrecan Knock-in Mice From Joint-Overuse," J. Biomech., **49**(9), pp. 1634–1640.
[35] Nia, H. T., Gauci, S. J., Azadi, M., Hung, H.-H., Frank, E., Fosang, A. J., Ortiz, C., and Grodzinsky, A. J., 2015, "High-Bandwidth AFM-Based Rheology Is a Sensitive Indicator of Early Cartilage Aggrecan Degradation Relevant to Mouse Models of Osteoarthritis," J. Biomech., **48**(1), pp. 162–165.
[36] Nia, H. T., Han, L., Li, Y., Ortiz, C., and Grodzinsky, A., 2011, "Poroelasticity of Cartilage at the Nanoscale," Biophys. J., **101**(9), pp. 2304–2313.
[37] Han, B., Nia, H. T., Wang, C., Chandrasekaran, P., Li, Q., Cherry, D. R., Li, H., Grodzinsky, A. J., and Han, L., 2017, "AFM-Nanomechanical Test: An Interdisciplinary Tool That Links the Understanding of Cartilage and Meniscus Biomechanics, Osteoarthritis Degeneration, and Tissue Engineering," ACS Biomaterials Sci. Eng., **3**(9), pp. 2033–2049.
[38] Nia, H. T., Han, L., Bozchalooi, I. S., Roughley, P., Youcef-Toumi, K., Grodzinsky, A. J., and Ortiz, C., 2015, "Aggrecan Nanoscale Solid-Fluid Interactions Are a Primary Determinant of Cartilage Dynamic Mechanical Properties," ACS Nano, **9**(3), pp. 2614–2625.
[39] Peffers, M. J., Thorpe, C. T., Collins, J. A., Eong, R., Wei, T. K. J., Screen, H. R. C., and Clegg, P. D., 2014, "Proteomic Analysis Reveals Age-Related Changes in Tendon Matrix Composition, With Age- and Injury-Specific Matrix Fragmentation," J. Biol. Chem., **289**(37), pp. 25867–25878.
[40] Riley, G. P., Harrall, R. L., Constant, C. R., Chard, M. D., Cawston, T. E., and Hazleman, B. L., 1994, "Glycosaminoglycans of Human Rotator Cuff Tendons: Changes With Age and in Chronic Rotator Cuff Tendinitis," Ann. Rheum. Dis., **53**(6), pp. 367–376.
[41] Esquisatto, M. A. M., Joazeiro, P. P., Pimentel, E. R., and Gomes, L., 2007, "The Effect of Age on the Structure and Composition of Rat Tendon Fibrocartilage," Cell Biol. Int., **31**(6), pp. 570–577.
[42] Moore, M. J., and De Beaux, A., 1987, "A Quantitative Ultrastructural Study of Rat Tendon From Birth to Maturity," J. Anat., **153**, pp. 163–169.
[43] Strocchi, R., De Pasquale, V., Guizzardi, S., Govoni, P., Facchini, A., Raspanti, M., Girolami, M., and Giannini, S., 1991, "Human Achilles Tendon: Morphological and Morphometric Variations as a Function of Age," Foot Ankle, **12**(2), pp. 100–104.
[44] Butler, S. L., Kohles, S. S., Thielke, R. J., Chen, C., and Vanderby, R., 1997, "Interstitial Fluid Flow in Tendons or Ligaments: A Porous Medium Finite Element Simulation," Med. Biol. Eng. Comput., **35**(6), pp. 742–746.
[45] Chen, C. T., Malkus, D. S., and Vanderby, R., 1998, "A Fiber Matrix Model for Interstitial Fluid Flow and Permeability in Ligaments and Tendons," Biorheology, **35**(2), pp. 103–118.
[46] Fessel, G., Cadby, J., Wunderli, S., van Weeren, R., and Snedeker, J. G., 2014, "Dose- and Time-Dependent Effects of Genipin Crosslinking on Cell Viability and Tissue Mechanics—Toward Clinical Application for Tendon Repair," Acta Biomater., **10**(5), pp. 1897–1906.
[47] Gautieri, A., Passini, F. S., Silván, U., Guizar-Sicairos, M., Carimati, G., Volpi, P., Moretti, M., Schoenhuber, H., Redaelli, A., Berli, M., and Snedeker, J. G., 2017, "Advanced Glycation End-Products: Mechanics of Aged Collagen From Molecule to Tissue," Matrix Biol., **59**, pp. 95–108.
[48] Pang, X., Wu, J. P., Allison, G. T., Xu, J., Rubenson, J., Zheng, M.-H., Lloyd, D. G., Gardiner, B., Wang, A., and Kirk, T. B., 2016, "Three Dimensional Microstructural Network of Elastin, Collagen, and Cells in Achilles Tendons," J. Orthop. Res., **35**(6), pp. 1203–1214.
[49] Fang, F., and Lake, S. P., 2016, "Multiscale Mechanical Integrity of Human Supraspinatus Tendon in Shear After Elastin Depletion," J. Mech. Behav. Biomed. Mater., **63**, pp. 443–455.
[50] Fessel, G., Wernli, J., Li, Y., Gerber, C., and Snedeker, J. G., 2012, "Exogenous Collagen Cross-Linking Recovers Tendon Functional Integrity in an Experimental Model of Partial Tear," J. Orthop. Res., **30**(6), pp. 973–981.
[51] Zhang, J., and Wang, J. H.-C., 2015, "Moderate Exercise Mitigates the Detrimental Effects of Aging on Tendon Stem Cells," PLoS One, **10**(6), p. e0130454.
[52] Yu, T.-Y., Pang, J.-H. S., Wu, K. P.-H., Chen, M. J.-L., Chen, C.-H., and Tsai, W.-C., 2013, "Aging Is Associated With Increased Activities of Matrix Metalloproteinase-2 and -9 in Tenocytes," BMC Musculoskeletal Disord., **14**, p. 2.
[53] Tsai, W.-C., Chang, H.-N., Yu, T.-Y., Chien, C.-H., Fu, L.-F., Liang, F.-C., and Pang, J.-H. S., 2011, "Decreased Proliferation of Aging Tenocytes Is Associated With down-Regulation of Cellular Senescence-Inhibited Gene and Up-Regulation of p27," J. Orthop. Res., **29**(10), pp. 1598–1603.
[54] Torricelli, P., Veronesi, F., Pagani, S., Maffulli, N., Masiero, S., Frizziero, A., and Fini, M., 2013, "In Vitro Tenocyte Metabolism in Aging and Oestrogen Deficiency," Age, **35**(6), pp. 2125–2136.
[55] Chen, C. T., McCabe, R. P., Grodzinsky, A. J., and Vanderby, R., 2000, "Transient and Cyclic Responses of Strain-Generated Potential in Rabbit Patellar Tendon Are Frequency and PH Dependent," ASME J. Biomech. Eng., **122**(5), pp. 465–470.