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ORIGINAL ARTICLE

Young's modulus and hardness of human trabecular bone with bisphosphonate treatment durations up to 20 years

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Abstract

Summary Bone modulus from patients with osteoporosis treated with bisphosphonates for 1 to 20 years was analyzed. Modulus increases during the first 6 years of treatment and remains unchanged thereafter.

Introduction Bisphosphonates are widely used for treating osteoporosis, but the relationship between treatment duration and bone quality is unclear. Since material properties partially determine bone quality, the present study quantified the relationship between human bone modulus and hardness with bisphosphonate treatment duration.

Methods Iliac crest bone samples from a consecutive case series of 86 osteoporotic Caucasian women continuously treated with oral bisphosphonates for 1.1–20 years were histologically evaluated to assess bone turnover and then tested using nanoindentation. Young's modulus and hardness were measured and related to bisphosphonate treatment duration by statistical modeling.

Results All bone samples had low bone turnover. Statistical models showed that with increasing bisphosphonate treatment duration, modulus and hardness increased, peaked, and plateaued. These models used quadratic terms to model modulus increases from 1 to 6 years of bisphosphonate treatment and linear terms to model modulus plateaus from 6 to 20 years of treatment. The treatment duration at which the quadratic–linear transition (join point) occurred also depended upon trabecular location. Hardness increased and peaked at 12.4 years of treatment; it remained constant for the next 7.6 years of treatment and was insensitive to trabecular location.

Conclusions Bone modulus increases with bisphosphonate treatment durations up to 6 years, no additional modulus increases occurred after 6 years of treatment. Although hardness increased, peaked at 12.4 years and remained constant for the next 7.6 years of BP treatment, the clinical relevance of hardness remains unclear.

Keywords Bone biomechanics · Bone hardness · Bone modulus · Bone quality · Nanoindentation

Introduction

Bisphosphonates (BPs) have an important role as primary medication for treatment of the large and increasing prevalence of osteoporosis [1, 2]. Patients are often reluctant to use these drugs fearing drug-related long-term complications. These complications have been attributed to decreased bone quality despite positive documented effects on bone mineral density and fracture reduction [1, 3–7].

Bone strength is determined by bone quantity and bone quality. Bone quality and its relationship to BP treatment were spotlighted in recent literature [8, 9]. Evidence has been provided showing that BP use is related to changes in bone mineral [10, 11], matrix properties [12, 13], bone structure [14, 15], and mechanical properties [16, 17]. This evidence was disputed by a publication stating "…no altered biomechanical examinations have ever provided a scientific answer linking bisphosphonate exposure to impairment in bone strength or bone quality" [8]. The present study addresses this important current controversy by providing new information directly linking BP treatment duration to bone quality. It also adds to our prior study of bone structure and BP treatment duration [15]. Specifically, the present study evaluates the relationship between the duration of BP treatment and some material properties of human bone.

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Materials and methods

Study design

This cross-sectional study evaluated the relationship between the duration of continuous oral bisphosphonate (BP) treatment and two biomechanical response variables, Young's modulus (hereafter modulus) and hardness. Nanomechanical testing quantified these two intrinsic (shape-independent) material properties in human cancellous bone obtained from an established model (the iliac crest) [18, 19]. Covariates included in the analyses of BP treatment duration vs. modulus or hardness were as follows: subject age, lumbar spine bone mineral density (BMD), proximal hip BMD, bone turnover, and bone volume/tissue volume (BV/TV). These parameters were measured only once at the time of bone biopsy following the various BP treatment durations as available from patient records. Design of this study conformed to the Declaration of Helsinki and had Institutional Review Board approval.

Sample selection

Patients previously diagnosed with osteoporosis and treated at other institutions with bisphosphonates who presented at the University of Kentucky Metabolic Bone Disease Clinic to assess options for continued treatment of osteoporosis were offered an anterior iliac crest bone biopsy for histological analyses of bone turnover [20, 21]. The initial diagnosis of osteoporosis was made by dual energy X-ray absorptiometry (DXA) of the femoral neck or lumbar spine or by the presence of a non-traumatic fracture. DXA measurements (GE Lunar, General Electric, Schenectady, NY) were performed shortly before bone sample procurement.

