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Aging leads to inferior Achilles tendon mechanics and altered ankle function in rodents

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Abstract

Spontaneous rupture of the Achilles tendon is increasingly common in the middle aged population. However, the cause for the particularly high incidence of injury in this age group is not well understood. Therefore, the objective of this study was to identify age-specific differences in the Achilles tendon-muscle complex using an animal model. Functional measures were performed *in vivo* and tissues were harvested following euthanasia for mechanical, structural, and histological analysis from young, middle aged, and old rats. Numerous alterations in tendon properties were detected across age groups, including inferior material properties (maximum stress, modulus) with increasing age. Differences in function were also observed, as older animals exhibited increased ankle joint passive stiffness and decreased propulsion force during locomotion. Macroscale differences in tendon organization were not observed, although cell density and nuclear shape did vary between age groups. Muscle fiber size and type distribution were not notably affected by age, indicating that other factors may be more responsible for age-specific Achilles tendon rupture rates. This study improves our understanding of the role of aging in Achilles tendon biomechanics and ankle function, and helps provide a potential explanation for the disparate incidence of Achilles tendon ruptures in varying age groups.

Keywords

Biomechanics; Injury; Fatigue; Skeletal Muscle; Elasticity; Orthopaedics; Age

INTRODUCTION

Achilles tendon injuries are most common in middle-aged men, especially those involved in recreational sports. Various studies have each reported a consistent increased incidence rate

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Conflicts of Interest

The authors have no conflicts of interest to report.

since the 1950s that is greater than the rate of population increase alone (Lantto et al., 2015; Leppilahti et al., 1996; Suchak et al., 2005). Furthermore, there is evidence that the median age and overall incidence in older individuals specifically of Achilles tendon ruptures have both increased over the past decade (Huttunen et al., 2014). The mechanisms underlying this age-specific disparity are not well understood, but have been suggested to include increased participation in demanding sports by older individuals combined with tissue degeneration associated with aging (Ng et al., 2011; Peffers et al., 2015).

Specifically, age-dependent changes in Achilles tendon physiology and mechanics, such as decreased blood flow and increased tissue stiffness, have been implicated as potential causes for the high incidence of ruptures in this age group. However, although peritendinous blood flow is decreased while at rest in older individuals, blood flow in Achilles tendons during exercise is similar throughout ages, suggesting that vascular factors are not likely solely responsible for the increased incidence with age of Achilles tendon injuries (Langberg et al., 2001). Importantly, *in vivo* human studies evaluating biomechanical changes in aging Achilles tendon have yielded contradictory results as to whether Achilles tendon stiffness and strain in older individuals is decreased, increased, or similar to younger individuals(Karamanidis and Arampatzis, 2006; Kubo et al., 2007; Onambele et al., 2006). Additionally, there has been suggestion that regular physical activity as athletes age can help to avoid degeneration by increasing tendon size, strength, and nutrient delivery (Smith et al., 2002). Conversely, a recent study found no differences in Achilles tendon cross-sectional area, collagen content, or mechanical properties between adult long distance runners who were physically active or inactive in young age, suggesting that physical activity during youth did not improve Achilles tendon properties (Lenskjold et al., 2015). In summary, previous clinical studies have differing results which do not establish a clear relationship between age and Achilles tendon ruptures, likely due to an incomplete fundamental understanding of how aging affects Achilles tendon physiology and biomechanics. Given the practical limitations of human studies, there exists a clear need for a rigorous animal study that leverages the benefits of using a non-human model to better and more comprehensively define the role of aging in Achilles tendon structure and function.

Animal models offer a highly controlled system to study Achilles tendon biomechanics, and have demonstrated a potential explanation for the contrasting incidence in Achilles tendon rupture across sex (Pardes et al., 2016). However, it is unknown if aging also alters Achilles tendon mechanical behavior that could help explain the particularly high frequency of Achilles tendon ruptures in middle-aged men. There is preliminary support for this hypothesis, as changes in the viscoelastic response of the gastrocnemius-Achilles (GC-AT) muscle-tendon unit have been identified with increasing age (Plate et al., 2013). However, more comprehensive investigation is needed in order to better define the impact of aging on Achilles tendon-muscle properties and ankle function *in vivo* and *ex vivo*, ultimately helping to elucidate the mechanism underlying the age-specific incidence of ruptures observed clinically.

Therefore, the objective of this study was to identify functional, mechanical, and structural differences among Achilles tendon-muscle units from young, middle aged, and old male rats. We hypothesized that middle aged and old rats would exhibit increased joint stiffness

and decreased Achilles tendon material quality compared to young rats, as well as decreased matrix organization.

METHODS

Design

All procedures were approved by the University of Pennsylvania's Animal Care and Use Committee. Young (8 mo), Middle Aged (19 mo), and Old (28 mo) male F344XBN rats, approximating respective human ages of 18, 43, and 63 years (Quinn, 2005), were acquired from the National Institute of Aging (n=16/group) and euthanized following *in vivo* functional testing. This animal model shows musculoskeletal changes similar to those observed in human aging and has been previously used specifically for the study of the rotator cuff and Achilles tendon-muscle units aging response (Mannava et al., 2011; Plate et al., 2013).

Gait analysis

Animals (n=12–16/group) were acclimated to an instrumented walkway, and spatial, temporal, and kinetic parameters were quantified during autonomous locomotion as previously described (Fryhofer et al., 2016). Briefly, maximum ground reaction forces (medial/lateral, braking, propulsion, vertical) were calculated following isolation of the hindlimb on either one of two six degree-of-freedom force plates and reported as percent of animal body weight (%BW). Spatiotemporal parameters (stride length, stride width, speed, and stance time) were determined by semi-automated analysis of images captured by digital camera during locomotion using MATLAB (Mathworks; Natick, MA).

Passive joint function

Passive ankle range of motion (ROM) and stiffness were measured using a custom device while animals (n=16/group) were anesthetized as previously described (Fryhofer et al., 2016). Bilinear fits were applied to torque-angle data (within a consistent torque range across all animals) in order to calculate toe and linear stiffness for both dorsiflexion and plantarflexion. Range of motion was measured relative to the geometric zero position (ankle and tibia oriented perpendicularly).

Tendon sample preparation

Animals were euthanized (mass [mean±SD]: Young 393±25g, Middle Aged 521±27g, Old 504±44g) and Achilles tendon-foot units were harvested and either processed for histological assays or frozen until preparation for structural and mechanical analysis. Prior to all *ex vivo* testing and analyses, specimens were randomized and blinded to the study investigators. High frequency ultrasound/mechanical testing specimens (same specimens were used for both assays) were fine dissected to remove non-tendinous soft tissue and measured for tendon midsubstance cross sectional area (CSA) using a custom laser-based device (Freedman et al., 2016). Stain dots were applied for optical strain tracking, the calcaneus-foot complex was embedded in PMMA, and the proximal end of the tendon was attached between sandpaper using cyanoacrylate to leave a gauge length of 12 mm (Freedman et al., 2016).

