ORIGINAL ARTICLE

JBMR

Bone Micromechanical Properties Are Compromised During Long-Term Alendronate Therapy Independently of Mineralization

Yohann Bala,[1,2] Baptiste Depalle,[1,2] Delphine Farlay,[1,2] Thierry Douillard,[3,4] Sylvain Meille,[3,4] Helene Follet,[1,2] Roland Chapurlat,[1,2] Jérôme Chevalier,[3,4] and Georges Boivin[1,2]

[1]INSERM, UMR1033, F-69372, Lyon, France
[2]Université de Lyon, F-69008 Lyon, France
[3]INSA Lyon, MATEIS UMR5510, F-69621 Villeurbanne, France
[4]Université de Lyon, CNRS, Villeurbanne, France

ABSTRACT

In the treatment of postmenopausal osteoporosis (PMOP), the use of alendronate (ALN) leads to a decrease in the risk of vertebral and nonvertebral fractures. To explore the possible adverse effects of prolonged ALN therapy, we studied the effects of 8 ± 2 years (6–10 years) of ALN treatment on the iliac cortical bone mineral and collagen quality and micromechanical properties; by design, our study examined these parameters, independent of the degree of mineralization. From six ALN-treated and five age-matched untreated PMOP women, 153 bone structural units have been chosen according their degree of mineralization to obtain the same distribution in each group. In those bone structural units, Fourier transform infrared spectroscopy, quantitative microradiography, and nanoindentation were used to assess bone quality. Irrespective of the degree of mineralization, ALN treatment was associated with higher collagen maturity (+7%, $p < 0.001$, c.v. = 13% and 16% in treated and untreated women, respectively) and lower mineral crystallinity than that observed in the untreated PMOP group (−2%, $p < 0.0001$, c.v. = 3% in both groups). Bone matrix from ALN-treated women also had lower elastic modulus (−12%, $p < 0.0001$, c.v. = 14% in both groups) and, contact hardness (−6%, $p < 0.05$, c.v. = 14% in both groups) than that of untreated women. Crystallinity (which reflects the size and perfection of crystals) was associated with both elastic modulus and contact hardness in treated women exclusively ($r = 0.43$ and $r = 0.54$, $p < 0.0001$, respectively), even after adjustment for the amount of mineral. We infer that long-term ALN treatment compromises micromechanical properties of the bone matrix as assessed *ex vivo*. The strength deficits are in part related to difference in crystallinity, irrespective of the mineral amount and mineral maturity. These novel findings at local levels of bone structure will have to be taken into account in the study of the pathophysiology of bone fragilities associated with prolonged ALN treatment. © 2012 American Society for Bone and Mineral Research.

KEY WORDS: BONE QUALITY; FOURIER TRANSFORM INFRARED MICROSPECTROSCOPY; LONG-TERM ALENDRONATE; NANOINDENTATION; POSTMENOPAUSAL OSTEOPOROSIS

Introduction

A bone is a hierarchically organized structure.[1] Bone strength is derived the interaction between the material composition and bone macrostructure.[2–5] Bone remodeling maintains the composition and structure of bone by removing damaged or old bone and replacing it with an equal volume of new bone. However, as age advances, the appearance of a negative bone balance for basic multicellular units (BMU) and accelerated remodeling produce structural deterioration.[6]

Alendronate (ALN), an antiresorptive drug, reduces remodeling activity and reduces the progression of structural decay and fracture risk.[7–10] With protracted suppression of remodeling, preservation of the structure may be compromised by alterations in the material composition of the bone. Indeed, under these conditions, secondary mineralization goes to completion, increasing the mean degree of mineralization and decreasing its heterogeneity in cortical and trabecular bone tissue.[11–13] Decreased remodeling also results in increased occurrence of microdamage and reduced microdamage removal as assessed in

Received in original form October 3, 2011; revised form November 22, 2011; accepted December 5, 2011. Published online December 20, 2011.
Address correspondence to: Yohann Bala, PhD, INSERM UMR 1033 Equipe "Qualité Osseuse et Marqueurs Biologiques," Université de Lyon, Faculté de Médecine Lyon Est, Claude Bernard (Domaine Laennec), 7-11 rue Guillaume Paradin, 69372 Lyon Cedex 08 France. E-mail: yohannbala@gmail.fr

Journal of Bone and Mineral Research, Vol. 27, No. 4, April 2012, pp 825–834
DOI: 10.1002/jbmr.1501

animal models.[14,15] Evidence of these effects in human subjects is less well established.[16–18] A study of bone biopsies of patients drawn from the cross-sectional FLEX trial suggested neither continued increases in tissue mineralization nor homogenization of mineral content over a 10-year treatment.[19]

Several factors determine matrix strength. In addition to the degree and homogeneity of tissue mineralization density, the extent of collagen crosslinking and the size and perfection of apatite crystals contribute to matrix strength; each of these can be influenced by prolonged suppression of remodeling.[20–24] Adverse events such as delayed fracture healing, osteonecrosis of the jaw, and atypical subtrochanteric fractures may be, in part, the result of prolonged bisphosphonate treatment.[25–27] Thus, in some instances, long-term bisphosphonate use may lead to impaired bone quality and their causes remain unclear. It is currently believed that the effects of ALN on material properties are mainly consequences of changes in remodeling activity.[13] However, recent findings suggest that bisphosphonates can exert effects on matrix quality independent of bone turnover.[28]

The aim of the study was to determine the effect of prolonged oral treatment with ALN on the material composition and micromechanical behavior of cortical bone matrix *ex vivo*. This study was designed to be performed independently of the degree of mineralization. Indeed, the nanostructural quality (mineral maturity, crystallinity, and collagen maturity) and micromechanical properties were assessed in bone structural units (BSUs), selected from treated and untreated postmenopausal osteoporotic (PMOP) women to share a similar degree of mineralization. At tissue level (ie, whole cortical), we hypothesized that the antiresorptive activity of ALN will lead to a higher cortical mean degree of mineralization with a narrower distribution compared with the untreated PMOP women. However, in BSUs of the same degree of mineralization, ALN treatment would result in an alteration in crystallinity independently of the amount of mineral, because of its interaction with bone apatite. We propose that these changes in crystallinity in long-term ALN-treated patients could lead to compromised local mechanical properties compared with untreated PMOP women.

Material and Methods

Iliac bone samples

Transiliac bone biopsies were performed in patients double labeled with demethylchlortetracycline (600 mg/day, 2 days on, 10 days off, 2 days on). Five biopsies were taken from 69 ± 2-year-old untreated PMOP.[29] The long-term ALN-treated group consisted of six biopsies from 72 ± 8-year-old PMOP women treated for 8 ± 2 years with oral ALN prescribed exclusively to treat osteoporosis (ALN_{LT}, 10 mg daily or 70 mg weekly).[16] Undecalcified samples were fixed in 70% alcohol, dehydrated in absolute alcohol, and then embedded in methyl methacrylate (MMA).

