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Bone collagen network integrity and transverse fracture toughness of human cortical bone

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ABSTRACT

Greater understanding of the determinants of skeletal fragility is highly sought due to the great burden that bone affecting diseases and fractures have on economies, societies and health care systems. Being a complex, hierarchical composite of collagen type-I and non-stoichiometric substituted hydroxyapatite, bone derives toughness from its organic phase. In this study, we tested whether early observations that a strong correlation between bone collagen integrity measured by thermomechanical methods and work to fracture exist in a more general and heterogeneous sampling of the population.

Neighboring uniform specimens from an established, highly characterized and previously published collection of human cortical bone samples (femur mid-shaft) were decalcified in EDTA. Fifty-four of the original 62 donors were included (26 male and 28 females; ages 21–101 years; aging, osteoporosis, diabetes and cancer). Following decalcification, bone collagen was tested using hydrothermal isometric tension (HIT) testing in order to measure the collagen's thermal stability (denaturation temperature, T_d) and network connectivity (maximum rate of isometric tension generation; Max.Slope). We used linear regression and general linear models (GLMs) with several explanatory variables to determine whether relationships between HIT parameters and generally accepted bone quality factors (e.g., cortical porosity, pentosidine content [pen], pyridinoline content [pyd]), age, and measures of fracture toughness (crack initiation fracture toughness, K_{init}, and total energy release/dissipation rate evaluated at the point of unstable fast fracture, J-int) were significant.

Bone collagen connectivity (Max.Slope) correlated well with the measures of fracture toughness (R^2 = 24–35%), and to a lesser degree with bound water fraction (BW; R^2 = 7.9%) and pore water fraction (PW; R^2 = 9.1%). Significant correlations with age, apparent volumetric bone mineral density (vBMD), and mature enzymatic [pyd] and non-enzymatic collagen crosslinks [pen] were not detected. GLMs found that Max.Slope and vBMD (or BW), with or without age as additional covariate, all significantly explained the variance in Kinit (adjusted-R^2 = 36.7–49.0%). Also, the best-fit model for J-int (adjusted-R^2 = 35.7%) included only age and Max.Slope as explanatory variables with Max.Slope contributing twice as much as age. Max.Slope and BW without age were also significant predictors of J-int (adjusted-R^2 = 35.5%).

In conclusion, bone collagen integrity as measured by thermomechanical methods is a key factor in cortical bone fracture toughness. This study further demonstrates that greater attention should be paid to degradation of the overall organic phase, rather than a specific biomarker (e.g. [pen]), when seeking to understand elevated fracture rates in aging and disease.

1. Introduction

There is still a need for a more comprehensive understanding of the determinant mechanisms of poor bone quality and causes of elevated fracture risk in aging and certain 'bone affecting' diseases because of the economic and societal burdens that fractures impose. Western populations are becoming older. For example, the proportion of Canada's population over 65 years old is expected to double, reaching approximately 8.7 million individuals over the next 25 years and approaching 25% of the population [1]. Associated with this increase will be an increased burden of aging and chronic 'bone affecting' diseases (e.g. frailty, diabetes [2–4], osteoporosis, chronic kidney/renal disease

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[5]) that can lead to fragility fractures and associated morbidity and mortality [6–8].

While current x-ray-based clinical tools have been successful in detecting cases of relatively severe bone loss (low areal bone mineral density or aBMD), they do a poor job of fracture prediction on an individual patient basis. Over half of all non-vertebral fractures in people over the age of 55 years occur in people with normal aBMD [9]. In normal aging, fracture risk increases 4× every 20 years of age, and does so independent of aBMD [10,11]. In fact, the decline in bone strength is disproportionately faster than the decline in bone volume [10]. Patients with bone affecting diseases such as diabetes or renal osteodystrophy do not necessarily present with low clinical aBMD but do experience higher rates of fractures [2,5,12].

One reason for the poor ability of aBMD or even volumetric BMD from quantitative computed tomography (CT) scans to predict a patient's risk to fracture is that x-ray based tools do not necessarily measure 'bone quality' and moreover do not 'see' the organic phase of the bone (which constitutes 45% of cortical bone volume) [13]. With respect to bone mechanics and bone quality, microstructure plays a definite role in bone fracture toughness [14], but additionally, the organic phase is widely acknowledged as being important - *if not critical* - to the fracture resistance of what is abstractly a micro-structurally complex, micro-cracking, quasi-brittle material that leverages numerous intrinsic and extrinsic mechanisms to inhibit and resist crack growth [15]. Modern mechanics studies of cortical bone indicate that organic factors, invisible to x-ray based clinical tools, contribute to fracture resistance [15–18]. In fact, they describe critical physical mechanisms (micro-damage process zone, fibril bridging of a crack) by which bone collagen plays key roles in stabilizing cracks against unstable growth [15,19]. Yet, most studies have neglected direct examination of the integrity and mechanical properties of the organic phase. A few exceptions exist, including work by Peter Zioupos [20], Xiaodu Wang [21], and recent studies probing bone with Raman spectroscopy [22,23]. The earlier studies from the 1990s to 2000s of human cadaveric bone demonstrated strong correlations between bone collagen nativity and connectivity and cortical bone fracture toughness, and captured the effects of the degradation that occurs with aging [20,21].