High frequency ultrasound (HFUS)

Prior to mechanical testing, sagittal B-mode images of tendons loaded at 1N in a 1X PBS bath(n=11–12/group) were captured at 0.25mm increments using a 40MHz scanner (MS550D; VisualSonics, CA) as previously described (Riggin et al., 2014). A custom MATLAB program was used to analyze the 3–4 central-most images and determine tendon matrix alignment (reported as circular standard deviation) and density (reported as echogenicity).

Mechanical testing

Samples (n=11–12/group/protocol) were tested to evaluate quasistatic properties (ramp to failure with optical strain tracking) or viscoelastic, dynamic, and fatigue properties (stress relaxation, low-strain frequency sweep, load-controlled fatigue testing) using one of two previously described protocols (Freedman et al., 2016; Pardes et al., 2016). Briefly, the Achilles tendon was maintained perpendicular to the foot to mimic *in vivo* loading and submerged in a 1X PBS bath at 37°C. The Electropuls E3000 testing frame (Instron; Norwood, MA) was used with a 250 N load cell, while images were captured by a digital camera (Basler; Exton, PA) and 200 mm lens (Nikon; Melville, NY). Fatigue samples were tested until failure or completion of 20,000 cycles. Mechanical properties were evaluated at 50 cycles (early response) and 1500 cycles (latest possible cycle number to include data from >90% of all specimens). Load-displacement data for all tests were acquired by WaveMatrix (Instron; Norwood, MA) and imported into MATLAB for calculation of mechanical properties (Freedman et al., 2016).

Tendon histology

Achilles tendons (n=4–8/group) were fixed, decalcified, and embedded in paraffin using standard techniques and sectioned sagitally at 7μm (Fryhofer et al., 2016). Sections were then stained with Hematoxylin-Eosin (H&E) or Safranin-O/Fast Green and imaged at 100X. Images were evaluated for cell density and nuclear shape factor (0= circle; 1=line) using a commercial software (Bioquant Osteo II; Nashville, TN) (Rooney et al., 2016). Safranin-O positive staining was determined using a previously described thresholding method in ImageJ (Fryhofer et al., 2016).

Muscle histology

Gastrocnemius-soleus muscle mid-belly (n=7/group) was excised at the time of sacrifice, flash frozen, and stored at −80°C until axial cryosectioning at 10μm, as described previously (Pardes et al., 2016). Immunofluorescence imaging was performed on sections stained for laminin and myosin heavy chain (MyHC) types 1, 2a, and 2b. The anti-MyHC antibodies developed by Stefano Schiaffino were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. MyHC2x expression was presumed from unstained fibers. After staining, three images were taken from both the superficial and deep regions of each muscle. The SMASH application in MATLAB was used to calculate muscle fiber size (minimum Feret diameter) and type distribution for each region (Smith and Barton, 2014).

Statistics

One-way ANOVAs were used to compare groups for functional, structural, and mechanical Achilles tendon-muscle properties, and significant relationships ($p<0.05$) were further evaluated using post hoc Student's t-tests with Bonferroni corrections ($\alpha = 0.05/3$), except for cycles completed and tendon histological properties. For cycles completed, ranks were assigned (1, 0–5000 cycles; 2, 5000–10000 cycles, 3, 10000–15000 cycles; 4, 15000–20000 cycles) and the non-parametric Kruskal-Wallis test with Dunn's post hoc tests was used. Non-parametric Kruskal–Wallis tests were also used to evaluate differences in tendon histological properties between groups ($\alpha = 0.05$). All data are presented as mean ± standard deviation except for fatigue cycles completed and tendon histology, which are reported as median (interquartile range).

RESULTS

Gait analysis revealed that lateral force increased and propulsion force decreased with increasing age (Fig. 1A–B), while no significant differences in braking or vertical forces were detected (Fig. 1C–D). Animals also took slower, shorter, and wider steps as they aged (Fig. 1E–G).

Evaluation of passive ankle joint function demonstrated no differences in plantarflexion range of motion (ROM) or toe stiffness across age groups, but plantarflexion linear stiffness was increased in Middle Aged animals compared to the other groups (Fig. 2A–B). Aging resulted in decreased dorsiflexion ROM and increased linear, but not toe, stiffness (Fig. 2C–D).

HFUS analysis revealed tendon matrix organization and density did not differ with increasing age (Fig. 3A–B). Tendon cross-sectional area, however, increased 13% and 10% from Young to Middle Aged and Old groups, respectively (Fig. 3C).

While maximum force was not different between groups, maximum stress was superior in Young animals compared to the Middle Aged and Old groups (Fig. 4A–B). Stiffness was not significantly different between groups, but tendons from the Young animals demonstrated an average modulus 35% and 26% greater than tendons from the Middle Aged and Old animals, respectively (Fig. 4C–D). Age was not a significant factor on the maximum strain and toughness (Fig. 4E–F).

No differences in the tendon viscoelastic properties percent relaxation or tan(6) were detected between groups (Fig. 5A–B). Conversely, the dynamic modulus, $|E^*|$, was 37% greater in the Young group compared to tendons from Middle aged and Old animals at all frequencies tested (Fig. 5C).

Tendons from Middle Aged and Old animals exhibited superior fatigue life compared to Young animals (Fig. 6A). Peak strain, laxity, and secant stiffness were not significantly different between groups after 50 or 1500 cycles of loading (Fig. 6B–D). Secant modulus was increased in Young animals, though this only reached statistical significance at 50 and

not 1500 cycles (Fig. 6E). Additionally, there was a trend towards decreased hysteresis in Middle Aged animals after 1500 cycles (Fig. 6F).

Histological analysis revealed that Achilles tendon cell density decreased from Young and Middle Aged animals to Old animals (Fig. 7A). Nuclear shape also became more rounded in the Old animals compared to more spindle shaped in the Middle Aged and Young animals (Fig. 7B). Very little proteoglycan content was detected and no differences were observed across age groups (Fig. 7C). Representative images for tendon histology are shown for Young, Middle Aged, and Old for both H&E and Safranin-O/Fast Green (Saf-O) staining (Fig. 7D–I).

Muscle fiber size and type distribution did not vary dramatically with age (Fig. 8A–C). However, there were trends toward decreased superficial fiber size and deep type 2a fiber content in the Old animals compared to Young animals.