Quantitative microradiography[30]

Sections (150 μm-thick) were cut in a plane perpendicular to the Haversian canal with a precision diamond wire saw (Well, Escil, Chassieu, France). Sections were progressively ground (silicium carbides) to a thickness of 100 ± 1 μm and polished with diamond suspension of 0.25 μm. Contact microradiography was performed on 100-μm-thick bone sections to assess the degree of mineralization of bone (DMB) using an X-ray diffraction unit (PW 1830/40; Philips, Limeil Brévannes, France). Nickel-filtered copper Kα radiation was used under 25 kV and 25 mA. Both bone sample and an aluminum standard X-ray absorption were recorded on a high-resolution film exposed for 20 minutes (Geola, Slavich International, Vilnius, Lithuania). Acquisitions of microradiographs were performed using a digital camera (image pixel size: 5.64 μm). After calibration using the aluminum standard, the mean gray levels of BSUs were converted into degrees of mineralization values (in g mineral/cm^3). The DMB was first measured for the whole cortical bone, resulting in the mean DMB value. The spatial distribution of DMB was assessed by measuring the heterogeneity index, which corresponds to the full width at half maximum (FWHM) of the distribution curves of DMB values for each sample.

Selection of bone structural units

From microradiographs obtained from the PMOP group, 20 BSUs were selected in each bone sample and qualitatively separated according their gray level in low-, medium-, and high-DMB tertiles. The range of DMB values obtained in each tertile was then used to select 20 BSUs in each bone sample from the ALN_{LT} group according the same classification scheme. These BSUs of known DMB were then tested using instrumented indentation and Fourier transform infrared microspectroscopy (FTIRM). Finally, the full set of variables was obtained for a total of 153 BSUs (55 and 98 osteons and interstitial cortical areas in the PMOP and ALN_{LT} groups, respectively). This procedure allowed us to obtain two groups of BSUs with similar DMBs (Table 1).

Instrumented indentation testing

Tests were carried out on BSUs of known DMB on microradiographed 100-μm-thick bone sections using a Nano Indenter II machine (Nano Instruments Inc., Oak Ridge TN, USA) equipped with a Berkovich diamond tip. Indentation tests were performed under displacement control according to the following protocol: a 0.05 $second^{-1}$ constant strain rate was applied to a peak of displacement of 5000 nm, followed by a 10-second holding at peak displacement to limit the viscous behavior of bone tissue, a 45-second withdrawal to 10% of maximum displacement, a 50-second hold period for thermal drift calculation, and final withdrawal to complete unload (Fig. 1*A*). The measurements were performed at a relatively high peak load (~500 mN) to overcome effects related to the heterogeneity of bone tissue at the lamellar level. The system was calibrated with fused silica according to the protocol of Oliver and Pharr.[31] In this study, bone was assumed to be isotropic with a Poisson ratio of 0.3. To avoid measurement artefacts, the indent location was chosen to be ≥10 μm from porosities (visible osteocyte lacunae and/or Haversian canal).

Indentation load–displacement curves (Fig. 1*A*) were analyzed using an in-house program developed using Matlab R2010a (The MathWorks Inc., Natik, MA, USA). For each indent, elastic modulus (E in GPa) and contact hardness (H_c in GPa) were calculated using

Table 1. Degree of Mineralization of Bone (DMB), Ultrastructural Quality, and Micromechanical Variables in 153 Bone Structural Units Selected According their DMB From Untreated Postmenopausal Women (PMOP) and PMOP Women Treated for 8 ± 2 Years with Alendronate (ALN_{LT})

	Groups									
	PMOP					ALN_{LT}				
Variables	Mean	SD	CV (%)	Min	Max	Mean	SD	CV (%)	Min	Max
Mineral amount										
DMB (g/cm^3)	1.101	0.080	7.3	0.925	1.278	1.112	0.078	7.0	0.831	1.310
Ultrastructure quality										
Mineral maturity	1.847	0.227	12.3	1.300	2.547	1.926	0.298	15.5	1.208	1.256
FWHM 604 cm$^{-1\Delta}$	24.775	0.698	2.8	22.846	26.086	25.290^c	0.763	3.0	23.066	27.841
Collagen maturity	4.676	0.758	16.2	2.784	5.993	4.992^b	0.653	13.1	3.394	7.085
Micromechanical variables										
E (GPa)	19.1	2.7	14.1	14.0	24.5	16.8^c	2.6	15.5	8.0	20.9
H_c (GPa)	0.63	0.09	14.3	0.48	0.84	0.59^a	0.08	13.6	0.39	0.74
H (GPa)	1.80	0.34	18.9	1.22	2.67	1.79	0.31	17.3	1.04	2.70
W_{tot} (mN · μm)	702	94	13.4	517	962	676	87	12.9	491	820
W_p (mN · μm)	526	66	12.5	384	699	501^a	65	13.0	342	611
W_e (mN · μm)	177	31	17.5	127	263	175	27	15.4	107	237

$^a p < 0.05$, $^b p < 0.001$, $^c p < 0.0001$ versus PMOP.
Δ FWHM of the 604 cm^{-1} peak is inversely proportional to crystallinity index.
Micromechanical variables: Elastic Modulus (E), Contact Hardness (H_c), True Hardness (H), and Total (W_{tot}), Irreversible (W_p), and Reversible (W_e) Work of Indentation.

the method described by Oliver and Pharr.[31] Because H_c is a complex parameter influenced by both elastic and plastic behavior, we also calculated the purely plastic hardness or *true hardness* (H in GPa) as defined by Sakaï.[32] H was obtained by considering bone to be an elastoplastic material with a behavior modeled, during the indentation loading, by a purely elastic element connected to a purely plastic element in series. The plastic element is represented by H, and the elastic element is represented by E.[32,33] The work of indentation was also obtained from the loading and unloading curves. The total work of indentation (W_{tot}) is defined as the area under the loading curve, the elastic (reversible) work (W_e) is defined as the area under the unloading curve, and the plastic (irreversible) work (W_p) is defined as the area enclosed by the loading and unloading curves (Fig. 1*B*).[32,34]

FTIRM

FTIRM was performed on a 30 × 30 μm area of BSUs that had been previously tested using instrumented indentation. Indeed, 100-μm-thick sections were cut into 2-μm-thick sections with a polycut E microtome (Leica, Wetzlar, Germany). The collection of spectra was gathered in transmission mode with a Spectrum 100 spectrometer equipped with an Auto-IMAGE microscope (Perkin-Elmer, Shelton, CT, USA). Each spectrum corresponded to 150 cumulated scans. The contributions of air and MMA were subtracted from the individual spectra, and a baseline correction was performed. The spectra were then curve-fitted using GRAMS/AI (Thermo galactic, Salem, NH, USA) to analyze the peaks of amides (1600–1700 cm^{-1}), of the ν_4PO_4 domain (500–650 cm^{-1}), and of the $\nu_1\nu_3PO_4$ domain (900–1200 cm^{-1}) (Fig. 2*A*). The collagen maturity was evaluated using the area ratio 1660 cm^{-1} over 1690 cm^{-1} peaks (Fig. 2*B*).[35] Mineral maturity was calculated as the area ratio 1030 cm^{-1} over 1110 cm^{-1} peaks (Fig. 2*C*).[36] The 1030 cm^{-1} peak corresponds to an apatitic phosphate, whereas the 1110 cm^{-1} peak corresponds to a nonapatitic phosphate. This variable reflects the transformation of immature precursors of the hydrated layer into a mature apatite. Finally, mineral crystallinity index was assessed by FWHM of the 604 cm^{-1} band in the ν_4PO_4 domain (Fig. 2C).[36] The mineral crystallinity index, which reflects the crystalline domain size and the organization of ions in the unit cells of the crystal, is inversely proportional to the FWHM of the 604 cm^{-1} peak.