If the integrity of the bone collagen is therefore critical to bone fracture resistance, and there is evidence of degradation with aging, then what are the key mechanisms of the degradation? Many post-translational organic phase modifications of bone collagen have been reported as being associated with aging and disease. Certainly, the most examined are covalent crosslinks – both those provided by the highly regulated action of the enzyme lysyl-oxidase (LOX) and those that form spontaneously and are associated with disease states involving elevated oxidative stress and/or hyperglycemia [24]. These are the Advanced Glycation Endproducts (AGEs). The importance of LOX-derived covalent crosslinking to bone strength and fracture toughness is well characterized. This is built upon evidence from lathyrism models [25] and recent fracture toughness studies on human bone by Gauthier et al. [26] who reported a significant correlation between enzymatic collagen crosslinking maturation and fracture toughness when pooling measurements from the femoral and radial diaphysis. On the other hand, the idea of over-crosslinking by AGEs is a widely accepted paradigm. This is built upon two pieces of evidence: 1) pentosidine, a readily measurable AGE crosslink, is known to accumulate in bone with aging and bone affecting diseases such as diabetes [21,24], and 2) in vitro ribation/glycation models (where bone is incubated in ribose or glucose solutions and accumulates pentosidine and other ribation products (adducts and crosslinks)) demonstrated that ribation can over stabilize and crosslink bone collagen, limit its deformation during loading and lead to loss of ductility and fracture toughness of bone [27–29].

However, these ideas of over-crosslinking of bone collagen leading to loss of toughness are inconsistent with a) the positive effect of LOX crosslinks on bone (and bone collagen) strength and fracture toughness, and b) the well documented degradation of bone collagen integrity, more specifically the degradation of the collagen molecules and their connectivity [20,21]. In fact, the concentrations of pentosidine measured in aged human bone are small compared to the concentration of all of the LOX-derived crosslinks found in bone (mmol per mol versus mol per mol respectively) [30,31]. The above facts highlight the unmet need for greater mechanistic understanding of the causes of bone fragility linked to the organic phase.

1.1. Objective

A) Test whether the observations by Zioupos [20] and Wang [21] are repeated in a more general and heterogeneous sampling of the population, including males and females, normal aging, osteoporosis, diabetes and cancer. B) Test the relative strengths of bone collagen integrity parameters, and various putative bone quality determinants mentioned above, towards explaining transverse cortical bone fracture toughness.

1.2. Hypothesis

A) A relatively strong correlation between cortical bone fracture toughness and bone collagen network integrity will be confirmed statistically. B) Bone collagen network integrity will contribute to explaining variation in fracture toughness more than all other determinants (including the advanced glycation end-product biomarker, pentosidine).

2. Materials and methods

2.1. Specimen preparation, micro-computed tomography, and fracture toughness testing

Femurs or femur mid-shafts from sixty-two human donors (30 male, age = 63.5 ± 23.7 [range 21–98] years and 32 female, age = 64.4 ± 21.3 [range 23–101] years) were obtained from the Musculoskeletal Transplant Foundation (Edison, NJ), the Vanderbilt Donor Program (Nashville, TN), and the National Disease Research Interchange (Philadelphia, PA) and stored fresh-frozen [14,32]. Fracture toughness testing was attempted for a cortical bone specimen from an additional male 80 year old donor, but the force dropped around the start of crack growth prior to the unloading compliance step. This did not occur for the other 62 mechanical tests. Since there were two male, 81 year old donors among the 62 donors, this donor with the improper test was excluded from the previous studies [14,32] and the present study. The heterogeneous group of donors included healthy donors as well as those with a history of cancer (including chemotherapy), diabetes types I and II, and diagnosed osteoporosis. From these donors, 54 specimens (28 male, age = 62.0 ± 24.7 [range 21–98] years and 26 female, age = 64.0 ± 21.9 [range 23–101] years) were successfully characterized in this study (see Results section). The micro-computed tomography, fracture toughness, bound and pore water, and pentosidine data described below are the same as those presented previously in Granke et al. [32] To this previously published data, we have now added novel hydrothermal isometric tension testing data, mature enzymatic collagen crosslinks, and new statistical analyses.

