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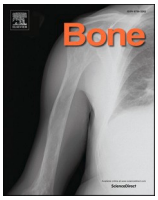

Full Length Article

Divergent mechanical properties of older human male femora reveal unique combinations of morphological and compositional traits contributing to low strength

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ABSTRACT

Bone strength is generally thought to decline with aging and prior work has compared traits between younger and older cohorts to identify the structural and compositional changes that contribute to fracture risk with age. However, for men, the majority of individuals do not fracture a bone in their lifetime. While fracture occurrence is multifactorial, the absence of fracture in the majority of males suggests that some individuals maintain bone strength or do not lose enough strength to fracture, whereas others do lose strength with aging. Consequently, not all structural and material changes observed with age may lead to strength-decline. We propose that consideration of different subgroups of older individuals will provide a more precise understanding of which structural and material changes directly contribute to strength-decline. We identified subgroups using latent profile analysis (LPA), which is a clustering-based algorithm that takes multiple continuous variables into account. Human cadaveric male femoral diaphyses (n = 33, 26–89 years) were subjected to whole bone and tissue-level mechanical tests. Morphological traits, porosity, and cortical tissue mineral density (Ct.TMD) were obtained, as were measures of enzymatic cross-links and the advanced glycation end product, pentosidine (PEN). A univariate analysis first identified a younger cohort (YNG, n = 11) and older cohort (n = 22). LPA was then conducted using three mechanical traits (whole bone strength, tissue-level strength, and tissue-level post-yield strain), resulting in a further stratification of the older group into two similarly aged groups ($p = 0.558$), but one with higher (OHM, n = 16) and another with lower mechanical properties (OLM, n = 6). The OLM group exhibited lower whole bone strength ($p = 0.016$), tissue-level strength ($p < 0.001$), and tissue-level post-yield strain ($p < 0.001$) compared to the YNG group. Meanwhile, the OHM only exhibited significantly lower tissue-level post-yield strain ($p < 0.001$), compared to the YNG group. Between the two older groups, the OHM group exhibited higher whole bone strength ($p = 0.037$), tissue-level strength ($p = 0.006$), and tissue-level post-yield strain ($p = 0.012$) than the OLM group. Probing the morphological and compositional relationships among the three groups, both OHM and OLM exhibited increased PEN content ($p < 0.001$, $p = 0.008$ respectively) and increased Log(cortical pore score) relative to YNG ($p = 0.003$, $p < 0.001$ respectively). Compared to the OHM group, the OLM also exhibited increased marrow area ($p = 0.049$), water content ($p = 0.048$), and decreased Ct.TMD ($p = 0.005$). The key traits that were unique to the OLM group compared to YNG were decreased Ct.TMD ($p < 0.001$) and increased Log(porosity) ($p = 0.002$). There were many properties that differed between the younger and older groups, but not all were associated with reduced mechanical properties, highlighting the relative importance of certain age-related traits such as porosity, Ct.TMD, water content, and marrow area that were unique to the OLM group. Overall, this work supports the hypothesis that there are subgroups of men showing different strength-decline trajectories with aging and establishes a basis for refining our understanding of which age-related changes are directly contributing to decreased strength.

Abbreviations: LPA, latent profile analysis; OHM, older group with higher mechanical properties; OLM, older group with lower mechanical properties; Ct.TMD, cortical tissue mineral density; DHLNL, dihydroxy-lysino-norleucine; HLNL, hydroxy-lysino-norleucine; PYD, pyridinoline; DPD, deoxypyridinoline; PEN, pentosidine; BMD, bone mineral density; AGEs, advanced glycation end products; Tt.Ar, total area; Ct.Ar, cortical area; Ma.Ar, marrow area.

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1. Introduction

Bone strength is generally thought to decline with aging [1–3]. Fragility fractures are multifactorial considering diminished bone mechanical properties, loss of neuromuscular control and other age-related changes. However, fracture risk statistics suggest heterogeneity exists within the population for these risk factors, and the extent to which strength declines with aging across a heterogeneous population is not clear. One in three women and one in five men above the age of 50 will fracture a bone [4]. Conversely, this means that the majority of women and men will not experience a bone fragility fracture in their lifetime. These statistics suggest that bone strength declines to the point of increasing fracture risk for some individuals but is maintained or does not decline to the point of increased fracture risk for other individuals. Given the hierarchical organization and adaptive nature of bone, it is possible there are structural and/or material changes that occur with age and do not deleteriously affect strength. While other factors such as muscle loss or neuromuscular control contribute to fall and fracture risk, we hypothesize that not all skeletal age-related structural and material changes in the skeleton contribute to strength-decline and these lower risk changes may be missed in a "one-size-fits-all" approach. The challenge is to identify the traits that directly contribute to strength-decline from those that do not. For example, sorting individuals based on a bone morphological phenotype (e.g., external size) exposed the heterogeneity that exists in skeletal aging. Wide bones showed a sharper decline in strength with age compared to narrow bones [5,6]. Traits like cortical tissue mineral density (Ct.TMD) declined in wide but not narrow bones, but other traits like advanced glycation end products (AGEs) increased with age for both groups. While Ct.TMD correlated positively with mechanical properties for both wide and narrow groups, increasing AGEs correlated negatively with mechanical properties only for the wide group [5]. Enzymatic collagen cross-linking is thought to have a positive effect on bone strength and toughness, whereas non-enzymatic AGE formation with age or disease like diabetes, is thought to have a deleterious effect [7–9]. However, the relative importance of these post-translational collagen modifications with age-related structural or morphological changes remains unclear. These prior analyses suggest that some trait changes are either not contributing to strength-decline or only affecting strength when combined with certain morphological phenotypes. There are many structural and material changes that occur with aging [10], but we do not know which contribute to strength-decline directly or which affect strength but only when contextualized based on other traits or adaptive processes.