Statistical analysis

Statistical analysis was performed using SPSS v16.0 (SPSS Inc., Chicago, IL, USA) with an alpha risk of 5%. The results were reported as the mean ± standard deviation (SD). The mean DMB and heterogeneity index were compared using nonparametric U of the Mann-Whitney test. At the BSU level, all of the variables were found to be normally distributed using the Shapiro-Wilk procedure. Means were compared using an unpaired t test. The influence of the microstructure on the micromechanical behavior was studied using linear regression analyses (r corresponds to Pearson's correlation coefficient). Fisher's transformation was used to calculate the 95% confidence intervals (CI) of r-values. Stepwise forward multiple regression models were used with the following equation: micromechanical variable = amount of mineral (DMB) + quality of mineral (Crystallinity) + quality of organic matrix (Collagen

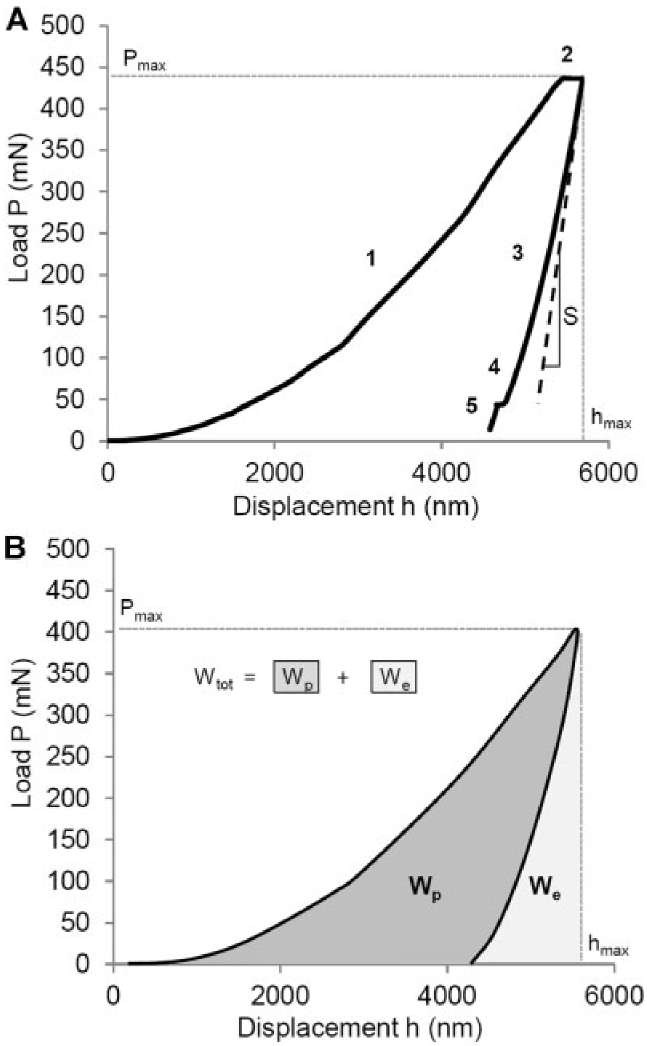

Fig. 1. Load–displacement curve obtained with indentation testing. The curve shows regions related to loading (1), 10-second holding segment (2), 90% unloading (3), 50-second holding segment allowing the thermal drift correction, (4) and complete unloading (5). S is the contact stiffness corresponding to the slope of the unloading curve tangent (*A*). After computerized extraction of the two holding segments, total work of indentation (W_{tot}) was measured as the sum of the reversible (W_e) and irreversible (W_p) work of indentation (*B*).

maturity). Values of β correspond to the standardized regression coefficient.

Results

Material composition

The mean DMB measured in whole cortical bone was higher in ALN_{LT}-treated women than in untreated PMOP women ($1.163 \pm 0.067\,g/cm^3$ vs. $1.044 \pm 0.079\,g/cm^3$, $p = 0.011$), and the heterogeneity index was lower ($0.166 \pm 0.033\,g/cm^3$ vs. $0.228 \pm 0.032\,g/cm^3$, $p = 0.035$) (Fig. 3). The DMB measured in specifically selected cortical BSUs in the two groups, ensured that they were matched by the mean and range (Table 1). In BSUs isolated from ALN_{LT}-treated patients, the mineral maturity was unchanged, while the collagen maturity was higher than in untreated PMOP women (+7%, $p < 0.001$). ALN treatment was associated with a lower crystallinity index than that observed measured in BSUs selected from untreated patients (−2%, $p < 0.0001$ with c.v. = 3% in both groups) (Table 1).

Micromechanical properties

In BSUs selected according to their DMB, both the elastic modulus and the contact hardness were lower in long-term ALN-treated women compared with the untreated group (−12%, $p < 0.0001$ and −6%, $p < 0.05$, respectively). Long-term ALN treatment also resulted in a plastic work of indentation that was lower than that in PMOP group (−5%, $p < 0.05$) (Table 1).

Associations between material composition and micromechanical properties

In both groups, DMB was associated with elastic modulus, contact hardness, purely plastic hardness, and works of indentation, explaining from 16% to 50% of their variability ($p < 0.001$ for all) (Table 2). However, correlations between ultrastructural properties and micromechanical variables differ in the two groups (Table 2). Contrary to our observations in the untreated PMOP group, in ALN_{LT}-treated women, crystallinity was correlated with elastic modulus, contact hardness and purely plastic hardness ($r = 0.43$, 0.54 and 0.46, respectively, $p < 0.001$ for all) (Table 2). These correlations remained significant after adjustment for DMB (*r* values became 0.34, 0.41, and 0.30, respectively, $p \leq 0.005$ for all).

The multiple regression model including the DMB, the collagen maturity, and the crystallinity, explained from 30% to 60% of the variability in elastic modulus, contact hardness or plastic hardness among groups (final *R* values ranging from 0.54–0.76). The respective roles of the selected independent variables in micromechanical properties were different in untreated PMOP and long-term ALN-treated women (Table 3). In the ALN_{LT}-treated group, the crystallinity was a significant predictor of the elastic modulus ($\beta = 0.28$, $p = 0.006$), contact hardness ($\beta = 0.30$, $p = 0.001$) and purely plastic hardness ($\beta = 0.22$, $p = 0.008$), while it was systematically not significant in the untreated PMOP group (Table 3).

