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# Quantifying the Effects of Aging on Morphological and Cellular Properties of Human Female Pelvic Floor Muscles

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## Abstract

Age-related pelvic floor muscle (PFM) dysfunction is a critical defect in the progression to pelvic floor disorders (PFDs). Despite dramatic prevalence of PFDs in older women, the underlying pathophysiology of age-related PFM dysfunction remains poorly understood. Using cadaveric specimens, we quantified aging effects on functionally relevant PFM properties and compared PFMs with the appendicular muscles from the same donors. PFMs, obturator internus, and vastus lateralis were procured from younger ($N = 4$) and older ($N = 11$) donors with known obstetrical and medical history. Our findings demonstrate that PFMs undergo degenerative, rather than atrophic, alterations. Importantly, age-related fibrotic degeneration disproportionally impacts PFMs compared to the appendicular muscles. We identified intramuscular lipid accumulation as another contributing factor to the pathological alterations of PFMs with aging. We observed

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SUPPLEMENTARY INFORMATION

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CONFLICT OF INTEREST

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a fourfold decrease in muscle stem cell (MuSC) pool of aged relative to younger PFMs, but the MuSC pool of appendicular muscles from the same older donors was only twofold lower than in younger group, although these differences were not statistically significant. Age-related degeneration appears to disproportionally impact PFMs relative to the appendicular muscles from the same donors. Knowledge of tissue- and cell-level changes in aged PFMs is essential to promote our understanding of the mechanisms governing PFM dysfunction in older women.

**Keywords**

Aging; Female; Pelvic floor disorders; Pelvic floor muscles

## INTRODUCTION

Pelvic floor disorders (PFDs), including pelvic organ prolapse and urinary and fecal incontinence, represent a major health problem in older women due to their substantial negative impact on quality of life, associated morbidities, and economic costs.[34] The etiology of PFDs is multifactorial, with vaginal childbirth and aging identified as the key risk factors.[34] After menopause, aging becomes the strongest risk factor and older women can develop PFDs independent of parity.[23] Prevalence of PFDs progressively escalates with age, with a rate of 53% in women 80 years and older.[50] The number of older women in the U.S. with PFDs is predicted to increase to 43.8 million by 2050, accompanied by a dramatic rise in costs of PFDs to individuals and society.[50] In the past, many older women accepted PFDs as a normal part of aging and too often did not seek help; however the desire and expectations of healthy aging are increasing, further expanding the demand for care.[12]

The pelvic floor skeletal muscles (PFMs) are integral components of the pelvic floor. PFMs provide support for abdominal and pelvic viscera by opposing intraabdominal pressures and gravitational forces, and facilitate urinary and fecal continence. The PFM complex includes the coccygeus and the components of the levator ani: iliococcygeus, pubococcygeus and puborectalis. PFM dysfunction is a critical defect in the progression to symptomatic PFDs.[7,10,30] Despite this, the underlying pathophysiology of PFDs and the cause of age-related PFM dysfunction remain elusive. As a result, there are currently no preventive measures, and existing treatments are limited to PFM rehabilitation that is less effective in older women and surgeries that have high rates of failure and adverse events.[44]

It is well established that aging negatively affects the function of limb and trunk muscles via loss of muscle mass (sarcopenia), increased intramuscular collagen (fibrosis), fatty infiltration, and a decrease in resident muscle stem cells.[9,18,24] Much less is known about the impact of aging on the intrinsic components of the female PFMs. Their location deep within the pelvis precludes direct examination, contributing to this scientific disparity. The existing understanding of the PFM response to mechanical and physiological alterations is mainly derived from radiological examinations that rely on volumetric assessments. These imaging studies have failed to identify age-related changes in the PFMs,[31] perhaps due to their inability to evaluate alterations in the muscle components. This limitation of the conventional radiological assessments of PFMs has left a large gap in knowledge regarding

functionally relevant age-related muscle changes. As an alternative, human cadaveric specimens can be harnessed to probe the mechanisms of PFM dysfunction in older women.

The need to elucidate the causes of age-related PFM dysfunction and the associated PFDs is clear: until basic mechanisms are better understood, preventative measures will remain non-existent, while treatments will continue to offer marginal promise at best. The objective of the current study was to conduct a comprehensive investigation of the histomorphological properties of pelvic and non-pelvic muscles obtained from younger and older female cadaveric donors to investigate changes due to aging at the tissue and cellular levels.

We selected several parameters based on their known changes in disease states and aging in non-PFMs. We wanted to qualitatively assess the myofibrillar shape and packing, given that in healthy skeletal muscles individual myofibers demonstrate polygonal appearance and tight packing.[21] We also wanted to assess the intramuscular extracellular matrix (ECM), a connective tissue network, predominantly composed of collagen, which drives muscle passive mechanical properties and determines muscle load-bearing capacity. Age-related pathological increases in ECM appear to play a significant role in the dysfunction of various muscles, including PFMs.[2,6,17] We aimed to assess intramuscular fat, which typically comprises only very small portion of a normal healthy muscle and increased intramuscular fat content is associated with decreased muscle strength in non-PFMs.[1] We further aimed to examine proportion of centralized nuclei. Myofibers are multinucleated cells, in which nuclei are predominantly spaced at the periphery in healthy adult muscles, with only 3-5% of nuclei located centrally.[21] An increased proportion of centralized nuclei is a characteristic of many muscle disorders and serves as a marker of muscle degeneration/regeneration.[15,21] We also wanted to examine cross sectional area (CSA) of individual myofibers, which is a predictor of active force production.[29] Although radiological assessment of whole muscle CSA is commonly performed, in contrast to the myofiber CSA, it is a poor predictor of active muscle force generating capacity. The above, in part, is due to the fact that the whole muscle CSA measurements do not distinguish between contractile and non-contractile muscle components and, therefore, can be increased by such pathological alterations as fibrosis and fatty infiltration.[31] We also set off to determine the fiber phenotype, since muscle fibers are dynamic structures capable of altering their phenotype under various conditions, such as aging.[36] The myofiber CSA of type II fibers has been shown to be disproportionally affected by aging in the limb muscles.[3] Myosin heavy chain (MHC) isoforms represent an ideal marker for fiber typing, as the predominant isoform is the main determinant of muscle overall phenotype.[37,40] Finally, muscle stem cells (MuSCs) are essential for skeletal muscle homeostasis and regeneration after injury. Upon an external stimulus, MuSCs activate, proliferate, and differentiate to form new myofibers or fuse with pre-existing ones. The limb muscles' regenerative capacity is negatively affected by aging due to the decline in MuSCs number and functionality.[32] Multiple studies identify age-associated contraction of MuSC reservoir with the limb muscle degeneration.[47,48]