Inclusion and exclusion criteria

Bone samples from a consecutive case series of osteoporotic Caucasian women treated with one of three types of oral BPs (alendronate, risedronate, or ibandronate) for durations varying from 1.1 to 20 years were included. Bone biopsies were performed to evaluate the effects of the duration of BP treatment on bone intercepted at the time of biopsy. Exclusion criteria applied at the time of patient enrollment were prior treatment with long-term steroids, anticonvulsants, or any pharmacologic agents known to alter bone metabolism (except oral BPs). Samples were also excluded from patients with chronic alcoholism, drug addiction, malignancy, malabsorption, Paget's disease, osteogenesis imperfecta, hemiplegia/paraplegia, reduced kidney function (GFR < 60 ml/min), uncontrolled systemic illness, or other organic illnesses with potential influence on bone metabolism.

Sample procurement and preparation

Bone biopsies were performed using an electric drill and a vertical approach. During the biopsy procedure, the drill remained centered in the ilium to avoid inclusion of the lateral or medial cortices [22]. Cylindrically shaped bone samples of approximately 0.4 cm diameter and 2.5–4.0 cm length were obtained. Harvested samples were fixed in ethanol at room temperature, dehydrated, and embedded in polymethylmethacrylate (PMMA). Seven-micron thick sections were cut from the undecalcified bone samples, stained by the modified Masson-Goldner stain, and evaluated by histomorphometry for assessment of bone quantity (BV/TV) and bone turnover (activation frequency) [22]. These measurements were made from regions of trabecular bone approximating the sites of subsequent nanomechanical testing.

Whole bone samples mounted in PMMA were prepared for nanomechanical testing by wet sanding with abrasive silicon carbide papers of decreasing grit size (ending in 1200) and polishing with suspended diamond abrasive media (0.3 and 0.05 μm) on rotating cloths lubricated with deionized water [23, 24]. After all sanding and polishing procedures, bone samples were immersed for a single 10-min period in an ultrasonic bath to remove sanding and polishing debris.

Nanomechanical testing

Two parameters were evaluated to quantify mechanical measures of bone quality: modulus and hardness. Modulus quantifies the shape-independent resistance of a material to elastic (reversible) deformation. Hardness quantifies the shape-independent resistance of a material to plastic (permanent) deformation resulting from local indentation. Nanoindentation was performed using a Nanoindenter G200 (Agilent, Oak Ridge, TN) equipped with a Berkovich three-sided pyramidal diamond tip indentor following established methods [23, 25]. Calibration of the nanoindentation system, done prior to each set of measurements, was performed by indenting fused silica of known modulus [26].

Each cancellous bone sample was indented 30 times (six different trabeculae each indented at five different locations). The six different trabeculae were chosen arbitrarily provided they met minimum size (width > 150 μm and length > 500 μm) requirements. Indentation sites were chosen using a standardized approach consisting of five separate indentations on each qualified trabeculum. A line perpendicular to the long axis of the trabeculae was chosen and used to position the sites for each of the five approximately equally spaced nanoindentation sites. This line was moved along the long axis until it covered a length that was devoid of surface irregularities. The five testing sites included the center of the trabeculum, the region near both edges (but at least 100 nm from the edge), and two midpoints between the center and the two edges (Fig. 1). These five

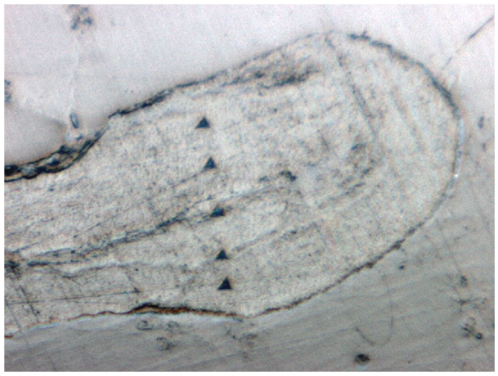


Fig. 1 Photomicrograph showing 5 nanoindentations across the width of a representative trabeculum. The five nanoindentations evaluate the spatial heterogeneity of each trabeculum by testing at the: left edge, left midpoint, center, right midpoint, and right edge

