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Low Bone Toughness in the TallyHO Model of Juvenile Type 2 Diabetes Does Not Worsen with Age

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Abstract

Fracture risk increases as type 2 diabetes (T2D) progresses. With the rising incidence of T2D, in particular early-onset T2D, a representative pre-clinical model is needed to study mechanisms for treating or preventing diabetic bone disease. Towards that goal, we hypothesized that fracture resistance of bone from diabetic TallyHO mice decreases as the duration of diabetes increases. Femurs and lumbar vertebrae were harvested from male, TallyHO mice and male, non-diabetic SWR/J mice at 16 weeks (n = 12 per strain) and 34 weeks (n = 13 per strain) of age. As is characteristic of this model of juvenile T2D, the TallyHO mice were obese and hyperglycemic at an early age (5 weeks and 10 weeks of age, respectively). The femur mid-shaft of TallyHO mice had higher tissue mineral density and larger cortical area, as determined by micro-computed tomography, compared to the femur mid-shaft of SWR/J mice, irrespective of age. As such, the diabetic rodent bone was structurally stronger than the non-diabetic rodent bone, but the higher peak force endured by the diaphysis during three-point (3pt) bending was not independent of the difference in body weight. Upon accounting for the structure of the femur diaphysis, the estimated toughness at 16 weeks and 34 weeks was lower for the diabetic mice than for non-diabetic controls, but neither toughness nor estimated material strength and resistance to crack growth (3pt bending of contralateral notched femur) decreased as the duration of hyperglycemia increased.

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With respect to trabecular bone, there were no differences in the compressive strength of the L6 vertebral strength between diabetic and non-diabetic mice at both ages despite a lower trabecular bone volume for the TallyHO than for the SWR/J mice at 34 weeks. Amide I sub-peak ratios as determined by Raman Spectroscopy analysis of the femur diaphysis suggested a difference in collagen structure between diabetic and non-diabetic mice, although there was not a significant difference in matrix pentosidine between the groups. Overall, the fracture resistance of bone in the TallyHO model of T2D did not progressively decrease with increasing duration of hyperglycemia. However, given the variability in hyperglycemia in this model, there were correlations between blood glucose levels and certain structural properties including peak force.

Keywords

Type 2 diabetes; fracture risk; bone quality; pentosidine; Raman spectroscopy; mechanical properties; micro-computed tomography

1. Introduction

Fracture risk is higher among individuals with diabetes compared to those without diabetes for a given T-score as determined by dual energy X-ray absorptiometry (DXA) [1, 2]. Multiple epidemiological studies have also found that the relative risk of fracture is higher among type 2 diabetics than age-matched non-diabetics [3–7]. This increase in fracture risk is most pronounced at the hip, and a meta-analysis by Janghorbani et al. estimated a 1.7 times greater risk of hip fracture for adults with type 2 diabetes (T2D) [3]. While risk factors unrelated to bone increase fracture incidence in T2D, elevated fracture risk is still observed when adjusting for diabetes-associated increase in the propensity to fall or in decreased muscle tone [3, 4, 8]. This elevated fracture risk is somewhat counterintuitive because a larger BMI and hyperinsulinemia in type 2 diabetics would generally favor a higher bone mass and strength, though the relation between obesity and femoral neck strength is complex [9]. The lack of an apparent loss in bone mineral density (BMD) with T2D suggests that the disease is negatively affecting bone quality [10].

Incidence of type 2 diabetes is rising. According to the CDC, an estimated 6.5% of people in the United States had diabetes in 2015 up from 5.5% in 2005 [11]. The increasing prevalence of T2D does not just occur among adults [12], as the population of obese children has increased [13, 14]. While this increase in obesity incidence may be leveling off in recent years, the continued rise in severe obesity could drive an increase in complications associated with juvenile-onset T2D [15]. Furthermore, as duration of diabetes increases, fracture risk appears to increase [6]. Thus, the increasing prevalence of T2D among juveniles may result in even higher relative risk of fracture later in life than current estimates. While there has yet to be a study examining this in juvenile-onset T2D, type 1 diabetes has been associated with increased fracture risk throughout lifespan [16].

There are multiple pathways through which T2D can lower bone quality and increase fracture risk [10, 17], but T2D is not necessarily causing a deterioration in cortical structure or trabecular architecture, at least not directly. Several cross-sectional studies involving high resolution, peripheral quantitative computed tomography (HR-pQCT) imaging of post-

menopausal women did not find lower cortical thickness, lower estimated failure load in compression, lower trabecular thickness, or lower trabecular bone volume fraction for subjects with T2D compared to non-diabetic controls [18–20]. In one recent HR-pQCT study involving men and women (Framingham cohort), the cross-sectional area of the tibia mid-shaft, not the distal radius, was significantly lower (by 2.4%) for those with T2D compared to non-diabetics [21]. There were no significant differences in the trabecular architecture, trabecular volumetric BMD, or estimated compressive failure load between the groups at these peripheral sites, though cortical thickness was lower in T2D individuals with prior fracture [21]. With respect to microstructure, cortical porosity (Ct.Po) has been observed to be higher in the distal radius [18, 22, 23] and tibia mid-shaft [23] for those with T2D, especially if the T2D patient had suffered a fracture [21, 24, 25], but an elevated Ct.Po with T2D has not always been observed [19, 20, 26]. With respect to material properties of bone, studies using a microindentation tool, designed to assess the resistance of cortical bone at the tibia mid-shaft to impact loading, have found lower bone material strength index (BMSi) in post-menopausal patients with T2D compared to age-matched non-diabetic women [19, 20, 26]. At the matrix level of the hierarchical organization of bone, an increase in advanced glycation end-products (AGEs) caused by elevated glucose levels could also result in more brittle bones [27, 28].

