

Dose-dependent effects of pharmaceutical treatments on bone matrix properties in ovariectomized rats

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ABSTRACT

As both anabolic and anti-catabolic osteoporosis drugs affect bone formation and resorption processes, they may contribute to bone's overall mechanical behavior by altering the quality of the bone matrix. We used an ovariectomized rat model and a novel fracture mechanics approach to investigate whether treatment with an anabolic (parathyroid hormone) or anti-catabolic (alendronate) osteoporosis drugs will alter the organic and mineral matrix components and consequently cortical bone fracture toughness. Ovariectomized (at 5 months age) rats were treated with either parathyroid hormone or alendronate at low and high doses for 6 months (age 6–12 months). Specifically, treatment groups included untreated ovariectomized controls ($n = 9$), high-dose alendronate ($n = 10$), low-dose alendronate ($n = 9$), high-dose parathyroid hormone ($n = 10$), and low-dose parathyroid hormone ($n = 9$). After euthanasia, cortical microbeams from the lateral quadrant were extracted, notched, and tested in 3-point bending to measure fracture toughness. Portions of the bone were used to measure changes in the 1) organic matrix through quantification of advanced glycation end-products (AGEs) and non-collagenous proteins, and 2) mineral matrix through assessment of mineral crystallinity. Compared to the ovariectomized group, rats treated with high doses of parathyroid hormone and alendronate had significantly increased cortical bone fracture toughness, which corresponded primarily to increased non-collagenous proteins while there was no change in AGEs. Additionally, low-dose PTH treatment increased matrix crystallinity and decreased AGE levels. In summary, ovariectomized rats treated with pharmaceutical drugs had increased non-collagenous matrix proteins and improved fracture toughness compared to controls. Further investigation is required for different doses and longer treatment periods.

1. Introduction

Bone's fracture resistance is influenced by properties related to bone quantity (e.g., mass) and bone quality (e.g., geometry, tissue matrix) (Boskey and Imbert, 2017). Although the effects of bone mass and geometry on skeletal mechanics and fragility have been investigated extensively (Karim et al., 2013a, 2013b), there is far less information available on the effects of the tissue matrix on bone's mechanical properties, particularly in cortical bone. Gaining insight into this relationship is important because the bone matrix can undergo substantial changes with aging or with the onset of clinical conditions such as post-menopausal osteoporosis (Sroga and Vashishth, 2012; Osterhoff et al., 2016).

Post-menopausal osteoporosis has commonly been simulated by an ovariectomized (OVX) animal model, in particular the OVX rat (Thompson et al., 1995). Osteoporosis can be treated by pharmaceutical drugs including bisphosphonates and parathyroid hormone, both of which can alter bone's matrix composition and have direct consequences on bone's mechanical behavior (Jerome et al., 2001; Allen and Burr, 2011). Bisphosphonates, such as alendronate (ALN), are a group of anti-remodeling drugs that impair the action of osteoclasts on the bone surface which suppress bone resorption (Masarachia et al., 1996; Boivin and Meunier, 2002). This class of drugs induces morphological changes in osteoclasts and forces early cell apoptosis to prevent further bone loss (Masarachia et al., 1996). The decreased resorption may consequently increase overall bone mass (Boivin and Meunier, 2002) and decrease

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fracture risk (Cummings et al., 1998; Black et al., 2000; Axelsson et al., 2017). In contrast, administration of intermittent parathyroid hormone (PTH), an anabolic hormone treatment, stimulates osteoblasts and subsequently increases bone formation, outweighing the resorption process (Girotra et al., 2006; Gesty-Palmer et al., 2009). As a result, PTH treatment can increase bone mass and decrease fracture risk in patients (Neer et al., 2001).

Both ALN and PTH directly affect bone's resorptive and formative activities, and consequently, bone's matrix components. The dominant constituents of bone's matrix are inorganic hydroxyapatite mineral and organic proteins including type I collagen and non-collagenous proteins (e.g., osteocalcin, osteopontin) (Burr and Allen, 2013). Type I collagen can undergo numerous biochemical modifications including non-enzymatic glycation, which is a spontaneous reaction involving extracellular sugars and amino acid residues that result in the formation of chemical adducts and crosslinks known as advanced glycation end-products (AGEs). The accumulation of AGEs may correlate with a deterioration in bone's overall mechanical properties (Vashishth et al., 2001; Hernandez et al., 2005; Tang et al., 2007; Garnero, 2012; Karim and Vashishth, 2012; Nyman and Makowski, 2012; Karim et al., 2013a, 2013b).

A number of studies indicate that AGE accumulation is associated with reduced bone formation (Yang et al., 2016), resorption (Valcourt et al., 2007), turnover (Allen et al., 2008), as well as deteriorated bone stiffness, toughness, other post-yield mechanical properties, and indentation properties (Wang et al., 2002; Nyman et al., 2006; Nyman et al., 2007; Viguet-Carrin et al., 2008; Tang et al., 2009; Poundarik et al., 2015; Karim et al., 2018; Hunt et al., 2019; Merlo et al., 2020). Additionally, the non-collagenous proteins osteopontin and osteocalcin can affect bone's mechanical behavior. These proteins are involved in mineralization (Glowacki et al., 1991; Goldstein et al., 1993; Ducy et al., 1996; Fantner et al., 2005), bone resorptive and formative activities (Boskey et al., 1998; Kavukcuoglu et al., 2009; Sroga et al., 2011a, 2011b; Sroga and Vashishth, 2012), and together act to link the organic-inorganic interface in bone and hold mineralized collagen fibers together thereby enhancing bone toughness (Hara et al., 2007; Poundarik et al., 2012; Bailey et al., 2017; Nikel et al., 2018; Poundarik et al., 2018).

The goal of this work was to understand how alterations in bone's organic matrix impact bone fracture toughness, a measure of bone's inherent ability to resist crack initiation and propagation (Vashishth, 2004; Ural and Vashishth, 2014). To accomplish this goal, we used an OVX rat model to stimulate bone resorption paired with different doses of ALN and PTH treatment to manipulate bone tissue quality across a wide spectrum. Specifically, using high doses of these drugs that are beyond clinically-relevant doses allowed us to investigate how alterations in bone's organic and inorganic matrix components may impact bone fracture toughness. Previous data demonstrated that administration of ALN and PTH in an OVX model or in osteoporosis can alter 1) bone's organic matrix through changes in type I collagen by the accumulation of AGEs (Saito et al., 2011; Kimura et al., 2017), 2) non-collagenous proteins such as osteocalcin and osteopontin (Kurland et al., 2000; Chiang et al., 2011; Shao et al., 2017), and/or 3) bone's mineral (Chesnut et al., 1995; Schwarz et al., 2012). We hypothesized that both ALN and PTH treatments will improve bone fracture toughness through alterations in bone matrix quality including both the mineral and organic matrix.

2. Methods

2.1. Experimental drug treatment

As the effects of ovariectomy on bone properties, including fracture toughness, are already well established in the literature (Camargos et al., 2015; Yu et al., 2015; Massie et al., 2021), we focused on comparing drug-treated OVX rats with non-treated OVX controls. The OVX

procedure was performed by Charles River Laboratories at 5 months of age for all groups and drug treatment administration began after acclimatization at 6 months of age by subcutaneous injection. Selection of drug treatment doses is supported by previous reports (Peter and Rodan, 1999; Vahle et al., 2002). Female Sprague-Dawley rats (n = 47) were divided by body weight into untreated (no injection) OVX controls (n = 9), high-dose alendronate (H-ALN, n = 10, 2 mg/kg, 2×/week), low-dose alendronate (L-ALN, n = 9, 10 µg/kg, 2×/week), high-dose PTH (H-PTH, n = 10, 75 µg/kg, 5×/week), and low-dose PTH (L-PTH, n = 9, 0.3 µg/kg, 5×/week) groups. Rats were given access to a standard rodent chow and water ad libitum, were subjected to a 12:12 hour light:dark cycle, and were housed in 2 rats/cage in the same room. Rats were sacrificed at 12 months of age. Following sacrifice via isoflurane inhalation and cervical dislocation, femurs were dissected for use in this study and stored in saline at -20 °C until use. All animal procedures were approved by the Stony Brook University's Institutional Animal Care and Use Committee.