We hypothesized that aged PFMs undergo untoward pathological changes of the intrinsic muscle components to a greater extent than non-PFMs, regardless of parity, potentially accounting for the discrepant negative effect of aging on passive mechanical properties of the PFMs but not of the appendicular muscles from the same donors.[6]

# METHODS

## Specimen Acquisition

Given significant constraints associated with PFM sampling in living women, specimens were obtained from fresh post-mortem cadaveric female donors with documented medical, surgical, and obstetrical history, including number and mode of deliveries, through the Bequest Body Donation Program at the University of Minnesota. The study was exempt from the Institutional Review Board approval. Donors were categorized as younger (≤ 52 years) or older (> 52 years) based on the United States women's average age of menopause, a marker of biological senescence. In addition, the existing epidemiologic studies indicate that after menopause, aging is the leading risk factor for PFDs.[34,35] Moreover, studies report pathological changes in the contractile compartment of the limb muscles in the 6th decade.[27,33] Donors with the following history were excluded: gynecologic or colorectal malignancy, pelvic metastasis, pelvic radiation, connective tissue disorder, myopathy, pelvic organ or rectal prolapse, colectomy, or proctectomy. Parous donors were characterized as vaginally parous or vaginally nulliparous, i.e. history of abdominal deliveries exclusively. The following grouping was based on the published evidence that PFM injury and dysfunction are rarely observed after Cesarean section.[20]

Muscle biopsies were obtained from younger (YVN, $N = 4$) and older (OVN, $N = 4$) vaginally nulliparous, as well as older vaginally parous (OVP, $N = 7$) cadaveric donors between 0-7 days post-mortem. All biopsies (one per each unilateral muscle) were taken from the same mid-belly location of each muscle in a standardized fashion. To distinguish site from disease-specific alterations, PFMs were compared to non-PFMs from the same donors. To evaluate the effect of aging without potential confounding effect of pelvic organ prolapse or mechanical unloading, only donors without gross avulsions or pelvic organ prolapse at or distal to the hymen with post-mortem Credé maneuver were included.

## Quantitative Histologic Analyses

Biopsies of the individual PFMs (coccygeus, iliococcygeus, pubococcygeus, and puborectalis) as well as the appendicular muscles, obturator internus (OI), which shares load with PFMs, and vastus lateralis (VL), part of the quadriceps femoris, were snap-frozen immediately upon procurement, and stored in −80 °C. Serial 10 $\mu$m cross-sections were processed for various outcomes of interest, as detailed below. Whole muscle cross-sections were visualized using a microscope with epifluorescence and bright field optics (Zeiss, Oberkochen, Germany). Images, obtained with image capture software (Leica, Buffalo Grove, Illinois, USA), were used for quantitative analyses, performed by the investigators blinded to the study group and muscle identity. For qualitative and quantitative assessments, one muscle-cross section per side (left and right) was analyzed, for a total of two cross-sections per muscle type per outcome per donor.

## Collagen Area Fraction

Hematoxylin and Eosin (H&E) stain was used for the qualitative evaluation of myofibrillar shape and packing. Gomori Trichrome stain was used to quantify muscle collagen content. Collagen content, expressed as the percent area fraction, was calculated from the total

positive blue (collagen) pixels divided by the muscle-cross sectional area using ImageJ software.

### Intramuscular Fat Content

Muscle-cross sections were stained with oil-red-O, which gives lipids an orange-red color, for the examination of intramuscular fat.[38] Intramuscular fat content was calculated from the total positive orange pixels divided by the muscle cross-sectional area using ImageJ software. Lipid content was expressed as percentage of fiber area occupied by lipid aggregates.

### Proportion of Centralized Nuclei

Centralized nuclei were identified by 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain. The number of fibers with centralized nuclei compared to the total number of fibers within the whole muscle cross-section was calculated using a custom macro on ImageJ software.[41]

### Muscle Fiber Cross-Sectional Area and Fiber Phenotype

Fiber-type specific fiber size was measured on laminin-stained sections overlapped with anti-myosin heavy chain (MHC) isoforms IIa and IIx specific antibodies. Briefly, slides were rinsed with Tris-buffered saline (TBS) × 5 min and incubated with blocking buffer for 1 h (5% milk diluted in TBS), and with primary antibodies for 3 h (Table S1). Slides were further rinsed in TBS, and incubated with secondary antibodies for 1 h, rinsed with TBS, water and cover-slipped. Fiber size was determined using a custom Macro script run on ImageJ software.[39,41]

Any fiber that was not stained with either the anti-MHC IIa or anti-MHC IIx antibody was counted as type I fiber. Given extremely sparse published data on PFM fiber phenotype, we employed an additional method to confirm the validity of our approach using an established gel electrophoresis technique.[36,45] MHC bands were identified and quantified with densitometry (GS-800, BioRad, USA), based on molecular mass relative to a protein standard containing all adult skeletal muscle myosin isoforms (I, IIA, IIX).