Several recent studies have employed cluster-based algorithms to identify bone phenotypes contributing to fracture risk, relying on fracture incidence and clinical imaging [11–14]. The objective of this cadaveric study was to identify subgroups in the cortical diaphysis of the male femur, based upon mechanical performance, and characterize the phenotypical traits of each subgroup. This characterization may allow us to differentiate the structural and material changes that are associated with strength-decline from those that change with age but do not directly affect strength. We used latent profile analysis (LPA), which is a clustering-based algorithm that examines multiple continuous variables, to identify underlying groups in a dataset [15,16]. While this technique has mainly been used in the social sciences, the ability to examine multiple continuous variables simultaneously to identify subgroups is well suited to the hierarchical nature of bone where many structural and material traits across length scales may contribute to bone strength [8]. Briefly, we have taken an unsupervised approach to sort individuals into subgroups based upon age, whole bone strength, and tissue-level mechanical outcomes, followed by a comparison of morphological and compositional properties among the subgroups to identify bone traits that are uniquely associated with lower strength of older bone.

2. Methods

2.1. Samples

The mechanical, morphological, and compositional traits were reported previously [5] [17],. However, this data is being further analyzed in the current study in a novel way using latent profile analysis to identify subgroups within a cohort of human femora from male donors stratified by mechanical and compositional properties. Pairs of fresh-frozen, male femora (n = 33, 26–89 years old) were obtained from the University of Michigan Anatomical Donations Program (Ann Arbor, MI, USA), Science Care (Phoenix, AZ, USA), and Anatomy Gifts Registry (Hanover, MD, USA). Human tissue use and handling were approved by the University of Michigan Institutional Biosafety Committee and deemed exempt by the Institutional Review Board. All methodologies, apart from the LPA analysis, can be found in the previously reported study but are briefly summarized below [5]. Throughout this study, the term "trait" is used to describe a specific compositional or morphological outcome; "phenotype" is defined as a set of multiple traits; mechanical properties are limited to whole bone strength, tissue-level strength, and tissue-level post-yield strain; and "subgroups" or "groups" (classes) refer to the LPA classifications into young (YNG), older with higher mechanical properties (OHM), or older with lower mechanical properties (OLM).

2.2. Morphology & mechanical testing

Left femora were imaged at the midshaft by a peripheral quantitative computed tomography (pQCT) system (XCT 2000 L, Stratec Medizintechnik, Pforzheim, Germany) with 161 μm in-plane pixel size. Calibration scans were run daily with a cortical bone phantom of known density (Bone Diagnostic LLC, Spring Branch TX, USA). MomentMacroJ (www.hopkinsmedicine.org/fae/mmacro.html) and ImageJ [18] were used to analyze 2D images of the cross-section to quantify all bone morphology measurements including cortical area (Ct.Ar), marrow area (Ma.Ar), total area (Tt.Ar), and cortical tissue mineral density (Ct.TMD). Bone length was measured from the distal articular surface of the condyles to the superior side of the femoral neck. After imaging, femora were loaded to failure at a rate of 0.1 mm/s under four-point bending using a material testing system (Model 8511, Instron, Inc., Norwood, MA, USA) with the anterior surface in tension. The upper span loading points were located at 25 % and 75 % of bone length and the lower span loading points were located at 33 % to 67 % of bone length. Whole bone strength was calculated from the load-deflection curves and adjusted for loading configuration to assess the maximum bending moment.

For the right femora, a 60 mm long sample was sectioned from the midshaft. A 5 mm proximal section of this midshaft sample was reserved for a higher resolution scan to assess porosity. From the remaining 55 mm portion of the midshaft sample, a 55 × 5 × 2.5 mm beam was milled from the anterior quadrant using a computer numerical controlled mill (Velox VR-1414 CNC; Velox CNC, Orange, CA, USA). The anterior quadrant was examined because this region shows large age-related changes in porosity [19]. These milled beams were tested under four point bending at 0.05 mm/s, with an upper span of 18 mm and a lower span of 42 mm while submerged in a calcium supplemented phosphate buffered saline solution (PBS) kept at 37 °C [6]. Stress and strain were calculated using bending equations and the 0.2 % offset method [20]. The maximum stress and post-yield strain (i.e., strain from yield to failure) of these milled beam tests are hereafter referred to as tissue-level strength and tissue-level post-yield strain, respectively.

2.3. Porosity, cortical pore score & ash content measures

The 5 mm cross-sections of the right femora were cleaned with an

oxidative detergent (OxiClean, Church & Dwight Co., Trenton, NJ, USA), sonicated and rinsed in PBS, and then dried at 37 °C. This section was then scanned using a nanoComputed Tomography system (nanotom-m; Waygate Technologies LP, Pasadena, TX, USA) with scan settings of 150 kV, 320 μA, 500 ms, 3 averages, filtering of 0.653 mm aluminum and 0.07 mm brass, 108 min scan time at a 13 μm voxel size. Calibration scans were collected daily using cortical bone phantoms. Using ImageJ [18] a 2 mm^2 circular region of interest was drawn in the anterior quadrant along the anterior-posterior axis at the same region from which the beams were milled. Images were thresholded to segment bone from background and porosity was calculated as the total pore area divided by the total area of the region of interest (ROI). Cortical pore score (CPS), which assesses the cumulative effect of pore size and location relative to the geometric centroid, was calculated as $CPS = \sum_i A_i d_i^2$, where A_i is the pore area and d_i is the distance from the pore to the geometric centroid of the cross-section. This same 5 mm cross section was divided into sextants using a diamond coated band saw, and the anterior sextant was defatted, hydrated, and weighed (hydrated weight), dried at 80 °C to a constant weight (dried weight, organic + mineral weight), ashed at 600 °C for 18 h, and then weighed (ash weight). Ash content was calculated as ash weight normalized by hydrated weight. Water content was calculated as the difference between hydrated and dried weights and normalized to hydrated weight.

