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Intramuscular connective tissue content and mechanical properties: Influence of aging and physical activity in mice

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ABSTRACT

Aging is accompanied by morphological and mechanical changes to the intramuscular connective tissue (IMCT) of skeletal muscles, but whether physical exercise can influence these changes is debated. We investigated the effects of aging and exercise with high or low resistance on composition and mechanical properties of the IMCT, including direct measurements on isolated IMCT which has rarely been reported.

Middle-aged (11 months, n = 24) and old (22 months, n = 18) C57BL/6 mice completed either high (HR) or low (LR) resistance voluntary wheel running or were sedentary (SED) for 10 weeks. Passive mechanical properties of the intact soleus and plantaris muscles and the isolated IMCT of the plantaris muscle were measured in vitro. IMCT thickness was measured on picrosirius red stained cross sections of the gastrocnemius and soleus muscle and for the gastrocnemius hydroxyproline content was quantified biochemically and advanced glycation end-products (AGEs) estimated by fluorometry.

Mechanical stiffness, IMCT content and total AGEs were all elevated with aging in agreement with previous findings but were largely unaffected by training.

Conclusion: IMCT accumulated with aging with a proportional increase in mechanical stiffness, but even the relatively high exercise volume achieved with voluntary wheel-running with or without resistance did not significantly influence these changes.

1. Introduction

Skeletal muscle mass and strength decrease with aging, and in addition the intramuscular connective tissue (IMCT) of skeletal muscle alters morphology and possibly mechanical properties (Kjaer, 2004; Pavan et al., 2020). Animal data suggest that aging is associated with elevated amounts of collagen in muscle (Alnaqeeb et al., 1984; Ramaswamy et al., 2011), which is possibly muscle specific (Zimmerman et al., 1993). Moreover, the IMCT appears to increase in thickness with age, which may be most pronounced in the perimysium (Alnaqeeb et al., 1984), and this increase contributes to an overall increase in passive muscle stiffness (Alnaqeeb et al., 1984; Gao et al., 2008). An increase in passive muscle stiffness with aging has also been reported in humans (Pavan et al., 2020), while reports of accumulation of muscle collagen content is inconsistent (Babraj et al., 2005; Haus et al., 2007; Mikkelsen et al., 2017; Pavan et al., 2020). It is possible that the discrepancy between animal and human data may reside in the absence of discriminating between endomysium, epimysium, perimysium and muscle fiber type when studying the IMCT (Kovanen et al., 1984b).

A sizeable portion of the force developed in muscle fibers is transmitted to the tendon via the IMCT (Street, 1983), and yet to what extent force transmission is affected by age related muscle loss is unclear. Only few studies have investigated the mechanical properties of IMCT related to aging (Haus et al., 2007; Wood et al., 2014; Zhang and Gao, 2014; Pavan et al., 2020), and the possible effects of physical training (Noonan et al., 2020), but little is known about differentiated effects on endomysium, epimysium and perimysium. Old age appears to reduce lateral force transmission to the epimysium in rodents (Ramaswamy et al., 2011; Zhang and Gao, 2014), indicating a weaker or more compliant IMCT. However, for longitudinal force transmission, indirect measures

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suggest that IMCT is stiffer in old mice compared to young (Wood et al., 2014), although this has not been corroborated in a human model (Pavan et al., 2020). These studies generally compared old with young adults, which makes it unclear to what extent the results are related to maturation or aging per se.

The mechanical properties of IMCT depend on the amount and type of collagen, and covalent enzymatic cross-links that form between collagen molecules, which are necessary to provide strength and stability of collagen fibrils (Bailey et al., 1998). In addition, there are non-enzymatic cross-links called advanced glycation end-products (AGEs) that are primarily formed when reducing sugars (glucose) bind to amino acids (Avery and Bailey, 2005), which is an unrestrained process that occurs at different locations along the collagen molecule (Tanaka et al., 1988). In aging human skeletal muscle, the density of AGEs appears to be markedly elevated (Haus et al., 2007), which may contribute to changes in mechanical properties of the muscle.

The effect of endurance training on IMCT is unclear. In humans, it has been shown that intramuscular collagen synthesis and turnover is elevated in response to exercise (Miller et al., 2005; Holm et al., 2010), but whether habitual exercise results in structural, biochemical or biomechanical changes in the intramuscular connective tissue remains unknown. In animals it has been shown that prolonged endurance training results in greater collagen content and passive muscle stiffness in muscles that contain primarily type 1 fibers (Kovanen et al., 1984a; Kovanen et al., 1984b). In contrast, others have demonstrated that the age associated elevation in passive muscle stiffness may be attenuated by endurance training (Gosselin et al., 1998). However, the mitigation was ascribed to a reduction in enzymatic cross-links rather than collagen content (Zimmerman et al., 1993; Gosselin et al., 1998), and the effect appeared to be specific to slow twitch (type 1) muscle (Zimmerman et al., 1993). It should be noted that very few studies have examined resistance training, which places greater forces on the intramuscular connective tissue. One animal study showed that jump training increased muscle stiffness and collagen content (Ducomps et al., 2003). To what extent habitual physical activity with high load influences collagen content, accumulation of AGEs and mechanical properties of the IMCT is currently unknown.

The present study investigated the effects of aging and of high and low load resistance training, respectively, on the passive mechanical properties of the IMCT, endomysium thickness, collagen content and AGEs density. We hypothesized that aging would be accompanied by thicker IMCT and increased contents of collagen and AGEs resulting in stiffer intramuscular tissue. Further, we hypothesized that training would attenuate these changes, and that high resistance would be more efficient in doing so than low resistance training.

2. Materials and methods

The experiments were conducted in accordance with Danish guidelines (Amendment #1306 of November 23, 2007) and approved by the Danish Animal Inspectorate, Ministry of Justice (permit #2014-15-0201-00326).

2.1. Animals

Middle-aged (11 months old) and old (22–23 months old) C57BL/6 mice (Janvier Labs, France) were randomly divided into three intervention groups – high resistance wheel running (HR), low resistance wheel running (LR) or sedentary (SED). The mice were housed individually under standard conditions on a 12 h light – 12 h darkness cycle with tap water and standard chow ad libitum. In addition, the wheel running groups had a custom made (described in details in (Olesen et al., 2021)) running wheel with adjustable resistance at their disposal throughout the 10 weeks intervention. At the end of the intervention, the mice were sacrificed by cervical dislocation and tissues were measured and weighed before being treated for their respective measurements as described in the following sections.