Various rodent models have been used for investigating diabetic bone disease, but as previously reviewed [29], the effect of T2D on fracture resistance has only been reported for a few mouse models and done so with several limitations. For example, in a MKR transgenic mouse model of early onset T2D (hyperglycemia by 6–8 weeks), the bones of MKR mice are structurally weaker than non-diabetic controls (FVB/N strain). Unlike humans with T2D, these mice have slender bones, are lean, and their insulin resistance is due to specific gene mutations, namely the loss of insulin and insulin-like growth factor 1 (IGF-1) receptors in skeletal muscle [30]. However, such gene mutations are rarely the clinical cause of T2D. Overcoming this limitation, the KK-A^y mouse is a polygenic model developing hyperglycemia spontaneously by 8 weeks [31]. When compared to non-diabetic male ddY mice, male KK-A^y mice had lower areal BMD at the proximal femur, not at the mid-shaft, by 18 weeks of age [32]. However, when using non-diabetic C57BL/6 for controls, Xu et al. observed lower volumetric BMD and lower structural strength in bending of the tibia mid-shaft for the diabetic KK-A^y mice [33]. Yet another study comparing male KK-A^y to male C57BL/6 mice observed higher areal BMD of the whole femur, lower volumetric BMD of trabecular bone in the distal femur, and higher volumetric BMD of cortical bone in the distal femur [34]. To the best of our knowledge, there is no study reporting on the fracture resistance phenotype of KK-A^y/a with respect to a/a littermate controls. Another commercially available, polygenic, spontaneous mouse model for T2D is the TallyHO strain. This mouse develops hyperglycemia before skeletal maturity [35] and is obese compared to its recommended control strain, SWR/J [36, 37]. As previously reported by Devlin et al., the femur diaphysis is structurally stronger in bending but brittle (lower post-yield displacement) for the diabetic TallyHO compared to non-diabetic SWR mice at 17 weeks of age [36].

To determine whether the TallyHO mouse can be a model of diabetic bone disease that represents what is known about the disease in humans (i.e., shows increase in fracture risk

with increase in duration of diabetes), we hypothesize that fracture resistance decreases as the duration of diabetes increases beyond skeletal maturity. We analyzed bones from both TallyHO mice and SWR/J controls at 16 weeks (short duration) and 34 weeks of age (long duration) using multiple techniques in order to establish whether bone parameters associated with greater bone fragility in humans. Specifically, we determined whether i) an increase in cortical porosity, ii) a deterioration in trabecular architecture, iii) an accumulation of matrix AGEs, and iv) alterations in the structure of collagen I reflective of crosslinking also accompany a T2D-related decrease in the mechanical properties of the bone in this mouse model.

2. Materials and Methods

2.1. Animal care and tissue collection

TallyHO/Jng (n=30) and SWR/J (n=30), male mice were purchased from Jackson Laboratory (Bar Harbor, ME). Female mice were not included because female TallyHO mice do not develop overt diabetes [35]. Animals were fed standard chow 5L0D with 13% kcal from fat (LabDiet, St Louis, MO) up until 16 weeks of age. Subsequently, all animals were switched to a 12450B purified diet with 10% kcal from fat (Research Diet, Inc., News Brunswick, NJ). Weekly, animals were weighed and their non-fasting glucose levels measured using a OneTouch UltraMini glucometer (LifeScan Inc., Milpitas, CA). Glycated hemoglobin (HbA1c) was measured for a sub-set of mice at 34 weeks of age using a Bayer DCA 2000 system by the VUMC Metabolic Phenotyping Core. Immediately prior to sacrifice, blood was collected by cardiac exsanguination while animals were under ketamine/xylazine anesthesia. Animals were either euthanized at 16 weeks of age or at 34 weeks of age. Due to 6 premature deaths from fungal nephritis (unexpected), 10 additional TallyHO mice were ordered. Mice were then housed in sterile cages. All animal protocols were approved by the local Institutional Animal Care and Use Committee. One SWR/J mouse was excluded from the study (34-week group) because lymphoma was observed at necropsy. Femurs and L6 vertebrae were frozen in phosphate buffer solution (PBS) for micro-computed tomography analysis (μ CT) and biomechanical testing. The posterior side of the right femur from each mouse was micro-notched at the mid-shaft using first a low speed diamond embedded saw, and then a razorblade coated with a diamond solution.

2.2. Micro-computed tomography analysis

Both femurs and the L6 vertebra were imaged with high-resolution μ CT scanner (μ CT50; Scanco Medical AG, Brüttisellen, Switzerland). The regions of interest (ROIs) for the left femurs included the mid-shaft (1.86 mm) at the point of loading (described in subsequent section) and distal metaphysis (3.72 mm) extending approximately 25% below the growth plate. The scan parameters were: 70 kVp/114 μ A, 0.5 Al filter, beam hardening (BH) correction, 1160 samples per 500 projections per 180° rotation, 600 ms integration time, and 6 μ m isotropic voxel size. For L6 VBs, the ROI was the bone between the cartilaginous end-plates. Scan parameters were: 55 kVp/200 μ A, 0.5 mm Al filter, beam-hardening correction, 500 samples per 500 projections per 180° rotation, 1200 ms integration time, and 12 μ m isotropic voxel size. The mid-shaft of each notched, right femur was imaged with another μ CT scanner (μ CT40; Scanco Medical AG, Brüttisellen, Switzerland). The scan parameters

were: 70 kVp/114 μ A, 0.5 mm Al filter, beam-hardening correction, 2048 samples per 1000 projections per 180° rotation, 300 ms integration time, 6 μ m isotropic voxel size. Images were analyzed and contoured as described previously [38]. Thresholds varied among bone type: i) for the intact left femur diaphysis, global threshold was 912.6 mgHA/cm³, ii) for the cortical compartment, an inverted threshold setting of 816.0 mgHA/cm³ was also used to determine porosity (Ct.Po), iii) for the trabecular bone of the left femur metaphysis, global threshold was 393.2 mgHA/cm³, iv) for the notched right femur, it was 863.1 mgHA/cm³, and v) for trabecular bone in the vertebral body, it was 514.0 mgHA/cm³ [38]. A sigma of 0.2 and support of 1 (Gaussian filter) were used with all thresholds to suppress image noise.

2.3. Mechanical testing

2.3.1 Three-point bend testing of left femurs—Hydrated left femurs were loaded to failure in a three-point (3pt) bending at a displacement rate of 3 mm/min using a servo-hydraulic material testing system (DynaMight 8841, Instron, Norwood, MA) fitted with a 100 N load cell (Honeywell, OH, Model no. 060-C863-02). All femurs were placed on the lower supports with a span of 8 mm such that the anterior side was down and the medial side was forward. Using a custom Matlab script (Mathworks, Natick, MA), the force vs. displacement curve (sampled at 50 Hz during 3pt bending) was processed to determine structural properties (stiffness or slope, peak force, yield force, post-yield displacement, work-to-failure) [38], and then material properties (bending strength, peak moment, toughness, modulus, etc.) were estimated using μ CT-derived structural parameters as previously described [39].