The preparation of the mechanical test specimens and measurement methods are extensively described elsewhere [14,32] and briefly summarized here. Single-edge notched beam (SENB) specimens (length × thickness × width: 19 to 31 mm × 1.9 to 3.3 mm × 4.0 to 6.8 mm) were machined from the lateral quadrant of each femur at the mid-shaft (Fig. 1(A)). A notch was cut at mid-span of the machined SENB specimen, using a low-speed, water-irrigated bone cutting saw with a diamond-embedded circular blade, such that it originated on the endosteal surface and traversed ~1/4th the sample width, W. The notch was further sharpened with a razor blade using a diamond

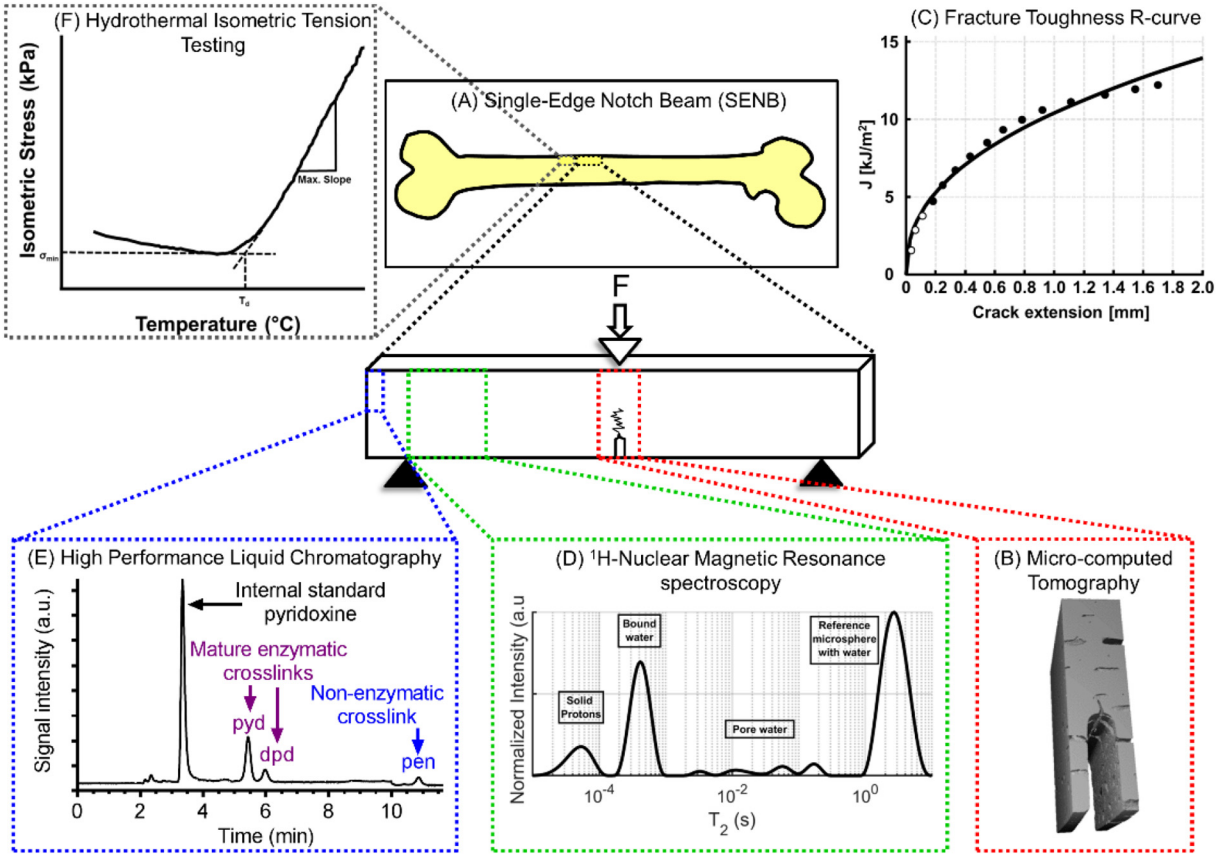

Fig. 1. Methods scheme for this study. (A) Specimens were harvested from the lateral mid-shaft diaphysis of 62 human femora. (B) After machining, the notch region was imaged with μCT in order to measure cortical porosity and vBMD. (C) Fracture toughness R-curves were measured using standard methods. (D) Water contents were measured using ^{1}H NMR relaxometry methods. (E) Bone collagen crosslinks were measured using HPLC methods. (F) Bone collagen integrity was assessed using Hydrothermal Isometric Tension (HIT) testing, measuring its thermal stability (Td) and network connectivity (Max.Slope).

suspension solution as coolant to create the micro-notch. The region of the bone specimen ahead of the sharpened notch was imaged with micro-computed tomography (μCT) to determine cortical porosity (Ct.Po.) and local volumetric bone mineral density (vBMD) [32]. Briefly, the μCT scans were conducted using a μCT50 from Scanco Medical AG using a 5 micron isotropic voxel size and the following x-ray beam settings (tube voltage = 90kVp; beam current = 200 μA; 2048 samples per 1000 projections per 180° rotation; integration time = 400 ms). See Fig. 1(B).