2.2. Assessment of specimen and crack geometry

Cortical microbeams (~0.70 mm × 11.3 mm × 0.85 mm) were cut from the lateral quadrant of rat femur diaphyses using a low-speed diamond wafer blade (South Bay Technology, Inc.) based on protocols we used previously (Tommasini et al., 2012). Uneven surfaces were polished using fine 1500 grit sandpaper. Microbeams were notched on the periosteal side to approximately 20% of the width, following ASTM (2009, 2011) standards as closely as possible. Notches were made using a diamond wafer blade and then manually sharpened with a scalpel blade. Microcomputed tomography (µCT) was used to measure bone geometry at 17.5 µm voxel size prior to testing as done previously (Scanco Medical AG, Brüttisellen, Switzerland) (Tommasini et al., 2012; Wallace et al., 2012). 3D rendered images of the area containing the bone notch were visualized and rotated directly in the microCT image viewer. Once the notch was identified, 2D images containing a bone slice showing full dimensions of the beams cross-section and showing the largest crack size were used to calculate crack dimensions. The notch depth was calculated as the difference in length from the edge of the microbeam to the notch, measured in the center of the microbeam. Specimen thickness, specimen width, and length were also measured from µCT images.

2.3. Measurement of mechanical properties

As measurement of rodent bone's material properties are difficult to evaluate due to size constraints, we used a fracture mechanics approach for small animal models to measure cortical bone toughness. Microbeams were tested in 3-point bending tests in anatomically correct configuration with the notched side placed in tension (Fig. 1) to determine critical stress intensity factor, K_{IC} or fracture toughness at crack initiation. The span length was fixed at 3.2 mm. Testing was conducted under constant hydration with saline (Ritchie et al., 2008, 2010) using a micromechanical testing system (Electroforce 3200, TA Instruments, New Castle, DE). Bending tests were performed at a loading rate of 0.001 mm/s until failure occurred. Force-deformation curves, specimen geometry, and notch dimensions were used to calculate fracture toughness at crack initiation using Eqs. (1) and (2) as described by ASTM (2009, 2011) standards:

$$K_{IC} = \frac{PS}{BW^{\frac{3}{2}}} f\left(\frac{a}{W}\right) \quad (1)$$

$$f\left(\frac{a}{W}\right) = 3\sqrt{\frac{a}{W}} \frac{1.99 - \frac{a}{W} \left(1 - \frac{a}{W}\right) \left(2.15 - 3.93 \frac{a}{W} + 2.7 \left(\frac{a}{W}\right)^2\right)}{2 \left(1 + 2 \frac{a}{W}\right) \left(1 - \frac{a}{W}\right)^{\frac{3}{2}}} \quad (2)$$

With the variables defined as: P = load at initiation (A secant line

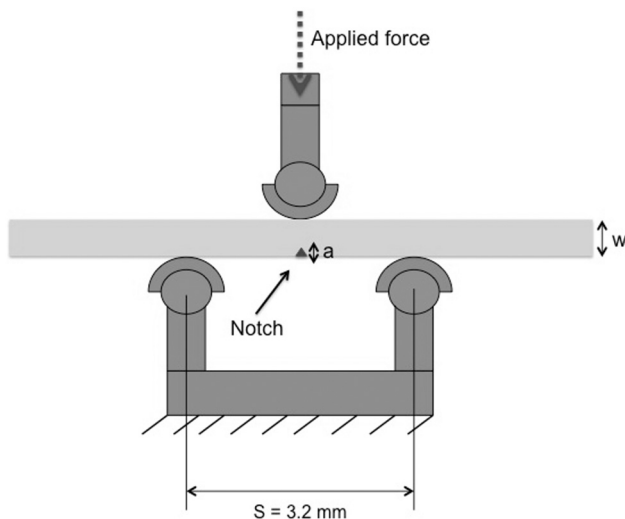


Fig. 1. Schematic diagram of the 3-point bending configuration for fracture toughness tests.

with 5% lower slope than the elastic modulus was plotted on the load-deformation curve, and its intersection with the curve was used to determine the load at initiation); S = span length; B = specimen thickness (measured along the side of the created notch); W = specimen width (measured along the side that is perpendicular to the created notch); a = crack size; and $f\left(\frac{a}{W}\right)$ = geometrical shape factor.

2.4. Protein extraction

A small portion of cortical bone beam ($7.45 \text{ mg} \pm 4.31 \text{ mg}$) was collected for protein extraction. Each specimen was first washed in cold nanopure water until free of blood. Specimens were defatted by three 15-minute washes in 500 μL of cold isopropyl ether under constant agitation. Liquid nitrogen was placed into a mortar and pestle unit and used to crush each specimen into a fine bone powder. The bone powders were then placed into microcentrifuge tubes.

Protein extraction and dialysis were conducted based on an established protocol (Sroga et al., 2011a, 2011b, Sroga and Vashishth, 2011). Briefly, 0.08 mL/mg of an extraction buffer (0.05 M tetrasodium EDTA, 4 M guanidine chloride, 30 mM Tris-HCL, 1 mg/mL BSA, 1 mL/L protease inhibitor, pH 7.4) was added to each microcentrifuge tube containing bone powders. A dialysis membrane (Spectrum Laboratories) was sealed between the tube and its lid (with hole), and the tubes were inverted into a beaker of extraction buffer. Protein extraction was conducted at 4 °C for 48 h. Specimen tubes were then transferred to beakers containing 1 \times PBS with protease inhibitor. Dialysis was conducted at 4 °C for 120 h. The 1 \times PBS solution was changed 3 times daily for the first 48 h, then once daily for the following 72 h. The extracts were centrifuged at 13,000 rpm for 30 min at 4 °C.

A portion of the supernatant was saved in -80 °C for enzyme-linked immunosorbent assays (ELISA). Another portion of the supernatant was vortexed and lyophilized for hydrolysis for AGEs measurement. Hydrolysis was conducted at 110 °C for 20 h (10 μL of 6 N HCL per 1 mg bone).

2.5. Quantification of non-collagenous proteins and advanced glycation end-products

Protein extracts were diluted 9370 \times and 1875 \times for measurement of osteopontin and osteocalcin, respectively, and proteins were quantified according to manufacturer's protocols included with rat ELISA kits (Osteopontin: IBL-America, cat# 27360, Osteocalcin: Biomedical

Technologies, cat# BT-490). A pre-assay with several dilutions of 2 randomly selected samples was performed to determine the dilution factors.

To measure total fluorescent AGEs, quinine standards and 200 \times diluted specimen hydrolysates were measured for fluorescence at 360/460 nm excitation/emission with a Synergy HT microplate reader (BioTek, Winooski, VT). A chloramine-T solution was added to hydroxyproline standards and specimen hydrolysates to oxidize hydroxyproline. A perchloric acid solution was then added to remove residual chloramine-T. Finally, a *p*-dimethylaminobenzaldehyde solution was added and incubated at 60 °C. Absorbance was measured on cooled specimens at 570 nm using the same microplate reader used in previous steps. Collagen content was calculated from hydroxyproline quantity for each specimen (Gross, 1958). Quinine fluorescence was then normalized to collagen content to measure total fluorescent AGEs.

2.6. Reflection-based Fourier Transform Infrared Microspectroscopy

Cortical bone from the lateral quadrant of the contralateral femur of each rat was analyzed using reflection-based Fourier Transform Infrared Microspectroscopy (FTIRM). Cortical bones were dehydrated in a series of ethanol washes and embedded in methyl methacrylate (PMMA) blocks. Hardened blocks were polished (Buehler, Lake Bluff, IL) with 1200 grit carbide paper and a cloth impregnated with diamond suspensions (particle size of 3, 1, 0.25, and 0.05 μm). Polishing on each sample was done for 3 min at each step. FTIRM was then conducted using a Nicolet Magna 860 Step-Scan FTIR spectrometer and Continuum IR microscope (Thermo Fisher Scientific, Waltham, MA). Data were collected in reflection mode (15 μm beam size, 4/cm resolution, 256 scans/pixel) from the surface of polished cortical bone. Spectra collected were analyzed for the ratio of non-reducible to reducible collagen crosslinks (peak height ratio 1660/1690 cm^{-1} , linear baseline 1800 cm^{-1}) and mineral crystallinity (peak height ratio 1020/1030 cm^{-1} , linear baseline 930–1150 cm^{-1}) using peak height ratios according to published protocols (Fig. 2) (Miller et al., 2007; Acerbo et al., 2014).

2.7. Statistical analyses

Shapiro-Wilk tests for normality were conducted for all variables. Fracture toughness, osteopontin, total fluorescent AGEs, and collagen maturity data were compared for 1) OVX vs ALN-treated groups and for 2) OVX vs PTH-treated groups with two separate One-Way ANOVA with Tukey post-hoc tests, while osteocalcin and mineral crystallinity were compared with two separate non-parametric Kruskal-Wallis with Dunn's post-hoc tests. Multiple regression analyses to predict fracture toughness with non-collagenous proteins, total fluorescent AGEs, collagen maturity, and crystallinity were conducted for OVX vs ALN-treated groups and for OVX vs PTH-treated groups. Significance was determined based on $\alpha = 0.05$, and any p-values between 0.05 and 0.1 were considered as borderline significant. All statistical analyses were completed using Stata/IC 13.1 (StataCorp) and SPSS 26 (IBM).