2.3.2 Fracture toughness testing—Notched right femurs were also tested in 3pt bending (DynaMight 8841, Instron, Norwood, MA) using the 100 N load cell while hydrated with PBS, but the span was adjusted to four times the mean outer anterior–posterior diameter (i.e., in the direction of loading) rounded to the nearest mm. The loading rate of these femurs was 0.06 mm/min. All tests were recorded using a high resolution DSLR camera (Canon EOS 7D) fitted with a macro lens to qualitatively monitor the crack propagation and to discard outliers if any. Again, force vs. displacement curves were processed using custom Matlab script to calculate the critical stress intensity factor, $K_{c,initial}$, using the peak force during the load-to-failure test and the initial notch angle (θ was determined using μ CT images) as previously described [40]. Cracking toughness was measured by dividing the work-to-failure (W_f) by Ct.Ar and adjusted for the given span [38].

2.3.3 Axial compression testing—Each hydrated L6 vertebra was loaded to failure at 3 mm/min between two custom compression platens with a moment relief [38]. VB strength was defined as the peak force. Axial stress was determined by the peak force divided by the cross-sectional area of the VB. Cross-sectional area was estimated by dividing the segmented bone volume of the VB (transverse processes transected) by the axial length of the ROI.

2.4. Raman spectroscopy

Raman spectra were collected from right femurs after fracture toughness testing by focusing a 830 nm laser several millimeters away from broken surface. Using a confocal Raman

microscope (Invia, Renishaw, Hoffman Estates, IL), an average of 10 consecutive spectra per bone (each collected for 5 s duration) was acquired through a 20X objective (NA=0.40). From the averaged Raman spectrum per bone sample, the background fluorescence was subtracted using a 4th order polynomial curve. Subsequently, spectra were smoothed using a 4th order Savitzky-Golay filter (LabSpec software, Horiba Jobin Yvon, Edison, NJ) to the signal to noise ratio. Peak ratios of $\nu_1\text{PO}_4$ /Amide I, $\nu_1\text{PO}_4$ /Amide III, $\nu_1\text{PO}_4$ /Proline, and $\text{CO}_3/\nu_1\text{PO}_4$ [41] as well as Amide I sub-peak ratios 1670/1640, 1670/1690, and 1670/1610 were calculated as previously described [42]. The crystallinity was calculated as the inverse of the width of the $\nu_1\text{PO}_4$ peak at half maximum.

2.5. High Performance Liquid Chromatography (HPLC) and fAGE

The proximal and distal half of broken left femurs were flushed, weighed separately (wet mass) and separately demineralized in 20% EDTA at 4 °C. After demineralization, the samples were dehydrated for 24 hours at room temperature under a vacuum. Samples were then hydrolyzed with 6 N HCl at 110 °C for 20 hours. Hydrochloric acid was removed using a SpeedVac Concentrator System (ThermoFisher). Samples were filtered through 0.2 μm syringe filters (Fisher) and then split so as to have ≈ 1 mg of dry weight of bone for each assay. The proximal half was used for crosslink analysis. The crosslinks pyridinoline (PYD) and pentosidine (PE) were measured with a high performance liquid chromatography (HPLC) system (Beckman Coulter System Gold 126, Brea, CA) involving a silica-based, reversed phase C18 column (Waters Spherisorb® 5 μm ODS2, Milford, MA) column and fluorescence detector (Waters 2475 Multi λ Fluorescence Detector) as we previously described [41]. Collagen was determined based on hydroxyproline measurements using a HPLC assay with a Waters PicoTag column (Waters, Milford, MA) and a UV detector (Beckman Coulter 168 Detector, Brea, CA) as previously described [41]. Crosslinks were calculated based on a standard curve using standards of known concentrations (PYD: Quidel®, TECOMedical group, Switzerland and PE: International Maillard Reaction Society).

The hydrolyzed samples from the distal half of the femur were suspended in 350 μL of 0.1 M H_2SO_4 . Samples were compared to a standard of quinine sulfate suspended in 0.1 M H_2SO_4 as previously described [43]. 150 μL was pipetted in duplicate for each sample and standard into a black-bottom 96 well plate. Fluorescence was measured with an excitation wavelength of 370 nm and an emission wavelength of 440 nm. Fluorescent advanced glycation end-products (fAGEs) were calculated based on the standard curve and normalized to an estimated measurement of collagen based on the ratio of mol of collagen to wet mass of the proximal half.

2.6 Serum analysis

Serum was separated by centrifugation in serum collection tubes. Insulin was measured using a radioimmunoassay (RIA) from Millipore and was performed by the VUMC Hormone Assay and Analytical Services Core. P1NP and TRAcP-5b were measured in serum using Rat/Mouse ELISAs (Immunodiagnostic Systems, Inc., Fountain Hills, AZ) [44] according to the manufacturer's protocols.

2.7 Statistical Analysis

Instead of two-way analysis of variance, which assumes normality and homoscedasticity, general linear models (GLMs) with bootstrapping (500 replicates) were analyzed (STATA, College Station, TX) to determine whether each bone property depended on mouse strain, age, and their interaction (inter.), appropriate post-hoc comparisons were done using Mann-Whitney tests (Matlab, MathWorks, Natick, MA) with a Holm-Sidak correction for 2 multiple comparisons (e.g., TallyHO vs. SWR/J within 16 weeks and TallyHO vs. SWR/J within 34 weeks when strain was a significant factor). Subsequently, body weight, calculated as an average over 10 weeks to 16 weeks or over 24 weeks to 34 weeks of age, was included as a covariate in the STATA GLMs to determine if body weight was the driving factor for strain differences in bone properties. These analyses of covariance were separated out by age group to limit the number of factors being analyzed at once. For each group, correlations between average blood glucose levels and bone properties were determined using linear regression analysis with bootstrapping (500 replicates). Averaged blood glucose was calculated from 8 weeks to 16 weeks for mice at 16 weeks of age and from 8 weeks to 33 weeks for mice at 34 weeks of age. Longitudinal body weight and glucose comparisons between strains were done at individual time points using Mann-Whitney.

3 Results

3.1 TallyHO mice weighed significantly more than control mice and exhibited variable hyperglycemia

TallyHO mice had a higher body weight than SWR/J mice as early as 5 weeks of age (Figure 1A), and so the body weight, calculated by taking the average over the last 6 weeks or 10 weeks of life, was significantly higher for TallyHO than for SWR/J mice at both ages (Table 1). Diabetes, as defined by persistent non-fasting glucose levels above 250 mg/dL, a common threshold in rodent studies [45], occurred for most TallyHO mice around 10 weeks of age (Figure 1B). One TallyHO mouse (green symbols in Figure 1B) was excluded from the study due to inconsistent hyperglycemia. Eight TallyHO mice experienced significant weight loss and were euthanized before the end of the study. These premature deaths were likely due to complications of overt, uncontrolled diabetes (>500 mg/dL) and occurred between 20 and 32 weeks of age. Glucose levels were higher in TallyHO mice compared to SWR/J mice, as expected, but the degree of hyperglycemia in TallyHO mice did not progressively increase with age (Figures 1B and 2A). Given the variability in non-fasting glucose measurements, HbA1c was measured for a 10–12 mice per group at 34 weeks. HbA1c was 33.3% higher in the TallyHO mice compared to SWR/J controls (Table 1), and this measure of glycated hemoglobin correlated with non-fasting glucose levels in TallyHO mice (Figure 2B).