### Muscle Stem Cell Reservoir

Muscle stem cells (MuSCs) were identified using fluorescence microscopy in a whole muscle cross-section by satisfying all of the following criteria: (1) Pax7 labeling, (2) localization in the sub-basal laminal region and (3) co-localization with a nucleus, according to previously published protocol[4] (Table S1). MuSCs were quantified using ImageJ software, and normalized to area of image to obtain cell density.

### Statistical Considerations

Data within and between groups were compared with one or two-way repeated measures ANOVA, respectively, with Tukey's *post-hoc* pairwise comparisons when indicated. Repeated measures were employed to account for the fact that different muscles were procured from the same donors. For type specific fiber area, log-transformed fiber area was analyzed using generalized estimating equations with an independent covariance structure clustered on donors, with Tukey–Kramer methods to adjust for multiple comparisons.

Significance was accepted at $p < 0.05$. All analyses were performed using GraphPad Prism 8 (San Diego, California, USA) or SAS 9.4 (Cary, North Carolina, USA).

## RESULTS

### Donor Characteristics

The mean (SD) ages of the cadaveric donors were 41.0 ± 11.9 years for the YVN group, 72.5 ± 15.2 years for the OVN group, and 73.0 ± 13.3 years for the OVP group. The mean age was significantly different between the young and two older groups ($p < 0.01$). The age of the OVN vs. OVP donors was comparable ($p = 0.9$). The mean body mass index (BMI) of the three groups was not significantly different (YVN = 20.8 ± 1.9 kg/m$^2$, OVN = 25.4 ± 4.9 kg/m$^2$, OVP = 21.3 ± 4.5 kg/m$^2$, $p > 0.2$). The median abdominal parity was 0 (0–2) for YVN and 0 (0–1) in OVN, with 6 out of 8 donors being nulliparous overall. The median parity for the OVP group was 3 (range 1–7).

### Intramuscular Collagen Content

We first performed qualitative assessment of the myofibrillar shape and packing. In both younger and older specimens, the appendicular muscles, consisted of tightly packed polygonal fibers (Figs. 1a and 1b). Myofiber packing of non-PFMs and PFMs appeared similar in the younger donors. In contrast, in the older specimens, whether OVN or OVP, PFMs demonstrated notable disrupted myofiber packing, with only moderate packing density compared with non-PFMs from the same donors.

The endomysial and perimysial intramuscular collagen content of the coccygeus, iliococcygeus, pubococcygeus and puborectalis were similar regardless of age group or parity (Table S2). Thus, the data from the individual components of the PFM complex were combined for comparisons with non-PFMs within each age group as well as between group analyses. To assess the effect of vaginal parity on the intramuscular ECM, the collagen content was compared between OVN and OVP specimens and was found to not differ significantly across all muscles examined ($p = 0.9$).

In the younger group, there was a trend towards a higher collagen content in PFMs compared to OI and VL, $p = 0.1$ (Fig. 2a). In contrast, in the older groups there was substantially higher collagen content in PFMs compared to OI and VL, $p < 0.0001$ (Fig. 2b). Interestingly, there was no difference in the intramuscular ECM content of OI and VL between the groups regardless of age ($p = 0.8$). On the other hand, the collagen content of the PFMs tended to be higher in older compared to younger specimens ($p = 0.10$).

### Intramuscular Fat Content

Similar to the findings regarding collagen content, intramuscular fat of the coccygeus, iliococcygeus, pubococcygeus and puborectalis did not differ within age groups (Table S3). Thus, these data were also combined in subsequent analyses. To assess the effect of vaginal parity on fatty degeneration of aged specimens, the intramuscular fat content was compared between OVN and OVP specimens, and similar to the collagen content, was found to not differ significantly across all muscles examined ($p = 0.6$).

In the younger group, the intramuscular fat content of the PFMs did not differ significantly from the fat content of OI or VL ($p > 0.2$). In contrast, increased fat content was observed in aged PFMs compared to aged OI ($p = 0.03$) and VL ($p = 0.004$) procured from the same older donors. The intramuscular fat content of OI and VL did not differ between younger compared to older specimens ($p = 0.8$). Contrary, significant fatty degeneration was observed in aged PFMs compared to younger specimens ($p = 0.02$; Figs. 2c and 2d).

## Proportion of Centralized Nuclei

Similar to the other indicators of muscle degeneration, discussed above, the proportion of centralized nuclei of the individual components of the PFM complex were similar within each group regardless of age or parity ($p = 0.2–0.9$, Table S4). In the younger and older groups, the proportion of the PFM fibers with centralized nuclei was significantly higher than that of the OI muscle ($p = 0.007$), Fig. 3a. The proportion of centralized nuclei of PFMs and VL did not differ in younger specimens ($p = 0.9$), while a trend towards increased number of fibers with centralized nuclei was observed in PFMs compared to VL in older specimens ($p = 0.09$). The proportion of centralized nuclei in non-PFMs did not differ between younger vs. older specimens ($p = 0.7$). With respect to the PFMs, we observed a trend towards increased percentage of centralized nuclei associated with aging ($p = 0.1$).

## Muscle Type Specific Fiber Size

The proportion of type I and type II fibers (Fig. 3b) did not differ significantly between the individual components of the PFM complex, with the exception of a higher percent of type I fibers in puborectalis compared to pubococcygeus in the older specimens ($p = 0.03$). No differences were observed between OVN and OVP groups for any of the muscles examined ($p = 0.5–0.9$) (Figs. 3c and 3d).