3. Results

3.1. Alendronate treatment

Fracture toughness of the H-ALN group was greater than that of the OVX (+82.5%, $p \leq 0.01$) and L-ALN (+82.5%, $p \leq 0.01$) groups (Table 1, Fig. 3A). No differences were observed in AGE content between OVX and ALN-treated rats (Table 1, Fig. 3B). Osteocalcin was significantly greater in H-ALN rats compared to OVX rats (+32.1%, $p \leq 0.05$) and in H-ALN rats compared to L-ALN rats (+39.8%, $p \leq 0.05$) (Table 1, Fig. 3C). Osteopontin was greater in H-ALN rats compared to OVX rats (+133.9%, $p \leq 0.01$) and compared to L-ALN rats (+74.7%, $p \leq 0.05$) (Table 1, Fig. 3D). No differences were observed in mineral crystallinity

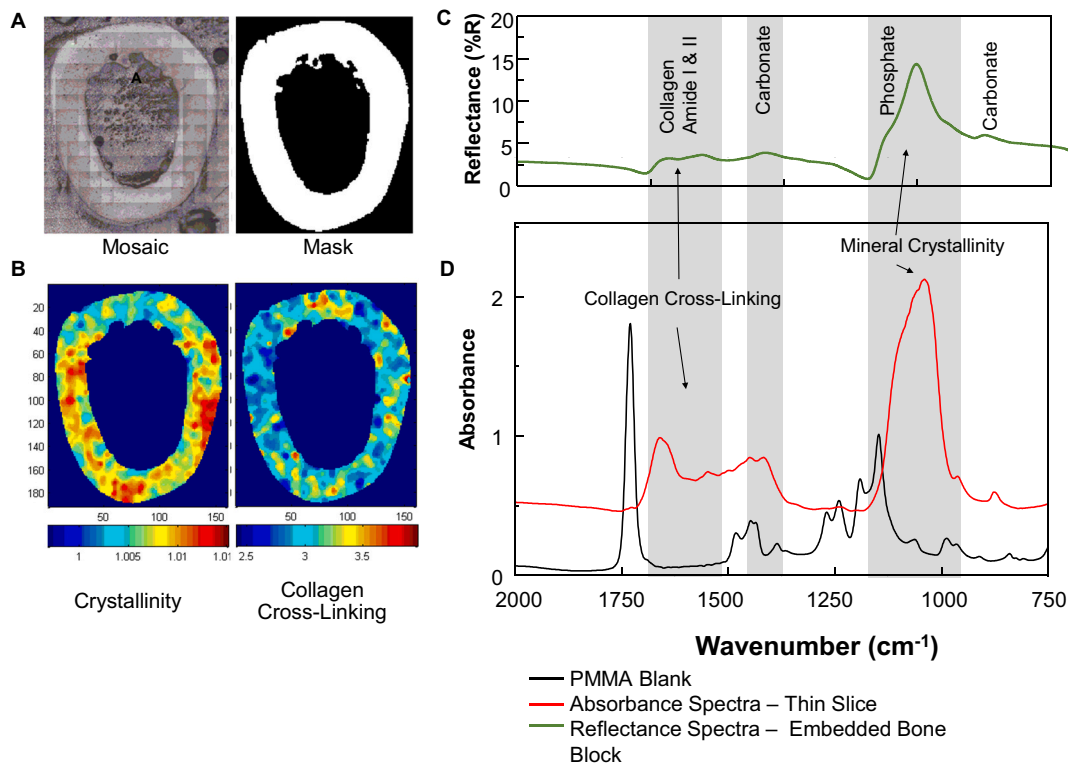


Fig. 2. Reflection-based Fourier Transform Infrared Microspectroscopy. (A) Bone blocks embedded in PMMA (green) were analyzed for (B) ratio of non-reducible to reducible collagen crosslinks (peak height ratio 1660/1690 cm^{-1} , linear baseline 1800 cm^{-1}) and mineral crystallinity (peak height ratio 1020/1030 cm^{-1} , linear baseline 930–1150 cm^{-1}) using peak height ratios, these methods were compared (D) to blank PMMA blocks (black), and a thin slice (red) cut from the same bone block as previously published (Acerbo et al., 2014). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Average and standard deviation of each group for fracture toughness, non-collagenous protein content, total fluorescent AGEs, collagen maturity, and crystallinity.

Group	Fracture toughness (kPa $\sqrt{\text{m}}$)	Osteocalcin (pg/ μg)	Osteopontin (pg/ μg)	AGEs (ng quinine/mg collagen)	Collagen maturity	Crystallinity
OVX	0.90 \pm 0.41	0.54 \pm 0.20	0.03 \pm 0.02	845.3 \pm 136.1	1.95 \pm 0.10	1.28 \pm 0.02
H-ALN	1.64 \pm 0.41 \diamond *	0.72 \pm 0.07 \diamond *	0.06 \pm 0.02 \diamond *	890.7 \pm 163.4	1.91 \pm 0.04	1.30 \pm 0.06
L-ALN	0.90 \pm 0.57	0.51 \pm 0.21	0.04 \pm 0.03	799.7 \pm 131.1	1.94 \pm 0.11	1.29 \pm 0.05
H-PTH	1.44 \pm 0.48 \diamond *	0.62 \pm 0.24	0.07 \pm 0.05	791.5 \pm 221.7*	1.92 \pm 0.05	1.31 \pm 0.03 \diamond
L-PTH	0.96 \pm 0.53	0.57 \pm 0.28	0.07 \pm 0.07	555.8 \pm 123.7 \diamond	1.86 \pm 0.09	1.37 \pm 0.08 \diamond

Significant differences between groups are reported by One-Way ANOVA with Tukey post-hoc tests for fracture toughness, osteopontin, AGEs, and collagen maturity, and by Kruskal-Wallis with Dunn's post-hoc tests for osteocalcin and crystallinity.

\diamond $p \leq 0.05$ compared to OVX group.

* $p \leq 0.05$ compared to low dose drug group.

or collagen maturity between OVX and ALN-treated groups (Table 1, Fig. 3E–F).

Multiple regression analyses indicated that in OVX and ALN-treated groups, 28% of the variance in fracture toughness was explained by non-collagenous proteins, total fluorescent AGEs, collagen maturity, and crystallinity as independent variables (full model: unadjusted $r^2 = 0.56$, adjusted $r^2 = 0.28$). Although it appeared that osteopontin alone was the significant predictor of fracture toughness ($p \leq 0.05$), the full multiple regression model itself was not significant ($p = 0.18$, Table 2).

3.2. Parathyroid hormone treatment

Fracture toughness for the H-PTH group was greater than that of the OVX (+60.2%, $p \leq 0.05$) and L-PTH (+49.6%, $p \leq 0.05$) groups (Table 1, Fig. 3A). Total fluorescent AGEs were significantly less in L-PTH rats compared to OVX (–34.2%, $p \leq 0.01$) and H-PTH rats (–42.4%, $p \leq 0.01$) (Table 1, Fig. 3B). There was no difference in osteocalcin between PTH groups and OVX (Table 1, Fig. 3C). Differences

in OPN between groups were borderline significant with OPN being greater in the H-PTH (+176.6%, $p = 0.071$) and L-PTH (+176.2%, $p = 0.077$) groups compared to the OVX group (Table 1, Fig. 3D). There was higher mineral crystallinity in both the L-PTH group (+7.0%, $p \leq 0.001$) and H-PTH group compared to OVX (+4.1%, $p \leq 0.05$) (Table 1, Fig. 3E). The 4.6% decrease in collagen maturity in the L-PTH group compared to the OVX group was borderline significant ($p = 0.051$, Table 1, Fig. 3F).

In OVX and PTH-treated groups, fracture toughness was predicted by the independent variables (full model: unadjusted $r^2 = 0.60$, adjusted $r^2 = 0.41$, $p \leq 0.05$, Table 2) with osteocalcin ($p \leq 0.05$), osteopontin ($p = 0.059$), collagen maturity ($p \leq 0.05$), and crystallinity ($p \leq 0.05$) all being predictors of fracture toughness. Total fluorescent AGEs did not correlate with fracture toughness in the PTH treated group.

4. Discussion

We investigated the effect of bone matrix changes on fracture

Table 2

Multiple regression models to predict fracture toughness (kPa \sqrt{m}) with non-collagenous proteins, total fluorescent AGEs, collagen maturity, and crystallinity as independent variables.