Discussion

As hypothesized, long-term ALN therapy led to a higher mean degree of cortical mineralization than that observed in untreated osteoporotic women with a more narrow distribution. However, we also observed that in BSUs of the same degree of mineralization, micromechanical properties such as elastic modulus and contact hardness were compromised by the prolonged use of ALN. The results suggest that for postmenopausal osteoporotic women treated in an outpatient clinic, protracted use of ALN leads to decreases in size/perfection of crystals that can impair local mechanical properties.

Most of the available biopsy studies suggest that suppression of remodeling by antiresorptive drugs increases the mean degree of mineralization and decreases the heterogeneity of the mineralization.[11,12,19] A higher degree of mineralization has

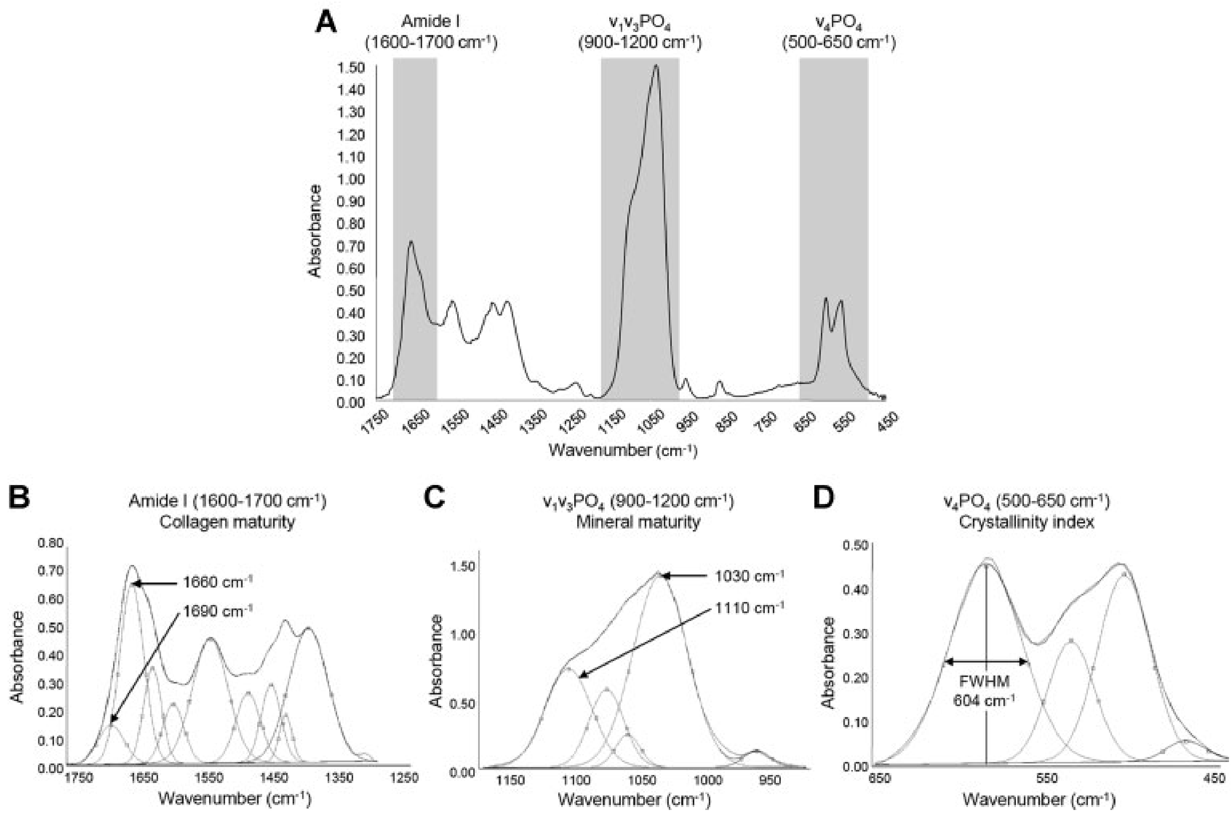

Fig. 2. Typical infrared spectra of a cortical bone structural unit (*A*). Detail of the domains deconvoluted to measure the collagen maturity (1660/1690 cm^{-1} area ratio) (*B*), the mineral maturity (1030/1110 cm^{-1} area ratio) (*C*), and the crystallinity index, which is inversely proportional to the FWHM of the 604 cm^{-1} peak (*D*).

been associated with a decrease in histological surrogates of remodeling activity.[37,38] This is explained by the fact that under treatment, BSUs have more time to complete their mineralization before being resorbed in the next remodeling cycle. This mechanism is supported by the present study. However, the remodeling suppression is only partial and new bone matrix is formed under treatment.[39] It has been shown that the mineralization of bone matrix formed during bisphosphonate therapy is normal.[40–42]

To limit the influence of the remodeling suppressor activity of ALN on the assessment of its effects on bone matrix, we measured bone material and mechanical properties in BSUs that

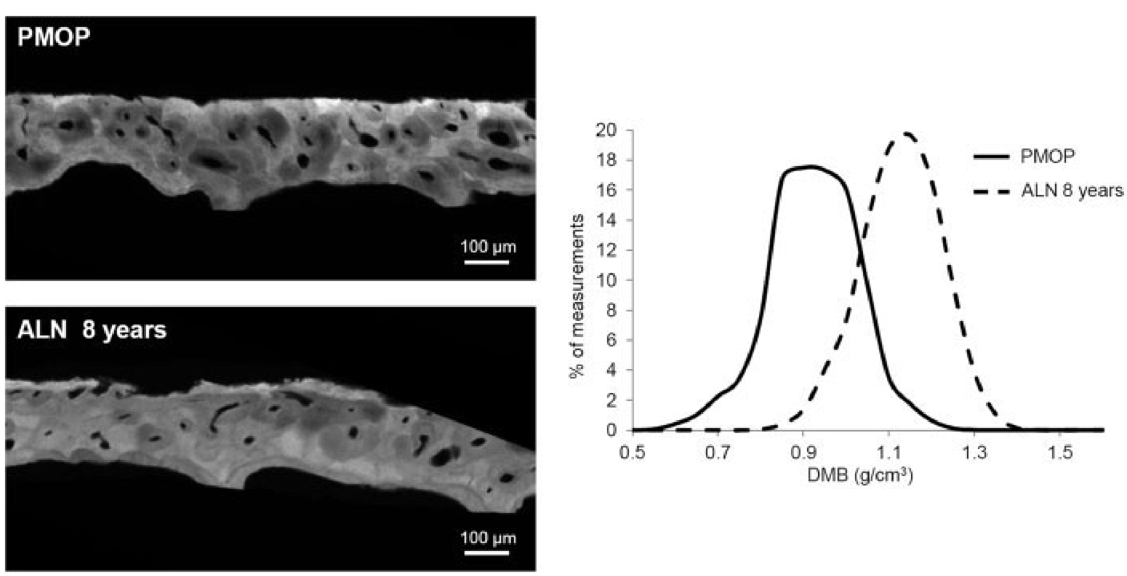

Fig. 3. Example of microradiographs obtained at the tissue level in the cortical bone of untreated postmenopausal osteoporotic woman (PMOP) and of PMOP woman treated for 8 years with oral alendronate (ALN, *left*). Distribution curves of degree mineralization (DMB) highlight a higher mean DMB and lower heterogeneity in the treated patient than in the untreated PMOP women (*right*).