measurement sites across each trabeculum were chosen to account for the spatially varying and heterogeneous properties of bone [12, 27]. Microscopic visualization ensured the desired indentor position for each nanoindentation test site. This visualization, plus the restriction that only "full width" trabeculae were tested, assured sufficient bone material under the nanoindentor. Mean values of the edge (12 total), midpoint (12 total), and center measurements (6 total) were computed for each bone specimen and used in subsequent analyses.

Each nanoindentation began by applying a peak load of 8 mN to the indentor at a constant loading rate of 0.4 mN/s. This peak load produced a maximum indentation depth of approximately 0.7 to 1 μm, ensured an accurate modulus value, and reduced the effect of residual surface roughness. This peak load was maintained for a 10-s hold time to ensure that the subsequent unloading was completely elastic [23, 28]. Then the indentor was unloaded to 10% of the maximum load (0.8 mN) and this load was maintained for 100 s for thermal drift correction [23]. Modulus was calculated from the slope of the first 50% of the unloading curve (Fig. 2) and standardized values for bone and indentor Poisson ratios (0.3 and 0.07) were used [25]. Hardness (H) was calculated from the maximum load applied divided by the projected area made by the indentor while applying this maximum load [25].

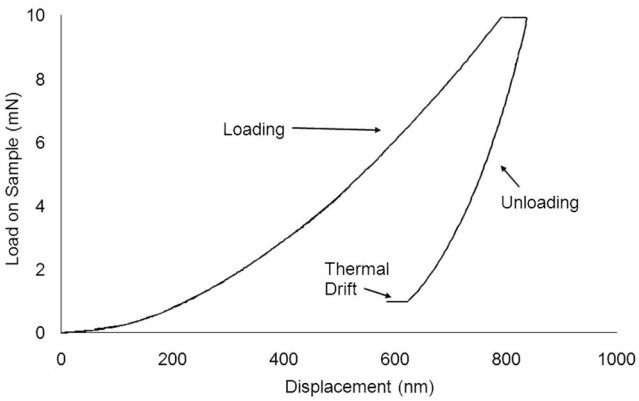


Fig. 2 Schematic illustration of nanoindentor displacement versus nanoindentor force on bone

Statistical analyses

The relationships between BP treatment duration and modulus or hardness were modeled using nonlinear regression adjusted for covariates; e.g., patient age, histomorphometrically determined BV/TV, activation frequency, and BMD at the spine and the hip determined at the time of biopsy (after the specified duration of BP treatment). Least Trimmed Squares Analyses at the 1% level was used to identify potential outliers. Identified points more than 2 standard deviations from the fitted models were excluded. Only statistically significant ($p < 0.05$) covariates and their interactions with BP treatment duration were retained in the models. Repeated measures analysis of variance was used to fit all linear models and was run in PROC GLM in SAS 9.3 (SAS Institute, Cary, NC). Nonlinear models were fit using PROC NLIN. Fit of the models was assessed through visual inspection of residuals and testing lack of fit to the models. Appropriate transformations were made to ensure homoscedasticity and normality of the residuals.

Results

Eighty-nine (89) samples (Table 1) were tested by nanoindentation; three samples were excluded due to limited bone quality or rejection by Least Trimmed Squares Analyses at the 1% level. The remaining 86 included samples had low or low-normal bone turnover and virtually all were treated with alendronate. Five patients had osteoporotic fractures and no hip or lumbar spine BMD data. Neither heteroscedasticity nor non-normality was observed in the biomechanical response data; therefore, no data transformations were performed at any time to obtain the models presented (Tables 2 and 3).