OVX and ALN groups		
Multiple regression model: $r = 0.75$, $r^2 = 0.56$, adjusted $r^2 = 0.28$, p -value = 0.18		
	Unstandardized coefficient	Standardized coefficient
AGEs (ng quinine/mg collagen)	0.001	0.373
Osteocalcin (pg/ μ g)	-1027.4	-0.219
Osteopontin (pg/ μ g)	14,636.1*	0.780*
Collagen maturity	3.2	0.366
Crystallinity	7.0	0.485
OVX and PTH groups		
Multiple regression model: $r = 0.77$, $r^2 = 0.60$, adjusted $r^2 = 0.41$, p -value ≤ 0.05		
	Unstandardized coefficient	Standardized coefficient
AGEs (ng quinine/mg collagen)	<0.001	0.035
Osteocalcin (pg/ μ g)	1682.4*	0.530*
Osteopontin (pg/ μ g)	7145.6 [†]	0.593 [†]
Collagen maturity	8.8*	1.196*
Crystallinity	7.1*	0.776*

* $p \leq 0.05$.

[†] $0.05 < p \leq 0.06$.

toughness properties in OVX rats induced by high and low doses of ALN and PTH treatments. Although the measurement of bone material properties in small rodent bones is challenging, we utilized a novel fracture mechanics approach that allowed the measurement of fracture toughness at crack initiation in rat cortical microbeams. In support of our hypothesis, ALN treatment did improve fracture toughness and this improvement was due to increased osteopontin content without any changes in AGEs or mineral crystallinity. Also supporting our hypothesis, PTH treatment improved fracture toughness and this improvement was due to increased mineral crystallinity, collagen maturity, and osteocalcin and osteopontin. However, although AGE content was significantly reduced in PTH-treated groups, reduced AGEs were not a major contributing factor according to our multiple regression analysis. Overall, changes in bone matrix quality components helped to explain the variance in fracture toughness with only osteopontin having a significant effect with ALN treatment, and non-collagenous proteins, collagen maturity, and mineral crystallinity all having a significant effect with PTH treatment.

The increased non-collagenous protein content in ALN-treated rats may be due to changes in bone cell activity induced by bisphosphonate treatment (Sahni et al., 1993; Russell and Rogers, 1999). Bisphosphonates adsorb to bone mineral at resorption sites, making them easily accessible to osteoclasts, which then internalize them via endocytosis. Hence, bisphosphonates disturb osteoclast cell metabolism and decrease survival, leading to inhibition of osteoclast proliferation and activity (Russell and Rogers, 1999; Komatsu et al., 2013). Although there are reports of ALN having no positive effect on osteoblast proliferation and activity (García-Moreno et al., 1998; Iwata et al., 2006), a number of studies illustrate that ALN stimulates osteoblastic differentiation, activity, and/or increases the average maturity of active osteoblasts in bone (Sahni et al., 1993; Altundal and Gursoy, 2005; Xiong et al., 2009; Komatsu et al., 2013; Ma et al., 2018; Xu et al., 2019). Mature osteoblasts demonstrate upregulated osteopontin and osteocalcin production in comparison to immature osteoblasts (Bellows et al., 1999). Inhibited bone resorption combined with unchanged or increased bone formation results in the bone formation rate surpassing the resorption rate. Consequently, there is more net osteoblast activity where osteoblasts produce both osteocalcin (Chenu et al., 1994; Barille et al., 1996) and

osteopontin (Reinholt et al., 1990), further contributing to increased non-collagenous protein content with ALN treatment. Our study was limited in that rats do not undergo intracortical remodeling and we did not directly assess the effects of ALN on bone cell response or conduct histomorphometric analysis that could indicate the amount of bone resorbed or newly formed, which should be done in our future work.

ALN treatment increased fracture toughness, which represents the inherent resistance of a material to initiate the formation of a crack. The change in toughness found here may result from the increased production of osteocalcin and osteopontin due to ALN treatment. In support, we conducted a multiple regression analysis to identify the predictors of fracture toughness when an OVX model was treated with ALN. This analysis showed that osteopontin was the primary predictor of fracture toughness rather than AGE content, osteocalcin, mineral crystallinity, and collagen maturity as other possible factors. Hence, it seems that ALN treatment preserves osteopontin in the matrix and/or promotes secretion of more osteopontin via stimulation of osteoblasts as described above. Non-collagenous matrix proteins play an important role in bone fracture toughness by a collective interaction between osteocalcin and osteopontin that results in dilatational band formation and a consequent crack initiation (Duvall et al., 2007; Thurner et al., 2010; Poundarik et al., 2012; Rodrigues et al., 2012; Poundarik et al., 2015). However, we found that only administration of a high dose of ALN, beyond clinical dosage, increased the fracture toughness of bone whereas fracture toughness of L-ALN treated rats was not significantly different from OVX controls. It is possible that the 6-month treatment at the lower, more clinically relevant dose of ALN, was not delivered for a long enough duration to measure the long-term effects of increased production of non-collagenous proteins and subsequent changes in toughness as was observed in the higher dose. Further work with low-dose ALN for longer treatment duration would clarify these results.

Intermittent delivery of PTH can stimulate bone growth through increased differentiation of osteoblasts or an anti-apoptotic effect on osteoblasts (Jilka et al., 1999; Datta and Abou-Samra, 2009; Osagie-Clouard et al., 2017), resulting in increased bone formation (Aslan et al., 2012). In support, we observed a trend for increased osteopontin in PTH-treated groups. Osteopontin is produced by osteoblasts (Reinholt et al., 1990), which can regulate mineral composition in the bone matrix (Poundarik et al., 2018), and plays an important role in fracture toughness through its interactions with osteocalcin (Duvall et al., 2007; Thurner et al., 2010; Poundarik et al., 2012; Rodrigues et al., 2012; Poundarik et al., 2015). Hence, because PTH stimulates osteoblasts, treatment with PTH will have a downstream effect of producing more osteopontin. As a result, bone will have increased ability to dissipate energy under applied loads to prevent crack production with a consequent improvement in bone fracture toughness (Hara et al., 2007; Poundarik et al., 2012; Bailey et al., 2017; Nikel et al., 2018; Poundarik et al., 2018). This assertion also supports the report of an increase in small pores in cortical bone in PTH-treated rats (Tommasini et al., 2012), where small pores can more effectively absorb shear forces than large pores (Cheng et al., 2009) and consequently prevent or reduce crack production (Herman et al., 2010).

PTH treatment increased fracture toughness, but only in the high PTH group. Given that PTH can increase bone mass and decrease fracture risk (Neer et al., 2001), this increase in bone fracture toughness is not surprising. The observed increase in non-collagenous protein content likely played a role for the increased fracture toughness in this group. In support, multiple regression analysis conducted to identify predictors of fracture toughness when starting in an OVX condition and then being treated with PTH showed that non-collagenous proteins, collagen maturity, and crystallinity were predictors of fracture toughness.

Although there were no differences in collagen components (i.e., AGEs), we observed that AGEs were reduced in L-PTH group compared to H-PTH or OVX groups, with no change in fracture toughness. Moreover, although not statistically significant, there tended to be lower