DISCUSSION

Aging is the single greatest risk factor for tendon disorders, yet the impact of aging on Achilles tendon-muscle properties remains incomplete (Cury et al., 2016; Stenroth et al., 2012). Previous studies have discovered microstructural changes in the tendon with increasing age, including decreased collagen fibril diameters, decreased crimp angle, and altered collagen type III and elastic content, but their link to macro-scale tendon and ankle mechanics is not intuitive or well understood (Slane et al., 2015; Slane and Thelen, 2015). Furthermore, human studies have reported conflicting results on the effects of aging on Achilles tendon mechanical function, which other investigators suggested may be in part due to low sample size, methodological differences, and spatial definitions of the tendon (Slane et al., 2016; Slane and Thelen, 2015; Stenroth et al., 2012). Therefore, the objective of this study was to leverage the advantages of a highly controlled animal model to comprehensively investigate functional, mechanical, and structural/compositional differences across Achilles tendon-muscle units from young, middle aged, and old male rats.

With regard to ankle joint function, Middle Aged and Old rats demonstrated similar deficits to those observed in humans as a consequence of aging. Specifically, increased passive stiffness and decreased range of motion are hallmark features of aging joints, including the ankle (Menz, 2015). It has been demonstrated that such joint-level changes are directly associated functional ability in human studies (Menz et al., 2005; Spink et al., 2011). This is in agreement with the results of our rodent gait analysis, which revealed that increasing age led to increased lateral force and decreased propulsion force, as well as shorter, slower, and wider strides during locomotion. Furthermore, our findings are highly consistent with human walking and running gait analysis studies (Devita et al., 2016; Franz, 2016; Ko et al., 2012). Given that greater calf muscle activation (both magnitude and variability) and metabolic cost of walking have been observed in older individuals or in cases of abnormal tendon mechanics, it is possible that these adaptations may represent a compensatory mechanism to overcome passive plantarflexion stiffness and achieve sufficient ankle range of motion during locomotion, at least in middle aged adults (Mian et al., 2007; Mian et al., 2006; Schmitz et al., 2009; Suydam et al., 2015). Further increases in muscle activation during

demanding push-off movements, such as jumping, could then lead to overstraining and subsequent injury of an already mechanically inferior tendon, as evidenced by our *ex vivo* mechanical analysis (Slane and Thelen, 2015). However, this potential injury mechanism requires future investigation in a more clinically relevant age group (40–50 years old rather than 65+ years old) and during challenging physical activities typically associated with spontaneous Achilles tendon rupture.

Mechanical testing revealed superior tendon material quality in the Young group, consistent with our initial hypothesis. Our results agree with previous animal and clinical studies which reported an increase in tendon compliance with increasing age, which may lead to subsequent injury (Cury et al., 2016; Dudhia et al., 2007; Joseph et al., 2014; Mian et al., 2007; Slane and Thelen, 2015; Svensson et al., 2016). It is important to note that these effects may vary across different tendons, as others have reported age-specific increases in the stiffness and modulus of the tibialis anterior tendon, for example (Slane et al., 2015; Wood et al., 2011; Zaseck et al., 2016). We did not identify any age-related differences in tendon viscoelastic properties, although such differences appear to be present in the entire Achilles tendon-muscle unit *in vivo* (Plate et al., 2013). Interestingly, we also observed relatively few differences in Achilles tendon fatigue properties, indicating that the tendon may perform similarly during lower intensity activities involving cyclical loading, regardless of age. However, as Achilles tendon ruptures generally occur spontaneously due to a single rapid, high intensity loading event, this suggests that quasi-static failure properties may better explain the mechanical etiology of acute injury, whereas fatigue resilience is more indicative of repetitive performance of daily functions like walking (Freedman et al., 2016; Maffulli et al., 2000). This concept is supported by the inferior max stress and modulus detected in the older groups of the current study, which likely have a role in the mechanism underlying the disparate incidence of ruptures in the middle aged population reported previously.

Contrary to our initial hypothesis, we did not observe consistent alterations in tendon matrix organization or composition with increasing age, though some differences were observed. Previous studies have reported changes in mRNA levels and activity of proteins involved extracellular matrix organization and turnover that could compromise the structural integrity of aged tendon (Thorpe et al., 2016; Yu et al., 2013; Zaseck et al., 2016). However, results from our HFUS analysis suggest that these distinct molecular profiles do not necessarily translate into macroscale age-specific differences in tendon matrix alignment or density. Cell density was inferior in the Old group, in agreement with previous work which noted decreased cellularity in Achilles tendons from elderly rats (Cury et al., 2016). Tendon cell shape has been reported to change from round to spindle shaped during tissue maturation, yet our results suggest that this phenomena may be relevant to the aging process as well (Svensson et al., 2016). The lack of differences in GAG staining between age groups was expected, given that proteoglycan content is a minor component of uninjured Achilles tendons and has only been shown to decrease with age in some, but not all, tendons and ligaments (Kostrominova and Brooks, 2013; Pardes et al., 2016; Svensson et al., 2016; Thornton et al., 2015). This is also consistent with our mechanical testing results, specifically that Achilles tendon viscoelasticity did not vary with age. The increase in Achilles tendon cross-sectional area (CSA) with age is consistent with previous studies,

which some have suggested may be due to increased water content or collagen-III content (Cury et al., 2016; Stenroth et al., 2012; Svensson et al., 2016). Yet other investigators have reported no difference or even decreased water content (perhaps corresponding to changes in GAG content) in tendons and ligaments with increasing age, indicating that collagen-III may be the predominant factor responsible for increased CSA (Svensson et al., 2016; Thornton et al., 2015). Given that collagen-III fibers are thinner and more compliant than collagen-I fibers, an age-related elevation of collagen-III content would also help to explain the decreased resistance to tensile force of older Achilles tendons observed in our study and should be further investigated in the future (Cury et al., 2016).

Aging is generally considered to result in deterioration of muscle mass, quality, and strength (sarcopenia) (Doherty, 2003; Larsson et al., 1979). While we did observe a trend towards decreased superficial fiber size with increasing age, widespread alterations of muscle composition were not detected. Interestingly, a recent study revealed that the decline in muscle size and strength is not solely responsible for the decreased propulsive power observed during gait in older adults, and that these individuals may actually underutilize their available muscular potential (Franz, 2016). Thus, the complex interplay between tendon material properties, ankle joint stiffness, and muscle sarcopenia is not fully understood, but has potentially significant impacts on mechanical performance and injury risk, warranting additional investigation in animal and human models (Danos et al., 2016; Slane and Thelen, 2015).