3.2 Mechanical properties of bone from TallyHO mice did not progressively worsen with age

To determine whether fracture resistance at the material level (i.e., independent of bone structure) decreased with the duration of T2D, we assessed multiple mechanical properties of cortical bone. For intact femur diaphysis, bending strength, or the estimated peak stress endured by the intact femur diaphysis, was higher for diabetic mice at 16 weeks of age

(Figure 3A), but not at 34 weeks of age. Toughness was lower for the TallyHO mice compared to non-diabetic controls, but it did not progressively worsen with age as anticipated (Figure 3B). Similarly, post-yield deflection was lower in the diabetic TallyHO compared to the control SWR/J mice, irrespective of age (Table 2). Body weight was a contributing factor to only bending strength and only at 34 weeks, but this did not change the lack of a T2D-related difference at this age (Supplemental Table 2).

When testing the notched right femurs for the ability of bone to resist crack initiation, $K_{c,initial}$ trended towards being lower in diabetic mice (Figure 3C), but this reduction was not statistically significant. Cracking toughness, or the energy dissipated during crack propagation, was not different between diabetic and control mice. However, cracking toughness was significantly lower at 34 weeks compared to 16 weeks in non-diabetic mice but not in diabetic TallyHO mice (Figure 3D). Body weight was not a significant contributor to either fracture toughness parameter, while strain became a significant explanatory variable ($p=0.038$) of cracking toughness at 34 weeks when body weight ($p=0.178$) was included as a covariate (Supplemental Table 2).

3.3 The TallyHO femurs were structurally stronger than SWR/J femurs but this greater strength was not independent of body weight

With respect to fracture resistance at the whole-bone level (i.e., dependent on bone structure), the peak force endured by the femur was higher for TallyHO than for SWR/J mice. This is likely due to the cross-sectional area of the femur diaphysis (Ct.Ar) and the cortical thickness (Ct.Th) being larger for diabetic mice than non-diabetic mice at both ages (Figure 4A and Figure 4B). However, minimum moment of inertia (I_{min}) was higher in diabetic mice only at 34 weeks of age (Figure 4C). The endosteal perimeter (Ec.Pm) was lower for TallyHO mice at 16 weeks, but this increased with age for TallyHO mice so as to not be lower at 34 weeks compared to the SWR/J mice. Cortical porosity was lower in diabetic mice than non-diabetic mice at both ages and did not change with duration of diabetes (Figure 5A). Body weight was a significant explanatory variable of I_{min} , Ct.Ar, and periosteal perimeter (Ps.Pm) (Supplemental Table 4) for both age groups. However, body weight significantly contributed to cortical thickness only at 16 weeks and to Ec.Pm at 34 weeks. Body weight was also significant factor in the linear models explaining peak force, yield force, and stiffness at both ages. Furthermore, when body weight was included as a covariate, the T2D-related difference in the peak force and the yield force was no longer significant.

3.4 Lower trabecular bone volume did not result in lower lumbar vertebra strength for the TallyHO mice compared to the control mice

In order to estimate the contribution of trabecular bone to whole-bone strength, compression tests of the L6 vertebra were performed. Neither strain nor age affected peak compressive force (Table 3). Despite the lack of difference in VB strength between the strains, trabecular bone volume fraction (BV/TV) of the vertebra was lower for the diabetic TallyHO compared to the non-diabetic mice at 34 weeks. As such, the apparent axial stress was higher for the TallyHO mice at both ages (Table 3). Similar strain-related differences in trabecular micro-architecture were observed in the distal femur metaphysis, though BV/TV was lower at 16

weeks for the TallyHO than for the SWR/J mice (Table 4). Body weight was a significant explanatory variable of BV/TV at 16 weeks of age for both the vertebra and femur, but strain remained a significant factor. Body weight affected only the strain-related difference in vertebral Tb.TMD at 16 weeks. It was not a significant contributor to peak force or any other compressive mechanical parameter (Supplemental Table 3).

3.6 The bone matrix of diabetic TallyHO mice had altered secondary structure of collagen I and higher mineralization compared to control bone matrix

To identify a potential cause of the brittleness phenotype in TallyHO mice, various matrix characteristics were assessed. As measured by Raman spectroscopy, all 3 sub-peak ratios of the Amide I band that reflects the secondary structure of collagen I – 1670/1640, 1670/1610, and 1670/1690 (cm^{-1}) – were higher for diabetic than for the non-diabetic mice (Table 5). There was no significant difference in PYD, a mature enzymatic crosslink, between strains. Additionally, matrix pentosidine concentration was not altered with either diabetes or age (Table 5). fAGEs were not significantly different between the strains, and significantly increased with age in only SWR/J mice (Table 5). Raman spectroscopy confirmed the higher mineralization in cortical bone of TallyHO mice (Figure 4D) with multiple measurements of the mineral-to-matrix ratio ($\nu_1\text{PO}_4/\text{Proline}$ and $\nu_1\text{PO}_4/\text{Amide I}$) being higher for the diabetic bone than for the non-diabetic bone at both 16 weeks and 34 weeks of age. However, $\nu_1\text{PO}_4/\text{Amide III}$ was only higher for the TallyHO mice than for the SWR/J mice at 34 weeks (Table 5). As for characteristics of the mineral phase, crystallinity increased with age for both strains (Table 5), but did not change with diabetes with respect to controls. $\text{CO}_3/\nu_1\text{PO}_4$ was lower for diabetic mice at both ages, but did not further progress with age (Table 5). Body weight significantly explained $\nu_1\text{PO}_4/\text{Proline}$ and crystallinity at 16 weeks, but not at 34 weeks, and strain was still a significant factor. Interestingly, the strain-related difference in the Amide I sub-band peak ratios at 34 weeks was no longer significant when body weight was included as a covariate (Supplemental Table 5).