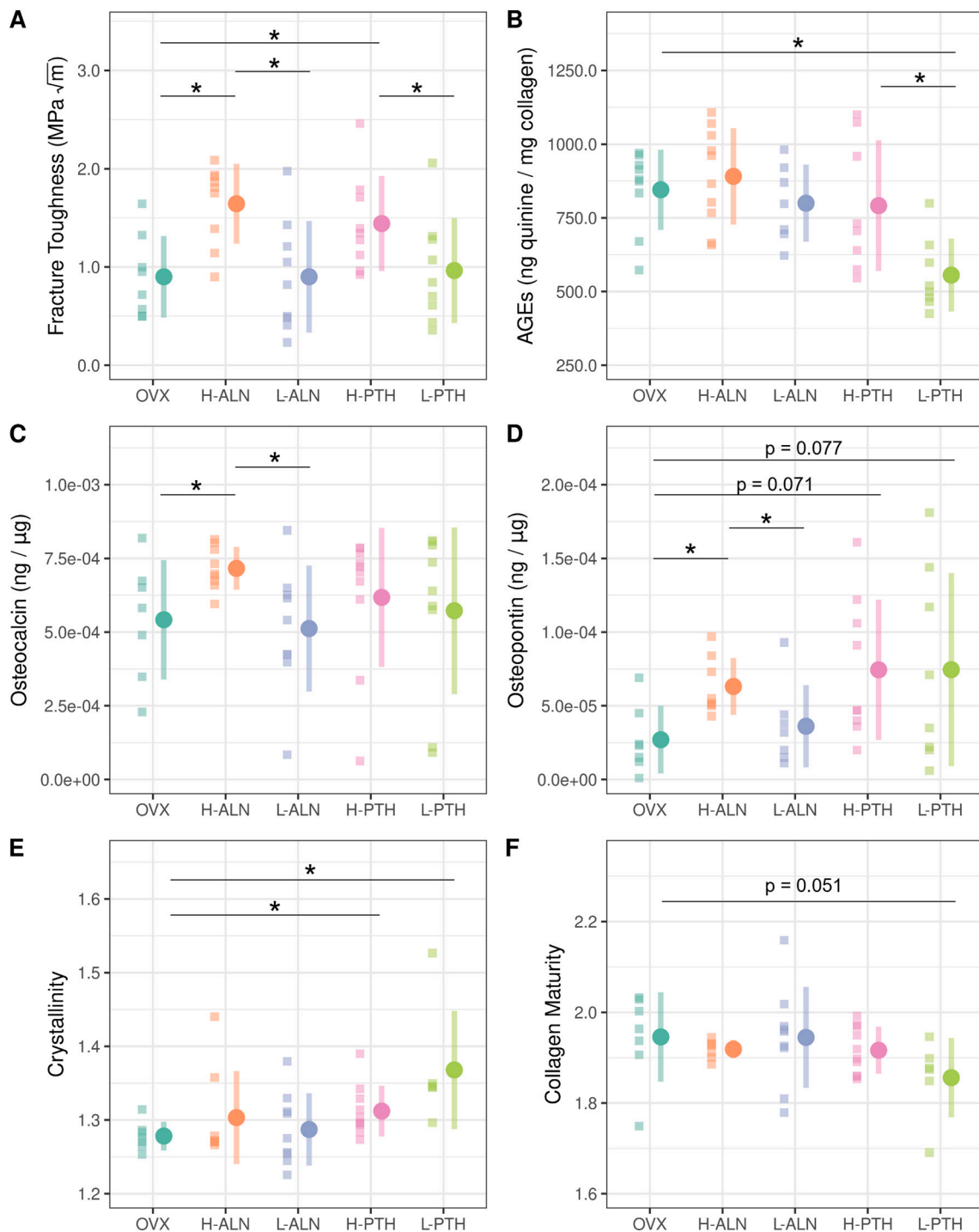


Fig. 3. (A) Fracture toughness, (B) AGEs, (C) osteocalcin, (D) osteopontin, (E) crystallinity, and (F) collagen maturity for OVX rats treated with alendronate and parathyroid hormone. Graphs depict individual data points (squares) and the mean (circles) \pm standard deviation for each group. Significant differences between groups ($p \leq 0.05$) are indicated with an asterisk.

collagen maturity in the L-PTH group compared to OVX controls. This suggests that 1) the L-PTH treated bone contained less mature collagen and consequently resulted in less AGE formation, and 2) AGEs do not significantly impact fracture toughness in an OVX rat model, which is supported by our multiple regression analyses (Table 2). Additionally, L-PTH rats had increased mineral crystallinity, which further supports the notion that PTH increases bone quantity in cortical bone, albeit through changes in mineral apposition (Sato et al., 2002). Increased mineral crystallinity as well as the decreased quantity of glycation-induced crosslinks found here would typically suggest improved bone

toughness (Vashishth et al., 2001; Vashishth, 2005; Tang et al., 2007). Additional work is needed with low dose PTH at longer durations of treatment to help explain these findings.

Our fracture toughness analysis provided information on how matrix level changes affect the resistance of bone as a material while eliminating the effect of idealized assumptions used in whole bone fracture toughness tests (i.e., assuming femurs as idealized hollow cylinders). Nevertheless, this method has some inherent limitations that should be considered in the interpretation of the results. For example, our method measured fracture toughness or resistance of bone against the initiation

of a crack. Because bone is known to crack and not fracture, based on this data we cannot directly predict whether higher resistance to crack initiation will increase or decrease bone fragility. Previous studies on bone have demonstrated that the tendency of bone to form micro-damage that does not coalesce into a fracture enhances toughness, while increased stress to initiate a crack that progresses into a fracture without collateral damage is the hallmark of brittle bone (Vashishth et al., 1997; Nalla et al., 2003).

In addition to the limitations stated above, further work is needed to assess other possibilities through which ALN or PTH treatment could impact fracture toughness including the impact of microarchitecture, degree of mineralization, other non-collagenous proteins, and specific AGEs. We previously reported in the same rats used in this study, that the lateral quadrant of femoral diaphyses had lower lacunae per tissue volume in ALN-treated rats compared to untreated OVX controls and comparatively more small pores in the tissue in PTH-treated rats (Tommasini et al., 2012). We also reported that ALN treatment but not PTH treatment mitigated the increased collagen alignment and reduced mineral crystal lengths due to OVX in new periosteal bone, while both treatments reduced mineral crystal width in existing intracortical bone (Acerbo et al., 2014). However, further work is needed to understand the direct effect of these factors on fracture toughness.

Despite these limitations, our results are largely consistent with previous clinical trials and prospective studies on post-menopausal women with bisphosphonates that show a reduction in fracture risk with an associated increase in bone mineral density (Bone et al., 2000; Reginster et al., 2000). While we found no change in glycation content under bisphosphonate treatment, work on other animal models showed that bisphosphonates increased glycation (Saito et al., 2008; Tang et al., 2009). AGEs accumulate in bone over time and previous studies used longer treatment duration (2–3 years) and a different animal model. Because AGE accumulation has been implicated in long-term treatment with bisphosphonate and in atypical femoral fractures, our data supports short-term treatment can minimize AGE accumulation in bone. This highlights the beneficial effects of non-collagenous proteins such as increase in content of osteocalcin and osteopontin with high ALN dose to increase fracture toughness while keeping accumulation of AGEs under control. In support, our multiple regression analyses indicated that fracture toughness can be predicted by non-collagenous proteins, collagen maturity, and crystallinity in OVX and PTH groups. Specifically, we found that the high-dose PTH group had increased fracture toughness that was explained in part by increased non-collagenous protein, osteopontin, in the matrix with no changes in AGEs. Low-dose PTH resulted in reduction of AGEs but no significant changes in osteocalcin and osteopontin content or increased fracture toughness. Such dose-specific differences have been previously reported in literature regarding the effect of PTH therapy on cortical bone (Compston, 2007). A few studies showed minimal or even negative effects while other studies showed beneficial effects of PTH treatment on cortical bone health (Jerome et al., 1999; Compston, 2007; Iwaniec et al., 2008; Ascenzi et al., 2012). Altogether, the current study demonstrated that treatments that enhance fracture toughness compared to OVX controls were attributed to increased presence of non-collagenous matrix proteins while minimizing the accumulation of AGEs in the cortical bone matrix.

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CRediT authorship contribution statement

Lamya Karim: Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Andrea Kwaczala:** Methodology, Investigation, Writing – original draft, Writing – review & editing. **Deepak Vashishth:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Stefan Judex:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

None of the authors have conflicts of interest to disclose.

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References

- 2009 ASTM E399-09 Standard Test Method for Linear-elastic Plane-strain Fracture Toughness K_{IC} of Metallic Materials, 2009, p. 33.
- 2011 ASTM E1820-11 Standard Test Method for Measurement of Fracture Toughness, 2011, p. 56.
- Acerbo, A.S., Kwaczala, A.T., Yang, L., Judex, S., Miller, L.M., 2014. Alterations in collagen and mineral nanostructure observed in osteoporosis and pharmaceutical treatments using simultaneous small- and wide-angle X-ray scattering. *Calcif. Tissue Int.* 95 (5), 446–456.
- Allen, M., Burr, D., 2011. Bisphosphonate effects on bone turnover, microdamage, and mechanical properties: what we think we know and what we know that we don't know. *Bone* 49, 56–65.
- Allen, M.R., Gineyts, E., Leeming, D.J., Burr, D.B., Delmas, P.D., 2008. Bisphosphonates alter trabecular bone collagen cross-linking and isomerization in beagle dog vertebra. *Osteoporos.Int.* 19 (3), 329–337.
- Altundal, H., Gursoy, B., 2005. The influence of alendronate on bone formation after autogenous free bone grafting in rats. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 99 (3), 285–291.
- Ascenzi, M.-G., Liao, V.P., Lee, B.M., Billi, F., Zhou, H., Lindsay, R., Cosman, F., Nieves, J., Bilezikian, J.P., Dempster, D.W., 2012. Parathyroid hormone treatment improves the cortical bone microstructure by improving the distribution of type I collagen in postmenopausal women with osteoporosis. *J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res.* 27 (3), 702–712.
- Aslan, D., Andersen, M.D., Gede, L.B., de Franca, T.K., Jorgensen, S.R., Schwarz, P., Jorgensen, N.R., 2012. Mechanisms for the bone anabolic effect of parathyroid hormone treatment in humans. *Scand.J.Clin.Lab.Invest.* 72 (1), 14–22.
- Axelsson, K.F., Wallander, M., Johansson, H., Lundh, D., Lorentzon, M., 2017. Hip fracture risk and safety with alendronate treatment in the oldest-old. *J.Intern.Med.* 282 (6), 546–559.
- Bailey, S., Karsenty, G., Gundberg, C., Vashishth, D., 2017. Osteocalcin and osteopontin influence bone morphology and mechanical properties. *Ann.N.Y.Acad.Sci.* 1409 (1), 79–84.
- Barille, S., Pellat-Deceunynck, C., Bataille, R., Amiot, M., 1996. Ectopic secretion of osteocalcin, the major non-collagenous bone protein, by the myeloma cell line NCI-H929. *J. Bone Miner.Res.* 11 (4), 466–471.
- Bellows, C.G., Reimers, S.M., Heersche, J.N., 1999. Expression of mRNAs for type-I collagen, bone sialoprotein, osteocalcin, and osteopontin at different stages of osteoblastic differentiation and their regulation by 1,25 dihydroxyvitamin D₃. *Cell Tissue Res.* 297 (2), 249–259.
- Black, D.M., Thompson, D.E., Bauer, D.C., Ensrud, K., Musliner, T., Hochberg, M.C., Nevitt, M.C., Suryawanshi, S., Cummings, S.R., 2000. Fracture risk reduction with alendronate in women with osteoporosis: the fracture intervention trial. *J. Clin. Endocrinol. Metab.* 85 (11), 4118–4124.
- Boivin, G., Meunier, P.J., 2002. Effects of bisphosphonates on matrix mineralization. *J. Musculoskelet. Neuronal Interact.* 2 (6), 538–543.
- Bone, H.G., Greenspan, S.L., McKeever, C., Bell, N., Davidson, M., Downs, R.W., Emkey, R., Meunier, P.J., Miller, S.S., Mulloy, A.L., Recker, R.R., Weiss, S.R., Heyden, N., Musliner, T., Suryawanshi, S., Yates, A.J., Lombardi, A., 2000.