There are limitations to this study. First, only male animals were used to study the role of aging in Achilles tendon biomechanics, structure, and ankle function. It has been previously demonstrated by our group and others that sex differences exist in Achilles tendon homeostasis, incidence of injury, and healing response (Fryhofer et al., 2016; Pardes et al., 2016). Given that men are much more likely to rupture their Achilles tendon, however, we chose to isolate the effects of age by performing the current study in a uniform male cohort. Additionally, the mean age for Achilles tendon rupture is similar between males and females (Pardes et al., 2016). Nevertheless, it is still possible that some aspects of the tendon's response to aging are sex-specific and this should be investigated in future studies. It is also important to note that the impacts of aging on tendon and ligaments appear to be tissue-specific, as described earlier and as evidenced by the distinct age groups most affected by specific injuries (e.g., ACL ruptures in younger adults, Achilles ruptures in middle-aged adults, and rotator cuff tears in the elderly).Thus, the results of the current aging study should be interpreted specifically for Achilles tendon physiology and may not be broadly generalizable as relevant to all tendons and ligaments Next, it is likely that the counterintuitive result of decreased fatigue life in the Young tendons is at least in part due to their decreased cross-sectional area, which resulted in greater peak cyclical stress experienced throughout load-controlled fatigue testing. All tendons were cycled between ~8–40% of their max force in order to ensure consistent loading regardless of age. It would be possible to instead perform fatigue loading based on age-specific max stress, but this could confound direct comparison of fatigue properties. Therefore, since both approaches have their respective benefits and shortcomings, we opted for the method that allowed for the most intuitive comparisons of mechanical properties between age groups. Finally, it should be noted that passive dorsiflexion of the ankle of uninjured rats at all ages resulted in

eventual contact between the foot and knee. Thus, it is possible that the true dorsiflexion ROM is greater than reported in this study due to anatomical hindrance. Despite this potential systematic limitation, the torque cutoffs used in the current and previous work allow us to be confident in the accuracy of the dorsiflexion stiffness calculations, given that the threshold used included a sufficient portion of the linear region for robust bilinear fit analysis (Fryhofer et al., 2016).

In conclusion, this study begins to define the role of aging in Achilles tendon biomechanics and ankle function in order to better understand the heightened injury risk in an increasingly active middle aged population. Specifically, we utilized a highly controlled animal model to investigate how Achilles tendon mechanical, structural, and functional properties vary from young to old age through a battery of *in vivo* and *ex vivo* assays. Key deficits in tendon material properties with increasing age were identified, which may help explain the increased rupture incidence of the middle aged population, especially when considered in tandem with the functional impairments detected in this study. Macroscale differences in tendon organization and composition were not observed, suggesting that other factors are primarily responsible for age-related deterioration of tendon mechanical strength. Additionally, our results provide a foundation for use of this animal model in future studies on the potential age-specific response of the Achilles tendon-muscle unit to loading or injury.

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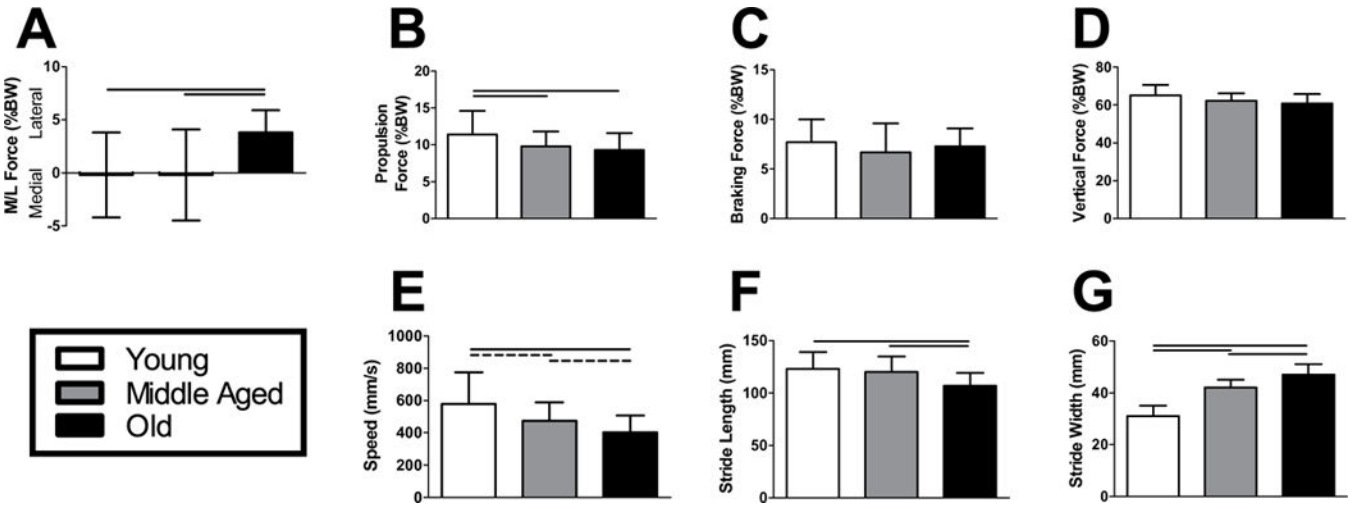

Figure 1. Kinetic and spatiotemporal gait analysis

Old animals had increased (A) lateral force and decreased (B) propulsion force during locomotion. Peak (C) braking and (D) vertical forces did not exhibit an age-dependent response. As animals increased in age, their (E) walking speed and (F) stride length declined, while their (G) stride width increased. n = 12–16/group. Data represented as means and error bars indicating standard deviation. Solid lines indicate significant differences ($p < 0.05/3$) and dashed lines indicate trends ($p < 0.10$).