Data unrelated to the current study from the same study sample have previously been published (Ziegler et al., 2019; Olesen et al., 2021). Measurements for the present study were successfully performed on 18 old and 24 middle-aged mice with slight differences in sample size compared to the previously reported data due to failed measurements postmortem.

2.2. Intervention

The low resistance wheels had a resistance of <2 g throughout the 10 weeks intervention. The high resistance wheels started at 5 g of resistance, which increased by 1 g per week the first two weeks, and then 1 g increments every second week for the rest of the intervention ending at 10 g. The resistance in each high resistance wheel was accurate within <1 g.

Running distance, bout duration, maximal speed and mean speed were extracted. Running wheel activity for the animal cohort of the present study has previously been published (Olesen et al., 2021). Thirty-six out of the 42 mice used for the present work are the same as in the previous study, but some mice were excluded from the present and previous study due to damaged samples, and therefore, the descriptive data differ a bit from those previously reported.

2.3. Passive mechanical test of intact muscles

The right soleus and plantaris muscles were collected and stored cold in PBS for max 4 h. The muscles were dissected with intact distal tendon and proximal insertion including a piece of the tibia or femur bone respectively. For the mechanical test, distal tendons were tied around a needle pin, the bones were pierced with a needle, and each needle pin was mounted on hooks in a tensile testing platform (Deben, Suffolk, UK) with the sample submerged in PBS. Start length was defined as the length where force increased by 0.01 N from the slack baseline. For preconditioning, muscles were lengthened by 25 % of the start length. The start length was then reassessed, and muscles were cyclically loaded and unloaded between start length and 20 % strain 3 times (0.1 mm/s). The last cycle was used for analysis. Due to shifts in the force onset over the cycles, the actual strain achieved in the last cycle varied and was <20 %, analyses were therefore made at a common strain of 10 %. Because the shape of the muscles was irregular, the average anatomical cross-sectional area was estimated as muscle mass divided by muscle density (1.0597 g/cm^3) (Mendez and Keys, 1960) and muscle slack length. The ACSA was used for calculating stress.

2.4. Lateral force transmission in the soleus muscle

After the three loading cycles, two incisions were made into the soleus muscle: The first incision was located at the middle of the distal aponeurosis, and the second at the middle of the proximal aponeurosis from the opposite side (Fig. 1A–B). The purpose was to leave no or very few fibers intact to assess lateral force transmission. Since the pennation angle in soleus muscle is very low (~8°) (Burkholder et al., 1994), all fibers would theoretical be disconnected by these incisions. Incisions were performed with the soleus muscle mounted in the mechanical setup. After each incision, the muscle was lengthened to 20 % strain once, and peak force was recorded.

2.5. Mechanical test of isolated plantaris IMCT

After the passive mechanical test of the intact PL muscle, the whole muscle (including bone, tendon and pins) was chemically decellularized to isolate the IMCT (Fig. 1C). The challenge of decellularizing the intact muscle was to penetrate the IMCT and cellular membrane in order to wash intracellular substances out. Thus, we modified the protocol of Stern et al., 2009 (Stern et al., 2009). The muscles were incubated three

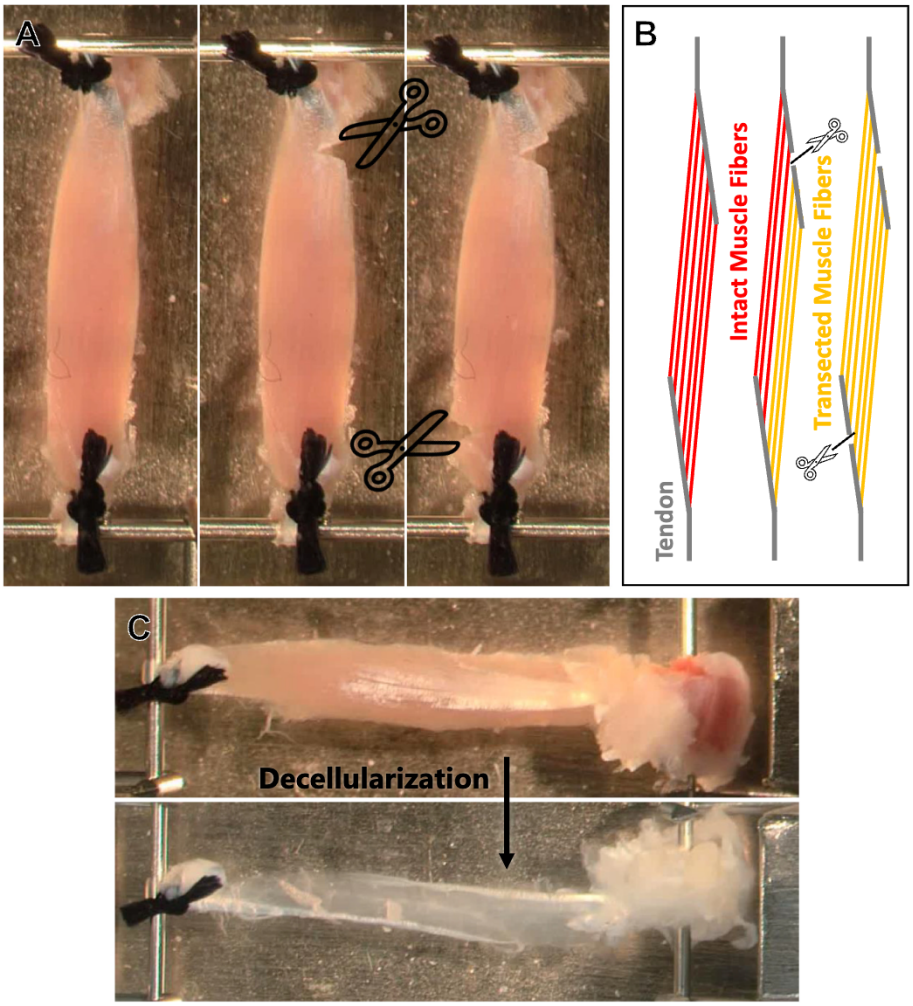

Fig. 1. Illustration of mechanical experiments. A) Lateral force transmission experiment in soleus muscle. Left: Intact muscle. Mid: Half of distal aponeurosis is transected. Right: Half of proximal aponeurosis is transected. B) Schematic illustration of the lateral force transmission experiment. C) Decellularization experiment in plantaris muscle. Top: Intact muscle. Bottom: Decellularized muscle representing only the IMCT.

days with 1 % Triton X-100, 4 h with 0.05 % Trypsin-EDTA (Sigma, T4049), followed by another three days with 1 % Triton X-100. The muscles were washed in distilled water over night after each incubation, and for 2 days after the last incubation. All steps were performed with agitation at 4 °C, except the Trypsin incubation which was at room temperature. The decellularized plantaris muscles were tested mechanically in a similar fashion as before. Initially, 3 stretch-shortening cycles were performed with the same absolute start length and deformation as before decellularization. These cycles barely developed any force. Therefore, a new start length was determined and the decellularized muscles were cyclically tested three times (0.1 mm/s) up to 20 % of the original muscle length. Due to shifts in the force onset over the cycles, and an increased starting length of the decellularized tissue, the actual strain achieved varied and was substantially <20 %, analyses were therefore made at a common strain of 6 %. Finally, the sample was tested to failure. Stress values were calculated based on the ACSA of the intact muscle to ensure comparability.