2.4. Cross-link measurements

From the milled beam used for tissue-level mechanical testing, a specimen (5 mm × 5 mm × 2.5 mm) distal to the fracture was sectioned using a low-speed diamond wafering saw (Buehler, Inc., Lake Bluff, IL USA). Samples were decalcified using Cal-Ex (CS510, Fisher, Waltham, MA), washed with phosphate buffered saline (PBS), diced and reduced by sodium borohydride. After denaturing, the samples were digested with trypsin. Half of the tryptic digest was set aside for pyrrolic cross-link quantification [21] and the other half was subjected to acid hydrolysis with hydrochloric acid. A small portion of the acid hydrolysate was diluted and used to quantify hydroxyproline content [22]. Another portion of the acid hydrolysate was purified via a solid phase extraction (SPE) column (Bond Elut-Cellulose, 12102095, Agilent, Santa Clara, CA) eluted, lyophilized, and reconstituted with a pyridoxine hydrochloride solution to be used as an internal standard.

Quantification of the immature cross-links: dihydroxy-lysino-norleucine (DHLNL), hydroxy-lysino-norleucine (HLNL), mature cross-links: pyridinoline (PYD), deoxypyridinoline (DPD), and advanced glycation end-product: pentosidine (PEN) was carried out on two high performance liquid chromatography (HPLC) runs under fluorescent detection (Waters 1525 Binary Pump, Waters Corp., Milford, MA, USA), using an Atlantis T3 column (Waters, PN: 186003728). Column and gradient specifications can be found in the previously published study [5] but briefly the first injection detected PYD, DPD, and PEN, while a second injection with a post-column addition of o-phthaldialdehyde solution detected DHLNL and HLNL. A standard curve was used to determine cross-link concentrations and normalized to collagen content based on hydroxyproline content. Standards included DHLNL, HLNL both a generous gift from Simon Robins (University of Aberdeen), PYD, DPD (Quidel, San Diego, CA) and PEN (Cayman Chemical, Ann Arbor, MI). Cross-link ratios were determined by grouping cross-links into immature (DHLNL, HLNL), mature (PYD, DPD, pyrroles), pyridinolines (PYD, DPD), and all enzymatic (DHLNL, HLNL, PYD, DPD, pyrroles).

2.5. Latent profile analysis (LPA) & statistical analysis

Latent profile analysis is a Gaussian finite mixture model, or clustering-based algorithm, used to identify underlying subgroups of data based upon multiple continuous variables for each sample, and assigning a probability that each sample belongs in a subgroup. LPA analyses were conducted using the tidyLPA package available with R [16]. Initial use of LPA indicated that there was a bimodal age distribution in the sample collection, and this bimodal distribution dominated the division into subgroups when combined with other traits. To overcome this, samples were first separated into younger and older age groups (clustering/univariate LPA), where 2 groups was the best statistical fit. Samples with >85 % probability of belonging to the older group were classified as older, all others being classified as younger (YNG).

LPA was then conducted on the older group using only mechanical properties (whole bone strength, tissue-level strength, tissue-level post yield strain) which identified two classes or sub-groups as the best fit. These three mechanical properties were chosen so the clustering accounted for differences in whole bone and tissue-level strength. We also included tissue-level post-yield strain as a measure of ductility (or brittleness) based on our prior work showing how differences in bone brittleness affect bone strength [17]. The two subgroups were an older group with higher mechanical properties (OHM) and an older group with lower mechanical properties (OLM). Each individual sample had more than a 95 % probability of belonging to either the OHM or OLM group, except for one sample (75 % probability of being in OLM). Conducting an LPA using a single mechanical property (whole bone strength, tissue-level strength or tissue-level post-yield strain) resulted in different groupings with a limited number in the lower performing group. Using just two traits such as whole bone strength and tissue-level strength, similar groupings to those presented here were observed. The number of classes assigned to each LPA was determined by a minimization of error between 1 and 5 classes following an analytic hierarchy process for cluster-based analysis incorporating Akaike's Information Criterion (AIC), Approximate Weight of Evidence (AWE), Bayesian Information Criterion (BIC), Classification Likelihood Criterion (CLC), and Kullback Information Criterion (KIC) defined by the tidyLPA package [23].

Bone traits were then compared across the three groups, (YNG, OHM, OLM) using one-way ANOVA with Tukey's post-hoc test. Significance was defined as $p < 0.05$, with marginal significance defined as $p < 0.10$. To simplify the presentation so similar traits could be presented on the same graph, individual traits were scaled to z-scores, centering on the mean of the YNG-group. A Student's *t*-test was also conducted to compare the YNG and older groups prior to division by LPA. An additional table with the fold-difference for significant differences between YNG, OHM, and OLM groups is included in the supplemental material (Supplemental Table 2). All data and R-code are provided in the supplemental material as well. Traits that failed the Shapiro-Wilk normality test were log transformed.

3. Results

3.1. Division into subgroups by latent profile analysis

Univariate clustering with age sorted the samples first into younger and older subgroups and then LPA sorted the older group into strong and weak subgroups (Fig. 1). Briefly, the single variate LPA (or univariate cluster analysis) was conducted with age, which split the data set into a younger (Y, n = 11) and an older group (n = 22) (Fig. 1B). For the older group, LPA was conducted using three mechanical properties (whole bone strength, tissue-level strength, and tissue-level post-yield strain) which identified two groups (Fig. 1C), an older group with higher mechanical properties (OHM, n = 16) and an older group with lower mechanical properties (OLM, n = 6). Fig. 1 B–C, depict the division into older and younger using age (Fig. 1B), then division of the older group by mechanical properties (Fig. 1C). All data in Figs. 1C through 7 are presented as z-scores, plotting each sample as it's standard deviation from the mean of the YNG group, for ease of comparing differences in multiple traits among groups. A table with the unscaled, averaged values and percent differences between each group is provided in the