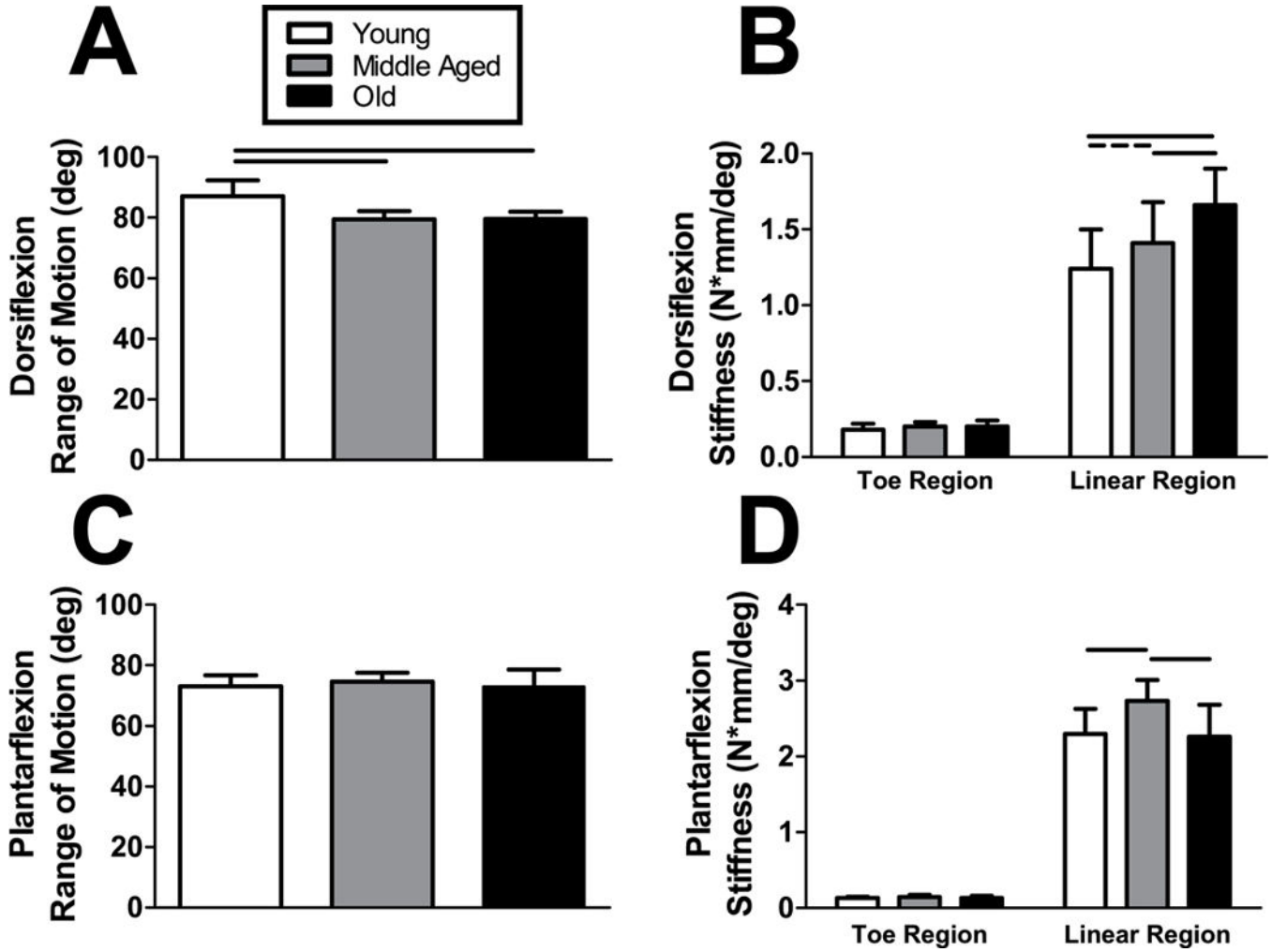

Figure 2. Passive ankle joint kinematics

(A) Dorsiflexion range of motion was inferior in both Middle Aged and Old animals compared to the Young group. Conversely, the linear region of (B) plantarflexion stiffness increased as animals aged. (C) There were no differences in plantarflexion range of motion across all groups, despite an increase in the linear region of (D) plantarflexion stiffness in Middle Aged animals. No differences in dorsiflexion or plantarflexion toe region stiffness were detected. n = 16/group. Data represented as means and error bars indicating standard deviation. Solid lines indicate significant differences ($p < 0.05/3$) and dashed lines indicate trends ($p < 0.10$).

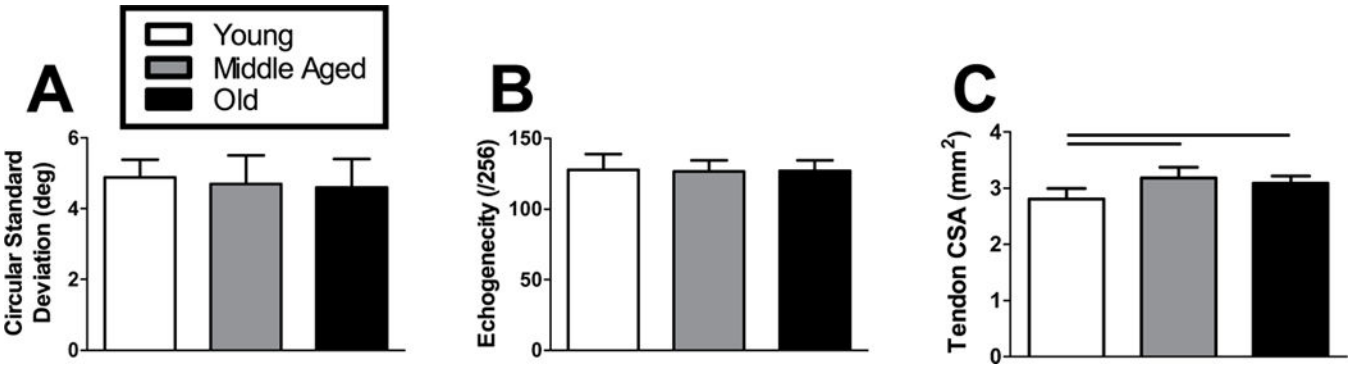

Figure 3. Achilles tendon structure

High frequency ultrasound (HFUS) assessment of tendon structure revealed no differences in (A) circular standard deviation (matrix organization) or (B) echogenicity (matrix density). A laser-based device was used to measure (C) tendon cross-sectional area, which was greater in Middle Aged and Old animals compared to the Young group. n = 11–12/group. Data represented as means and error bars indicating standard deviation. Solid lines indicate significant differences ($p < 0.05/3$).