Next, a progressive, loading/partial unloading/reloading scheme in a three-point bending configuration was used to initiate and propagate a crack from the razor sharpened notch through the cortical bone such that crack extension was transverse to the osteonal direction and in the radial direction (starting from the endocortex). A non-linear fracture mechanics approach based on the analysis of the resulting R-curve (J-integral vs. crack length) provided the resistance (elastic plus 'inelastic' contributions) to initiate (K_{init} based in J_{1c}) a crack from the notch as well as the total energy dissipation rate during fracture evaluated at the point of unstable fast fracture (J-int; analogous to work-to-fracture measures). See Fig. 1(C). These methods are consistent with ASTM Standard E1820-15a (2015) and established in the authors' labs [14,18,32]. Evaluation of crack propagation toughness (d(J-int)/da) was not fruitful due to high variability. After mechanical testing, two segments from each SENB specimen were extracted for further analysis by ^{1}H NMR spectroscopy for quantification of bound water (BW) and pore water (PW) (Fig. 1(D)) and high performance liquid chromatography (HPLC) for pentosidine crosslink content ([pen]) (Fig. 1(E)), respectively. See below. The specimens were stored in phosphate-buffered saline at −20 °C between phases of the experimental protocol.

2.2. ^{1}H NMR and HPLC

Pore water and bound water volumes were measured from one segment of each SENB specimen using our previously published protocols in which transverse relaxation time constant (T_2) from ^{1}H nuclear magnetic resonance (NMR) relaxometry distinguishes proton signals from collagen bound water (~400 ms) and pore water (~1 ms–1 s) [32–34] These volumes were divided by the specimen volume (calculated from Archimedes' principle) to give bound water (BW) and pore water (PW) volume fractions as percentage. Likewise, pentosidine concentration ([pen]) came from using previously published measurements [32] in which another segment of bone (~10 to 50 mg) from each SENB specimen was demineralized in 20% EDTA (0.68 M, pH 7.4) and then hydrolyzed (110 °C, 20 to 24 h) in 6 N HCl (10 mL/mg bone) for high performance liquid chromatography (HPLC) assays. The same assays also provided measurements of pyridinoline (PYD) and deoxypyridinoline (DPD) (supplemental materials). The moles of Hyp per mass of bone calculated from the chromatograms was divided by 14% (amount of Hyp in type I collagen) and by 0.3 (molecular weight of collagen in mg/pmol) to give pentosidine, PYD, and DPD concentration or content as mmol/mol of collagen ([pen],[pyd],[dpd]) [31].

2.3. Hydrothermal Isometric Tension testing

The bone collagen network integrity, in terms of thermal stability and connectivity, were measured using a Hydrothermal Isometric Tension (HIT) testing instrument designed for testing bone collagen [18]. HIT allows evaluation of bone collagen degradation with regards

to a loss of thermal stability and connectivity [17,18,35]. Specimens cut distal of the SENB specimens (Fig. 1(A)) reported above were decalcified using the same protocol as given for HPLC above. Once decalcified, the bone collagen was trimmed with a razor blade into two rectangular beams 1.5 × 1.5 × 20 mm in size. The length was aligned with the long axis of the diaphysis. The decalcified collagen specimens were held at fixed length (i.e. isometric) with a pair of tissue clamps and placed in a distilled water bath. A pre-load of 3 N was applied to each specimen prior to the test beginning. The bath was heated from room temperature to 90 °C at a rate of ~1.5 °C/min. Throughout the test, the contractile behavior of each collagen specimen was monitored with a 722 N load-cell (InterfaceMB-5, Scottsdale, Arizona, USA). Upon heating above a certain temperature, collagen will begin to denature (i.e., melt). As it melts, its semi-crystalline triple helix structure is driven to a random amorphous coil conformation and this change in molecular structure drives the specimen to shrink. However, if the collagen is held under isometric constraint, a contractile force is generated [36,37]. The denaturation (melting) temperature, Td, and maximum rate of isometric tension generation after Td (Max.Slope) are measures of the stability and connectivity of the collagen network, respectively. Max.Slope is thought to be a function of the cross-link density and peptide chain lengths [18,35,38]. See Fig. 1(F).