3.7 Body weight and several bone structural parameters of the TallyHO mice negatively correlated with averaged blood glucose levels

Given the high variability in blood glucose levels within the TallyHO group, we tested for significant relationships between hyperglycemia and bone properties. First, averaged blood glucose levels negatively correlated with body weight for the TallyHO mice at 34 weeks (Figure 6A) indicating that mice with severe hyperglycemia either failed to gain weight or lost weight. Over the long duration of T2D, P1NP, a marker of bone formation, also negatively correlated with blood glucose levels at 34 weeks (Figure 6B) suggesting that poorly controlled diabetes suppressed bone formation. P1NP was lower for TallyHO mice compared to SWR/J mice at both ages (Table 1). Although the TallyHO femur diaphysis was stronger, peak force (Figure 6C) and Ct.Ar (Figure 6D) decreased as hyperglycemia increased in these mice. At the material level, blood glucose levels correlated with toughness at 16 weeks in TallyHO mice such that those with high blood glucose levels unexpectedly had higher toughness (Supplemental Table 7). On the other hand, glucose levels negatively correlated with cracking toughness at 16 weeks of age for TallyHO mice (Supplemental Table 7). No such correlations were found among 34-week-old TallyHO mice. For the bone matrix properties, glucose negatively correlated with 1670/1640 ($r = -0.597$, $p = 0.009$) and

1670/1610 ($r = -0.555$, $p = 0.022$) ratios in TallyHO mice at 34 weeks, albeit the correlations were weak. Furthermore, when looking at the mineral phase, glucose levels correlated with crystallinity in TallyHO animals at 34 weeks. Surprisingly, pentosidine levels in the bone matrix did not correlate with blood glucose levels (Supplemental Table 10) nor did fAGEs levels at 34 weeks (Supplemental Table 10).

4. Discussion

There is a need for rodent models that mimic diabetic bone disease in humans because such animal models can be used in pre-clinical studies to prevent the diabetes-related decrease in fracture resistance. Moreover, by having an animal model in which BMD is not lower in the T2D rodents compared to non-diabetic rodents but instead the fracture resistance is progressively worse with diabetes duration, new pathogenic mechanisms leading to increased fracture risk could be identified as potential therapeutic targets as well as new matrix-sensitive tools for diagnosing high fracture risk could be assessed. While overall volumetric BMD was not lower in the diabetic TallyHO mice compared to their recommended non-diabetic strain, none of the mechanical properties, whether determined at the whole-bone level or the material level, decreased as the duration of T2D progressed. Thus, this model of juvenile-onset T2D does not mimic the likely increase in fracture risk that occurs as the duration in type 2 diabetes increases among humans. Nonetheless, a subset of TallyHO who experience severe hyperglycemia may experience dysfunction in other organs (e.g., kidney) that contribute to a loss of fracture resistance.

The TallyHO mouse strain was developed from selectively breeding male mice that presented with hyperglycemia within a colony of Theiler Original mice. In the initial study describing the development of this mouse model of T2D [46], male mice at 26 weeks were overweight with an average body mass of 45 ± 2 g (mean $\pm$ SEM) and hyperglycemic with a non-fasting, plasma glucose levels of 544 ± 24 mg/dL (mean $\pm$ SEM). Compared to C57BL/6J mice (arbitrarily chosen), female TallyHO mice were also obese but were not hyperglycemic [46]. In a follow-up study by the Jackson Laboratory, the male TallyHO mice had significantly higher body weight by 4 weeks and were diabetic (plasma glucose levels above 250 mg/dL) by 10 weeks of age compared to male C57BL/6J mice [35]. These differences were similar to the present study comparing the body mass and glucose levels of the TallyHO mice to non-diabetic, male SWR/J mice. Because the SWR/J strain has greater genetic similarity to the TallyHO strain (86.8% homology), SWR/J mice are now recommended for use as the non-diabetic control by the Jackson Laboratory [47]. Nonetheless, the lack of a littermate control makes strain a possible contributor to the observed differences in bone between the non-diabetic and diabetic mice.

In this study, the phenotype of the diabetic TallyHO mice, when compared to the non-diabetic SWR/J mice at 16 weeks of age, was similar to the one previously described by Devlin et al. at 17 weeks of age [36]. Specifically, in both studies, there was high variability in glucose levels among the TallyHO mice, and the onset of persistent hyperglycemia occurred by 10 weeks of age. With respect to the skeletal phenotype, cortical TMD and cortical thickness were higher in the TallyHO mice compared to the SWR/J mice in both studies. Devlin et al. also observed that the structural strength of femur diaphysis was higher

for the diabetic mice, while post-yield displacement was lower. We additionally found that toughness (i.e., the ability of cortical bone to dissipate energy during fracture) did not change between 16 weeks and 34 weeks of age in both diabetic and non-diabetic mice. Furthermore, as with the Devlin study, we did not observe differences in FAGEs within bone between diabetic and non-diabetic mice. Notably, in a recent study analyzing hydrolysates from proximal femora of diabetic KK-Ay/a mice and non-diabetic a/a littermates at 20 weeks of age (12 weeks of hyperglycemia) [48], there were no significant difference in pentosidine concentration similar to our findings with the TallyHO mice.

The early onset of obesity and diabetes makes it difficult to determine whether or not there are differences in fracture resistance between the strains prior to hyperglycemia. Furthermore, as the SWR/J strain is not obese, certain differences between the strains may be due to obesity as suggested by the covariate analysis of bone structural properties (Supplemental Tables). Notably, other T2D rodent models such as the ZSD rat have strain-related differences in bone structure and strength before the onset of T2D [41]. Moreover, while the cortical bone of the femur from Zucker Diabetic Fatty rats (ZDF^{fa/fa}) was structurally weaker with lower areal BMD than those from the lean ZDF non-diabetic controls (ZDF^{fa/+}) at 33 weeks of age, no significant differences in material estimates of strength and toughness between the two groups were reported [49]. Effects of T2D on the fracture resistance of long bones at the material-level in rodent models tends depend on the duration of hyperglycemia, though few studies investigated multiple time points during the progression of the disease [50].