In the younger group, fiber type distribution was similar in PFMs (Tables S5–S6) compared to OI or VL, with the exception of a statistical trend towards a higher proportion of type I fibers in iliococcygeus compared to VL ($p = 0.07$). On the other hand, in older specimens a higher percent of type I fibers was identified in coccygeus, pubococcygeus, and puborectalis compared to OI ($p = 0.01$) and VL ($p = 0.01$), Fig. 3c. As expected, this was accompanied by a lower proportion of type II fibers in PFMs compared to non-PFMs ($p = 0.01$). The fiber type distribution did not differ between younger vs. older specimens for any muscles examined ($p = 0.9$), (Figs. 3c and 3d). The fiber phenotype distribution of the PFMs, identified using immunohistochemistry, was confirmed by the relative proportion of MHC I and II identified using gel electrophoresis (Fig. S1).

The fiber-type specific CSA data are summarized in Table S7. Irrespective of age or parity, there were no differences in fiber size between the individual components of the PFM complex for either fiber type ($p > 0.2$) with the exception of a smaller type I CSA in coccygeus relative to iliococcygeus in the younger group ($p = 0.008$). In the younger group, comparisons of PFMs vs. non-PFMs did not show any differences in CSA of type I fibers ($p > 0.3$). In contrast, CSA of type II fibers of iliococcygeus and puborectalis exceeded that of OI ($p < 0.0001$). With respect to the older specimens, type I fiber CSA did not differ between PFMs and OI ($p > 0.8$) but was significantly lower in the PFMs compared to VL

($p = 0.007$). CSA of type II fibers did not differ between aged PFMs and aged OI or VL ($p > 0.2$). The fiber CSA did not differ significantly between younger vs. older specimens regardless of the fiber phenotype for any muscles examined ($p > 0.3$). Surprisingly, a trend towards increased CSA of type I fibers was noted in aged VL compared to younger VL ($p = 0.08$); with no differences identified in the CSA of type II fibers ($p > 0.9$; Fig. 3).

### Muscle Stem Cell Reservoir

MuSC pool did not differ significantly between the individual components of the PFM complex in either the younger or the older groups, $p > 0.1$ (Table S8). In the younger and older groups, the density of the $Pax7^+$ MuSCs did not differ between PFMs and non-PFMs, $p = 0.9$ and $p = 0.65$, respectively (Figs. 4a and 4b). The MuSC reservoir of OI and VL did not differ between younger compared to older specimens ($p > 0.5$). Interestingly, MuSC density was fourfold higher in the younger PFMs compared to older PFMs, however this difference did not reach statistical significance ($p = 0.1$; Fig. 4).

## DISCUSSION

Using human cadavers, we have previously begun to uncover the effects that aging on the functionally relevant components of the female PFMs.[2,6,8] We demonstrated that aging, independent of vaginal parity, results in a marked decrease in muscle physiological cross-sectional area - a predictor of maximum force generating capacity. Furthermore, aged PFMs exhibit a dramatic increase in total collagen content, determined by hydroxyproline assay, compared to the younger specimens, which is deleterious to muscle mechanical properties.[2] We observed similar but of lesser magnitude age-related changes in the obturator internus muscle, a non-PFM closely associated with PFMs via aponeurotic attachments.[8] Interestingly, our subsequent assessments of the muscles' passive mechanical properties, which are primarily governed by the intramuscular ECM, revealed increased stiffness of the aged relative to young cadaveric PFMs, but not of the appendicular muscles from the same donors.[6] The above suggests that PFMs' passive mechanical properties are particularly negatively affected by aging even in the absence of symptomatic PFDs.

In the current study, we conducted a comprehensive evaluation of the morphometric muscle properties. Our findings demonstrate that PFMs undergo degenerative, rather than atrophic, alterations. As in prior investigations, we found that aging was associated with muscle fibrosis.[2,6,17] One of the major findings of this study is that age-related fibrotic degeneration disproportionally impacts PFMs relative to the appendicular muscles from the same donors. While fibrosis has long been viewed as the major marker of muscle degeneration, recent studies in the limb muscles suggest that functional age-related muscle dysfunction is also tied to intramuscular adipose tissue.[1] The link between aging and fatty degeneration of the PFMs has never been explored. We identified lipid accumulation as another potential contributing factor to the pathological alterations of the PFMs with aging. To our knowledge this is the first time this has been reported. The trend towards increased proportion of fibers with centralized nuclei observed in the PFMs of older donors also suggests ongoing degenerative processes associated with aging.

Consistent with the published studies examining the effect of aging on limb muscles, we found that fiber type distribution did not change with aging in either PFMs or non-PFMs.[22] Importantly, we did not observe fiber atrophy with aging. Taken together, our novel findings provide potential explanation for the published radiological observations that the total PFM cross-sectional area does not differ between younger and older nulliparous women.[31] The fibrotic and fatty degeneration of the PFMs, identified in the current study, are the likely mechanisms driving deterioration of the PFM function with age, despite unchanged whole muscle volume.[31] Another potential mechanism for age-related PFM dysfunction might be due to the changes in neural drive and PFM fiber recruitment with aging. However, age-associated PFM weakness and impaired response to rehabilitation[42] have been shown to be myogenic in origin,[11] consistent with analogous studies in limb muscles.[46]

The current results also provide a mechanistic explanation for our prior finding that aging disproportionally impacts passive mechanical properties of the PFMs, with significantly increased stiffness observed in older PFMs but not in the appendicular obturator internus or vastus lateralis muscles procured from the same donors.[6] Taken together, these findings at least partially explain the clinically identified decrease in aged PFM strength and diminished responsiveness of older women to PFM rehabilitation.[43]