2.4. Statistical analyses

Since not all the data passed normality (Shapiro-Wilk), linear regressions between max slope and selected bone properties as well as multivariate general linear models (GLMs) with a fracture toughness property as the dependent variable were bootstrapped with 500 replicates. After confirming that the interaction terms were not significant (successively removed in a backward stepwise manner), we used GLMs determine max slope and a selected bone property with and without age were significant covariates ($p < 0.05$) explaining the variance in a fracture toughness property. In the event a covariate was not significant in a GLM, a new GLM was analyzed without it. If 2 variables were not significant, the independent variable with highest p-value was removed. All statistical analyses were performed with STATA (v11.0, StatCorp, College Station, TX). Descriptive statistics of each selected variable (e.g., mean, median, range) can be found in the supplemental materials (Table S1).

3. Results

Of the 62 donors originally sourced, only 54 neighboring lateral femoral diaphysis specimens were successfully characterized for this study. Two specimens were not testable with HIT due to their small size. Another six specimens were excluded due to issues with the HIT testing (premature grip pullout, excessive load cell noise and drift) which were promptly resolved before testing of the remaining specimens. Finally, one specimen had very high porosity at the crack tip (an outlier in terms of porosity), and so linear regressions were analyzed with and without it.

As similarly observed for the original group of human femur specimens [32], both K_{init} ($R^2 = 0.208$, $p < 0.001$) and J-int ($R^2 = 0.114$, $p = 0.008$) declined with donor age. Previous work highlighted good correlations between these two fracture toughness measures and porosity and osteonal area [14]. Since increased porosity lowers the apparent local volumetric density of bone (vBMD) and raises the volume of voids that can be occupied by interstitial fluids (i.e. Pore Water), Ct.Po was not considered in further analyses as it is a strong covariate with volume fraction of pore water (PW) and vBMD.

Bone collagen connectivity, Max.Slope, correlated weakly with volume fraction of bound water (BW) and PW. Including and excluding one donor with excessive porosity (43%), linear correlations were detected ($p < 0.05$) between both fracture toughness measures (Kinit, J-int) and Max.Slope (Table 1 and Fig. 2). Also, the thermal stability, Td, of the bone collagen measured with HIT did not correlate with the fracture toughness measures (Table S2). Max.Slope did not correlate with pentosidine content (Fig. 3) or the mature enzymatic crosslinks (Table 1). The variance for each crosslink measurement was higher than the variance for Max.Slope, BW, and vBMD (Table S1) indicating that the lack of correlation is not due to a limited range in the values of crosslink content.

Table 1
Linear regression results of Max.Slope from HIT testing versus other bone quality factors, fracture toughness properties, and donor age.

Linear regression	N = 54		N = 53 (excludes high Ct.Po)	
Max.Slope vs.	R^2	(p-Value)[a]	R^2	(p-Value)[a]
vBMD	na	(0.927)	na	(0.411)
BW	**6.6**	**(0.045)**	**7.9**	**(0.032)**
PW	**5.5**	**(0.074)**	**9.1**	**(0.013)**
K_{init}	**17.1**	**(0.001)**	**24.4**	**(< 0.001)**
J_{int}	**30.7**	**(< 0.001)**	**35.1**	**(< 0.001)**
age	na	(0.370)	na	(0.338)
[pyd]	na	(0.323)	na	(0.336)
[dpd]	na	(0.624)	na	(0.618)
[pen]	na	(0.283)	na	(0.348)

na = not applicable. Bold indicates a statistically detectable correlation.
[a] Slope is different than zero for p-value < 0.05.

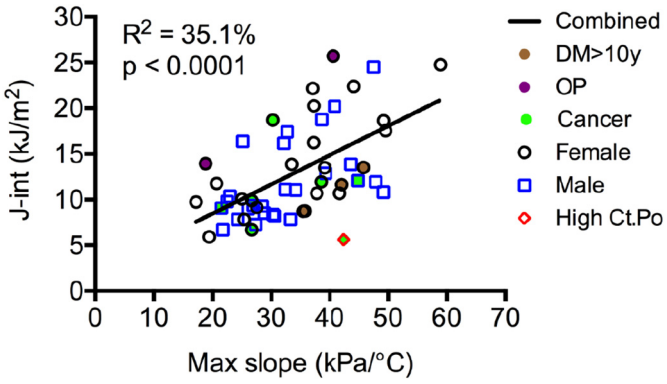

Fig. 2. J-integral fracture toughness at unstable failure (J-Int) versus bone collagen connectivity measure (Max.Slope). A relatively strong correlation was detected over a large range of toughness and connectivity measures from a heterogeneous group of donors. DM = diabetes mellitus, OP = osteoporosis. Circles are females and squares are males. Unfilled circles and squares did not have a documented disease (such as DM, OP or cancer).