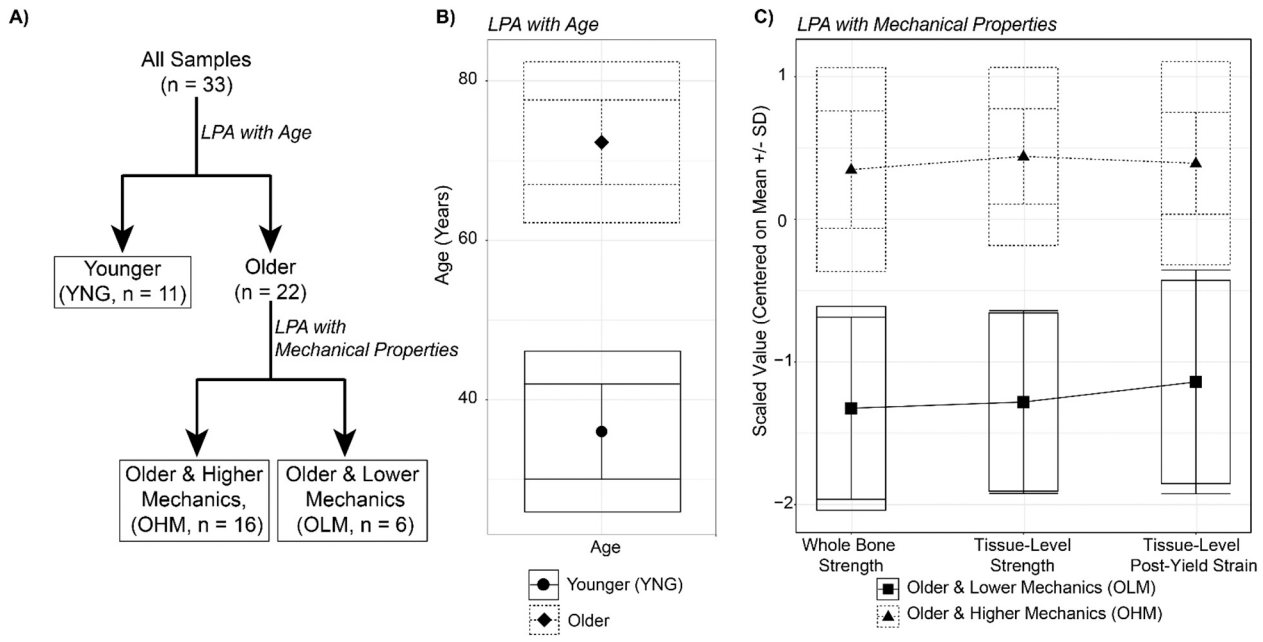

Fig. 1. Identification of subgroups by LPA: A) Graphic illustrating the division of samples by latent profile analysis (LPA) into B) younger (YNG) or older classes based on age, and C) older with higher mechanical properties (OHM) or older with lower mechanical (OLM) properties using whole bone strength, tissue-level strength, and tissue-level post-yield strain as the determinative properties. When examining the LPA of multiple traits (C), the data is scaled to center the mean of the YNG group at 0 and plotted as the z-score, each standard deviation from the mean. Bars = 95 % CI; Box = +/−1 Std. Dev.

supplemental (Supplemental Table 1) and Table 1 provides the unscaled averaged values for each trait where significant differences were observed between YNG and OHM or OLM.

Comparing the younger group to the older group before dividing into the OLM and OHM sub-groups, the older group demonstrated no significant difference in whole bone strength ($p = 0.259$), but did demonstrate 22.4 % lower tissue-level strength ($p = 0.022$), and 37.0 % lower tissue-level post-yield strain ($p = 0.002$) compared to the younger group (Table 1, Supp Table 2). Additionally significant differences between this undivided older group and YNG were detected, including a 20.1 % greater Log(Porosity) ($p = 0.004$), 8.4 % greater Log(CPS) ($p < 0.001$), 2.3 % lower Ct.TMD ($p = 0.018$) and 9.7 % greater Log(PEN) ($p < 0.001$). No significant difference between the YNG and older group was observed for marrow area ($p = 0.471$), ash content ($p = 0.446$), and water content ($p = 0.123$).

3.2. Comparison of mechanical, morphological, and compositional traits

The average age of the OHM (71.8 ± 10.2 years, $p < 0.001$) and OLM (75.6 ± 10.0 years, $p < 0.001$) groups was significantly greater than the age of the younger group (37.5 ± 9.5 years), but there was no significant difference ($p = 0.558$) in age between OHM and OLM groups (Fig. 2). Body weight, height and BMI did not differ among the groups.

Comparing the mechanical properties between the OLM and OHM groups confirmed the LPA sorted the samples into groups with significantly different mechanical properties; the OLM group compared to YNG showed a 33.6 % lower whole bone strength ($p = 0.016$), 48.4 % lower tissue-level strength ($p < 0.001$), and 59.9 % lower tissue-level post-yield strain ($p < 0.001$, Fig. 3). Likewise, compared to OHM, the OLM group exhibited 29.5 % lower whole bone strength ($p = 0.037$), 39.8 % lower tissue-level strength ($p = 0.006$), and 42.8 % lower tissue-

Table 1
Comparison of key traits with unscaled values: younger (YNG, n = 11), older (n = 22), older with higher mechanics (OHM, n = 16), and older with lower mechanics (OLM, n = 6). Data is presented as the mean (standard deviation) for each group. a: significantly different from YNG, $p < 0.05$, between YNG and Older with Student's *t*-test. b: significantly different from YNG, $p < 0.05$. c: significant difference between OHM and OLM, $p < 0.05$, 1-way ANOVA with Tukey's post-hoc for YNG, OHM, OLM. Asterisks indicate marginal significance $p < 0.10$. Ct.TMD – cortical tissue mineral density. CPS – cortical pore score. PEN – pentosidine.