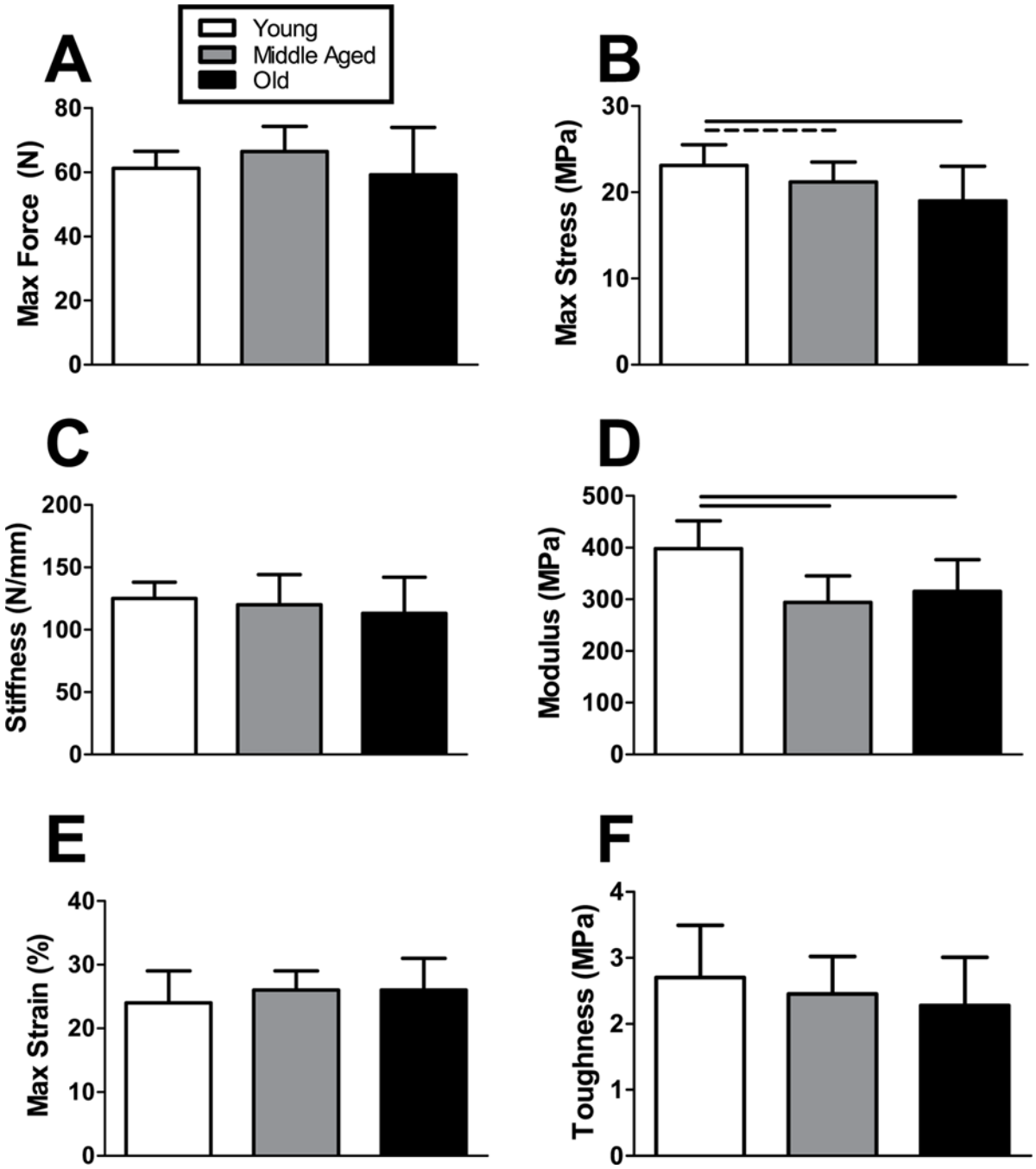

Figure 4. Achilles tendon quasi-static mechanical properties

(A) Max force did not differ across age groups, but (B) max stress was superior in the Young animals during a ramp-to-failure protocol. Similarly, (B) stiffness did not vary consistently with age but (C) modulus was greatest in the Young group. (E) Max strain and (F) toughness were not significantly different across age groups, but were strongly correlated ($R=0.80$, $p<0.0001$). $n = 11–12$/group. Data represented as means and error bars indicating standard deviation. Solid lines indicate significant differences ($p < 0.05/3$) and dashed lines indicate trends ($p < 0.10$).

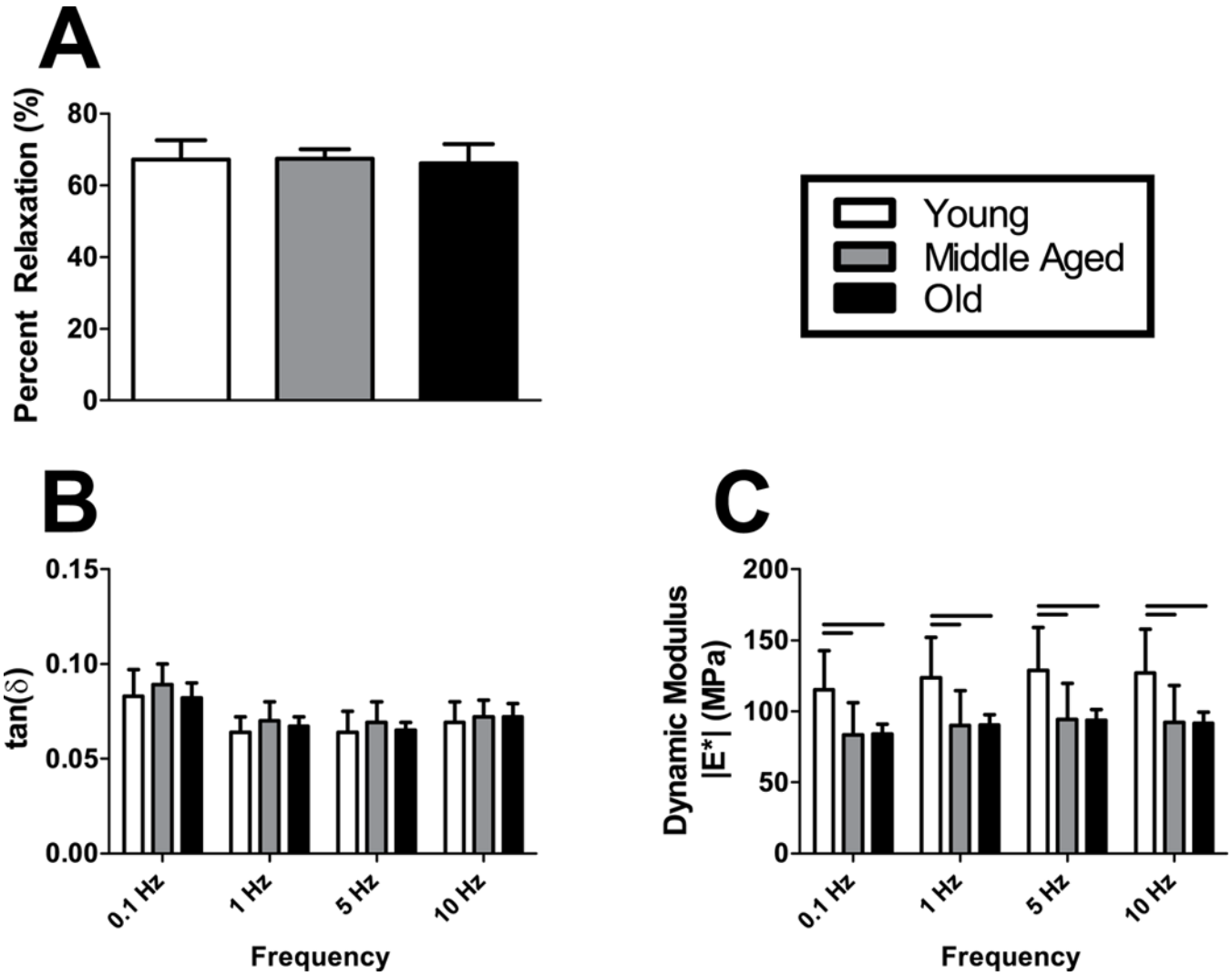

Figure 5. Achilles tendon viscoelastic and dynamic mechanical properties

Stress relaxation testing revealed no differences in (A) percent relaxation across age groups. A low-strain frequency sweep detected no changes in (B) tan(6) in response to aging, but (C) dynamic modulus was superior in the Young group at all frequencies tested. n = 11–12/ group. Data represented as means and error bars indicating standard deviation. Solid lines indicate significant differences ($p < 0.05/3$).