The ability of myofibers to regenerate throughout life relies on endogenous muscle stem cells (MuSCs), which maintain a functionally optimal proportion of contractile and ECM components.[26] Adult muscle damage occurs continuously, but the loss of function becomes evident only after the number of MuSCs and their capacity for repair decline with age, leading to the replacement of functional muscle by fibrotic and fatty tissue.[5] In this study, we evaluated, for the first time, the impact of aging on the PFMs' stem cell reservoir. Overall, we observed an approximately fourfold decrease in MuSC pool of aged PFMs compared to younger specimens. These differences did not reach statistical significance, possibly due to our small sample sizes and variability in the younger group. In contrast, MuSC pool of aged appendicular muscles from the same donors was only twofold lower than their counterparts in the younger group. The disproportionate reduction of MuSC reservoir of the female PFMs points towards one of the potential mechanisms governing the preponderant degeneration of these muscles with aging. Our future studies will focus on investigating the impact of aging on the regenerative capacity of the PFMs. Based on the previous successful isolation of MuSCs up to 17 days post-mortem from human cadaveric samples[25], cadaveric PFMs can be harnessed for *in vitro* MuSC studies.

The extremely limited number of published studies examining morphometric and cellular properties of the female PFMs include a few histological details or do not specifically address changes related to aging.[11,51] Yiou and colleagues examined histologic levator ani specimens from women with pelvic organ prolapse and found notable myofilament loss compared to deltoid muscle from the same patients.[51] However, only women with pelvic organ prolapse were evaluated, precluding assessment of the independent effects of aging on the PFMs. Heit *et al.* sampled levator ani of women with and without pelvic floor disorders at the time of gynecologic surgery. The authors' assessments were limited to fiber type distribution and fiber size, with no differences identified between groups.[19] Despite women being older in the pelvic floor disorder group, similar to the study by Yiou *et al.*,

conclusions regarding aging effects independent from potential changes induced by pelvic organ prolapse could not be drawn.

The strengths of our study include assessment of PFMs procured from cadaveric donors with known obstetrical and medical history. We performed a comprehensive evaluation of a full complement of the morphometric muscle properties at tissue and cellular levels rather than relying on indirect radiologic examination. By procuring and analyzing PFMs and non-PFMs from the same donors using the same techniques, we were able to draw direct comparisons of the effect of age on muscles by location, while minimizing differences at the individual level. In addition, examining PFMs of donors without a diagnosis of pelvic floor disorders, evidence of pelvic organ prolapse on post-mortem examination, active medications at the time of death for urge urinary incontinence, or prior surgery for pelvic floor disorders, we were better able to isolate the effects of aging apart from potential morphologic changes associated with pelvic floor disorders, especially pelvic organ prolapse. However, it is possible that the donors may have had pelvic floor disorders not reported in the medical record or which had been previously treated non-surgically.

The main limitations of our study were related to our use of cadaveric muscle specimens. However, due to their location deep within the pelvis, obtaining analogous PFM specimens from asymptomatic living women is not possible. Due to the limited availability of nulliparous donors, the sample sizes of the OVN and YVN groups are small. Similarly, we did not have a young parous group due to rarity of this type of donor, and so we could not fully examine the interaction between age and parity. Also, because the specimens were from cadaveric donors, we had to rely on review of the medical records for information about medical history. Specific information regarding menopausal status was not available, and therefore we chose 52 years as the age cut-off between groups based on average age of menopause in the U.S. It is, therefore, possible that some of the women in the younger group were postmenopausal and vice versa. Moreover, because we used cadaveric tissue, it is possible that some post-mortem artifact may have been present, but we expect this would have affected PFMs and non-PFMs equally. Additionally, we did examine a wide array of histomorphological properties of the muscle specimens. In the current study we chose to focus on the intramuscular collagen and fat content for the following reasons: (1) collagen is a major constituent of the intramuscular ECM,[1] (2) fibrosis is defined as pathological increase in collagen content[1], (3) fibrotic and adipogenic degeneration has been implicated in the deterioration of the limb muscles' function with age,[1,17] and (4) pathological increase in collagen content has been implicated as the primary driver of the increased muscle stiffness that we have previously demonstrated in the aged PFMs.[6] However, investigations focused on the aging effects on other constituents of the intramuscular ECM, such as elastin, represent a fruitful avenue for future studies. Lastly, we did not assess muscles' active mechanical properties, which requires testing of the whole muscle in the immediate post-mortem period. However, we have previously assessed passive mechanical properties of these muscles and found increased stiffness in aged compared to younger specimens in the PFMs but not in the appendicular muscles procured from the same donors.[6]

Our finding that aging is associated with degenerative changes of the PFMs provides impetus for using PFM rehabilitation in asymptomatic women as a potential strategy to

prevent or mitigate the untoward effects of aging of PFM structure and function, and the associated pelvic floor disorders. Indeed, orthopedic literature supports the use of muscle training in mitigating the effects of aging. Furthermore, preemptive antepartum PFM rehabilitation in continent women has been shown to decrease incidence of urinary incontinence postpartum.[16,49] Moreover, pathological ECM accumulation has the potential to stress-shield the myofibers, contributing to progressive atrophy and worsening fibrosis, as seen in dystrophic muscles.[13] Our study was purposefully performed on PFMs procured from donors without evidence of pelvic floor disorders, thus, the observed degenerative changes are likely to be even more pronounced in older women with pelvic floor disorders.