	Units	Younger (YNG)	Older	Older higher mechanics (OHM)	Older lower mechanics (OLM)
		n = 11	n = 22	n = 16	n = 6
Age	*Years*	37.5 (9.5)	72.7 (10.0)[a]	71.8 (10.2)[b]	75.6 (10.0)[b]
Whole bone strength	*N·m*	390.7 (120.6)	342.1 (69.8)	367.9 (51.4)[c]	259.4 (57.0)[b,c]
Tissue-level strength	*MPa*	136.4 (43.0)	105.8 (26.5)[a]	116.9 (16.7)[c]	70.4 (20.1)[b,c]
Tissue-level post-yield strain		0.019 (0.006)	0.012 (0.004)[a]	0.013 (0.003)[b,c]	0.008 (0.003)[b,c]
Marrow area	cm^2	172.1 (66.7)	185.9 (47.5)	171.4 (37.9)[c]	232.5 (48.3)[b*,c]
Log (Porosity)	*Log(%)*	−1.29 (0.23)	−1.03 (0.33)[a]	−1.08 (0.33)[c*]	−0.85 (0.26)[b,c*]
Log (CPS)		3.44 (0.16)	3.73 (0.19)[a]	3.68 (0.19)[b,c*]	3.87 (0.11)[b,c*]
Ct.TMD	*mg/HA*	1196.1 (29.7)	1168.3 (49.1)[a]	1180.9 (43.7)[c]	1127.9 (47.0)[b,c]
Ash content	*%*	58.2 (1.4)	58.5 (1.3)	58.8 (1.1)[c*]	57.4 (1.6)[c*]
Water content	*%*	10.3 (1.1)	9.5 (1.7)	9.0 (1.6)[c,b*]	10.8 (1.6)[c]
Log(PEN)	*Log(mol/mol collagen)*	−3.46 (0.18)	−3.13 (0.18)[a]	−3.09 (0.18)[b]	−3.25 (0.10)[b]
Log(PEN/total enzymatic)		−3.29 (0.19)	−2.92 (0.12)[a]	−2.90 (0.13)[b]	−2.99 (0.08)[b]
Log(PEN/mature)		−3.10 (0.18)	−2.74 (0.12)[a]	−2.72 (0.12)[b]	−2.80 (0.08)[b]
Log(PEN/total pyridinoline)		−2.72 (0.14)	−2.38 (0.13)[a]	−2.36 (0.13)[b]	−2.42 (0.11)[b]

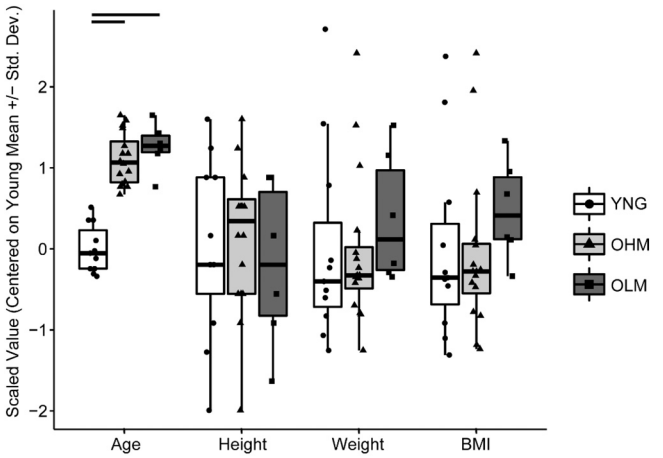

Fig. 2. Comparison of anthropometric traits: younger (YNG, n = 11), older with higher mechanical properties (OHM, n = 16), and older with lower mechanical properties (OLM, n = 6). Solid lines indicate $p < 0.05$ and dashed lines indicate $p < 0.10$ for a one-way ANOVA with Tukey's post-hoc. Data were scaled to the z-score, centering on the mean of the YNG group for each variable.

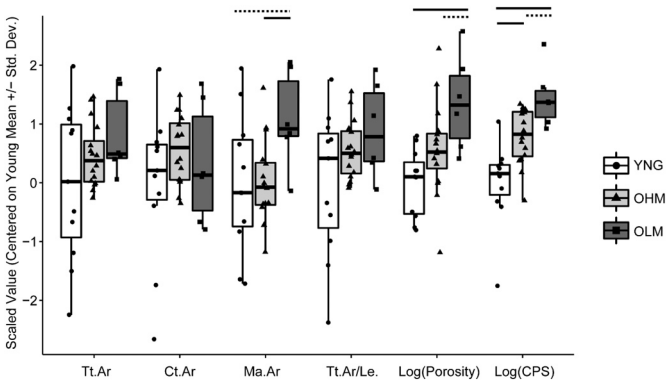

Fig. 4. Comparison of morphological traits: younger (YNG, n = 11), older with higher mechanical properties (OHM, n = 16), and older with lower mechanical properties (OLM, n = 6). Solid lines indicate $p < 0.05$ and dashed lines indicate $p < 0.10$ for a one-way ANOVA with Tukey's post-hoc. Data were scaled to the z-score, centering on the mean of the YNG group for each variable. Tt.Ar = total area; Ct.Ar = cortical area; Ma.Ar = marrow area, Le. = length, CPS = Cortical Pore Score.

level post-yield strain ($p = 0.012$). In contrast, the OHM group did not show a difference in either whole bone strength ($p = 0.767$) or tissue-level strength ($p = 0.084$) compared to the YNG group. The OHM group did show a 29.9 % lower post-yield strain compared to the YNG, ($p = 0.007$). Thus, in comparison to the YNG group, the OLM group was weaker and more brittle, whereas the OHM group was only slightly more brittle, but not weaker.

The three groups did not show significant differences in Tt.Ar, Tt.Ar/Le, or Ct.Ar, but did show differences in Ma.Ar and porosity (Fig. 4). Ma.Ar was 35.7 % greater ($p = 0.049$) for the OLM group compared to the OHM group (Fig. 4). The OLM group showed 34.2 % greater Log(porosity) compared to the YNG group ($p = 0.002$), whereas the OHM group did not show significant a difference in Log(porosity) relative to YNG ($p = 0.138$). When accounting for the size and geometric location of the pores, the OHM ($p = 0.004$) and OLM ($p < 0.001$) groups showed 7.1 % and 12.6 % greater Log(CPS) values compared to the YNG group, respectively.