Table 2. Correlations (Bivariate Pearson's Coefficient) Among the Degree of Mineralization (DMB), the Mineral and Collagen Maturities, the Crystallinity Index, and the Micromechanical Variables

Group	Variables	Micromechanical variables					
		E	H_c	H	W_{tot}	W_p	W_e
PMOP	DMB	0.68[b]	0.70[b]	0.57[b]	0.55[b]	0.56[b]	0.47[b]
		[0.50; 0.80]	[0.53; 0.81]	[0.36; 0.72]	[0.33; 0.71]	[0.34; 0.71]	[0.23; 0.65]
	Mineral maturity	0.48[b]	0.34[a]	0.20	0.19	0.21	0.12
		[0.25; 0.66]	[0.08; 0.56]	[−0.07; 0.44]	[−0.08; 0.43]	[−0.06; 0.45]	[−0.15; 0.37]
	FWHM 604 cm$^{-1\Delta}$	0.15	−0.25	−0.25	−0.33[a]	−0.31[a]	−0.33[a]
		[−0.13; 0.39]	[−0.48; 0.02]	[−0.49; 0.01]	[−0.54; −0.07]	[−0.05; −0.53]	[−0.07; −0.54]
	Collagen maturity	0.35[a]	0.55[b]	0.55[b]	0.49[b]	0.45[b]	0.52[b]
		[0.09; 0.56]	[0.34; 0.71]	[0.34; 0.71]	[0.26; 0.67]	[0.20; 0.64]	[0.30; 0.69]
ALN_{LT}	DMB	0.40[b]	0.67[b]	0.66[b]	0.59[b]	0.52[b]	0.66[b]
		[0.22; 0.55]	[0.54; 0.76]	[0.52; 0.76]	[0.45; 0.71]	[0.34; 0.65]	[0.53; 0.76]
	Mineral maturity	−0.13	−0.18	−0.07	−0.09	−0.09	−0.08
		[-0.32; 0.07]	[−0.31; 0.08]	[−0.26; 0.13]	[−0.29; 0.11]	[−0.28; 0.11]	[−0.28; 0.12]
	FWHM 604 cm$^{-1\Delta}$	−0.43[b]	−0.54[b]	−0.46[b]	−0.53[b]	−0.51[b]	−0.48[b]
		[−0.58; −0.25]	[−0.67; −0.38]	[−0.58; −0.26]	[−0.66; −0.37]	[−0.64; −0.34]	[−0.62; −0.32]
	Collagen maturity	0.25[a]	0.23[a]	0.15	0.23[a]	0.24[a]	0.17
		[0.06; 0.43]	[0.04; 0.41]	[−0.05; 0.34]	[0.03; 0.41]	[0.04; 0.41]	[−0.03; 0.35]

Pearson coefficient of correlation r [95% IC], $^a p < 0.05$, $^b p < 0.001$.
Δ FWHM of the 604 cm^{-1} peak is inversely proportional to crystallinity index.
Micromechanical variables: Elastic Modulus (E), Contact Hardness (H_c), True Hardness (H), and Total (W_{tot}), Irreversible (W_p), and Reversible (W_e) Work of Indentation.

had a similar mean and range in degree of mineralization in the treated and untreated groups. This decision was prompted by the fact that it is unlikely that ALN influence the mineral apposition rate, that is, the kinetics of primary mineralization, even after 6.4 years of treatment.[37] Furthermore, it has been shown in an animal model that neither ALN nor risedronate had an effect on the chronology of the mineralization or the relative durations of primary and secondary mineralization periods.[43] This result suggests that a given DMB value reflects a bone matrix of the same age in the treated and untreated groups. This hypothesis was supported in the present study as the mineral maturity, which directly reflects the age of the mineral phase,[36] did not differ between the populations of cortical BSUs selected in treated and untreated women.

Previous studies using FTIR to explore the effect of bisphosphonates on bone material properties have been performed using an imaging mode that implies areas of bone tissue including several BSUs along with interstitial tissue. This method did not reveal changes in collagen maturity in bone tissue from osteoporotic women after 3 years of ALN or risedronate treatment.[13,44] Those findings contrast with the results of biochemical experiments that concluded that treatment with bisphosphonates causes changes in collagen maturity. Indeed, significant decreases, 52% and 38%, in the αα/ββ-CTX ratio have been observed in PMOP women treated for 2 years with ALN and ibandronate, respectively.[45] These apparent discrepancies might be explained by differences among methodologies. The maturation of collagen is complex and includes not only enzymatic crosslinking but also nonenzymatic process.[23] The assessment of 1660/1690 cm^{-1} ratio by FTIR spectroscopy reflects a global index of collagen maturity, including changes in its secondary structure,[35] whereas chromatographic techniques allow the characterization of components of the whole process and has a much higher sensitivity.[46,47]

Our results revealed that in BSUs that were selected so that their DMB did not differ in mean or range, the collagen maturity was higher in ALN-treated compared with untreated women while the mineral maturity was not different. This result may be explained by direct effects of the interaction between ALN and bone apatite. It has been reported that once ALN binds to apatite crystal, its nitrogen is expected to confer an electrical charge at mineral surface (ie, a change in the zeta potential).[48] As recently hypothesized by others, cells being generally sensitive to changes in electrochemical potential, osteoblastic function might be qualitatively altered after the bisphosphonate binding to the apatite.[28]

The decrease in crystallinity observed under treatment represents a decrease in size/perfection of crystals that is caused by ALN independently of its antiresorptive effect. Our results are consistent with those described for zoledronate.[28] In fact, when bone matrix was of the same age (as shown by tetracycline labeling), crystallinity was lower in women who had been treated with zoledronate for 3 years than in those receiving placebo.[28] Results from the FLEX study highlighted that average mineral particle width increased linearly with tissue mineralization in iliac bone from long-term ALN-treated women.[19] This correlation supports the idea that the lower crystallinity observed in the present study at the BSU level might be more related to an impairment in the perfection rather than in the size of crystals.