In summary, delineation of precise alterations due to aging in female cadaveric PFMs provides specific parameters to be targeted by specialized non-invasive imaging to investigate the extent of such changes in living women. Cadaveric donors can be used to validate these imaging modalities. Subsequent correlation of the degree of change with PFM function and response to rehabilitation can potentially lead to the development of a non-invasive tool to identify older women at high risk for progression to symptomatic pelvic floor disorders and failure to respond to PFM exercises. The above would provide clinicians with objective criteria to base individualized counseling to their patients regarding prevention and treatment. Additionally, our findings suggest that fibroadipose progenitor cells may be a novel target in the goal of mitigating the negative impact of aging on PFMs. Significant advances in the treatment of muscular dysfunction became possible once precise muscle structure-function relationships, physiology, and pathophysiology were established in orthopedics and cardiology.[14,28] The current study is the first step in the continuum of investigations that have a high potential to shift the current research paradigm in female pelvic medicine towards mechanistic studies by directly and systematically examining age-related changes in the major determinants of PFM function.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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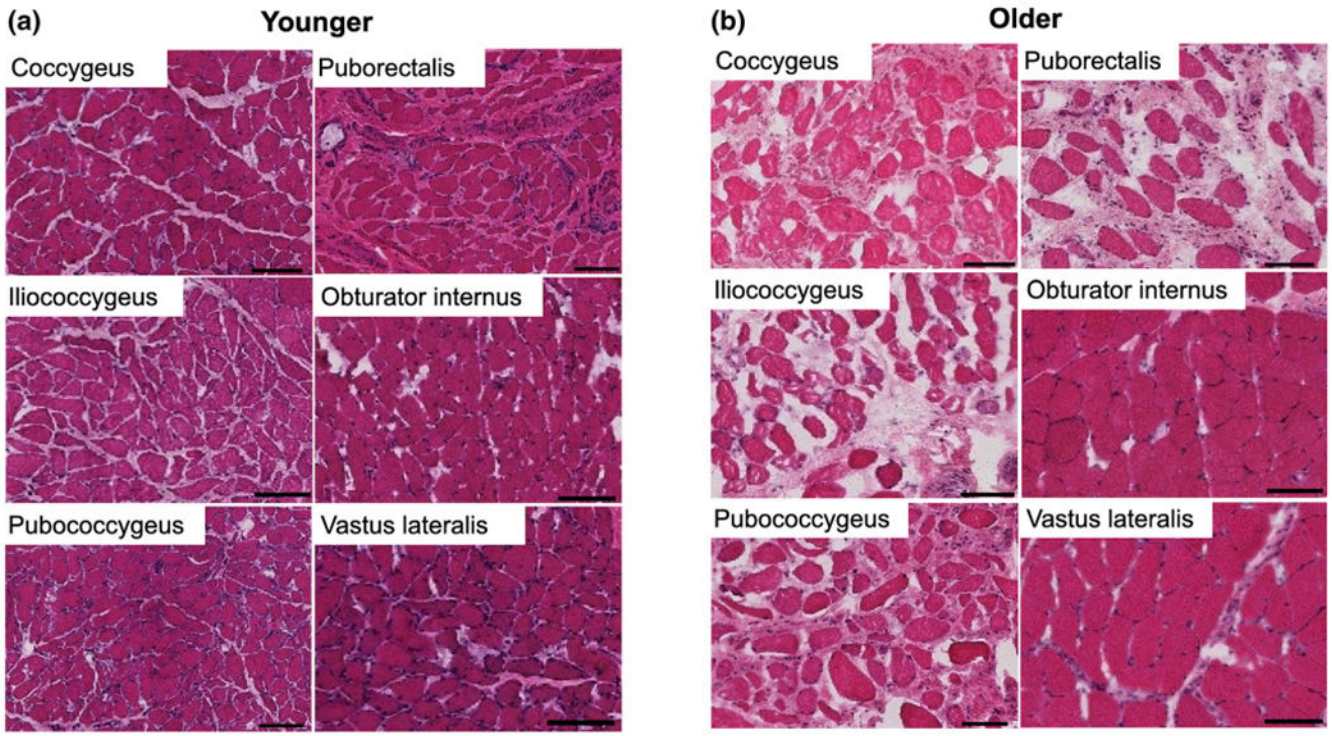


**FIGURE 1.**

Morphologic appearance of the younger and older female cadaveric pelvic floor muscles (PFMs) and appendicular obturator internus (OI) and vastus lateralis (VL) muscles stained with Hematoxylin and Eosin. In younger (a) and older (b) specimens, OI and (VL) consisted of tightly packed polygonal fibers. In contrast, aged PFMs demonstrated pronounced disruption of the myofiber packing compared to younger PFM specimens and to non-PFMs from the same older donors. Scale bar: 100 $\mu$m.