Regression models relate mean values for each trabecular location of the response variables, i.e., modulus and hardness, to BP treatment duration, following adjustment for covariates (Tables 2 and 3). Modulus varied with BP treatment duration ($p < 0.001$). Models relating modulus to BP treatment duration required both linear and quadratic terms. Hip BMD, spine BMD, activation frequency, and patient age were not significant covariates and thus were not included in any models. BV/TV was a weak but significant covariate and was thus included in models relating modulus ($p = 0.0499$) and hardness ($p = 0.0259$) to BP treatment duration (Tables 2 and 3) to clarify these relationships. Modulus also varied with trabecular location and was least near trabecular edges but greatest at trabecular centers.

Table 1 Subject characteristics

	Mean	Standard deviation	Range	*n*
Patient age at time of bone sample procurement (years)	62.3	8.6	34 to 82	86
Femoral neck bone mineral density (T-score)[a]	− 1.98	0.83	− 3.6 to 0	84
Lumbar spine bone mineral density (T-score)[a]	− 1.98	1.28	− 5.5 to 2.2	83
Bone volume/tissue volume (%)	15.5	4.8	4.7 to 30.9	86
Activation frequency (years^{-1})	0.151	0.116	0.005 to 0.560	71[b]
Duration of bisphosphonate treatment (years)	6.88	3.6	1.1 to 20	86

[a] Measured at the time of bone sample procurement and after a period of bisphosphonate treatment. Initial diagnosis of osteoporosis and commencement of bisphosphonate use were based upon the following: (1) DXA-derived T-score < −2.5 at either the femoral neck or spine or (2) an osteoporotic hip or spine fracture regardless of T-score

[b] Number of samples with tetracycline double-labeling for turnover assessment

Models relating modulus with BP treatment durations of 1.1 to 20 years are shown in compact form (Tables 2 and 3). Models for trabecular edges and midpoints are illustrated in Fig. 3a. Models for mean values of modulus at trabecular edges and trabecular midpoints are identical in form, the sole difference is the offset (y-axis intercept). This offset is quantified by a difference term (d) that separates the modulus-BP treatment duration model at trabecular edges from the modulus-BP treatment duration model at trabecular midpoints (Table 2). This difference term is significant ($p < 0.03707$) and reflects the model results that modulus values at trabecular midpoints are greater than modulus values at trabecular edges. This applies over the full range of BP treatment durations included in the study.

These models (Tables 2 and 3) show that modulus increases with increasing BP treatment duration and then plateaus. These models quantify the observed relationship between modulus and BP treatment duration using two terms, a quadratic term representing modulus increases associated with BP treatment of 1.1 years to the join point, and a linear term representing a modulus plateau associated with BP treatment from the join point to 20 years. The join point, shown by the solid vertical lines in the figures, marks the transition from the quadratic term to the linear term and occurs at varying BP treatment durations depending on biomechanical response parameter and trabecular location.

The modeled relationship between hardness and BP treatment duration is shown (Tables 2 and 3). The model relating hardness to BP treatment duration also used quadratic and linear terms to quantify the initial increase and plateau in hardness. There were, however, four noteworthy differences between this model and the model used for modulus. First, no difference was observed between hardness at trabecular edges and hardness at trabecular midpoints. This is indicated by the nonsignificant difference term (d) in the hardness model (Table 2). Thus, the model graphically illustrated (Fig. 4a) has only one solid line representing hardness averaged from trabecular edges and trabecular midpoints. Second, the strength of the relationship between initial hardness increase and BP treatment duration is substantially less than the strength of the relationship between

Table 2 Statistical models relating Young's modulus and hardness (GPa), measured at edges and midpoints, of trabecular bone to the duration of bisphosphonate treatment