There are various hypotheses for the effect of bisphosphonates binding to apatite on crystallinity. Numeric simulation has shown that ALN binds not only to a Ca of apatite with its P-C-P backbone (Ca I site) but also to a Ca II site, where the nitrogen

Table 3. Multiple Regression Analysis Describing Micromechanical Variables as a Function of the Degree of Mineralization of Bone (DMB), Collagen Maturity, and Crystallinity Index in Untreated Postmenopausal Women (PMOP) and Alendronate Long-Term Treated PMOP Women (ALN_{LT})

PMOP group					
Variables					
Dependent	Independent	Stepwise regression rank	Final *R*	β	*p* value
E	DMB	1	0.68	0.68	<0.001
	Collagen maturity	out		—	—
	FWHM 604 $cm^{-1\Delta}$	out		—	—
H_c	DMB	1	0.74	0.46	<0.001
	Collagen maturity	2		0.33	0.004
	FWHM 604 $cm^{-1\Delta}$	out		—	—
H	DMB	out	0.58	—	—
	Collagen maturity	1		0.58	<0.001
	FWHM 604 $cm^{-1\Delta}$	out		—	—

ALN_{LT} group					
Variables					
Dependent	Independent	Stepwise regression rank	Final *R*	β	*p* value
E	DMB	2	0.54	0.30	0.002
	Collagen maturity	out		—	—
	FWHM 604 $cm^{-1\Delta}$	1		−0.28	0.006
H_c	DMB	1	0.76	0.57	<0.0001
	Collagen maturity	3		0.15	0.031
	FWHM 604 $cm^{-1\Delta}$	2		−0.30	0.001
H	DMB	1	0.71	0.58	<0.0001
	Collagen maturity	out		—	—
	FWHM 604 $cm^{-1\Delta}$	2		−0.22	0.008

A significant effect of the crystallinity index was observed on all micromechanical properties measured in the ALN_{LT} group.
Δ FWHM of the 604 cm^{-1} peak is inversely proportional to crystallinity index.
Micromechanical variables: Elastic Modulus (*E*), Contact Hardness (H_c), and True Hardness (*H*).

atom of the R2 lateral chain participates in a N-H-O hydrogen bond with an OH coordinated to the calcium ion.[49,50] This double *anchoring* may influence the properties of apatite, including its crystallinity.[51] In vitro assays showed that the binding of ALN to mineral causes changes in the zeta potential as well as in the interfacial tension.[48] It is known that both the shape and the growth kinetics of the crystals are influenced by the electric environment.[48,52]

In the present study, irrespective of the degree of mineralization, nanoindentation highlighted a lower elastic modulus and contact hardness in long-term ALN-treated women compared with untreated women. This is consistent with data from our previous studies performed at the tissue level.[37] In this latter work, PMOP women treated with oral ALN for a mean duration of 6.4 years, the mean cortical microhardness was 7% lower, while the DMB was 9% higher than those in age-matched untreated PMOP women.[37] More generally, there is a lack of data concerning the impact of ALN treatment on bone micromechanical properties. Moreover, those obtained in animal models with Haversian remodeling seem contradictory.[53]

Bone apatite is a rigid material that cannot dissipate much energy, and it has been widely suggested that the collagen matrix plays a key role in bone toughness.[54] The fact that the collagen maturity measured by FTIR has been positively linked with both elastic modulus and contact hardness in animal models[55,56] as well as in our patients limits its potential effect on the decrease in these mechanical properties observed in the ALN_{LT} group. Interestingly, collagen maturity played different roles in purely plastic hardness between groups. In untreated PMOP women, plastic hardness (*H*) was exclusively explained by collagen maturity, whereas the contribution of collagen maturity was not significant in the ALN_{LT} group. From studies performed on isolated type I collagen, it has been shown that collagen has an almost perfectly elastic tensile behavior.[57,58] Therefore, our interpretation of the relative roles of the bone matrix components in bone's mechanical properties is limited because interactions between the mineral and collagen. These interactions define indeed how mineral and collagen, two mostly elastic elements, form a composite material with a complex viscoelastoplastic behavior.[54,59] Differences in the plastic behavior of bone tissue between the untreated PMOP and ALN_{LT} groups might result from changes in the interactions between collagen and mineral.

Our work highlights, a direct correlation between the crystallinity index and the elastic modulus, the contact hardness, and the plastic hardness at the BSU level in ALN_{LT} group only. This result supports the idea that alterations in crystallinity are directly involved in the observed decrease in micromechanical properties. Our results support the notion that the ability to dissipate plastic energy under local loading, which has been correlated with compressive work to failure in rat vertebrae,[60] is significantly lower in ALN_{LT} than in untreated PMOP women. In ribs from dogs tested in three-point bending assays, ALN treatment has been shown to produce a decrease in the postyield toughness.[61] Studies have failed to link this decrease in toughness with changes in microdamage accumulation, mineralization, or collagen crosslinking under bisphosphonates.[18] Our results suggest that local changes as impairments in mineral properties may also contribute to this phenomenon.

Our study has some limitations: the main ones are the limited number of samples analyzed and the cross-sectional design. We were not able to characterize the time-dependent effect of ALN because no baseline values were available. Moreover, the indentation modulus assumes an isotropic, elastic–plastic behavior of bone tissue with a defined Poisson ratio ($\nu = 0.3$).[34] Because transverse isotropy has been documented with microhardness[62] and because indentation tests were performed exclusively along the transversal axis of the BSUs, we believe this will not affect the trends documented in this study.

In conclusion, this study provides an analysis of the effects of long-term ALN treatment on the intrinsic properties of bone at the level of the bone structural unit. Our data suggest an association between long-term ALN treatment and a decrease in micromechanical properties that is mainly because of a decrease in mineral crystallinity. Although long-term ALN-treatment preserves bone mineral density at structural level,[63] the present study suggests that ALN may impair bone quality at lower levels of the bone organization. It is clinically important to further test the roles of alterations in crystallinity and micromechanics in patients suffering from atypical fractures associated with prolonged treatment with bisphosphonates.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

The authors are grateful to Professor Ego Seeman (Austin Health, University of Melbourne, Melbourne, Australia) for his assistance in the preparation and editing of the manuscript. We also thank Anne-Sophie Bravo Martin for her technical expertise and assistance (INSERM, UMR1033, F-69008, Lyon, France), and Philippe Clément for the development of the indentation's data processing software (INSA Lyon, MATEIS, CNRS 5510, F-69621, Villeurbanne, France).

Authors' roles: Data collection: YB, BD, and TD. Data analysis: YB and BD. Data interpretation: YB, BD, DF, SM, JC, and HF. Drafting manuscript: YB, HF, GB. Revising manuscript content: YB, BD, DF, TD, SM, HF, RC, JC, and GB. Approving final version of manuscript: YB, BD, DF, TD, SM, HF, RC, JC, and GB. YB takes responsibility for the integrity of the data analysis.