2.6. Stress-length curves and tangent modulus

The measured length and force from whole intact muscles and decellularized muscles were fitted to 3rd order polynomials. The polynomial fits were used to obtain stress-strain curves and tangent/elastic modulus. Tangent modulus for whole muscles was calculated as the slope at 10 % strain. For decellularized muscles the slope was determined at 6 % strain.

2.7. Picrosirius red staining

The left soleus and medial gastrocnemius muscles were embedded in Tissue-Tek (Sakura Finetek, Netherlands), frozen in liquid nitrogen cooled isopentane and stored at −80 °C. Transverse cry-sections (10 μm) were cut from the mid-belly of the soleus and medial gastrocnemius muscle. Sections were mounted on SuperFrost® Plus slides (Menzel-Gläser, Termo Scientific) and stored at −80 °C. For histology, sections were thawed, dried, fixed in Bouin's fluid (70 % saturated aqueous picric acid, 5 % acetic acid, 25 % formalin) for 30 min, rinsed in distilled water and stained with 0.15 % Fast Blue (F0500; Sigma-Aldrich) in a magnesium borate buffer (7 mM magnesium sulfate, 28 mM sodium metaborate) for 10 min to enhance the contrast. After rinsing in distilled water, the sections were stained with 0.1 % Direct Red 80 (365548; Sigma-Aldrich) in saturated aqueous picric acid for 60 min. Finally, sections were rinsed in acidified water (0.5 % glacial acetic acid) and picric alcohol (20 % absolute ethanol, 10 % saturated picric acid), dehydrated in 95 % ethanol for 30 min followed by two rinses in 100 % ethanol and one rinse in xylene before mounting with Pertex.

2.8. Endomysium histology

All images were captured within 24 h after staining using a 20×

objective with identical settings for all samples (Olympus BX51 microscope, Olympus DP71 camera). Enough images were taken to cover the whole section (1–2 for the soleus and 2–4 for the gastrocnemius muscle). A custom made semi-automated macro analyzed the images in ImageJ as follows: The circumference of each myofiber was identified by a threshold procedure followed by manual corrections of any poorly outlined fibers. A region of interest (ROI) was determined as a 15 pixels (6.4 μm) wide band around the myofiber starting at the circumference (Fig. 2). The number of picrosirius Red stained pixels was counted in the ROI and converted to an area (μm^2). The area was then divided by the circumference, to normalize for fiber size, thereby expressing the average thickness of the endomysium around each fiber. Collagen density of the endomysium was estimated by the PSR intensity (inverse brightness) of the stained pixels. The outermost layer of myofibers was often not representative of the rest of the myofibers and, therefore, these fibers along with damaged regions were excluded manually in all images. Perimysium was not explicitly excluded from the analyses because it is difficult to clearly distinguish from the endomysium, but because only connective tissue directly at the fiber surface is included, the contribution from larger perimysial structures is minimized. Using fiber type information obtained in a previous study on the same samples (Olesen et al., 2021), the endomysium was also analyzed for slow (type 1 and 2a) and fast (type 2× and 2b) fibers separately. The fibers were grouped as indicated due to only small numbers of type 1 fibers in the gastrocnemius (6 %) and type 2b fibers in soleus (0.4 %). Cryosectioning, staining, imaging and analyses were performed by investigators blinded to the age and training group.

2.9. Collagen and AGE cross-links

Collagen and AGEs were estimated by hydroxyproline quantification and fluorometry, respectively. The IMCT was chemically isolated for this purpose to avoid fluorescence from intracellular substances. After cutting sections of the gastrocnemius muscle for picrosirius red staining, the remaining tissue was washed to remove the Tissue-Tek and the epimysium manually peeled off. After obtaining the blotted wet-weight, tissue was decellularized in 0.5 ml 1 % SDS for 4 days (solution change at day 2) with agitation. Samples were washed thoroughly, and the isolated IMCT was digested with papain [0.125 mg/ml papain (P3125, Sigma) in 100 mM sodium phosphate buffer, 10 mM Na_2EDTA, and 10 mM L-cysteine (pH 6.5)] for 3 h at 60 °C with vortexing at the start and end. Aliquots of the papain digests were hydrolyzed and analyzed by a hydroxyproline assay to estimate collagen content as previously described (Heinemeier et al., 2016). Auto-fluorescence of the papain digest at 340 nm/460 nm (excitation/emission) was measured as an estimate of AGE cross-link content (Labella, 1965).

2.10. Statistics

Graphs and statistical analyses were made using the software GraphPad Prism (v.9.3.0, GraphPad Software LLC, CA, USA). Data was analyzed by a two-way linear mixed model ANOVA with age (middle-aged and old) and training (sedentary, LR or HR) as factors, using a compound symmetric covariance matrix. The tested outcomes were: muscle mass and size of plantaris, soleus and gastrocnemius; passive mechanical properties of intact plantaris and soleus; passive mechanical properties of decellularized plantaris and partially transected soleus; histological measures of IMCT content in soleus and gastrocnemius; biochemical measures of hydroxyproline and autofluorescence in gastrocnemius. For comparing IMCT content between fiber types, the same model was used but with age and fiber type (slow (type 1 + 2a) and fast (type 2x + 2b)) as factors. For the analyses of age and training there were no repeated measures, which essentially simplified the model to a 2-way ANOVA, while the fiber type analysis had fiber-type as a repeated measure. Post hoc tests were Tukey corrected. Significance level was set at $P < 0.05$ and trends at $P < 0.10$. If interactions (age x training or age x fiber-type) were not significant, they were removed from the model and main effects were tested. Mean and standard error of the mean (SEM) are reported in tables and graphs. P-values and the relative magnitude of significant differences are reported in the text. Unless otherwise stated, 6 old SED, 6 old LR, 6 old HR, 7 middle-aged SED, 8 middle-aged LR and 9 middle-aged HR mice were included in the analyses.