- Alendronate and estrogen effects in postmenopausal women with low bone mineral density. Alendronate/estrogen studygroup. *J.Clin.Endocrinol.Metab.* 85 (2), 720–726.
- Boskey, A.L., Gadaleta, S., Gundberg, C., Doty, S.B., Ducky, P., Karsenty, G., 1998. Fourier transform infrared microspectroscopic analysis of bones of osteocalcin-deficient mice provides insight into the function of osteocalcin. *Bone* 23 (3), 187–196.
- Boskey, A.L., Imbert, L., 2017. Bone quality changes associated with aging and disease: a review. *Ann.N.Y.Acad.Sci.* 1410 (1), 93–106.
- Burr, D.B., Allen, M.J., 2013. Academic Press.
- Camargos, G.V., Bhattacharya, P., van Lenthe, G.H., Del Bel Cury, A.A., Naert, I., Duyck, J., Vandamme, K., 2015. Mechanical competence of ovariectomy-induced compromised bone after single or combined treatment with high-frequency loading and bisphosphonates. *Sci.Rep.* 5 (1), 10795.
- Cheng, Z., Yao, W., Zimmermann, E.A., Busse, C., Ritchie, R.O., Lane, N.E., 2009. Prolonged treatments with antiresorptive agents and PTH have different effects on bone strength and the degree of mineralization in old estrogen-deficient osteoporotic rats. *J. Bone Miner.Res.* 24 (2), 209–220.
- Chenu, C., Colucci, S., Grano, M., Zigrino, P., Barattolo, R., Zamboni, G., Baldini, N., Vergnaud, P., Delmas, P.D., Zallone, A.Z., 1994. Osteocalcin induces chemotaxis, secretion of matrix proteins, and calcium-mediated intracellular signaling in human osteoclast-like cells. *J. Cell Biol.* 127 (4), 1149–1158.
- Chesnut 3rd, C.H., McClung, M.R., Ensrud, K.E., Bell, N.H., Genant, H.K., Harris, S.T., Singer, F.R., Stock, J.L., Yood, R.A., Delmas, P.D., et al., 1995. Alendronate treatment of the postmenopausal osteoporotic woman: effect of multiple dosages on bone mass and bone remodeling. *Am.J.Med.* 99 (2), 144–152.
- Chiang, T.I., Chang, I.C., Lee, H.S., Lee, H., Huang, C.H., Cheng, Y.W., 2011. Osteopontin regulates anabolic effect in human menopausal osteoporosis with intermittent parathyroid hormone treatment. *Osteoporos.Int.* 22 (2), 577–585.
- Compston, J.E., 2007. Skeletal actions of intermittent parathyroid hormone: effects on bone remodelling and structure. *Bone* 40 (6), 1447–1452.
- Cummings, S.R., Black, D.M., Thompson, D.E., Applegate, W.B., Barrett-Connor, E., Musliner, T.A., Palermo, L., Prineas, R., Rubin, S.M., Scott, J.C., Vogt, T., Wallace, R., Yates, A.J., LaCroix, A.Z., 1998. Effect of alendronate on risk of fracture in women with low bone density but without vertebral fractures: results from the fracture intervention trial. *JAMA* 280 (24), 2077–2082.
- Datta, N.S., Abou-Samra, A.B., 2009. PTH and PTHrP signaling in osteoblasts. *Cell. Signal.* 21 (8), 1245–1254.
- Ducky, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., Smith, E., Bonadio, J., Goldstein, S., Gundberg, C., Bradley, A., Karsenty, G., 1996. Increased bone formation in osteocalcin-deficient mice. *Nature* 382 (6590), 448–452.
- Duval, C.L., Taylor, W.R., Weiss, D., Wojtowicz, A.M., Guldborg, R.E., 2007. Impaired angiogenesis, early callus formation, and late stage remodeling in fracture healing of osteopontin-deficient mice. *J. Bone Miner.Res.* 22 (2), 286–297.
- Fantner, G.E., Hassenkam, T., Kindt, J.H., Weaver, J.C., Birkedal, H., Pechenik, L., Cutroni, J.A., Cidade, G.A.G., Stucky, G.D., Morse, D.E., Hansma, P.K., 2005. Sacrificial bonds and hidden length dissipate energy as mineralized fibrils separate during bone fracture. *Nat.Mater.* 4 (8), 612–616.
- García-Moreno, C., Serrano, S., Nacher, M., Farré, M., Díez, A., Mariñoso, M.L., Carbonell, J., Mellibovsky, L., Nogués, X., Ballester, J., Aubia, J., 1998. Effect of alendronate on cultured normal human osteoblasts. *Bone* 22 (3), 233–239.
- Garnero, P., 2012. The contribution of collagen crosslinks to bone strength. *Bonekey Rep.* 1, 182.
- Gesty-Palmer, D., Flannery, P., Yuan, L., Corsino, L., Spurney, R., Lefkowitz, R.J., Luttrell, L.M., 2009. A beta-arrestin-biased agonist of the parathyroid hormone receptor (PTH1R) promotes bone formation independent of G protein activation. *Sci. Transl.Med.* 1 (1), 1ra1.
- Girotra, M., Rubin, M.R., Bilezikian, J.P., 2006. The use of parathyroid hormone in the treatment of osteoporosis. *Rev.Endocr.Metab.Disord.* 7 (1–2), 113–121.
- Glowacki, J., Rey, C., Glimcher, M.J., Cox, K.A., Lian, J., 1991. A role for osteocalcin in osteoclast differentiation. *J.Cell.Biochem.* 45 (3), 292–302.
- Goldstein, S.A., Goulet, R., McCubrey, D., 1993. Measurement and significance of three-dimensional architecture to the mechanical integrity of trabecular bone. *Calcif. Tissue Int.* 53 (Suppl. 1), S127–S132 discussion S132–123.
- Gross, J., 1958. Studies on the formation of collagen. I. Properties and fractionation of neutral salt extracts of normal guinea pig connective tissue. *J. Exp. Med.* 107 (2), 247–263.
- Hara, K., Kobayashi, M., Akiyama, Y., 2007. Influence of bone osteocalcin levels on bone loss induced by ovariectomy in rats. *J. Bone Miner.Metab.* 25 (6), 345–353.
- Herman, B.C., Cardoso, L., Majeska, R.J., Jepsen, K.J., Schaffler, M.B., 2010. Activation of bone remodeling after fatigue: differential response to linear microcracks and diffuse damage. *Bone* 47 (4), 766–772.
- Hernandez, C.J., Tang, S.Y., Baumbach, B.M., Hwu, P.B., Sakke, A.N., van der Ham, F., DeGroot, J., Bank, R.A., Keaveny, T.M., 2005. Trabecular microfracture and the influence of pyridinium and non-enzymatic glycation-mediated collagen cross-links. *Bone* 37 (6), 825–832.
- Hunt, H., Torres, A., Palomino, P., Marty, E., Saiyed, R., Cohn, M., Jo, J., Warner, S., Sroga, G., King, K., Lane, J., Vashishth, D., Hernandez, C., Donnelly, E., 2019. Altered tissue composition, microarchitecture, and mechanical performance in cancellous bone from men with type 2 diabetes mellitus. *J. Bone Miner. Res.* 34 (7), 1191–1206.
- Iwaniec, U.T., Trevisiol, C.H., Maddalozzo, G.F., Rosen, C.J., Turner, R.T., 2008. Effects of low-dose parathyroid hormone on bone mass, turnover, and ectopic osteoinduction in a rat model for chronic alcohol abuse. *Bone* 42 (4), 695–701.
- Iwata, K., Li, J., Follet, H., Phipps, R.J., Burr, D.B., 2006. Bisphosphonates suppress periosteal osteoblast activity independently of resorption in rat femur and tibia. *Bone* 39 (5), 1053–1058.