References

1. Rho JY, Kuhn-Spearing L, Zioupos P. Mechanical properties and the hierarchical structure of bone. Med Eng Phys. 1998;20(2):92–102.
2. Bouxsein ML. Determinants of skeletal fragility. Best Pract Res Clin Rheumatol. 2005;19(6):897–11.
3. Seeman E, Delmas PD. Bone quality—the material and structural basis of bone strength and fragility. N Engl J Med. 2006;354(21):2250–61.
4. Fratzl P, Weinkamer R. Nature's hierarchical materials. Prog Mater Sci. 2007;52:1263–334.
5. Jepsen KJ. Functional interactions among morphologic and tissue quality traits define bone quality. Clin Orthop Relat Res. 2011; 469(8):2150–9.
6. Zebaze RM, Ghasem-Zadeh A, Bohte A, Iuliano-Burns S, Mirams M, Price RI, Mackie EJ, Seeman E. Intracortical remodelling and porosity in the distal radius and post-mortem femurs of women: a cross-sectional study. Lancet. 2010;375(9727):1729–36.
7. Black DM, Cummings SR, Karpf DB, Cauley JA, Thompson DE, Nevitt MC, Bauer DC, Genant HK, Haskell WL, Marcus R, Ott SM, Torner JC, Quandt SA, Reiss TF, Ensrud KE. Randomised trial of effect of alendronate on risk of fracture in women with existing vertebral fractures. Fracture Intervention Trial Research Group. Lancet. 1996; 348(9041):1535–41.
8. Black DM, Thompson DE, Bauer DC, Ensrud K, Musliner T, Hochberg MC, Nevitt MC, Suryawanshi S, Cummings SR. Fracture risk reduction with alendronate in women with osteoporosis: the Fracture Intervention Trial. FIT Research Group. J Clin Endocrinol Metab. 2000; 85(11):4118–24.
9. Cummings SR, Black DM, Thompson DE, Applegate WB, Barrett-Connor E, Musliner TA, Palermo L, Prineas R, Rubin SM, Scott JC, Vogt T, Wallace R, Yates AJ, LaCroix AZ. Effect of alendronate on risk of fracture in women with low bone density but without vertebral fractures: results from the Fracture Intervention Trial. JAMA. 1998;280(24):2077–82.
10. Liberman UA, Weiss SR, Broll J, Minne HW, Quan H, Bell NH, Rodriguez-Portales J, Downs RW Jr, Dequeker J, Favus M. Effect of oral alendronate on bone mineral density and the incidence of fractures in postmenopausal osteoporosis. The Alendronate Phase III Osteoporosis Treatment Study Group. N Engl J Med. 1995;333(22):1437–43.
11. Boivin GY, Chavassieux PM, Santora AC, Yates J, Meunier PJ. Alendronate increases bone strength by increasing the mean degree of mineralization of bone tissue in osteoporotic women. Bone. 2000; 27(5):687–94.
12. Roschger P, Rinnerthaler S, Yates J, Rodan GA, Fratzl P, Klaushofer K. Alendronate increases degree and uniformity of mineralization in cancellous bone and decreases the porosity in cortical bone of osteoporotic women. Bone. 2001;29(2):185–91.
13. Boskey AL, Spevak L, Weinstein RS. Spectroscopic markers of bone quality in alendronate-treated postmenopausal women. Osteoporos Int. 2009;20(5):793–800.
14. Allen MR, Iwata K, Phipps R, Burr DB. Alterations in canine vertebral bone turnover, microdamage accumulation, and biomechanical properties following 1-year treatment with clinical treatment doses of risedronate or alendronate. Bone. 2006;39(4):872–9.
15. Mashiba T, Hirano T, Turner CH, Forwood MR, Johnston CC, Burr DB. Suppressed bone turnover by bisphosphonates increases microdamage accumulation and reduces some biomechanical properties in dog rib. J Bone Miner Res. 2000;15(4):613–20.

16. Chapurlat RD, Arlot M, Burt-Pichat B, Chavassieux P, Roux JP, Portero-Muzy N, Delmas PD. Microcrack frequency and bone remodeling in postmenopausal osteoporotic women on long-term bisphosphonates: a bone biopsy study. J Bone Miner Res. 2007;22(10):1502–9.

17. Stepan JJ, Burr DB, Pavo I, Sipos A, Michalska D, Li J, Fahrleitner-Pammer A, Petto H, Westmore M, Michalsky D, Sato M, Dobnig H. Low bone mineral density is associated with bone microdamage accumulation in postmenopausal women with osteoporosis. Bone. 2007;41(3):378–85.

18. Allen MR, Burr DB. Bisphosphonate effects on bone turnover, microdamage, and mechanical properties: what we think we know and what we know that we don't know. Bone. 2011;49(1):56–65.

19. Roschger P, Lombardi A, Misof BM, Maier G, Fratzl-Zelman N, Fratzl P, Klaushofer K. Mineralization density distribution of postmenopausal osteoporotic bone is restored to normal after long-term alendronate treatment: qBEI and sSAXS data from the fracture intervention trial long-term extension (FLEX). J Bone Miner Res. 2010;25(1):48–55.

20. Allen MR, Gineyts E, Leeming DJ, Burr DB, Delmas PD. Bisphosphonates alter trabecular bone collagen cross-linking and isomerization in beagle dog vertebra. Osteoporos Int. 2008;19(3):329–37.

21. Tang SY, Allen MR, Phipps R, Burr DB, Vashishth D. Changes in non-enzymatic glycation and its association with altered mechanical properties following 1-year treatment with risedronate or alendronate. Osteoporos Int. 2009;20(6):887–94.

22. Tang SY, Zeenath U, Vashishth D. Effects of non-enzymatic glycation on cancellous bone fragility. Bone. 2007;40(4):1144–51.

23. Viguet-Carrin S, Garnero P, Delmas PD. The role of collagen in bone strength. Osteoporos Int. 2006;17(3):319–36.

24. Yerramshetty JS, Akkus O. The associations between mineral crystallinity and the mechanical properties of human cortical bone. Bone. 2008;42(3):476–82.

25. Khosla S, Burr D, Cauley J, Dempster DW, Ebeling PR, Felsenberg D, Gagel RF, Gilsanz V, Guise T, Koka S, McCauley LK, McGowan J, McKee MD, Mohla S, Pendrys DG, Raisz LG, Ruggiero SL, Shafer DM, Shum L, Silverman SL, Van Poznak CH, Watts N, Woo SB, Shane E. Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for Bone and Mineral Research. J Bone Miner Res. 2007;22(10):1479–91.

26. Odvina CV, Zerwekh JE, Rao DS, Maalouf N, Gottschalk FA, Pak CY. Severely suppressed bone turnover: a potential complication of alendronate therapy. J Clin Endocrinol Metab. 2005;90(3):1294–301.

27. Shane E, Burr D, Ebeling PR, Abrahamsen B, Adler RA, Brown TD, Cheung AM, Cosman F, Curtis JR, Dell R, Dempster D, Einhorn TA, Genant HK, Geusens P, Klaushofer K, Koval K, Lane JM, McKiernan F, McKinney R, Ng A, Nieves J, O'Keefe R, Papapoulos S, Sen HT, van der Meulen MC, Weinstein RS, Whyte M. Atypical subtrochanteric and diaphyseal femoral fractures: report of a task force of the American Society for Bone and Mineral Research. J Bone Miner Res. 2010;25(11):2267–94.