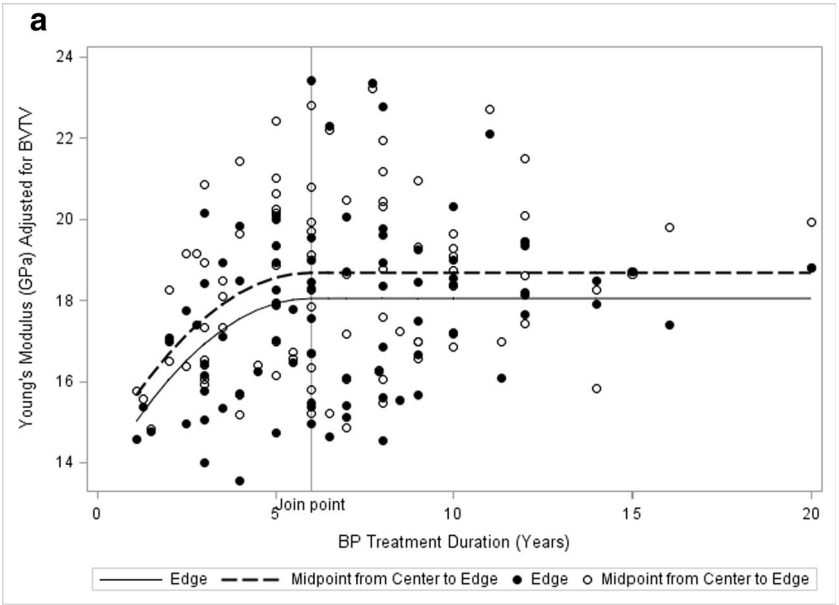
Biomaterial response variable	Intercept (a)	Coefficient of BV/TV term (b)	Coefficient of BP treatment duration term (c)	Join point of quadratic plateau (x_0)	Difference between edge and midpoint (d)
Young's modulus	13.8000 $p < 0.0001$	− 0.1017 $p = 0.0499$	1.5199 $p = 0.0092$	5.9898	− 0.3181 $p < 0.0307$
Hardness	0.5276 $p < 00001$	− 0.0045 $p = 0.0259$	0.0222 $p = 0.0051$	12.4191	NS

Values presented are repeated measures coefficients of Prediction Equations (form shown below) relating the biomechanical response variables (Young's modulus (GPa) and hardness (GPa)) at trabecular edges and midpoints to: bone volume/tissue volume (BV/TV) and linear as well as quadratic bisphosphonate (BP) treatment duration terms as determined from polynomial regression analyses. Prediction equation edge biomechanical response variable = a + b (BV/TV) + c (BP Rx duration) + c (BP Rx duration)^2 /x_0 + Difference, for BP Treatment duration less than x_0; and a + b (BV/TV) + cx_0/2 + Difference, for BP Treatment duration greater than x_0. Prediction equation midpoint biomechanical response variable = a + b (BV/TV) + c (BP Rx duration) + c (BP Rx duration)^2 /x_0 − Difference, for BP Treatment duration less than x_0; and a + b (BV/TV) + cx_0/2 + Difference, for BP Treatment duration greater than x_0. *p* values shown under the join point designate the significance of the quadratic plateau model (c and x_0). NS denotes not significantly different from 0

Table 3 Statistical models relating Young's modulus and hardness (GPa), measured at trabecular centers, to the duration of bisphosphonate treatment

Biomaterial response variable	Intercept (a)	Coefficient of BV/TV term (b)	Coefficient of BP treatment duration term (c)	Join point of quadratic plateau (x_0)
Young's modulus	6.3549 $p < 0.0001$	− 0.1023 $p = 0.0357$	9.6219 $p = 0.0374$	2.5804
Hardness	0.6542 $p < 0.0001$	− 0.0555 $p = 0.0044$		NS

Values presented are repeated measures coefficients of prediction equations relating the biomechanical response variables (Young's modulus (units of GPa) and hardness (units of GPa)) at the edge and midpoints of the trabeculae to: bone volume/tissue volume (BV/TV) and linear as well as quadratic bisphosphonate (BP) treatment duration terms as determined from polynomial regression analyses. Prediction equation trabecular center Young's modulus = a + b (BV/TV) + c (BP Rx duration) + c (BP Rx duration)^2 /x_0, for BP Treatment duration less than x_0; and a + b (BV/TV) + cx_0/2, for BP Treatment duration greater than x_0. *p* value shown under the join point provides the significance of the quadratic plateau model (c and x_0). NS denotes not significantly different from 0