The OLM group showed 5.7 % lower Ct.TMD compared to the YNG ($p < 0.001$) and 4.5 % lower Ct.TMD compared to OHM ($p = 0.005$, Fig. 5). No difference in Ct.TMD was observed between the YNG and OHM group ($p = 0.517$). The OLM group also showed a 20.0 % greater water content compared to the YNG group ($p = 0.048$), but no significant difference in either ash content or organic content.

There was no difference in individual enzymatic cross-links (Fig. 6) or in any ratio of enzymatic cross-links (Supplemental Fig. 1) among the subgroups. However, levels of Log(PEN) (Fig. 6, $p < 0.001$, $p = 0.008$), Log(PEN/total enzymatic cross-links), Log(PEN/mature cross-links) and Log(PEN/pyridinolines) were significantly greater for both OHM and OLM subgroups compared to the younger group ($p < 0.001$ for all, Fig. 7). These levels of Log(PEN) and PEN ratios did not differ between the OHM and OLM subgroups.

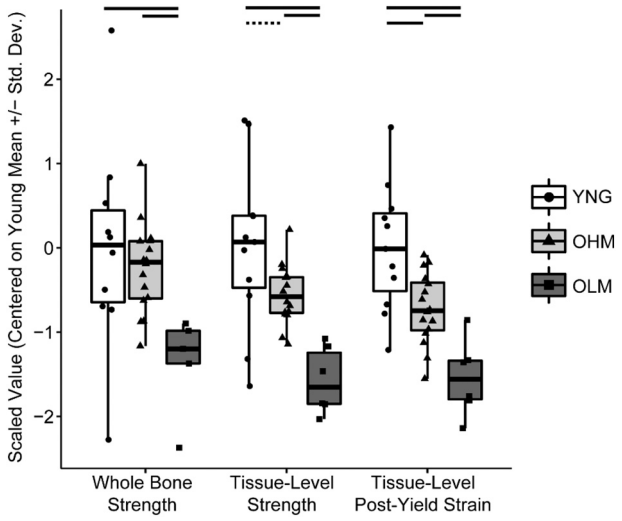

Fig. 3. Comparison of whole-bone and tissue-level mechanical properties: younger (YNG, n = 11), older with higher mechanical properties (OHM, n = 16), and older with lower mechanical properties (OLM, n = 6). Solid lines indicate $p < 0.05$ and dashed lines indicate $p < 0.10$ for a one-way ANOVA with Tukey's post-hoc. Data were scaled to the z-score, centering on the mean of the YNG group for each variable.

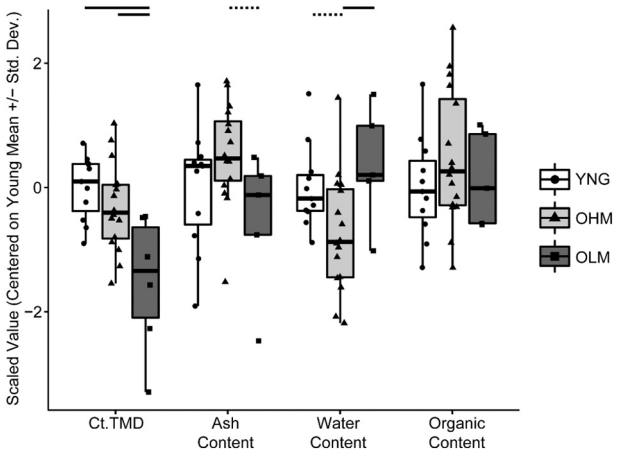

Fig. 5. Comparison of mineral density and ash/water/organic content: younger (YNG, n = 11), older with higher mechanical properties (OHM, n = 16), and older with lower mechanical properties (OLM, n = 6). Solid lines indicate $p < 0.05$ and dashed lines indicate $p < 0.10$ for a one-way ANOVA with Tukey's post-hoc. Data were scaled to the z-score, centering on the mean of the YNG group for each variable. Ct.TMD – cortical tissue mineral density.

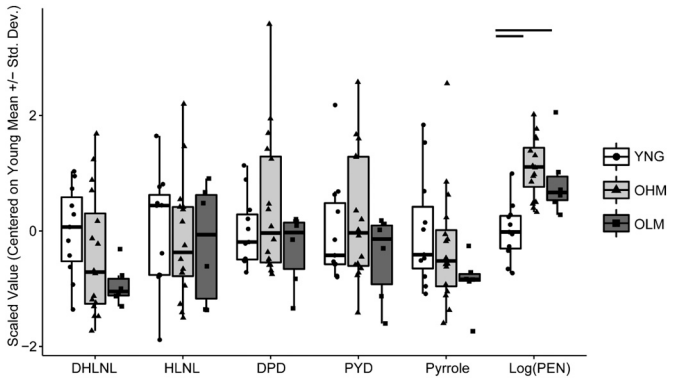

Fig. 6. Comparison of collagen cross-links: younger (YNG, n = 11), older with higher mechanics (OHM, n = 16), and older with lower mechanics (OLM, n = 6). Divalent immature enzymatic cross-links: dihydroxy-lysinonor-leucine (DHLNL), hydroxy-lysino-norleucine (HLNL), mature tri-valent enzymatic cross-links: pyridinoline (PYD), deoxypyridinoline (DPD), pyrroles and advanced glycation end-product pentosidine (PEN). Data were scaled to the z-score, centering on the mean of the YNG group for each variable. Solid lines indicate $p < 0.05$ for a one-way ANOVA with Tukey's post-hoc.