With regards to the vertebrae, the peak force did not decrease with diabetes despite a lower trabecular bone volume at 34 weeks compared to age-matched non-diabetic mice. Unlike with Devlin et al. study [36], which reported differences in trabecular architecture within the lumbar vertebra, not VB strength, at 17 weeks of age between SWR/J and TallyHO mice, we did not find a difference in trabecular bone volume in the L6 VB at 16 weeks between these 2 strains of mice. At the 34 weeks of age, the higher Tb.TMD (Table 3) could have compensated for the lower BV/TV and give added strength to the L6 VB of the TallyHO mice. However, while Tb.TMD increased with age for both mouse strains, peak force was not different between 16-week and 34-week old mice suggesting other factors within the lumbar vertebra (e.g., related to the matrix or the structure of the cortical shell) existed between the diabetic and non-diabetic mice. In the distal femur metaphysis, there was significantly lower BV/TV for the TallyHO mice at both ages (Table 4), and this is consistent with the previous study [36] and another study comparing male TallyHO to male C57BL/6J mice at 8 weeks of age [51]. T2D in humans does not appear to be a problem of poor trabecular architecture, at least not when assessed by HR-pQCT of peripheral sites [20, 21]. With regards to cortical micro-structure, porosity or pore number did not increase with duration of hyperglycemia as observed in ZSDS diabetic male rats [41]. There is however a limit to the size of pores that μ CT analysis of mouse bones can resolve. Furthermore, unlike with human cortical bone, extensive remodeling of the cortex does not occur in mice, and so higher porosity in the diaphysis of mouse long bone is typically a reflection of elevated resorption near the endosteum (Fig. 5). Possibly, with higher body weight, this resorption during diaphyseal expansion was lower for TallyHO mice.

Regardless of the cause (either strain-related or T2D-related), there are several possible explanations for the brittle bone phenotype of the TallyHO diabetic mice. The tissue mineral density (μ CT) and the mineral-to-matrix ratio (Raman spectroscopy) were higher for TallyHO cortical bone than for SWR/J cortical bone. It has been suggested that there is a non-linear relationship between degree of mineralization and toughness such that hyper-mineralization is thought to confer low bone toughness [52–54]. In the present study though, as mineralization increased with age for both mouse strains, toughness did not significantly differ between the age groups. Cracking toughness (Figure 3D) and post-yield displacement (Table 2), other indicators of brittleness, did decrease with age, namely for the non-diabetic mice. Also, the age-related increase in Ct.TMD only resulted in a higher bending strength at 34 weeks compared to 16 weeks of age for the SWR/J mice.

An alternative explanation relates to the organic matrix. The Amide I sub-peak ratio 1670/1640 (intensity ratio at these wavenumbers) was higher in diabetic mice. Unal et al. previously showed that the Amide I sub-peak ratio 1670/1640 (cm^{-1}) was negatively correlated with toughness and post-yield toughness of bovine cortical bone and was sensitive to thermally induced denaturation and mechanical denaturation in bovine cortical bone (i.e., a marker of helical structure of collagen I) [42]. For the WBN/Kob rat model of T2D, Saito et al. [55] observed a decrease in immature enzymatic collagen crosslink concentration (before the onset of hyperglycemia) with the progression of diabetes, but no T2D-related changes in mature enzymatic pyridinoline (as observed in the present study). While we did not measure immature crosslinks, our Raman analysis also found that the Amide I sub-peak ratio 1670/1690 (cm^{-1}), known as the matrix maturity ratio, was also higher in bones of TallyHO mice. This change could reflect a difference in the enzymatic crosslinking profile. Thus, it is possible that the increased brittleness of bone in TallyHO mice is due to an altered collagen structure in the bone matrix.

Finally, multiple mice experienced a loss in body weight as the duration of T2D increased. One TallyHO mouse in the 16-week group and three TallyHO mice in the 34-week group lost more than 10% of their peak body mass (other mice lost more than 20% of body mass and were euthanized before completion of the study), even though mice were fed a purified diet with a relatively low-fat content. The negative correlation between body weight and glucose levels (Figure 6A) suggests that this loss was related to uncontrolled hyperglycemia. Diabetic mice with the high glucose levels at 34 weeks of age also had reduced structural strength in bending (Figure 6C), reduced cortical area (Figure 6D), and reduced serum marker of bone formation (Table 1) suggesting additional complications potentially affecting the bone. Another limitation of the study is the lack of bone histomorphometry to confirm an inverse association between hyperglycemia and bone formation. Nonetheless, poor kidney function, a known complication of T2D, could alter bone turnover leading to structural deficits. Potentially, by counting on high variability in hyperglycemia from moderate to severe, the TallyHO model of T2D could be used to investigate the effect of glycemic control by standard (e.g., metformin) therapies and newer therapies (e.g., gliflozins) on bone strength.

5. Conclusions

Overall, the fracture resistance of bone from TallyHO mice did not progressively worsen with the duration of T2D, and the low toughness of cortical bone for these diabetic mice compared to the non-diabetic SWR/J mice existed at skeletal maturity (16 weeks of age) and in adulthood (34 weeks of age). It is unclear whether strain or early-onset of elevated glucose levels or both contribute to the differences in toughness and organic matrix seen between the two groups. While the TallyHO model mimics several features of type 2 diabetes in humans (glucose levels were variable and bone mass was not lower than normal), cortical porosity and trabecular number and thickness in long bones was lower compared to the non-diabetic mice when diabetic humans can have elevated cortical porosity with otherwise normal trabecular architecture. Moreover, AGEs did not accumulate in bone with diabetic progression as occurs in other tissues of diabetic humans. The lower trabecular bone volume fraction existing in this model juvenile-onset of T2D also did not confer lower lumbar vertebral body strength when comparing TallyHO to SWR/J mice at 34 weeks of age. The higher structural strength of femur mid-shaft for the diabetic mice was not independent of the difference in body weight between TallyHO and SWR/J mice. There is variability in individual blood glucose levels within the TallyHO group, and such levels negatively correlate with certain structural properties of the femur mid-shaft.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The elevated glucose levels in obese TallyHO mice are variable compared to SWR/J controls.
- The mechanical properties of cortical bone for TallyHO mice do not progressively worsen with age.
- The reduction in trabecular bone associated with diabetes in the TallyHO mice do not confer a decrease in the strength of lumbar vertebra.
- Glucose levels for the 34-week old TallyHO mice negatively correlate with several structural properties of cortical bone.

Figure 1.

Age-related changes (mean $\pm$ SD) in body weight (A) and non-fasting blood glucose levels (B). TallyHO mice (n=13) were obese compared to SWR/J controls (n=16) starting at 5 weeks of age. Glucose levels in TallyHO mice were significantly higher than control mice at an early age but achieved persistent diabetic status (consecutive weekly levels > 250 mg/dL; purple dashed line) at 10 weeks of age. One TallyHO mouse (green triangle) was not consistently diabetic and was excluded from the study.

Figure 2.