3. Results

3.1. Descriptive data

The old were lighter than the middle-aged mice (age $P = 0.001$), but there was no difference between the running groups (training $P = 0.67$). The tibia bone length was similar for all groups except the old sedentary (interaction $P = 0.028$) (Table 1) that had significantly shorter tibia bones than the old LR (−6 %, $P = 0.007$) and HR (−5 %, $P = 0.013$) groups. Plantaris and soleus muscle mass and ACSA were reduced by 14 % to 21 % in the old mice (age $P < 0.001$), with no significant effect of training in either plantaris ($P > 0.97$) or soleus, although for soleus both the muscle mass (training $P = 0.052$) and ACSA (training $P = 0.094$) tended to be greater by about 10 % with exercise, driven mainly by the HR group.

3.2. Mechanical properties of intact muscles

The average stress-strain curves of the intact soleus and plantaris muscles are shown in Fig. 3. The old soleus muscle had a significantly

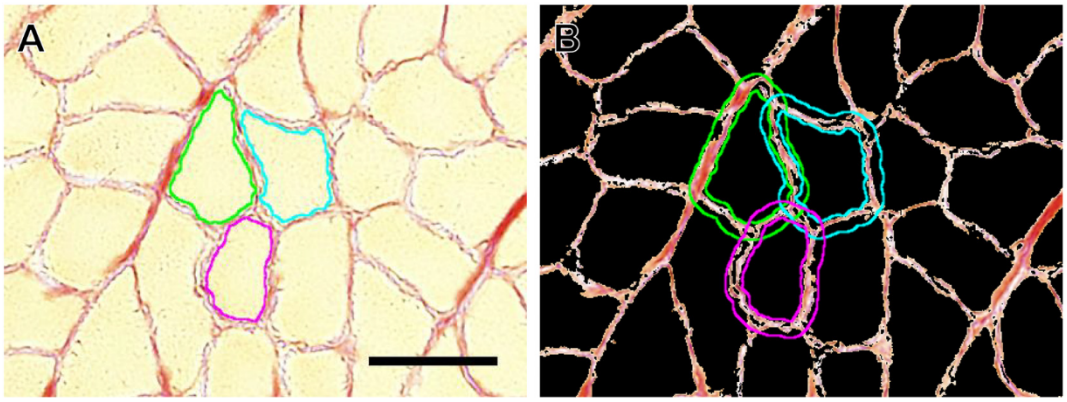

Fig. 2. Example of quantification of muscle endomysium. A) Muscle fibers (yellow) automatically outlined (colored outlines) in a picrosirius red stained section. B) The Sirius red positive parts of the image have been isolated by a color threshold and 15-pixel (6.4 μm) wide bands around each fiber are outlined. Scalebar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Descriptive data. Mean ± SEM.

	Middle-aged			Old		
	Sedentary	LR running	HR running	Sedentary	LR running	HR running
	n = 7	n = 8	n = 9	n = 6	n = 6	n = 6
Body weight (g)	33.4 ± 0.8	33.2 ± 1.1	32.9 ± 1.0	28.2 ± 1.3[a]	30.7 ± 1.2[a]	30.6 ± 1.5[a]
Tibia length (mm)	19.5 ± 0.2	19.3 ± 0.2	19.8 ± 0.2	18.8 ± 0.3[i]	19.9 ± 0.1	19.8 ± 0.3
Plantaris muscle:						
Mass (mg)	22.6 ± 1.9	23.4 ± 1.0	22.9 ± 0.8	18.3 ± 1.1[a]	18.0 ± 1.0[a]	18.3 ± 1.3[a]
Length (mm)	12.7 ± 0.3	12.7 ± 0.3	12.9 ± 0.2	12.3 ± 0.2	12.7 ± 0.2	12.6 ± 0.4
ACSA (mm^2)	1.68 ± 0.12	1.74 ± 0.07	1.67 ± 0.05	1.41 ± 0.09[a]	1.34 ± 0.08[a]	1.39 ± 0.13[a]
Soleus muscle:						
Mass (mg)	11.7 ± 0.5	12.8 ± 0.6[(t)]	12.8 ± 0.5[(t)]	9.7 ± 0.6[a]	10.5 ± 0.6[a(t)]	11.2 ± 0.7[a(t)]
Length (mm)	10.4 ± 0.3	10.2 ± 0.3	10.0 ± 0.2	10.0 ± 0.2	9.8 ± 0.1	10.2 ± 0.2
ACSA (mm^2)	1.06 ± 0.05	1.18 ± 0.05[(t)]	1.21 ± 0.05[(t)]	0.92 ± 0.07[a]	1.01 ± 0.06[a(t)]	1.03 ± 0.07[a(t)]
Gastrocnemius muscle:						
Mass (whole) (mg)	158.6 ± 4.8	159.3 ± 5.3	164.7 ± 2.1	118.3 ± 5.9[a]	120.4 ± 4.5[a]	125.3 ± 9.1[a]
Mass (medial) (mg)	60.6 ± 2.3	60.0 ± 1.5	60.4 ± 0.6	46.5 ± 3.0[a]	46.5 ± 1.2[a]	46.3 ± 3.5[a]

Note: ACSA: Estimated anatomical cross-sectional area. a: Age main-effect. (t): Trend for training main-effect. i: Interaction effect (see text for details).

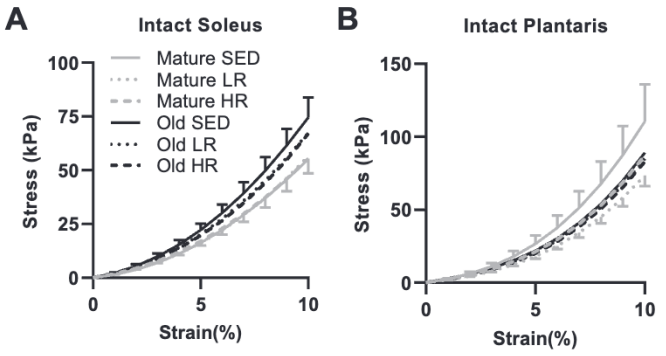

Fig. 3. Mean passive stress-strain response of whole muscles. A) Mechanical response of the intact soleus muscle. B) Mechanical response of the intact plantaris muscle. Mean ± SEM.

higher modulus at 10 % strain than the middle-aged (age P = 0.02) by 26 %, but there was no effect of wheel running (P = 0.94). Modulus of the intact plantaris muscle was not affected by age (P = 0.82) or training (P = 0.35).