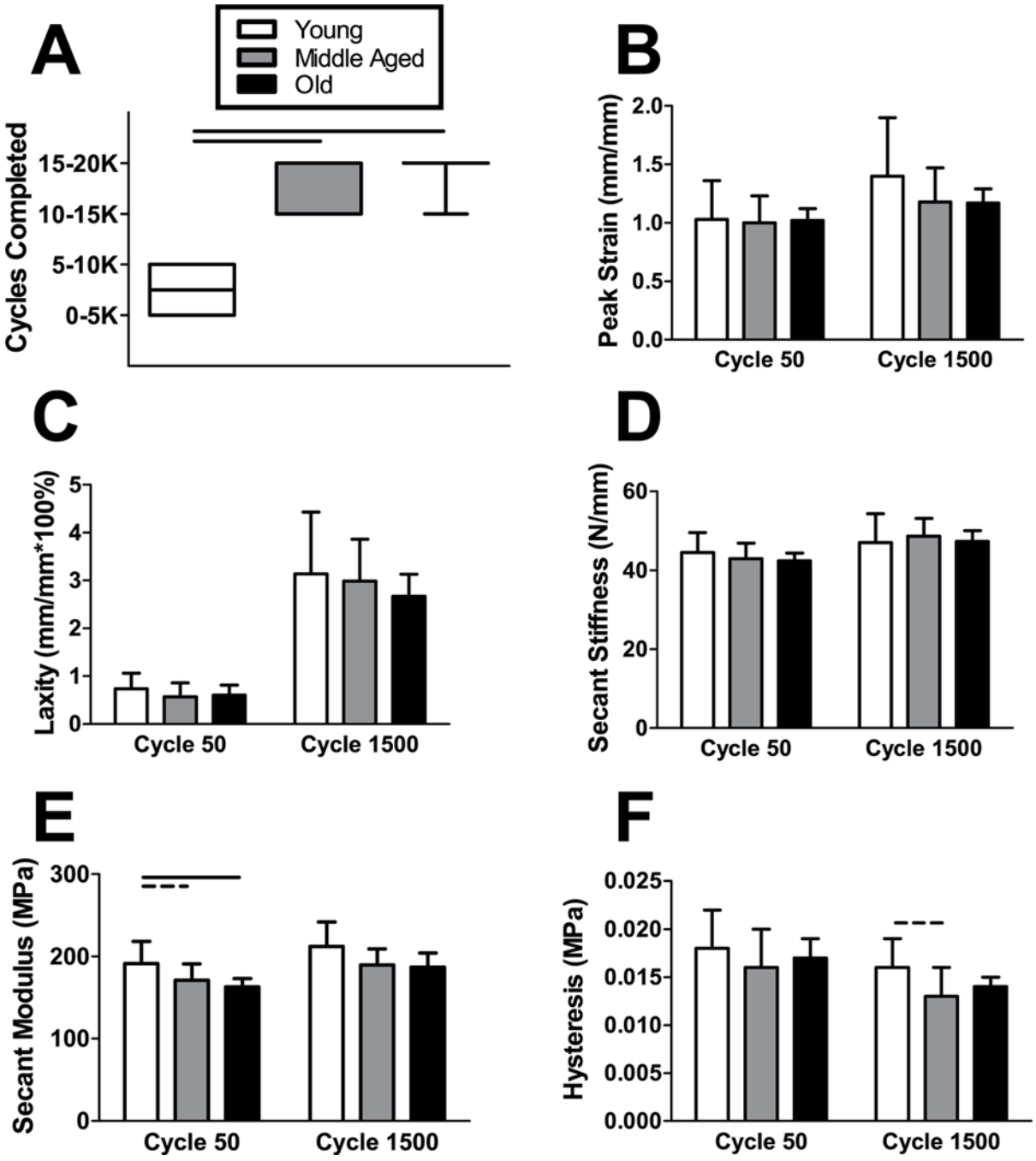

Figure 6. Achilles tendon fatigue mechanical properties

Tendons from Young animals had a shorter (A) fatigue life than the Middle Aged and Old groups. After 50 and 1500 cycles of fatigue loading, there were no differences in (B) peak strain, (C) laxity), or (D) secant stiffness between groups. (E) Secant modulus was increased in Young animals, though this only reached statistical significance at 50 and not 1500 cycles. There was a trend towards decreased (F) hysteresis in Middle Aged animals after 1500 cycles only. n = 11–12/group. Data represented as means and error bars indicating standard

deviation, except fatigue cycles completed (five number summary box plots). Solid lines indicate significant differences ($p < 0.05/3$) and dashed lines indicate trends ($p < 0.10$).