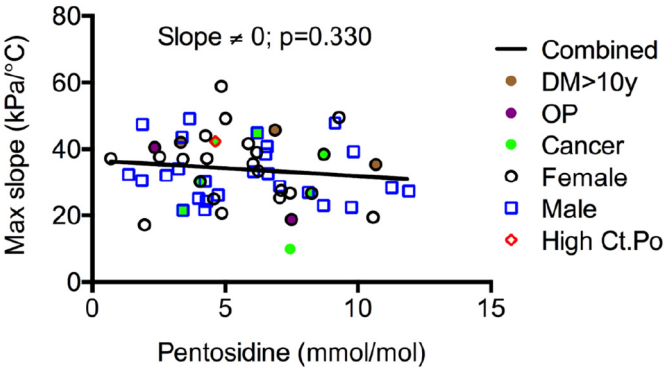

Fig. 3. Bone collagen connectivity (Max.Slope; kPa/°C) versus bone collagen pentosidine content (mmol/mol collagen). Surprisingly, no correlation was detected ($p = 0.477$) over a large range of pentosidine contents due to the heterogeneous group of donors. DM = diabetes mellitus, OP = osteoporosis. Circles are females and squares are males. Unfilled circles and squares did not have a documented disease (such as DM, OP or cancer).

Table 2
General linear model results showing multiple combinations of parameters (age, volumetric bone mineral density (vBMD), bound water (BW), pore water (PW) and connectivity (Max.Slope)) that explain the variance in the fracture toughness properties (K_{init} and J-int) of human cortical bone undergoing transverse fracture.

Fracture property	Linear model[a]			Adj-R^2 (%)
K_{init}	**Age**	**vBMD**	**Max.Slope**	49.0
	($\beta = -0.45$, $p < 0.001$)	**($\beta = 0.43$, $p < 0.001$)**	**($\beta = 0.37$, $p < 0.001$)**	
		vBMD	**Max.Slope**	29.6
		($\beta = 0.39$, $p = 0.012$)	**($\beta = 0.42$, $p < 0.001$)**	
	Age	**BW**	**Max.Slope**	36.7
	($\beta = -0.25$, $p = 0.039$)	**($\beta = 0.31$, $p = 0.023$)**	**($\beta = 0.30$, $p = 0.012$)**	
		BW	**Max.Slope**	33.4
		($\beta = 0.45$, $p < 0.001$)	**($\beta = 0.30$, $p = 0.023$)**	
	Age	**PW**	**Max.Slope**	45.9
	($\beta = -0.34$, $p = 0.001$)	**($\beta = -0.40$, $p = 0.001$)**	**($\beta = 0.28$, $p = 0.022$)**	
		PW	**Max.Slope**	35.7
		($\beta = -0.47$, $p < 0.001$)	**($\beta = 0.30$, $p = 0.021$)**	
J-int	**Age**	*vBMD*	**Max.Slope**	36.5
	($\beta = -0.29$, $p = 0.003$)	*($\beta = 0.14$, $p = 0.467$)*	**($\beta = 0.52$, $p < 0.001$)**	
		vBMD	**Max.Slope**	29.4
		($\beta = 0.12$, $p = 0.497$)	**($\beta = 0.56$, $p < 0.001$)**	
	Age	*BW*	**Max.Slope**	36.6
	($\beta = -0.18$, $p = 0.107$)	*($\beta = 0.18$, $p = 0.164$)*	**($\beta = 0.49$, $p < 0.001$)**	
		BW	**Max.Slope**	35.5
		($\beta = 0.28$, $p = 0.013$)	**($\beta = 0.48$, $p < 0.001$)**	
	Age	*PW*	**Max.Slope**	36.6
	($\beta = -0.25$, $p = 0.007$)	*($\beta = -0.15$, $p = 0.191$)*	**($\beta = 0.49$, $p < 0.001$)**	
		PW	**Max.Slope**	31.8
		($\beta = -0.20$, $p = 0.046$)	**($\beta = -0.51$, $p < 0.001$)**	
	Age		**Max.Slope**	35.7
	($\beta = -0.28$, $p = 0.005$)		**($\beta = 0.52$, $p < 0.001$)**	

Bold indicates a statistically significant explanatory parameter. Italics indicates the parameter is not statistically significant.
[a] β is the standardized coefficient for each independent covariate in the general linear model.

Results from the multivariate GLMs are summarized in Table 2. Note the relatively strong β standardized coefficients for Max.Slope (K_{init}: β = 0.37; J-int: β = 0.52). K_{init} was best explained by a combination of age, vBMD and Max.Slope. J-int was best explained by a combination of only age and Max.Slope. The addition of BW or PW, instead of vBMD, also helped age and max slope explain K_{init} (Table 2). Without age as a covariate, Max.Slope was still a significant predictor of K_{init} and J-int (Table 2). However, vBMD and the NMR measurements provided a negligible marginal improvement in the adjusted coefficient of determination (adjusted-R [2]) for J-int, and the inclusion of BW made age and itself not statistically significant. In the J-int vs. age and Max.Slope model, we found that bone collagen connectivity (Max.-Slope) and age were the important contributors (Table 2). Nonetheless, the ability of BW and Max.Slope without age to explain the variance J-int was similar to that of age and Max.Slope (Table 2).