3.3. Force transmission in the soleus muscle

Peak passive force of the intact and partly transected soleus muscle is shown in Fig. 4. There was no effect of age (P = 0.23) or training (P = 0.27). After the first transection, the passive force was reduced to ~80 % of that in the intact muscle, and the reduction tended to be greater in the running groups (P = 0.089, largely driven by the old HR group) but was unaffected by age (P = 0.91). After the second transection, the passive force dropped to ~70 % of the intact value, but no age (P = 0.60) or training (P = 0.27) effects were present.

3.4. Decellularized plantaris muscle

The decellularized plantaris muscles had 39 % greater modulus at 6 % strain in the old than the middle-aged mice (age P < 0.001, Fig. 5A–B). Modulus did not differ significantly between the running groups (training P = 0.55). The IMCT had similar failure strain in all groups (age P = 0.81, training P = 0.21) but failed at 19 % higher stress in old than middle-aged mice (age P = 0.006) with no significant effect of training (P = 0.14) (Fig. 5C–D).

3.5. Endomysial structure

The old mice had thicker endomysium than the middle-aged in both the gastrocnemius (24 %, age P < 0.001) and soleus (10 %, age P = 0.013) muscle (Fig. 6). There was no effect of wheel running on endomysial thickness in either the gastrocnemius (training P = 0.26) or soleus (training P = 0.14) muscle. The endomysium was generally thicker in the soleus muscle and less affected by age. The density (picrosirius red color intensity) of the gastrocnemius endomysium was lower in the old than the middle-aged animals (−15 %, age P = 0.002), but was unaffected by running (training P = 0.66). For the soleus there was an interaction (P = 0.039). Post-hoc analyses indicated that staining

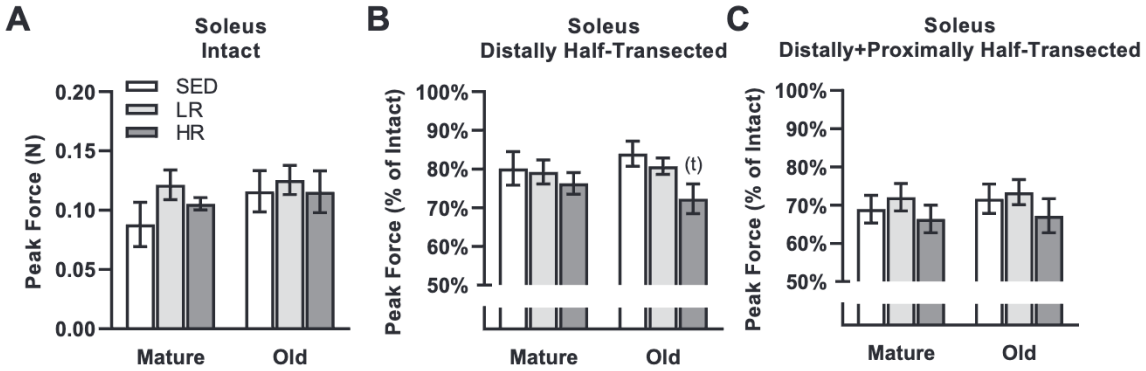

Fig. 4. Soleus muscle mechanics and intramuscular force transmission. A) Peak passive force of the intact soleus. B) The relative amount of passive force at the same absolute extension, following transaction of half the distal muscle. C) Same as B after also transecting the opposite half of the muscle at the proximal end. Mean ± SEM. (t): Trend for a training main-effect.

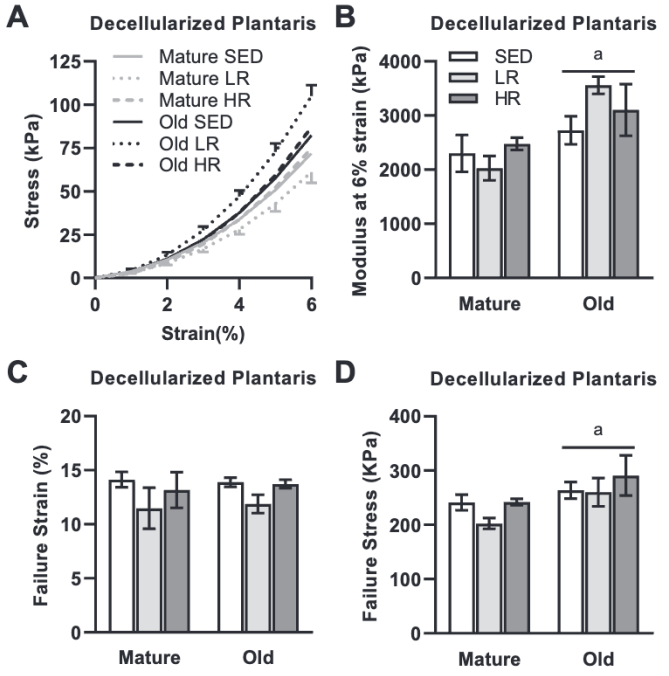

Fig. 5. Mechanical properties of the decellularized plantaris (IMCT). A) Mean sub-maximal stress-strain curves. B) Modulus at 6 % strain in sub-maximal tests. C) Failure strain in maximal test. D) Failure stress in maximal test. Mean ± SEM. a: Main effect of age.

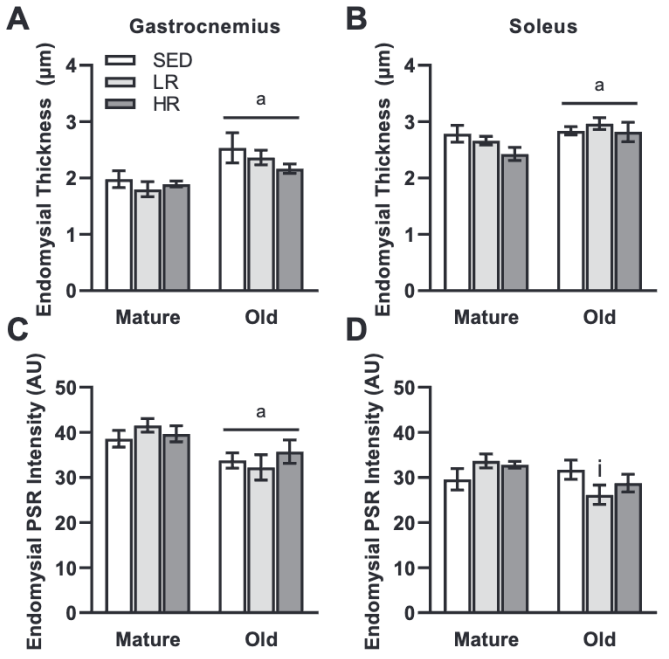

Fig. 6. Measurements of endomysial structure on picrosirius red (PSR) stained sections. A) Thickness of the endomysium in the gastrocnemius and B) the soleus muscle. C) Mean color staining intensity of the PSR stained pixels in the endomysium of the gastrocnemius and D) the soleus muscle (AU = arbitrary unit). Mean ± SEM. a: Main effect of age. i: Interaction effect. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