Blood glucose levels as an average between 8 weeks and 16 weeks for 16-week-old (wks) and between 8 weeks and 33 weeks for 34 wks (median $\pm$ interquartile range with GLM p-values) (A) and HbA1c vs. glucose levels (B). TallyHO mice had significantly higher glucose levels at both 16 weeks and 34 weeks, and these levels did not increase with age partly due to high variability among each age group. HbA1c values correlated strongly with average blood glucose levels. The data points from animals with blood glucose levels consistently higher than 450 mg/dL are shown in purple. Brackets indicate significant differences between strain within age group, and letters indicate significant differences between age groups within strain.

Figure 3.

Estimated material properties from bending tests (median $\pm$ interquartile range with GLM p-values). Compared to control mice, bending strength of the femur diaphysis (A) was higher for diabetic mice initially at 16 weeks, but was no longer different at 34 weeks of age. Toughness (B) was lower for diabetic mice at both ages, but did not further decrease with the duration of diabetes. $K_{c,initial}$ (C) did not significantly vary with age or strain, though showed a tendency to be less for diabetic mice. Cracking toughness (D) did not differ between the strains, but was less for control mice at 34 weeks than for control mice at 16 weeks. The data points from animals with blood glucose levels consistently higher than 450 mg/dL are shown in purple. Brackets indicate significant differences between strain within age group, and letters indicate significant differences between age groups within strain.

Figure 4.

Structural properties and volumetric tissue mineral density of the femur diaphysis (median $\pm$ interquartile range with GLM p-values). Cortical area (A) and thickness (B) were higher for TallyHO mice than for the control mice at both ages, and these bone properties increased with age for both strains. Minimum moment of inertia (C) was not higher for diabetic mice until 34 weeks of age. TMD differences were similar to the strain- and age-related differences in cortical area and thickness (D). The data points from animals with blood glucose levels consistently higher than 450 mg/dL are shown in purple. Brackets indicate significant differences between strain within age group, and letters indicate significant differences between age groups within strain.

Figure 5.

Cortical porosity (A) and pore number (B) for the femur diaphysis (median $\pm$ interquartile range with GLM p-values). Overall, cortical porosity was lower for diabetic mice compared to controls, but did not change with age for either diabetic or non-diabetic mice. Pore number was also lower for the TallyHO mice at both ages and decreased with aging for both strains. Representative 3D renderings of cortical bone depict the porosity distribution. The data points from animals with blood glucose levels higher than 450 mg/dL are shown in purple. Brackets indicate significant differences between strain within age group, and letters indicate significant differences between age groups within strain.

Figure 6.

Linear regression of averaged blood glucose levels with body weight (A), P1NP (B), peak force (C) and cortical area (D) for TallyHO mice at 34 weeks of age. The data points from animals with blood glucose levels higher than 450 mg/dL are shown in purple.

Table 1
Selected characteristics (mean $\pm$ SD) of diabetic TallyHO mice and non-diabetic SWR/J mice.

Property	Unit	16 weeks				34 weeks				GLM p-values		
		SWR/J (n 7)	TallyHO (n 7)	SWR/J (n 11)	TallyHO (n 11)	SWR/J (n 10)	Strain	Age	Interaction			
Averaged body weight	g	24.6 $\pm$ 0.9	34.9 $\pm$ 2.4	#	30.2 $\pm$ 1.5	<i>a</i>	43.7 $\pm$ 5.6	#, <i>b</i>	<0.0001	<0.0001	N/A	0.096
HbA1c	%				3.9 $\pm$ 0.2		5.2 $\pm$ 1.3	#				
Insulin	ng/mL	0.278 $\pm$ 0.239	0.836 $\pm$ 0.565		0.129 $\pm$ 0.085		1.80 $\pm$ 1.21	#	0.013	0.109	0.011	
PINP	ng/mL	23.9 $\pm$ 6.5	12.3 $\pm$ 2.9	#	22.3 $\pm$ 5.2		10.8 $\pm$ 5.7	#	<0.0001	0.577	0.976	
TRAcP-5b	U/L	11.58 $\pm$ 3.41	8.95 $\pm$ 3.16		6.33 $\pm$ 2.20	<i>a</i>	7.08 $\pm$ 1.21		0.174	0.001		0.090

: adjusted p-value less than 0.05 for strain comparison within age group.

a & *b* : adjusted p-value less than 0.05 for age comparison within strain.

Table 2

Biomechanical properties (mean $\pm$ SD) as determined from three-point bending tests of the left femur mid-shaft.

Property	Unit	16 weeks				34 weeks				GLM p-values		
		SWR/J (n 13)	TallyHO (n 13)	TallyHO (n 12)	SWR/J (n 15)	TallyHO (n 15)	TallyHO (n 13)	Strain	Age	Interaction		
Modulus	GPa	12.4 $\pm$ 0.8	12.8 $\pm$ 1.5	#	13.1 $\pm$ 1.1	13.1 $\pm$ 1.3		0.432	0.062	0.522		
PYD	mm	0.391 $\pm$ 0.081	0.275 $\pm$ 0.123	#	0.292 $\pm$ 0.072	0.187 $\pm$ 0.065	#	0.006	0.001	0.833		
Yield Force	N	16.6 $\pm$ 1.4	19.1 $\pm$ 1.7	#	21.1 $\pm$ 2.3	23.2 $\pm$ 2.4	<i>b</i>	<0.0001	<0.0001	0.711		
Peak Force	N	17.9 $\pm$ 1.4	20.5 $\pm$ 1.7	#	24.4 $\pm$ 1.9	27.3 $\pm$ 2.6	<i>#b</i>	<0.0001	<0.0001	0.819		
Stiffness	N/mm	112 $\pm$ 9	115 $\pm$ 10		154 $\pm$ 12	172 $\pm$ 18	<i>#b</i>	0.417	<0.0001	0.026		
Work-to-failure	Nmm	8.07 $\pm$ 0.97	7.33 $\pm$ 2.01		8.77 $\pm$ 1.54	7.12 $\pm$ 1.68		0.248	0.168	0.320		

: adjusted p-value less than 0.05 for strain comparison within age group.

a & b : adjusted p-value less than 0.05 for age comparison within strain.

Table 3

Selected properties (mean ± SD) from micro-computed tomography evaluations and compression tests of the 6th lumbar vertebra.