- Jerome, C.P., Burr, D.B., Van Bibber, T., Hock, J.M., Brommage, R., 2001. Treatment with human parathyroid hormone (1–34) for 18 months increases cancellous bone volume and improves trabecular architecture in ovariectomized cynomolgus monkeys (*Macaca fascicularis*). *Bone* 28 (2), 150–159.
- Jerome, C.P., Johnson, C.S., Vafai, H.T., Kaplan, K.C., Bailey, J., Capwell, B., Fraser, F., Hansen, L., Ramsay, H., Shadoan, M., Lees, C.J., Thomsen, J.S., Mosekilde, L., 1999. Effect of treatment for 6 months with human parathyroid hormone (1–34) peptide in ovariectomized cynomolgus monkeys (*Macaca fascicularis*). *Bone* 25 (3), 301–309.
- Jilka, R.L., Weinstein, R.S., Bellido, T., Roberson, P., Parfitt, A.M., Manolagas, S.C., 1999. Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. *J.Clin.Invest.* 104 (4), 439–446.
- Karim, L., Hussein, A.I., Morgan, E.F., Boussein, M.L., 2013. In: Marcus, R., Feldman, D., Dempster, D.W., Luckey, M., Cauley, J.A. (Eds.), *The Mechanical Behavior of Bone. Osteoporosis*, Fourth edition. Academic Press, San Diego, pp. 431–452.
- Karim, L., Moulton, J., Van Vliet, M., Velie, K., Robbins, A., Malekipour, F., Abdeen, A., Ayres, D., Boussein, M.L., 2018. Bone microarchitecture, biomechanical properties, and advanced glycation end-products in the proximal femur of adults with type 2 diabetes. *Bone* 114, 32–39.
- Karim, L., Tang, S.Y., Sroga, G.E., Vashishth, D., 2013. Differences in non-enzymatic glycation and collagen cross-links between human cortical and cancellous bone. *Osteoporos.Int.* 24 (9), 2441–2447.
- Karim, L., Vashishth, D., 2012. Heterogeneous glycation of cancellous bone and its association with bone quality and fragility. *PLoS One* 7 (4), e35047.
- Kavukcuoglu, N.B., Patterson-Buckendahl, P., Mann, A.B., 2009. Effect of osteocalcin deficiency on the nanomechanics and chemistry of mouse bones. *J.Mech.Behav. Biomed.Mater.* 2 (4), 348–354.
- Kimura, S., Saito, M., Kida, Y., Seki, A., Isaka, Y., Marumo, K., 2017. Effects of raloxifene and alendronate on non-enzymatic collagen cross-links and bone strength in ovariectomized rabbits in sequential treatments after daily human parathyroid hormone (1–34) administration. *Osteoporos.Int.* 28 (3), 1109–1119.
- Komatsu, K., Shimada, A., Shibata, T., Wada, S., Ideno, H., Nakashima, K., Amizuka, N., Noda, M., Nifuji, A., 2013. Alendronate promotes bone formation by inhibiting protein prenylation in osteoblasts in rat tooth replantation model. *J.Endocrinol.* 219 (2), 145–158.
- Kurland, E.S., Cosman, F., McMahon, D.J., Rosen, C.J., Lindsay, R., Bilezikian, J.P., 2000. Parathyroid hormone as a therapy for idiopathic osteoporosis in men: effects on bone mineral density and bone markers. *J.Clin.Endocrinol.Metab.* 85 (9), 3069–3076.
- Ma, X., Xu, Z., Ding, S., Yi, G., Wang, Q., 2018. Alendronate promotes osteoblast differentiation and bone formation in ovariectomy-induced osteoporosis through interferon- β /signal transducer and activator of transcription 1 pathway. *Exp. Ther. Med.* 15 (1), 182–190.
- Masarachia, P., Weinreb, M., Balena, R., Rodan, G.A., 1996. Comparison of the distribution of 3H-alendronate and 3H-etidronate in rat and mouse bones. *Bone* 19 (3), 281–290.
- Massie, C., Knapp, E., Chen, K., Berger, A.J., Awad, H.A., 2021. Improved prediction of femoral fracture toughness in mice by combining standard medical imaging with raman spectroscopy. *J.Biomech.* 116, 110243.
- Merlo, K., Aaronson, J., Vaidya, R., Rezaee, T., Chalivendra, V., Karim, L., 2020. In vitro-induced high sugar environments decrease human cortical bone elastic modulus and fracture toughness. *J.Orthop.Res.* 38 (5), 972–983.
- Miller, L.M., Little, W., Schirmer, A., Sheik, F., Busa, B., Judex, S., 2007. Accretion of bone quantity and quality in the developing mouse skeleton. *J. Bone Miner.Res.* 22 (7), 1037–1045.
- Nalla, R.K., Kinney, J.H., Ritchie, R.O., 2003. Mechanistic fracture criteria for the failure of human cortical bone. *Nat.Mater.* 2 (3), 164–168.
- Neer, R.M., Arnaud, C.D., Zanchetta, J.R., Prince, R., Gaich, G.A., Reginster, J.Y., Hodsman, A.B., Erikson, E.F., Ish-Shalom, S., Genant, H.K., Wang, O., Mitlak, B.H., 2001. Effect of parathyroid hormone (1–34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N.Engl.J.Med.* 344 (19), 1434–1441.
- Nikel, O., Poundarik, A.A., Bailey, S., Vashishth, D., 2018. Structural role of osteocalcin and osteopontin in energy dissipation in bone. *J.Biomech.* 80, 45–52.
- Nyman, J.S., Makowski, A.J., 2012. The contribution of the extracellular matrix to the fracture resistance of bone. *Curr. Osteoporos. Rep.* 10 (2), 169–177.
- Nyman, J.S., Roy, A., Acuna, R.L., Gayle, H.J., Reyes, M.J., Tyler, J.H., Dean, D.D., Wang, X., 2006. Age-related effect on the concentration of collagen crosslinks in human osteonal and interstitial bone tissue. *Bone* 39 (6), 1210–1217.
- Nyman, J.S., Roy, A., Tyler, J.H., Acuna, R.L., Gayle, H.J., Wang, X., 2007. Age-related factors affecting the postyield energy dissipation of human cortical bone. *J.Orthop. Res.* 25 (5), 646–655.
- Osagie-Clouard, L., Sanghani, A., Coathup, M., Briggs, T., Bostrom, M., Blunn, G., 2017. Parathyroid hormone 1–34 and skeletal anabolic action. *Bone Joint Res.* 6 (1), 14–21.
- Osterhoff, G., Morgan, E.F., Shefelbine, S.J., Karim, L., McNamara, L.M., Augat, P., 2016. Bone mechanical properties and changes with osteoporosis. *Injury* 47 (Suppl 2(Suppl 2)), S11–20.
- Peter, C., Rodan, G.A., 1999. Preclinical safety profile of alendronate. *Int. J. Clin. Pract. Suppl.* 101, 3–8.
- Poundarik, A.A., Boskey, A., Gundberg, C., Vashishth, D., 2018. Biomolecular regulation, composition and nanoarchitecture of bone mineral. *Sci.Rep.* 8 (1), 1191.
- Poundarik, A.A., Diab, T., Sroga, G.E., Ural, A., Boskey, A.L., Gundberg, C.M., Vashishth, D., 2012. Dilatational band formation in bone. *Proc.Natl.Acad.Sci.U.S.A.* 109 (47), 19178–19183.
- Poundarik, A.A., Wu, P.C., Evis, Z., Sroga, G.E., Ural, A., Rubin, M., Vashishth, D., 2015. A direct role of collagen glycation in bone fracture. *J.Mech.Behav.Biomed.Mater.* 52, 120–130.