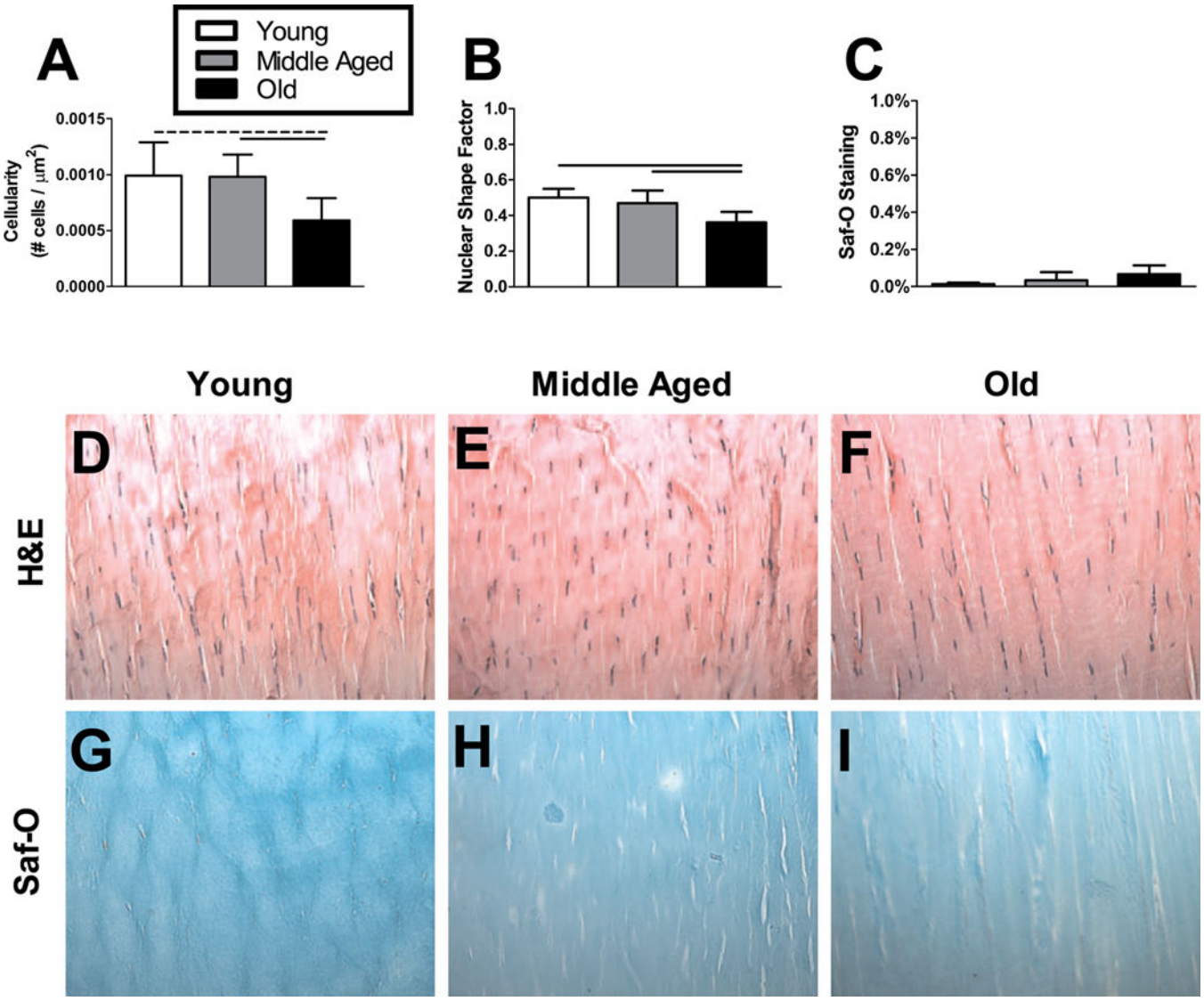

Figure 7. Achilles tendon histological properties

Young and Middle Aged animals had greater (A) cell density in their Achilles tendon midsubstance compared to Old animals. (B) Cell shape was more rounded in the tendons from Old animals than Middle Aged or Young animals. There was no significant change in (C) proteoglycan content across age groups. n = 4–8/group. Data represented as means and error bars indicating standard deviation. Solid lines indicate significant differences ($p < 0.05/3$) and dashed lines indicate trends ($p < 0.10$). Representative images are shown for each group (D–I).

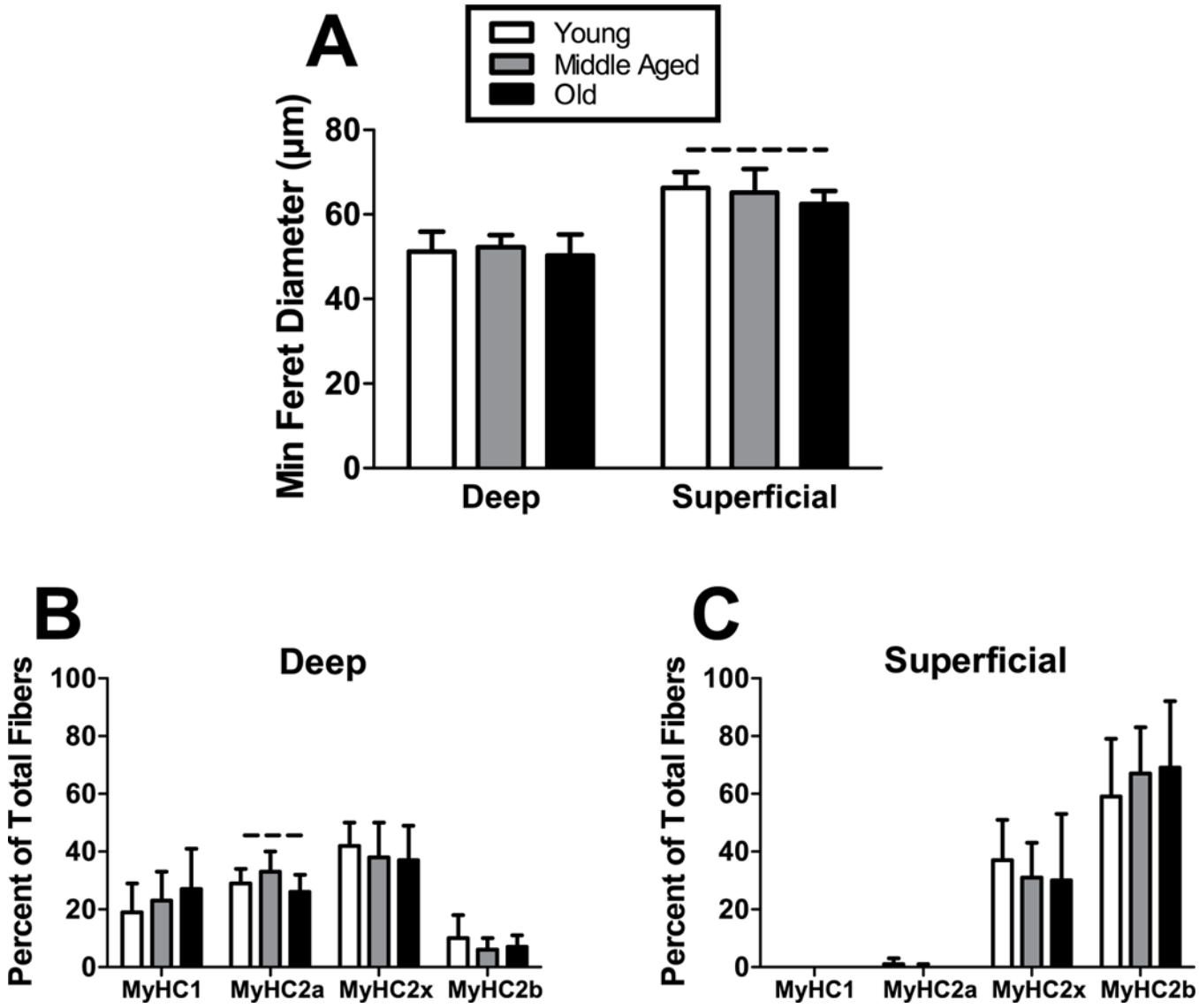

Figure 8. Muscle fiber size and type distribution

(A) Fiber size was not different across age groups in the deep region, but there was a trend towards inferior fiber size in Old animals in the superficial region. In the deep region, (B) fiber type distribution generally did not vary across age, except for a trend towards decreased type 2a in Old animals. Fiber type distribution in the (C) superficial region was not significantly altered by age. n = 7/group. Data represented as means and error bars indicating standard deviation. Solid lines indicate significant differences ($p < 0.05/3$) and dashed lines indicate trends ($p < 0.10$).