Property	Unit	16 weeks				34 weeks				GLM p-values		
		SWR/J (n 13)	TallyHO (n 12)	SWR/J (n 16)	TallyHO (n 13)	Strain	Age	Interaction				
BV/TV	%	27.0 ± 2.2	25.0 ± 4.1	28.0 ± 2.0	23.0 ± 3.0	#	0.330	0.044				
Tb.TMD	mgHA/cm ³	919 ± 9	968 ± 20	#	951 ± 5	<i>a</i>	<0.0001	0.380				
ConnD	mm ⁻³	226 ± 30	182 ± 20	#	197 ± 19	<i>a</i>	<0.0001	0.040				
SMI		0.315 ± 0.276	0.742 ± 0.342	#	0.294 ± 0.211	#	0.001	0.080				
Tb.N	mm ⁻¹	4.79 ± 0.24	4.66 ± 0.22		4.89 ± 0.29		0.158	0.925				
Tb.Th	mm	0.054 ± 0.002	0.058 ± 0.005	#	0.055 ± 0.001		0.008	0.268				
Tb.Sp	mm	0.212 ± 0.013	0.211 ± 0.011		0.201 ± 0.013		0.806	0.447				
Axial stress	MPa	37.6 ± 4.9	44.8 ± 5.3	#	38.5 ± 8.3		<0.0001	0.245				
Peak force	N	29.3 ± 3.6	30.3 ± 4.1		28.0 ± 6.4		0.553	0.207				
Work-to-peak force	Nmm	6.25 ± 1.58	5.97 ± 2.60		5.41 ± 2.30		0.732	0.620				

: adjusted p-value less than 0.05 for strain comparison within age group.
a & b : adjusted p-value less than 0.05 for age comparison within strain.

Table 4

Selected properties (mean $\pm$ SD) from micro-computed tomography evaluations of the left femur including caliper measurements of femur length.

Property	Unit	16 weeks			34 weeks			GLM p-values		
		SWR/J (n 13)	TallyHO (n 13)	SWR/J (n 12)	SWR/J (n 16)	TallyHO (n 16)	Strain	Age	Interaction	
Diaphysis										
Tt.Ar	mm ²	1.49 ± 0.05	1.45 ± 0.09		1.66 ± 0.09	1.71 ± 0.06	b	0.114	<0.0001	0.016
Ct.Po.Dn	mm ⁻³	2.16 ± 0.22	1.51 ± 0.20	#	1.57 ± 0.15	1.14 ± 0.14	#b	<0.0001	<0.0001	0.038
Length	mm	14.1 ± 0.2	14.8 ± 0.2	#	14.8 ± 0.7	15.3 ± 0.3	#b	<0.0001	<0.0001	0.295
Ps.Pm	mm	4.65 ± 0.08	4.64 ± 0.14		4.93 ± 0.13	5.02 ± 0.10	b	0.812	<0.0001	0.100
Ec.Pm	mm	3.23 ± 0.06	2.93 ± 0.21	#	3.33 ± 0.19	3.32 ± 0.15	b	<0.0001	0.047	0.001
Distal Metaphysis										
BV/TV	%	20.3 ± 2.8	10.0 ± 2.5	#	10.3 ± 2.6	5.6 ± 1.6	#b	<0.0001	<0.0001	<0.0001
Tb.TMD	mgHA/cm ³	969 ± 11	981 ± 18		1027 ± 16	1038 ± 23	b	0.104	<0.0001	0.531
ConnD	mm ⁻³	347 ± 55	170 ± 67	#	122 ± 51	63 ± 22	#b	<0.0001	<0.0001	<0.0001
SMI		1.08 ± 0.28	2.39 ± 0.35	#	2.01 ± 0.22	2.94 ± 0.24	#b	<0.0001	<0.0001	0.012
Tb.N	mm ⁻¹	5.31 ± 0.31	3.87 ± 0.24	#	3.37 ± 0.55	3.08 ± 0.47	b	<0.0001	<0.0001	<0.0001
Tb.Th	mm	0.046 ± 0.002	0.048 ± 0.005		0.048 ± 0.002	0.049 ± 0.008		0.206	0.019	0.897
Tb.Sp	mm	0.186 ± 0.012	0.256 ± 0.016	#	0.305 ± 0.058	0.329 ± 0.060	b	<0.0001	<0.0001	0.039

#; adjusted p-value less than 0.05 for strain comparison within age group.

a & b; adjusted p-value less than 0.05 for age comparison within strain.

Table 5

Bone matrix composition (mean $\pm$ SD) from Raman Spectroscopy and HPLC analysis of the femur.

Property	Unit	16 weeks				34 weeks				GLM p-values		
		SWR/J (n 12)	TallyHO (n 12)	SWR/J (n 15)	TallyHO (n 13)	Strain	Age	Interaction				
<i>Raman</i>												
ν_1 PO ₄ /Amide I	-	28.6 ± 2.5	31.6 ± 4.0	#	30.7 ± 4.1	38.3 ± 3.7	# <i>b</i>	0.023	0.109	0.024		
ν_1 PO ₄ /Amide III	-	26.1 ± 2.6	21.9 ± 4.9		18.2 ± 3.8	14.6 ± 4.5	# <i>b</i>	0.007	< 0.0001	0.781		
ν_1 PO ₄ /Pro	-	16.1 ± 0.8	17.6 ± 2.4	#	18.3 ± 3.0	23.5 ± 4.4	# <i>b</i>	0.041	0.005	0.028		
CO ₃ /ν ₁ PO ₄	-	0.160 ± 0.003	0.152 ± 0.007	#	0.156 ± 0.006	0.146 ± 0.010	#	0.001	0.077	0.412		
Crystallinity	-	0.054 ± 0.0	0.054 ± 0.001		0.056 ± 0.003	0.056 ± 0.001	<i>b</i>	0.174	0.014	0.880		
1670/1640	-	1.55 ± 0.05	1.76 ± 0.18	#	1.65 ± 0.18	2.01 ± 0.33	#	< 0.0001	0.040	0.198		
1670/1690	-	1.78 ± 0.07	1.89 ± 0.06	#	2.13 ± 0.14	2.29 ± 0.20	# <i>b</i>	< 0.0001	< 0.0001	0.400		
1670/1610	-	2.89 ± 0.20	4.25 ± 1.84	#	3.36 ± 1.13	5.87 ± 2.52	#	0.016	0.103	0.209		
<i>HPLC</i>												
PYD	mol/mol	0.123 ± 0.059	0.138 ± 0.069		0.215 ± 0.053	0.226 ± 0.048	<i>b</i>	0.547	< 0.0001	0.894		
Pentosidine	mmol/mol	203 ± 120	141 ± 49		135 ± 66	151 ± 98		0.087	0.082	0.111		
<i>Fluorescence</i>												
fAGEs	ng/mg	92 ± 31	118 ± 44		127 ± 34	129 ± 33		0.095	0.004	0.217		

#; adjusted p-value less than 0.05 for strain comparison within age group.

a & *b*; adjusted p-value less than 0.05 for age comparison within strain.