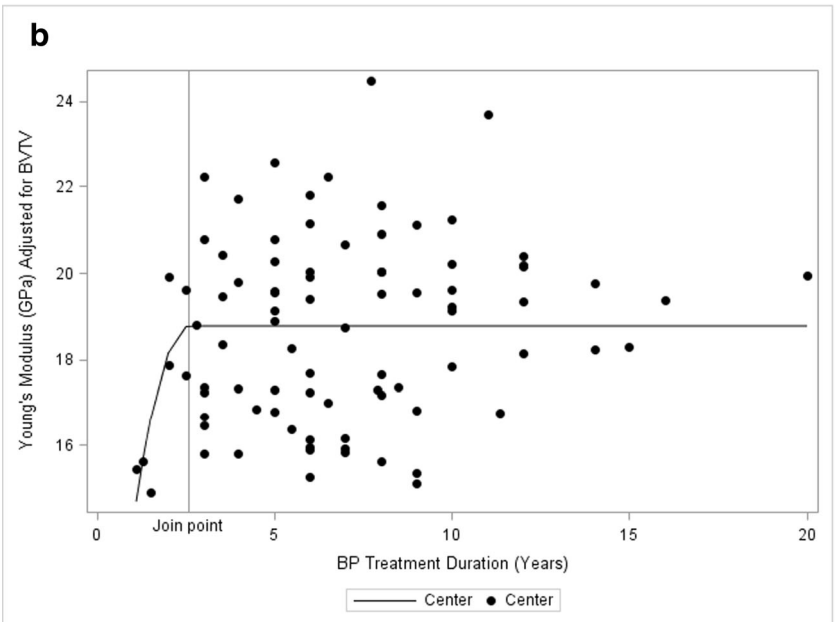


Fig. 3 **a** Young's modulus of human bone measured at trabecular edges (solid dots) and trabecular midpoints (hollow dots) vs. BP treatment duration. Each dot represents the mean of 12 measurements at trabecular edges and 12 measurements at trabecular midpoints. The y-axis offset between the two models is quantified by the Difference Term shown in Table 2. **b** Young's modulus of human bone measured at trabecular centers. Each dot represents the mean of six measurements