intensity was reduced with age in the LR group (−22 %, P = 0.017) but not the SED (P = 0.80) or HR (P = 0.30) groups. Endomysial structures were also investigated at a fiber-type specific level (Fig. 7). No

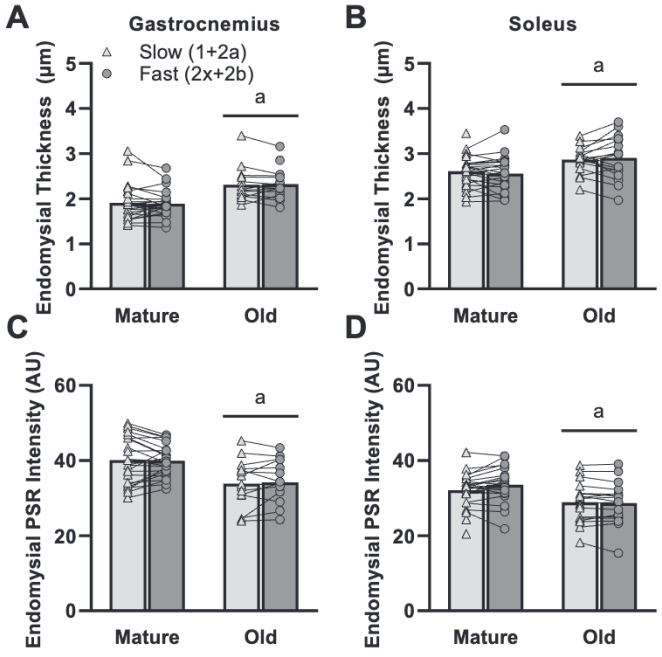

Fig. 7. Fiber type specific measurements of endomysial structure on picrosirius red (PSR) stained sections. A) Thickness of the endomysium in the gastrocnemius and B) the soleus muscle. C) Mean color staining intensity of the PSR stained pixels in the endomysium of the gastrocnemius and D) the soleus muscle (AU = arbitrary unit). All training groups were combined. Mean shown as bars with individual animals shown as dots. Light grey triangles = slow muscle fibers (Type 1 and 2a), dark grey circles = fast muscle fibers (Type 2x and 2b). a: Main effect of age. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

differences between slow (type 1 + 2a) and fast (type 2x + 2b) fibers were observed in either the gastrocnemius (Fig. 7A+C) or soleus (Fig. 7B+D) muscle (p > 0.3 for all measures).

3.6. Hydroxyproline/collagen and AGE content

Compared to the middle-aged animals, the old had greater hydroxyproline content (as a measure of collagen) (41 %, age P = 0.006) and autofluorescence (as a measure of AGEs) (44 %, age P < 0.001) normalized to muscle mass, while there was no influence of training on either hydroxyproline (P = 0.75) or autofluorescence (P = 0.56) (Fig. 8A–B). When AGEs were normalized to hydroxyproline there were no significant differences (age P = 0.67, training P = 0.97) (Fig. 8C).

4. Discussion

In the present study we assessed the effect of voluntary high and low resistance running on the properties of the intramuscular connective tissue in old and middle-aged mice. The data show that, in agreement with our hypotheses, aging was associated with a larger quantity of intramuscular connective tissue and greater mechanical stiffness of the isolated connective tissue. Contrary to our hypotheses, 10 weeks of voluntary wheel running was not able to significantly counteract these changes, even with progressively increasing wheel resistance.

4.1. Aging

In several animal studies, characteristics of aging IMCT have been investigated relative to a young adult group (Gosselin et al., 1998; Gao et al., 2008; Zhang and Gao, 2014; Guzzoni et al., 2018), whereas the present and some previous studies avoid the possible confounding effects of maturation and growth by using a middle-aged group for

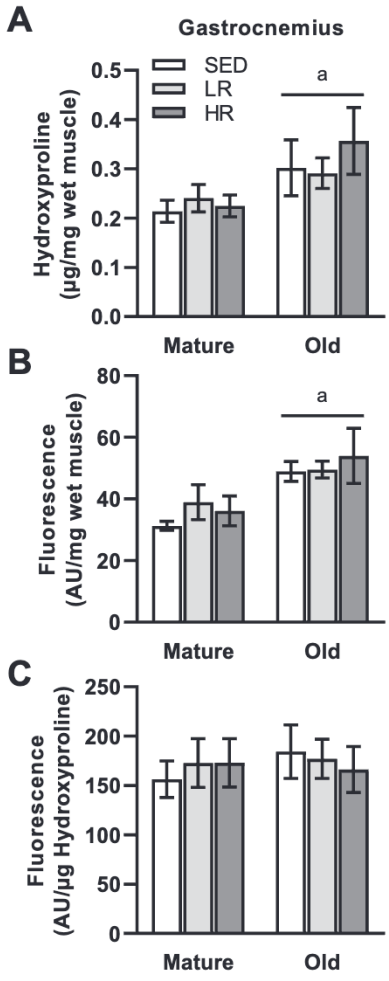

Fig. 8. Biochemical measurements on decellularized gastrocnemius muscle. A) Hydroxyproline normalized to intact muscle wet weight. B) Autofluorescence normalized to intact muscle wet weight. C) Autofluorescence normalized to hydroxyproline content. Mean ± SEM. a: Main effect of age.