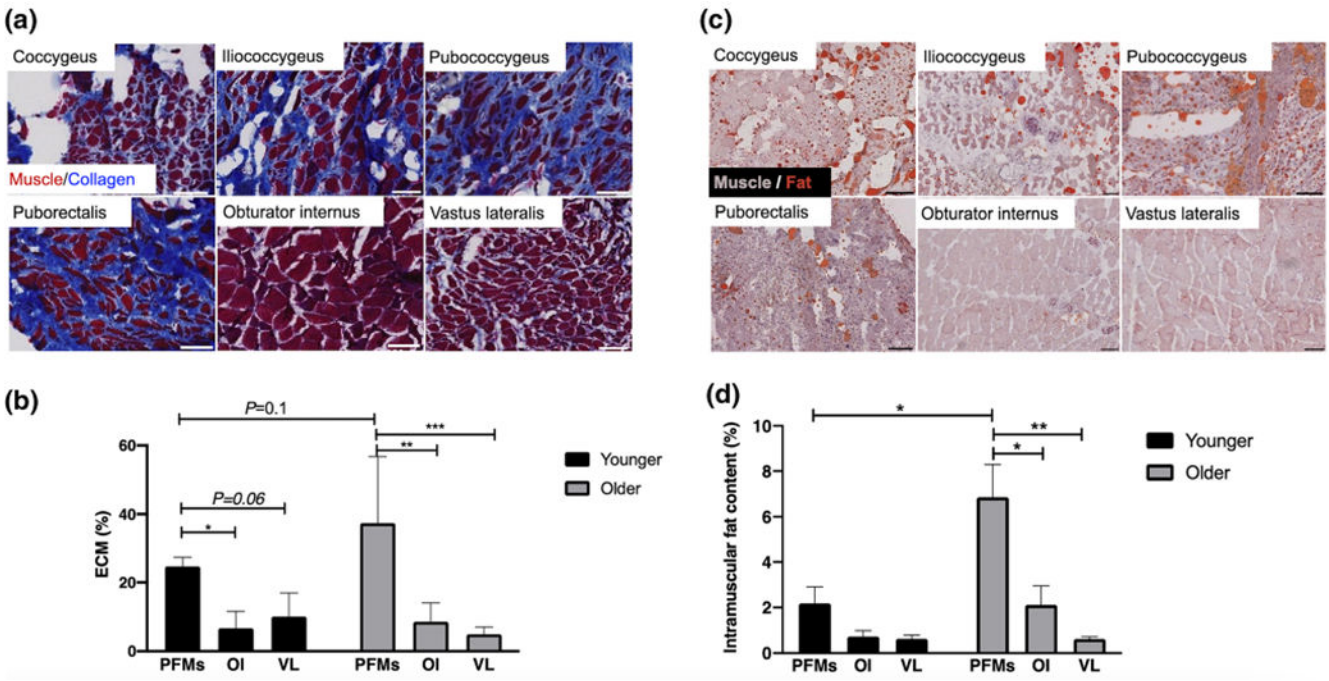


**FIGURE 2.**

Pelvic floor muscles (PFMs) are more prone to fibrotic and fatty degeneration with aging compared to appendicular obturator internus (OI) and vastus lateralis (VL) muscles. Gomori Trichrome staining of muscle cross-sections of the individual components of the PFM complex – coccygeus, iliococcygeus, pubococcygeus, and puborectalis, as well as OI and VL procured from older donors (a), demonstrating substantially higher collagen content in PFMs compared to OI and VL (b). In contrast, quantification of collagen content in the younger group demonstrated a much smaller difference between PFMs and non-PFMs (b). Oil-red-O staining of muscle cross-sections of the individual components of the PFM complex – coccygeus, iliococcygeus, pubococcygeus, and puborectalis, as well as OI and VL procured from older donors (c), demonstrating substantially higher lipid content in PFMs compared to OI and VL (d). In contrast, intramuscular fat content of PFMs did not differ from OI or VL in the younger group (d). While intramuscular fat content of non-PFMs did not differ between younger vs. older group, significant fatty degeneration was observed in aged PFMs compared to younger specimens (d). Footnote: Significantly different p values derived from 2-way repeated measures ANOVA. $*p < 0.05$, $*p < 0.01$, $***p < 0.001$, mean ± SEM. Scale bar: 100 $\mu$m.