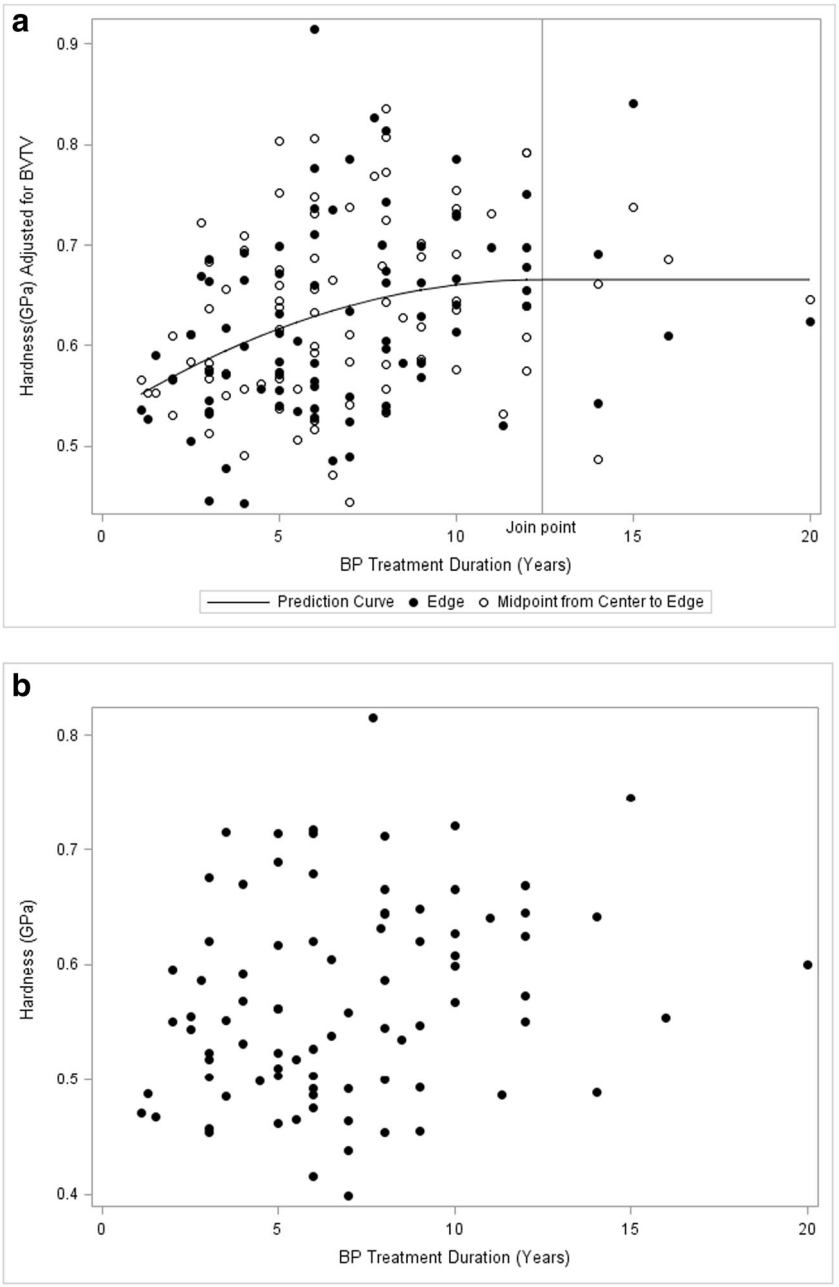


Fig. 4 **a** Hardness of human bone measured at trabecular edges (solid dots) and trabecular midpoints (hollow dots) vs. BP treatment duration. Each dot represents the mean of 12 measurements at trabecular edges and 12 measurements at trabecular midpoints. A single model is shown (solid line) due to the nonsignificant difference between these two locations. **b** Hardness of human bone measured at trabecular centers. Each dot represents the mean of six measurements. Since regression modeling of these data generates a line with a slope not significantly different from zero, no line is included on this figure

initial modulus increase and BP treatment duration. The models (Table 2) quantify the relative strength of these initial increases; note that the c-coefficient (c = 0.0222) of the hardness model (Table 2), is approximately 1.5% of the c-coefficient of the modulus model (c = 1.5199). Third, the join point for the hardness model, occurring at 12.4 years of BP treatment duration, differs from the join points of the modulus models (approximately 6 years for trabecular edges or midpoints, approximately 2.6 years for trabecular centers). Finally, no relationship was observed between hardness at trabecular centers and BP treatment duration, hence the absence of a c-coefficient (Table 3) and the absence of a solid line (Fig. 4b).

Discussion

The key findings of this study are the increase and plateau of modulus and hardness measured in trabecular bone with BP treatment durations of 1 to 20 years. While increases in both response parameters are quadratic, the modulus join point occurs at 2.6 or 6 years of BP treatment, depending upon trabecular location, while the hardness join point occurs at approximately 12 years of BP treatment irrespective of trabecular site.

Modeled increases in modulus and hardness with BP treatment durations less than 6 years are consistent with known increases in mineralization accompanying BP treatment

durations of 5 years and are associated with BP-mediated bone turnover reduction [29–31]. Differences observed between moduli at trabecular edges and midpoints are expected given prior reports and may be explained by considering variations in bone maturity and resulting time available for mineralization [12, 27]. Bone at trabecular centers is older and has had more time to mineralize than younger bone at trabecular edges [32]. This may help explain the observed trabeculae site differences in the reported modulus models.

The finding that BV/TV is a covariate in models relating modulus and hardness to BP treatment duration could be a manifestation of a negative feedback mechanism involving bone architecture (extrinsic bone properties) and bone material (intrinsic bone properties). Whole bone resistance to deformation under physiologic loading is governed by architecture and material properties, the latter is quantified by modulus. These two factors efficiently provide whole bone with deformation resistance. Increased modulus means decreased need for architecture and vice versa. This is supported in the models by the negative sign of the BV/TV covariate (Tables 2 and 3). This finding does not contradict the known increases in BMD with BP treatment up to 5 years because increases in BMD are mainly due to increases in mineral content not bone volume [33, 34].