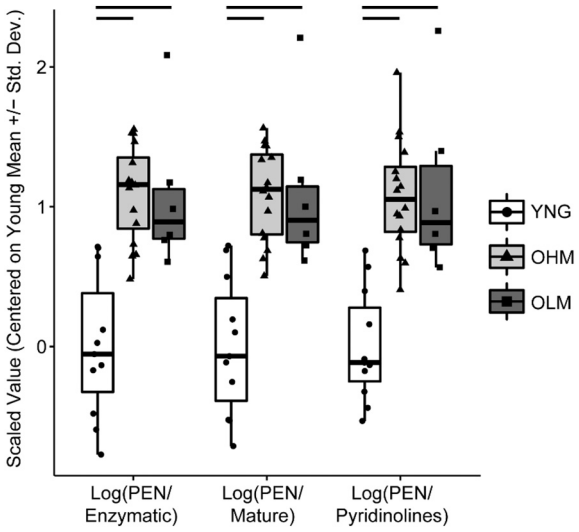

Fig. 7. Comparison of pentosidine (PEN) cross-link ratios: younger (YNG, n = 11), older with higher mechanics (OHM, n = 16), and older with lower mechanics (OLM, n = 6). Data were scaled to the z-score, centering on the mean of the YNG group for each variable. Solid lines indicate $p < 0.05$ for a one-way ANOVA with Tukey's post-hoc.

4. Discussion

This is the first study we are aware of that uses experimentally measured mechanical properties of human cadaveric bone as functional outcomes to cluster and identify underlying subgroups. Using an unsupervised statistical approach for group stratification, we identified two subgroups within the older cohort of cadaveric femora, one with higher mechanical properties (OHM) and one with lower mechanical properties (OLM) (Fig. 1c). The probability of group inclusion was >95 % for all samples except one which had a 75 % probability of being in OLM, indicating that the stratification was robust. Prior studies have typically treated older individuals as a homogenous group with the goal of identifying structural and material traits contributing to the strength-decline of bone with aging [3,24,25]. When comparing the mechanical, structural and compositional traits between the younger and older groups prior to LPA division, the differences found were consistent with prior studies [26,27]. Dividing samples by age is not devoid of utility as many traits were different between the older and younger groups (Table 1). However, identification of subgroups within the older group provides more granularity to observe increased Log(Porosity), and decreased Ct.TMD as changes unique to the OLM group whereas other traits like increased Log(PEN) were increased in both OHM and OLM groups. The majority of studies examining decreased bone strength with age have focused on population mean values, not taking into account the inter-individual variation in strength decline. Without this division into OHM and OLM subgroups, phenotypical differences between the older subgroups that may impact mechanical properties would be missed, such as the increased Ma.Ar and water content in the OLM compared to OHM. The existence of these separate subgroups and their subsequent differences in composition or morphology necessitate a shift away from a "one-size-fits-all" approach for fracture risk identification.

The OHM group showed no significant difference in whole bone strength or tissue-level strength compared to the YNG group, but did show a small but significant reduction in post-yield strain. The OHM group had femoral diaphyses that were as strong as the younger group, but with a slightly more brittle tissue (Fig. 3). Although cadaveric tests do not capture longitudinal age-related changes in bone properties, the lack of strength differences between the OHM and YNG groups, and between the YNG and older group prior to LPA division (Table 1) does suggest that a large proportion of male bones maintain strength with aging. In contrast, the 6 samples comprising the OLM subgroup were identified as having lower whole bone strength, tissue-level strength and tissue-level post-yield strain compared to the YNG and OHM group (Fig. 3). Although the fracture history of our donor population is not known outside of the lack of evidence of a prior fracture callus in the bones examined, the proportion of samples stratified into the OLM group (6/22 or ~27 %) is consistent with the 25 % residual lifetime risk of fracture for men over 60 [28]. Thus, our analysis revealed that only a small subgroup of older male bone samples showed reduced bone strength compared to the younger group. This outcome supports the general concept that an older population is not homogenous, but is heterogeneous regarding age-related changes in bone strength [5,6,11]. Our results suggest that it is important to first stratify a population into subgroups when attempting to identify structural and material traits contributing to strength-decline. This approximate 25:75 ratio of samples stratified into the OLM and OHM subgroups may be confirmed with future studies with a larger dataset but should also be expanded to include both sexes and a more ethnically diverse donor population.

We next compared the structural and material properties of the YNG, OHM and OLM groups to refine our understanding of the age-related changes that may contribute to strength-decline. Compared to the younger group, both the OHM and OLM exhibited increased Log(CPS) (Fig. 4), increased PEN (Fig. 6), and increases in all PEN cross-link ratios (Fig. 7). The structural and material trait differences that were unique to the OLM group compared to the YNG included decreased Ct.TMD (Fig. 5), and increased Log(Porosity) (Fig. 4). The greater porosity of the OLM group may help explain why this group also showed a small, yet marginally significant increase in Ma.Ar ($p = 0.065$) relative to the YNG group, but significantly greater Ma.Ar than the OHM group (Fig. 4). Age-related increases in porosity are concentrated near the endosteum [29], resulting in reductions in cortical thickness and increases in marrow area [30,31].

The importance of porosity in reducing bone strength has been well established [3]. Alternatively, these results may be interpreted as the increase in CPS in the OHM group compared to YNG did not increase to an amount that would negatively affect whole-bone strength, suggesting there is a threshold level of porosity that is needed to cause a decline in bone strength. CPS provides a measure of both the amount and location of pores [6], but by itself does not distinguish whether the increased porosity is located in a geometric position to contribute to strength-decline. The endocortical location of porosity helps to minimize the impact of porosity on strength. Finding an increase in CPS for the OHM

group, but without a concurrent difference in strength suggests further research is needed to better refine our understanding of the type and location of pores that contribute to strength-decline. In this dataset, porosity only significantly correlated with tissue-level strength and tissue-level strain, not whole bone strength [5]. Porosity and cross-sectional morphology can vary greatly between individuals of the same age [31]. These age-related trait changes depend on whole bone geometry, even for tissue-level mechanical properties [5,6]. Additionally, there were several individuals in the OHM group with high porosity values, but these individuals did not fall into the OLM group, highlighting the importance of multivariate, rather than univariate, predictors to identify fracture risk. This finding prompts the question of what other traits besides porosity can explain the difference in the age-related decline in strength.