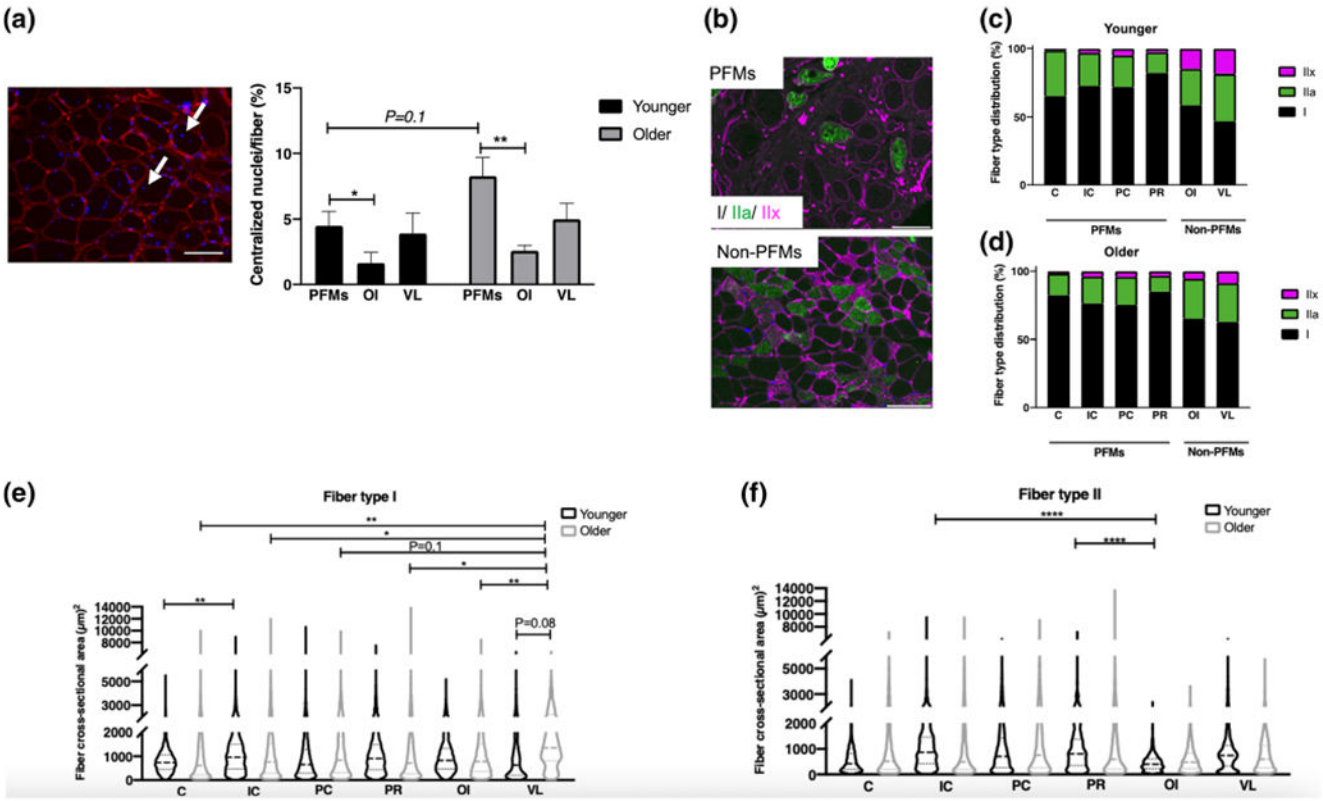


**FIGURE 3.**

Quantification of centralized nuclei, a marker of degeneration/regeneration, identified with DAPI (blue) staining on muscle cross-sectional images, demonstrating significantly higher proportion of fibers with centralized nuclei in the pelvic floor muscles (PFMs) compared to the appendicular obturator internus (OI) muscle in both, younger and older, groups (a). Muscle phenotype determined by laminin-stained sections overlapped with anti-myosin heavy chain (MHC) isoforms IIa and IIx specific antibodies and not labeled type I fibers (b) demonstrating similar fiber type distribution in the younger and older PFMs (c, d). In the younger group, no differences in fiber type distribution were observed in PFMs compared to non-PFMs (c). In contrast, a higher percent of type I fibers and a lower proportion of type II fibers were identified in coccygeus (C), pubococcygeus (PC), and puborectalis (PR) compared to OI and vastus lateralis (VL) in the older group (d). Fiber type specific fiber size was determined with a custom macro on ImageJ, and represented in violin plots (e, f). Fiber size did not differ between younger and older specimens regardless of fiber type for any muscle examined. Irrespective of age, there were no differences in fiber size between PFMs for either fiber type, with the exception of a smaller type I fiber size in C relative to iliococcygeus (IC) in the younger group. In the younger group, PFMs vs. non-PFMs were not different in fiber size of type I fibers (e), but IC and PR fiber size type II exceeded that of OI (f). For older specimens, type I fiber size did not differ between PFMs and OI but was lower in the PFMs vs. VL. Type II fiber size was not different in aged PFMs compared to aged non-PFMs. Footnote: Significantly different p values for centralized nuclei, fiber type distribution derived from 2-way repeated measures ANOVA, while for fiber size general estimated equations clustered by specimens was used. *$p < 0.05$, *$p < 0.01$, ***$p < 0.001$,

****$p < 0.0001$, mean ± SEM. Scale bar: 100 $\mu$m. *C* coccygeus, *IC* iliococcygeus, *PC* pubococcygeus, *PR* puborectalis, *OI* obturator internus, *VL* vastus lateralis.

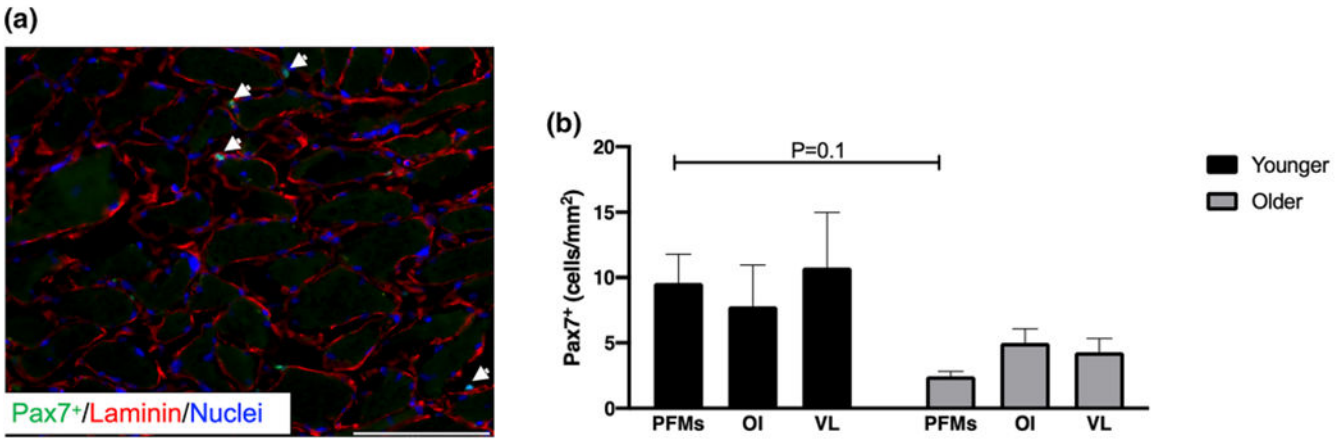


**FIGURE 4.**

Pelvic Floor Muscles' (PFMs) stem cell pool. Muscle stem cells (MuSCs), identified by Pax7 labeling, localization in the sub-basal laminal region, and co-localization with a nucleus (a) demonstrating similar MuSC reservoir in PFMs compared to non-PFMs in the younger and older group. The MuSCs pool of non-PFMs did not differ between younger compared to older specimens. In the older group, a trend towards decreased MuSCs reservoir was observed in the PFMs relative to OI and VL. MuSCs density was fourfold higher in the younger PFMs compared to older PFMs (b). Footnote: Significantly different p values derived from 2-way repeated measures ANOVA. mean ± SEM. Scale bar: 100 $\mu$m. *OI* obturator internus, *VL* vastus lateralis.