4. Discussion and conclusion

In this study, we tested whether early observations by Zioupos et al. [20] - that bone collagen integrity measured by thermomechanical methods correlated very well with work to fracture (r = 0.83) and fracture toughness measures (r = 0.65 for K_c and r = 0.86 for J-integral) - could be repeated in a more general and heterogeneous sampling of the population. Furthermore, we tested the relative strengths of contributions of bone collagen parameters to cortical bone fracture resistance in terms of the magnitudes of the standardized coefficients (β) for each factor in the general linear models.

From our heterogeneous donor group including donors for whom available information indicated osteoporosis, type 2 diabetes, cardiovascular diseases, and cancer (as well as other conditions), over a wide age range, it is evident that the transverse fracture resistance of human cortical bone is meaningfully correlated with the bone collagen network connectivity. Our correlations are weaker than those reported by Zioupos et al. [20] and this result is likely due to the fact that they used a much more controlled donor group (10 middle-aged males; with three measurements per donor). This correlation is presumably due to the key role collagen plays in plastic deformation and micro-damage stabilization within the process zone prior to true (rather than apparent) crack growth and in providing closing tractions during crack propagation whilst collagen fibril bridges stretch, tear and the molecules themselves perhaps unravel [15,39,40].

Crack initiation fracture toughness, K_{init}, was best explained by a combination of age, volumetric bone mineral density (vBMD) at the crack tip, and bone collagen connectivity (Max.Slope) resulting in an adjusted R^2 of 49.0%. This is similar to the ability of the linear combination of age, BW, PW, and the average loading slope (avgLS) from cyclic reference point indentation (cRPI) to explain the variance in K_{init} (adjusted R^2 of 53.3%) for the original cohort of 62 donors [32]. It is not clear how surface measurements of tissue-level stiffness below the periosteum relate to how cortical bone resists crack growth and so avgLS was not included as another bone quality variable. Replacing vBMD with pore water (PW) resulted in an adjusted R^2 of 45.9% (Table 2), presumably because cortical porosity would affect the K_{init} value by lowering the apparent elastic modulus ($K_{init} = \mathrm{sqrt}(J_{1c} * E)$) and by stress concentration effects [41]. Notably, factors relating to the bulk stiffness and porosity of the tissue (vBMD and PW) did not contribute significantly to the J-int general linear model. The variation in J-int was best explained by a model only including Max.Slope and age, though the linear combination of Max.Slope and BW as predictors was comparable (Table 2). The adjusted R^2 of 35.7% for J-int dependent on Max.Slope and age was similar to the adjusted R^2 of 35.2% for our previous best-fit model involving clinically compatible measurements (i.e., J-int was best explained by the linear combination of age, PW, and avgLS) [32] The ability of Max.Slope to explain J-int is presumably due to the dominant contribution of the organic phase to the non-linear energy dissipation toughening mechanisms that engage in the process zone that moves as the main crack tip advances and also continues to contribute in the crack wake (collagen fibril bridging in which collagen stretches and tears) [15,39,40]. The ability of bone collagen (and other organic phase proteins) to absorb and dissipate mechanical energy during fracture, through viscoelastic-plastic mechanisms [42], tearing of fibril bridges and perhaps even molecular unravelling, is thought to

be critical to its contribution to cortical bone fracture toughness [15]. These mechanisms are presumably compromised by degradation occurring with aging and disease; degradation that is evident in our measure of bone collagen integrity, the bone collagen connectivity parameter, Max.Slope.

The above findings and discussion strengthen the thesis that collagen network connectivity (a key factor in bone collagen integrity) is important towards understanding cortical bone fragility; a fact that has been somewhat overshadowed by recent perseverations on advanced glycation end-products (AGEs). In recent years, AGEs have been blamed somewhat dogmatically and to varying degrees for causing poor bone quality in aging [43], various diseases, drug treatments [44], and even irradiation sterilization [16]. However, causation has not been proven. The very popular in vitro models of ribation of bone collagen in situ demonstrate suppressed "plasticity" and fracture toughness, and increased stiffness and strength of the bone collagen, due to non-enzymatic glycation (NEG) [27,28]. The ideas from this in vitro model have been extended to in vivo studies without due consideration of other factors like the loss of collagen integrity earlier demonstrated by Peter Zioupos and Xiaodu Wang [20,21]. Certainly, certain AGEs (the crosslink, pentosidine, and the adduct, carboxymethyllysine) accumulate in bone collagen with aging and disease [24,45,46]. However, the in vitro NEG model results in increased connectivity, thermal stability, and stiffening and strengthening of the bone collagen, partially due to AGE crosslinking [28]. This is opposite to connectivity and stability measurements in studies of aging (like the present study), an earlier thermomechanical study by Zioupos [20], and opposite to results from mechanical testing of decalcified bone collagen in aging studies by X. Wang [21]. Therefore, the NEG model does not mimic the losses of bone collagen stability, connectivity and mechanical properties that are known to occur in aging (and are thought to occur in osteoporosis, diabetes and renal osteodystrophy).