- Reginster, J., Minne, H.W., Sorensen, O.H., Hooper, M., Roux, C., Brandi, M.L., Lund, B., Ethgen, D., Pack, S., Roumagnac, I., Eastell, R., 2000. Randomized trial of the effects of risedronate on vertebral fractures in women with established postmenopausal osteoporosis. *Vertebral efficacy with risedronate therapy (VERT) study group. Osteoporos.Int.* 11 (1), 83–91.
- Reinholt, F.P., Hulthén, K., Oldberg, A., Heinegård, D., 1990. Osteopontin—a possible anchor of osteoclasts to bone. *Proc.Natl.Acad.Sci.U.S.A.* 87 (12), 4473–4475.
- Ritchie, R.O., Koester, K.J., Ionova, S., Yao, W., Lane, N.E., Ager, J.W., 2008. Measurement of the toughness of bone: a tutorial with special reference to small animal studies. *Bone* 43 (5), 798–812.
- Ritchie, R.O., Koester, K.J., Ionova, S., Yao, W., Lane, N.E., Ager, J.W., 2010. Erratum: measurement of the toughness of bone: a tutorial with special reference to small animal studies [*Bone*, vol. 43 (5), Nov. 2008, pp. 798–812]. *Bone* 47 (3), 706.
- Rodrigues, A.M., Caetano-Lopes, J., Vale, A.C., Vidal, B., Lopes, A., Aleixo, I., Polido-Pereira, J., Sepriano, A., Perpétuo, I.P., Monteiro, J., Vaz, M.F., Fonseca, J.E., Canhão, H., 2012. Low osteocalcin/collagen type I bone gene expression ratio is associated with hip fragility fractures. *Bone* 51 (6), 981–989.
- Russell, R.G., Rogers, M.J., 1999. Bisphosphonates: from the laboratory to the clinic and back again. *Bone* 25 (1), 97–106.
- Sahni, M., Guenther, H.L., Fleisch, H., Collin, P., Martin, T.J., 1993. Bisphosphonates act on rat bone resorption through the mediation of osteoblasts. *J.Clin.Invest.* 91 (5), 2004–2011.
- Saito, M., Marumo, K., Kida, Y., Ushiku, C., Kato, S., Takao-Kawabata, R., Kuroda, T., 2011. Changes in the contents of enzymatic immature, mature, and non-enzymatic senescent cross-links of collagen after once-weekly treatment with human parathyroid hormone (1–34) for 18 months contribute to improvement of bone strength in ovariectomized monkeys. *Osteoporos.Int.* 22 (8), 2373–2383.
- Saito, M., Mori, S., Mashiba, T., Komatsubara, S., Marumo, K., 2008. Collagen maturity, glycation induced-pentosidine, and mineralization are increased following 3-year treatment with incadronate in dogs. *Osteoporos.Int.* 19 (9), 1343–1354.
- Sato, M., Vahle, J., Schmidt, A., Westmore, M., Smith, S., Rowley, E., Ma, L.Y., 2002. Abnormal bone architecture and biomechanical properties with near-lifetime treatment of rats with PTH. *Endocrinology* 143 (9), 3230–3242.
- Schwarz, P., Jorgensen, N.R., Mosekilde, L., Vestergaard, P., 2012. Effects of increasing age, dosage, and duration of PTH treatment on BMD increase—a meta-analysis. *Calcif. Tissue Int.* 90 (3), 165–173.
- Shao, Y., Hernandez-Buquer, S., Childress, P., Stayrook, K.R., Alvarez, M.B., Davis, H., Plotkin, L.I., He, Y., Condon, K.W., Burr, D.B., Warden, S.J., Robling, A.G., Yang, F.-C., Wek, R.C., Allen, M.R., Bidwell, J.P., 2017. Improving combination osteoporosis therapy in a preclinical model of heightened osteoanabolism. *Endocrinology* 158 (9), 2722–2740.
- Sroga, G.E., Karim, L., Colon, W., Vashishth, D., 2011. Biochemical characterization of major bone-matrix proteins using nanoscale-size bone samples and proteomics methodology. *Mol.Cell. Proteomics* 10 (9).
- Sroga, G.E., Karim, L., Colón, W., Vashishth, D., 2011. Biochemical characterization of major bone-matrix proteins using nanoscale-size bone samples and proteomics methodology. *Mol.Cell. Proteomics* 10 (9).
- Sroga, G.E., Vashishth, D., 2011. UPLC methodology for identification and quantitation of naturally fluorescent crosslinks in proteins: a study of bone collagen. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 879 (5–6), 379–385.
- Sroga, G.E., Vashishth, D., 2012. Effects of bone matrix proteins on fracture and fragility in osteoporosis. *Curr. Osteoporos. Rep.* 10 (2), 141–150.
- Tang, S.Y., Allen, M.R., Phipps, R., Burr, D.B., Vashishth, D., 2009. Changes in non-enzymatic glycation and its association with altered mechanical properties following 1-year treatment with risedronate or alendronate. *Osteoporos.Int.* 20 (6), 887–894.
- Tang, S.Y., Zeenath, U., Vashishth, D., 2007. Effects of non-enzymatic glycation on cancellous bone fragility. *Bone* 40 (4), 1144–1151.
- Thompson, D.D., Simmons, H.A., Pirie, C.M., Ke, H.Z., 1995. FDA guidelines and animal models for osteoporosis. *Bone* 17 (4, Supplement), S125–S133.
- Thurner, P.J., Chen, C.G., Ionova-Martin, S., Sun, L., Harman, A., Porter, A., Ager, J.W., Ritchie, R.O., Alliston, T., 2010. Osteopontin deficiency increases bone fragility but preserves bone mass. *Bone* 46 (6), 1564–1573.
- Tommasini, S.M., Trinward, A., Acerbo, A.S., De Carlo, F., Miller, L.M., Judex, S., 2012. Changes in intracortical microporosities induced by pharmaceutical treatment of osteoporosis as detected by high resolution micro-CT. *Bone* 50 (3), 596–604.
- Ural, A., Vashishth, D., 2014. Hierarchical perspective of bone toughness – from molecules to fracture. *Int.Mater.Rev.* 59 (5), 245–263.
- Vahle, J.L., Sato, M., Long, G.G., Young, J.K., Francis, P.C., Engelhardt, J.A., Westmore, M.S., Linda, Y., Nold, J.B., 2002. Skeletal changes in rats given daily subcutaneous injections of recombinant human parathyroid hormone (1–34) for 2 years and relevance to human safety. *Toxicol.Pathol.* 30 (3), 312–321.
- Valcourt, U., Merle, B., Gineys, E., Viguet-Carrin, S., Delmas, P.D., Garnero, P., 2007. Non-enzymatic glycation of bone collagen modifies osteoclastic activity and differentiation. *J.Biol.Chem.* 282 (8), 5691–5703.
- Vashishth, D., 2004. Rising crack-growth-resistance behavior in cortical bone: implications for toughness measurements. *J.Biomech.* 37 (6), 943–946.
- Vashishth, D., 2005. Collagen glycation and its role in fracture properties of bone. *J. Musculoskelet. Neuronal Interact.* 5 (4), 316.
- Vashishth, D., Behiri, J.C., Bonfield, W., 1997. Crack growth resistance in cortical bone: concept of microcrack toughening. *J.Biomech.* 30 (8), 763–769.
- Vashishth, D., Gibson, G.J., Khoury, J.I., Schaffler, M.B., Kimura, J., Fyhr, D.P., 2001. Influence of nonenzymatic glycation on biomechanical properties of cortical bone. *Bone* 28 (2), 195–201.
- Viguet-Carrin, S., Farlay, D., Bala, Y., Munoz, F., Bouxsein, M.L., Delmas, P.D., 2008. An in vitro model to test the contribution of advanced glycation end products to bone biomechanical properties. *Bone* 42 (1), 139–149.
- Wallace, L.J., Tommasini, S.M., Judex, S., Garland Jr., T., Demes, B., 2012. Genetic variations and physical activity as determinants of limb bone morphology: an experimental approach using a mouse model. *Am.J.Phys.Anthropol.* 148 (1), 24–35.
- Wang, X., Shen, X., Li, X., Agrawal, C.M., 2002. Age-related changes in the collagen network and toughness of bone. *Bone* 31 (1), 1–7.
- Xiong, Y., Yang, H.J., Feng, J., Shi, Z.L., Wu, L.D., 2009. Effects of alendronate on the proliferation and osteogenic differentiation of MG-63 cells. *J. Int. Med. Res.* 37 (2), 407–416.
- Xu, Z., Zhu, C., Xia, R., Shang, X., 2019. Alendronate stimulates osteoblast differentiation through PKA-STAT3 and STAT1 in an osteoporosis rat model. *Int.J.Clin.Exp.Med.* 12 (10), 12150–12157.
- Yang, X., Mostafa, A.J., Appleford, M., Sun, L.W., Wang, X., 2016. Bone formation is affected by matrix advanced glycation end products (AGEs) in vivo. *Calcif. Tissue Int.* 99 (4), 373–383.
- Yu, Z., Wang, G., Tang, T., Fu, L., Yu, X., Zhu, Z., Dai, K., 2015. Long-term effects of ovariectomy on the properties of bone in goats. *Exp. Ther. Med.* 9 (5), 1967–1973.