comparison (Kovanen et al., 1987; Zimmerman et al., 1993; Ramaswamy et al., 2011; Ballak et al., 2014; Wood et al., 2014). Our results showed a higher intramuscular collagen content, elevated AGE cross-linking and a larger endomysial connective tissue thickness in the old mice (Figs. 6A-B and 8A-B). These findings are consistent with the description of aging intramuscular connective tissue in previous studies (Ballak et al., 2014; Wood et al., 2014; Pavan et al., 2020). Aging was also accompanied by increased passive modulus of the intact soleus but not the plantaris muscle (Fig. 3). This is to some extent in agreement with previous work that has shown an increase in soleus muscle passive stiffness with aging in sedentary rats (Gosselin et al., 1998), however, in that study no effect of aging was seen in treadmill trained animals, while the present work shows an age effect in both sedentary and trained animals. Due to the presence of both IMCT and contractile tissue components, the interpretation of intact muscle passive mechanical properties is complicated. Direct measurements on the IMCT have not commonly been performed, but in the present work a more specific assessment of the soleus IMCT properties was performed by measuring lateral force transmission. In spite of the age differences in tensile properties of the intact soleus, relative force transmission was unaffected by age (Fig. 4). This finding is somewhat in contrast with results by Ramaswamy et al. (2011) who reported lower lateral force transmission during active contraction of the extensor digitorum longus (EDL) muscle of very old rats (36–38 months). However, it is worth noting that this previous study reported a five-fold greater IMCT thickness of the EDL, measured by a similar picrosirius red staining method to the present one, whereas we only observed 10 % greater ICMT thickness of the soleus. In addition, the intensity of the picrosirius red stain appeared lower in the old animals (the age effect was significant for the gastrocnemius but only for the LR group in soleus), indicating a reduction in endomysial collagen density concomitant to the increase in thickness. To the extent that IMCT content affects lateral force transmission, the modestly greater IMCT thickness of the present old soleus may therefore have been insufficient to influence lateral force transmission in contrast to that reported for the EDL.

For the plantaris, isolated IMCT mechanics were assessed following decellularization. The results showed that although no age differences were present in the passive stiffness of the whole muscle, the isolated IMCT had significantly greater tensile modulus and ultimate strength (Fig. 5) in the old group. Similar results have previously been reported by comparing mechanical properties of single-fibers and bundles from human vastus lateralis (Pavan et al., 2020) where it was estimated that the change in IMCT mechanics could largely be explained by the increase in IMCT quantity. While the present study did not directly measure IMCT content in the plantaris muscle, the 40 % greater modulus compares well with the 41 % greater hydroxyproline content (as a measure of IMCT) of the old gastrocnemius muscle (Fig. 8A). It seems reasonable to expect the properties of the IMCT to be comparable between the plantaris and the gastrocnemius muscles since they have a similar fiber type composition (primarily type IIB) and operate synergistically (DeNies et al., 2014; Olesen et al., 2021). If mechanical changes are entirely explained by changes in IMCT quantity, it suggests that IMCT quality is unaffected. One parameter that could affect the mechanical quality of IMCT with age are cross-links formed by AGEs, which are known to accumulate with aging in other connective tissue rich structures such as tendons (Thorpe et al., 2010). In the present gastrocnemius IMCT, absolute autofluorescence (as a measure of AGEs) was greater in the old group (Fig. 8B) but did not differ with age when normalized to the IMCT quantity (hydroxyproline) (Fig. 8C). This agrees with previous findings (Wood et al., 2014), and thereby supports the notion that mainly quantity and not quality is altered with aging. This contrasts somewhat with human data showing increased AGEs even when normalized to collagen content (Haus et al., 2007), which may be due to the much greater absolute aging in humans (~50 years) and a fast turnover of muscle connective tissue in rodents (Bechshoft et al., 2017).

It is worth considering the relative contributions of the endomysium, perimysium and epimysium. Histology based on PSR staining was designed to specifically measure the endomysium by only accounting for connective tissue immediately surrounding each muscle fiber, while biochemical measurement of hydroxyproline include both the endomysium and perimysium but not the epimysium, which was peeled off prior to processing. The magnitude of the increase with age in hydroxyproline was 41 % while the increase in endomysial thickness was only 24 % (Fig. 6A), indicating that perimysium likely also increased with aging, possibly to an even greater extent than the endomysium. Perimysium has been reported to form cable-like structures along muscle fibers and may be more important for longitudinal force transmission than the endomysium (Gillies et al., 2017). As for the epimysium, the quantity was not assessed in the present study, but could in principle have contributed to the mechanical changes observed for the plantaris IMCT since it was not possible to remove from those samples and previous work reported greater stiffness and strength of the epimysium in old rats (Gao et al., 2008).

4.2. Training adaptations

There was a tendency for greater mass and ACSA of the soleus muscles in the training groups (Table 1) but, contrary to our hypotheses, there were no consistent adaptations of IMCT to either of the present training regimens. The rationale behind our training protocol based on

voluntary wheel running, was to enable much greater total exercise volumes than supervised treadmill running typically does. The present protocol resulted in approximately 215 h of running with a total distance of around 250 km (Olesen et al., 2021). This can be compared to a typical treadmill protocol (60 min/day, 5 days/week, 8 weeks) (Carroll et al., 2015), which results in about 40 h of running and a distance of around 48 km, although it is possible to find treadmill studies that have applied even greater exercise volumes (60 min/day, 5 days/week, 2 years) resulting in around 500 h and 720 km of running (Kovanen et al., 1987). Treadmill running is typically performed at an incline to increase the load magnitude, and high wheel resistance in the present HR group results in a similar effect since greater resistance causes the mice to run at an inclined angle within the running wheel. It is worth noting that despite the high running volume in the present study, the activity in the training groups is likely close to the normal state for a mouse in the wild and could therefore arguably be considered a study of sedentarism rather than exercise. However, the animals were not trained during their life leading up to the study and training is therefore still the intervention stimulus even if it represents a "return to normal".