The present study shows that age is not a significant covariate relating modulus to BP treatment duration. While it may be argued that this finding could be due to coincidence of BP treatment duration with concomitant patient age and the inability to distinguish these two simultaneous events, removal of BP treatment duration from the analysis reveals that age is a non-significant variable. This is consistent with the findings and conclusions of an analogously conducted nanoindentation study of human cadaveric proximal femoral bone [35]. Although age-related declines in macroscopically measured tibial trabecular bone modulus have been reported [36], these declines may be due to age-related reductions in structure not age-related declines in intrinsic material properties. The finding that age is not a significant covariate may also be partially explained by the cross-sectional study design. The finding that neither age nor BMD were significant covariates in models relating bone mechanical response variables with BP treatment duration suggests that the effects of BPs on bone can be expected regardless of patient age or degree of osteoporosis.

Vickers indentation of iliac crest cancellous bone from 32 post-menopausal women treated with alendronate for 3–10 years (mean 6.4 years, SD 2.0 years) showed a 7.8% lower hardness in the BP-treated group and no relationship between hardness and the duration of alendronate treatment [11]. Nanoindentation assesses resistance to combined elastic (reversible) and plastic (permanent) deformation, but Vickers assesses resistance to plastic deformation only. As shown by the present statistical models, hardness changes with BP treatment duration were substantially smaller than modulus changes with BP treatment duration. No data exist indicating that hardness is a predictor of bone strength or fracture resistance. Clearly, the usefulness of hardness as bone quality metric requires further study [12, 37, 38].

The present study is limited to information available from a one-time measurement of embedded cancellous bone without baseline data. Also, the study was not powered to detect differences among various oral BPs. Compliance with prescribed BP treatment was assumed based on patient records and self-reported information. These data were supported by objective histological assessment of bone turnover. The role of PMMA embedding on nanoindentation remains unclear. One publication notes that embedding bone in PMMA preparatory to nanomechanical testing does not influence the elastic modulus [39], another study reports an increase in modulus with PMMA embedding [40]. Also, it is known that thorough air drying reduces the elastic modulus of interstitial bone by 9.7% [41]. Nevertheless, the modulus values presently reported are in keeping with published values for trabecular bone modulus [23, 42]. While the number of samples treated with BPs for 10 or more years is small, the important finding is that the join point, i.e., the duration of BP treatment at which the quadratic relationship changes to a linear relationship with zero slope (plateau), occurs at approximately 6 years. There are sufficient bone biopsies near this 6-year time point to provide the reported statistical confidence in the models shown. Samples near this 6-year BP treatment duration have a substantial role in determining the form of the four plateau models (three models for modulus, one model for hardness) shown. The addition of further samples from patients treated for 10 or more years with BPs is unlikely to change the form of these plateau models. Furthermore, the probability of obtaining samples treated with BPs > 10 years is small given present concerns regarding long-term BP treatment [43]. While it is clear that there are no changes in modulus at trabecular centers after 2.6 years, the paucity of data between 1.1 and 2.6 years of BP treatment needs to be considered when interpreting the early quadratic portion of this model. Finally, it is important to note that the models shown are applicable only over the period of BP treatment duration actually observed, i.e., 1.1 to 20 years. Extrapolation of these models to BP treatment durations less than 1.1 years or greater than 20 years is unsupported by the results of this study.

Conclusions

Bone modulus increases with bisphosphonate treatment durations up to 6 years and no additional modulus gain can be expected from treatment durations exceeding 6 years. Hardness increased, peaked at 12.4 years, and remained constant up to 20 years of BP treatment, but the clinical relevance of hardness remains unclear.

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Compliance with ethical standards

Conflicts of interest David Pienkowski, Constance Wood, and Hartmut Malluche declare that they have no conflict of interest. This study was supported by awards from the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health (R01 AR061578) and the Kentucky Nephrology Research Trust.

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