Increased pentosidine (Fig. 7), and increased Log(CPS) were the only trait differences detected between the OHM and the younger group. Given that the OHM group did not exhibit a decrease in whole bone strength but did have a marginal decrease ($p = 0.084$) in tissue-level strength and significantly decreased tissue-level post-yield strain relative to YNG, traits like PEN and Log(CPS) may be important to the age-related decline in tissue-level mechanical properties. PEN correlates with age [5], and given that there was no significant difference in age between the OHM and OLM groups, the lack of any significant difference in PEN content between OLM and OHM is logical. The lack of difference in PEN content between OLM and OHM suggests increased PEN (as a representative AGE) may not be sufficient alone to affect whole bone strength. This is contradictory to the current understanding of the role of AGEs in bone mechanics [8]. Increased PEN may still contribute to the decline in tissue-level mechanical properties with age observed here, but may only be detrimental to whole bone strength in combination with other factors like increased porosity. In this study, the samples were screened to exclude individuals with a disease known to impact bone metabolism, including diabetes where increases in PEN at higher levels may be more consequential [32]. Additionally, PEN is used as a surrogate marker for all AGEs. PEN may not be the most critical AGE for mechanical performance, and is present at lower concentrations compared to other AGEs such as carboxymethyl lysine in human tibiae [33]. Prior studies have also examined fractured vs. non-fractured cohorts [34–37]. PEN concentrations and ratios of PEN/total enzymatic cross-links are higher in a fractured cohort compared to a non-fractured cohort, but there are minimal differences in enzymatic cross-linking between fractured and non-fractured cohorts [35].

The increase in water content in the OLM group compared to the OHM was surprising as the water content of human cortical femoral bone decreases with age [38] and decreasing water content in cadaveric human cortical bone is associated with lower yield strength, work to fracture and increased stiffness [39]. This increase in water content in the OLM group may be secondary to the decreased Ct.TMD, and increased porosity, however neither of these variables correlated against water content [5]. The water content of the OHM group was slightly lower ($p = 0.087$, Fig. 5) compared to YNG, as would be expected with an increase in age, but this too was surprising given the increased water content of OLM, and lower mechanical properties in the OLM group.

The goal of the current study was to test whether an older cohort of human male femora could be stratified into subgroups with different mechanical properties. The scope of this study is constrained to experimentally measured mechanical properties of cadaveric bone as outcomes. Numerous factors beyond the mechanical properties presented here, such as bone fracture toughness, neuromuscular control, vision changes, and many others can contribute to an increase in falls and subsequent fracture risk. By isolating the structural and material properties of bone, these results support that heterogeneity in bone strength exists within similarly aged cohorts. Studies examining the relative importance of bone strength contextualized with other age-related changes in muscle loss or diminished motor control as they relate to fall and fracture risk are warranted.

A recent study employed a clustering technique to a large cohort, relying on HR-pQCT and clinical outcomes, revealing a healthy (normal volume and density), a low-volume, and a low-density phenotype [11]. In conjunction with the results reported herein, this highlights the need to avoid a "one-size fits all" approach based on areal BMD alone, and emphasizes a need to look at other morphological and compositional traits as indicators of fracture risk. The identification of the key traits that negatively contribute to the decline in bone mechanical properties with age, not just traits that are altered with age is critical for the identification of individuals at greatest risk of fracturing. Predictive tools like FRAX® incorporate not only femoral neck BMD, but add other predictors like height, weight, sex, and lifestyle behaviors [40]. However, these predictive tools do not identify fracture risk well in men [41], opening up the possibility for developing future predictive tools that identify key traits that lead to decreased bone mechanical properties and increased fracture risk. This LPA approach presents a new tool for the bone biomechanics field to sort complex hierarchical traits among diverse populations and distill these into the predominant traits driving the most adverse functional outcomes. Additionally, incorporating clinical measures of fracture risk with DXA scans to these cadaveric studies would allow us to directly identify which individuals may be missed by current diagnostic tools and drive the field towards improved fracture risk identification.

Although the small sample size in the OLM group may limit the statistical power of the study, by segmenting the older group into two groups the overall variability for each mechanical property decreased compared to a pooled older group combining OHM and OLM (Table 1). Therefore, the relative overall power of this study was not compromised following the LPA sorting. The younger group exhibited high variability in mechanical properties as well as bone morphology (Fig. 4). This observation was explored in the previous publication of this data set, where stratification of whole bone strength, tissue-level strength and tissue-level post-yield strain were apparent between wide and narrow bone phenotypes before 50 years of age [5]. Therefore, subgroups may also exist within a younger population and could be probed in a larger dataset. Differences observed between the OHM and OLM groups such as increased Ma.Ar, if confirmed in a larger dataset may serve as leading or lagging indicators of an OLM phenotype, refining the scope of clinical traits to examine for earlier treatment options.

5. Conclusions

A priori sorting of mechanical performance using latent profile analysis among a wide range of ages in male femora highlighted an age-dependent split in the dataset, presenting 3 distinct groups: younger, older with higher mechanical properties and older with lower mechanical properties. Taking these 3 groups and testing for phenotypic differences revealed several bone morphometric and compositional traits that were unique to low mechanical performance with age, including increased porosity, marrow area, water content and decreased cortical tissue mineral density. Meanwhile, pentosidine and cortical pore score - a measure of pore distribution - were the only traits that were significantly increased in both the OHM and OLM groups compared to YNG. This LPA approach presents a new tool for the bone biomechanics field to sort complex hierarchical traits within populations with the goal of identifying structural and material property changes contributing to age-related strength-declines and fracture risk.

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CRediT authorship contribution statement

Morgan W. Bolger: Methodology, Investigation, Formal analysis, Writing – original draft, Visualization. **Genevieve E. Romanowicz:** Methodology, Writing – review & editing. **Erin M.R. Bigelow:** Methodology, Investigation, Formal analysis, Writing – review & editing.

Ferrous S. Ward: Investigation, Writing – review & editing. **Antonio Ciarelli:** Investigation, Writing – review & editing. **Karl J. Jepsen:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **David H. Kohn:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts of interest.

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