While no consistent differences were seen with training, there was an interaction for the picrosirius staining intensity (measure of collagen density), which was lower in the soleus of the old LR group. This could indicate that more endurance type running may reduce IMCT content in the old but considering that such an effect was not evident in any of the other parameters, the finding should be considered tentative. Previous studies have reported mixed results of exercise on the quantity of IMCT, with some treadmill running studies in rats showing no effect on soleus, gastrocnemius or rectus femoris collagen content (Zimmerman et al., 1993; Gosselin et al., 1998) while another study reported almost a doubling in both soleus and gastrocnemius (Carroll et al., 2015). Life-long treadmill running increased collagen content in the soleus but not the rectus femoris (Kovanen et al., 1987), while resistance training has been reported to decrease the IMCT content as measured by histological staining in both soleus and gastrocnemius (Guzzoni et al., 2018). The variability observed in IMCT deposition with exercise may be related to the dynamic nature of this response. There is evidence to suggest that exercise results in a marked acute up-regulation of structural IMCT components (Takala et al., 1983; Guzzoni et al., 2018; Kritikaki et al., 2021), however, this is not necessarily associated with increased net accumulation, likely due to concomitant up-regulation of matrix degrading factors such as matrix metalloproteinases (MMPs) (Takala et al., 1983; Myllyla et al., 1986; Guzzoni et al., 2018). This suggests that exercise increases IMCT turnover, but whether the long-term result is an increase or decrease in total connective tissue deposition would depend critically on the exact balance of anabolic and catabolic processes. Despite the running protocols of the present study being ineffective, it therefore remains possible that other training modalities like the resistance exercises reported by Guzzoni et al. (Guzzoni et al., 2018) could counteract age related increases in IMCT. However, if very specific exercise regimes are required, then translation from rodent models may be difficult and future studies should therefore focus on specific exercises in humans. In lieu of effective exercise interventions, anti-fibrotic pharmaceuticals could present an alternative clinical approach. Such pharmaceuticals have shown some effect in animal models of muscle injury (Garg et al., 2015), but to our knowledge have not been investigated in relation to age-related changes and the potential benefits may not outweigh the risk of side effects. It is difficult to say whether a greater or lesser amount of IMCT would be desirable in any given situation as the functional optimum must balance between an overly restrictive fibrotic matrix at one extreme and an overly flexible and mechanically incompetent one at the other. The fact that aging is simultaneously associated with reduced muscle function and increased IMCT content may indicate that a reduction (change towards young state) would be desirable, however, increased IMCT could also be a compensatory mechanism. It has for example been suggested that a stiffer musculoskeletal system is associated with improved running economy (Gleim et al., 1990).

In agreement with our histological results (Fig. 6), most studies find that IMCT content of the soleus muscle (slow twitch) is greater than that of more fast twitch muscles like gastrocnemius and rectus femoris (Kovanen et al., 1984a; Kovanen et al., 1987; Zimmerman et al., 1993; Guzzoni et al., 2018). The same difference has also been reported between fast and slow twitch regions of the gastrocnemius muscle (Kovanen et al., 1984b), however, when the present data was analyzed in a fiber-type specific manner, no differences were observed between individual fast and slow fibers within the same muscle (Fig. 7). A likely explanation for this discrepancy is that the slow fibers are generally smaller than the fast (Olesen et al., 2021), which means that even though the IMCT thickness around each fiber is the same, the total quantity will be greater in regions with slow fibers because of a larger number of fibers per unit area.

Aside from quantitative changes to the IMCT, exercise related changes to the quality or function have received less research. The passive stiffness of whole muscles (including both IMCT and contractile components) has been reported to be lower with exercise in the soleus of old but not young rats (Gosselin et al., 1998), while others reported greater passive modulus and ultimate stress in the soleus of young rats with exercise (Kovanen et al., 1984a). More specific measurements on the isolated IMCT are scarce. Similar to the present results on the entire plantaris IMCT, the mechanical properties of isolated tibialis anterior epimysium has been reported to increase with age (Gao et al., 2008), but we are not aware of any previous studies of exercise effects on the mechanical function of endo- or peri-mysium.

A different approach to assess the mechanical properties of the IMCT (mainly endomysium) is to compare passive mechanical properties between single fibers and bundles. One such study showed that four weeks of downhill running increased intramuscular connective tissue stiffness in the extensor digitorum longus muscle, but not in the soleus and vastus lateralis muscles, and not after uphill running (Noonan et al., 2020). Downhill running requires eccentric contractions, while uphill running and wheel running predominantly requires concentric contractions. The variety in training effects indicates that the properties of IMCT are extremely sensitive to the specific muscle and training type amongst others.

4.3. Limitations

The sample size was relatively small, especially in the old group due to a greater loss of animals in this group (being close to their individual life length) than we had expected. The reason for this loss of animals is unclear but could be related to greater than expected stress from individual housing and the tests performed (Olesen et al., 2021). The use of voluntary wheel running should also be noted as a limitation since it leads to some heterogeneity in terms of the training volume within the groups, which may have led to greater variance in the results. In particular a few of the old animals ran less than the rest but these did not deviate in muscle mass or size (Olesen et al., 2021). The 10-week training duration could possibly be too short for significant remodeling of the IMCT, although a relatively large total exercise volume was achieved during this period. Due to the destructive nature of some measurements, it was not possible to perform all measurements within the same muscle and as a result any information inferred from results on different muscles must be considered tentative. The study only investigated passive and not active muscle mechanics, which may result in a suboptimal recruitment of connective tissue structures especially in the decellularized plantaris, where the IMCT organization deviates substantially from that of an intact contracting muscle. However, passive conditions should be more sensitive to changes in IMCT since the contribution of contractile components is minimized. Finally, it should be acknowledged that autofluorescence is only an indirect marker of advanced glycation, and thus discrete changes in AGEs (especially non-fluorescent compounds) could be overlooked.

5. Conclusion

The quantity of intramuscular connective tissue (endo-and perimysium) was greater in the soleus and gastrocnemius of old mice than in middle-aged animals and the mechanical properties of isolated connective tissue from the plantaris muscles were also greater at an advanced age. The data suggest that aging affects connective tissue quantity more so than quality, since the increase in mechanical properties was similar in magnitude to the increase in quantity. Ten weeks of voluntary wheel running at either low or high resistance did not counteract these age-related changes in intramuscular connective tissue in spite of a high exercise volume. While short term exercise was ineffective in the present study, connective tissue changes may be slow and future studies of life-long exercise, especially in humans, are warranted. Variability between muscle groups indicates that matrix adaptation may relate to function, future studies should therefore systematically investigate different muscles in terms of matrix mechanics and composition, including the impact of other components than collagen, such as adipose infiltration.

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CRediT authorship contribution statement

Annesofie Thorup Olesen: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Project administration, Funding acquisition, Writing - original draft, Writing - review & editing. **Lasse Malchow-Møller**: Investigation, Formal analysis. **Rune D. Bendixen**: Investigation, Formal analysis. **Michael Kjær**: Conceptualization, Supervision, Writing - review & editing. **Abigail L. Mackey**: Conceptualization, Methodology, Writing - review & editing. **S. Peter Magnusson**: Conceptualization, Methodology, Funding acquisition, Supervision, Writing - review & editing. **Rene B. Svensson**: Conceptualization, Methodology, Software, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization.

Declaration of competing interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Data availability

Data will be made available on request.

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