In the current study, the lack of correlation between bone collagen connectivity (Max.Slope) and pentosidine content ([pen]) suggests that a) pentosidine is not a robust bone-tissue-specific biomarker for poor bone quality and fracture risk in our specimen population, and b) lend to the thesis that other collagen factors supersede AGE crosslinks in contributing to low fracture toughness. Perhaps this is explained by the fact that pentosidine is a covalent crosslink which occurs in relatively low concentrations compared to the total content of normal lysyl oxidase catalysed collagen crosslinks [30,31,45,47]. Crosslinks in general, and including AGE crosslinks created in vitro or in vivo, should contribute to increasing bone collagen network connectivity measures [18,28,38], but enzymatic pyridinoline crosslinks do not necessary correlate with this connectivity in bone (see in this study and a previous study by Zioupos et al. [20]). A recent study by Gauthier et al. demonstrates positive correlations between J-integral fracture toughness, namely the energy dissipated at the start of crack growth ($J_{process}$) and during crack propagation (J_{prop}), and the ratio of mature-to-immature enzymatic collagen crosslink ratio when pooling specimens from adult human femurs and radii [26]. Despite this correlation between toughness and collagen maturation, the authors concluded that the enzymatic crosslinks and the non-enzymatic pentosidine crosslink only have a modest influence on these mechanical properties of human cortical bone. This suggests that factors stronger than pentosidine and pyridinoline content are more important to cortical bone fracture toughness. Clearly, bone collagen network connectivity does a much better job explaining transverse fracture toughness and the positive correlation between the two indicates that if fracture toughness is degrading in aging and disease, one should look to mechanisms that degrade bone collagen network connectivity. Decreases in overall covalent crosslinking contents and also increases in collagen molecular denaturation and fragmentation are logical candidates based on the literature. Certainly animal models have demonstrated the importance of overall covalent crosslinking contents to bone strength; a positive correlation [25,48]. Elevated pentosidine content in serum and urine is more likely indicative of elevated remodeling/turnover than poor bone quality [45,47]. Furthermore, recent work using ribation as a protectant against the degradation (collagen molecular denaturation and fragmentation) caused by gamma irradiation sterilization demonstrates that NEG/AGE crosslinks can actually counter loss of bone collagen network connectivity; in this case, loss caused by gamma irradiation sterilization at relatively high doses (25–35 kGy) [17,18,38].

The use of HIT to study bone quality is not without limitations. Firstly, HIT requires decalcified tissue. Decalcification likely leads to changes in the hydration status of the bone collagen, and this may vary between donors due to other unknown variations in bone collagen biochemistry. Furthermore, HIT testing does not provide a complete assessment of structural integrity of the bone collagen because thermomechanical testing is not the same as mechanical testing.

In conclusion, transverse fracture toughness of human cortical bone is strongly related to collagen network integrity, specifically measures of its connectivity. Due to the key role collagen plays in intrinsic and extrinsic fracture toughness mechanisms, collagen network connectivity could be an important target for preventing and predicting fracture, and determining fracture risk when combined with clinical BMD and cortical porosity measurements. If further progress is to be made, we propose that tools based on assessing bone collagen integrity should be important targets moving forward and towards developing better tools for preventing fractures and assessing risk of fracture. The lack of correlation between collagen network connectivity and both pyridinoline content and pentosidine content suggests that greater attention should be paid to degradation of the overall organic phase, rather than to a specific molecule or crosslink, when seeking to understand elevated fracture rates in aging and disease.

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Author contributions

TLW: Devised, proposed and funded the study of bone collagen using HIT, supervised the HIT experiments and data analysis, wrote the first draft of the manuscript and worked with the other authors to finalize the manuscript for submission.

DYD: Conducted the HIT experiments and data analysis, and worked with the other authors to finalize the manuscript for submission.

SU: Conducted the fracture toughness testing and micro-computed tomography evaluations as well as provided suggested changes to the manuscript.

MG: Analyzed the data from the fracture toughness tests to determine crack initiation toughness and J-integral as well as acquired the measurements of bound water volume fraction and pore water volume fraction.

JSN: Devised, proposed and funded the larger study, conducted the statistical analyses, revised and edited the manuscript. He also oversaw the collagen crosslink analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2018.10.024.

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