28. Gamsjaeger S, Buchinger B, Zwettler E, Recker R, Black D, Gasser JA, Eriksen EF, Klaushofer K, Paschalis EP. Bone material properties in actively bone-forming trabeculae in postmenopausal women with osteoporosis after three years of treatment with once-yearly Zoledronic acid. J Bone Miner Res. 2011;26(1):12–8.

29. Ott SM, Oleksik A, Lu Y, Harper K, Lips P. Bone histomorphometric and biochemical marker results of a 2-year placebo-controlled trial of raloxifene in postmenopausal women. J Bone Miner Res. 2002;17(2):341–8.

30. Boivin G, Bala Y, Doublier A, Farlay D, Ste-Marie LG, Meunier PJ, Delmas PD. The role of mineralization and organic matrix in the microhardness of bone tissue from controls and osteoporotic patients. Bone. 2008;43(3):532–8.

31. Oliver WC, Pharr GM. An improved technique for determining hardness and elastic modulus. J Mater Res. 1992;7(6):1564–83.

32. Sakai M. The Meyer Hardness: A measure for plasticity?. J Mater Res. 1999;14(9):3630–9.

33. Oyen ML. Nanoindentation hardness of mineralized tissues. J Biomech. 39(14):2699–702.

34. Bala Y, Depalle B, Douillard T, Meille S, Clément P, Follet H, Chevalier J, Boivin G. Respective roles of organic and mineral components of human cortical bone matrix in micromechanical behavior: an instrumented study. J Biomechan Behav Biomed Mater. 2011;4:1473–82.

35. Farlay D, Duclos M-E, Gineyts E, Bertholon C, Viguet-Carrin S, Nallala J, Sockalingum G, Bertrand D, Roger T, Hartmann DJ, Chapurlat R, Boivin G. The ratio 1660/1690cm-1 measured by Infrared Microspectroscopy is not specific of enzymatic collagen cross-links in bone tissue. PLoS ONE. 2011;6(12):e2873.

36. Farlay D, Panczer G, Rey C, Delmas PD, Boivin G. Mineral maturity and crystallinity index are distinct characteristics of bone mineral. J Bone Miner Metab. 2010;28(4):433–45.

37. Bala Y, Farlay D, Chapurlat R, Boivin G. Modifications of bone material properties in postmenopausal osteoporotic women long-term treated with alendronate. Eur J Endocrinol. 2011;165(4):647–55.

38. Borah B, Ritman EL, Dufresne TE, Jorgensen SM, Liu S, Sacha J, Phipps RJ, Turner RT. The effect of risedronate on bone mineralization as measured by micro-computed tomography with synchrotron radiation: correlation to histomorphometric indices of turnover. Bone. 2005;37(1):1–9.

39. Seeman E. Bone morphology in response to alendronate as seen by high-resolution computed tomography: through a glass darkly. J Bone Miner Res. 2010;25(12):2553–7.

40. Chavassieux PM, Arlot ME, Reda C, Wei L, Yates AJ, Meunier PJ. Histomorphometric assessment of the long-term effects of alendronate on bone quality and remodeling in patients with osteoporosis. J Clin Invest. 1997;100(6):1475–80.

41. Recker RR, Weinstein RS, Chesnut CH 3rd, Schimmer RC, Mahoney P, Hughes C, Bonvoisin B, Meunier PJ. Histomorphometric evaluation of daily and intermittent oral ibandronate in women with postmenopausal osteoporosis: results from the BONE study. Osteoporos Int. 2004;15(3):231–7.

42. Recker RR, Delmas PD, Halse J, Reid IR, Boonen S, Garcia-Hernandez PA, Supronik J, Lewiecki EM, Ochoa L, Miller P, Hu H, Mesenbrink P, Hartl F, Gasser J, Eriksen EF. Effects of intravenous zoledronic acid once yearly on bone remodeling and bone structure. J Bone Miner Res. 2008;23(1):6–16.

43. Fuchs RK, Faillace ME, Allen MR, Phipps R, Miller LM, Burr D. Bisphosphonates do not alter the rate of the secondary mineralization. Bone. 2011;49(4):701–5.

44. Durchschlag E, Paschalis EP, Zoehrer R, Roschger P, Fratzl P, Recker R, Phipps R, Klaushofer K. Bone material properties in trabecular bone from human iliac crest biopsies after 3- and 5-year treatment with risedronate. J Bone Miner Res. 2006;21(10):1581–90.

45. Byrjalsen I, Leeming DJ, Qvist P, Christiansen C, Karsdal MA. Bone turnover and bone collagen maturation in osteoporosis: effects of antiresorptive therapies. Osteoporos Int. 2008;19(3):339–48.

46. Sroga GE, Vashishth D. 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. 2011;879(5–6):379–85.

47. Viguet-Carrin S, Gineyts E, Bertholon C, Delmas PD. Simple and sensitive method for quantification of fluorescent enzymatic mature and senescent crosslinks of collagen in bone hydrolysate using single-column high performance liquid chromatography. J Chromatogr B Anal Technol Biomed Life Sci. 2009;877(1–2):1–7.

48. Nancollas GH, Tang R, Phipps RJ, Henneman Z, Gulde S, Wu W, Mangood A, Russell RG, Ebetino FH. Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite. Bone. 2006;38(5):617–27.

49. Ebetino FH, Barnett BL, Russell RG. A computational model delineates differences in hydroxyapatite binding affinities of bisphosphonates in clinical use. J Bone Miner Res. 2005;20(Suppl 1): S259.

50. Russell RG, Watts NB, Ebetino FH, Rogers MJ. Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos Int. 2008; 19(6):733–59.

51. Li ZY, Lam WM, Yang C, Xu B, Ni GX, Abbah SA, Cheung KM, Luk KD, Lu WW. Chemical composition, crystal size and lattice structural changes after incorporation of strontium into biomimetic apatite. Biomaterials. 2007;28(7):1452–60.

52. Wu W, Gerard DE, Nancollas GH. Nucleation at surfaces: the importance of interfacial energy. J Am Soc Nephrol. 1999;10(Suppl 14): S355–8.

53. Wang X, Shanbhag AS, Rubash HE, Agrawal CM. Short-term effects of bisphosphonates on the biomechanical properties of canine bone. J Biomed Mater Res. 1999;44(4):456–60.

54. Fratzl P, Gupta HS, Paschalis EP, Roschger P. Structure and mechanical quality of the collagen-mineral nano-composite in bone. J Mater Chem. 2004;14:2115–23.

55. Gourion-Arsiquaud S, Burket JC, Havill LM, DiCarlo E, Doty SB, Mendelsohn R, van der Meulen MC, Boskey AL. Spatial variation in osteonal bone properties relative to tissue and animal age. J Bone Miner Res. 2009;24(7):1271–81.

56. Isaksson H, Malkiewicz M, Nowak R, Helminen HJ, Jurvelin JS. Rabbit cortical bone tissue increases its elastic stiffness but becomes less viscoelastic with age. Bone. 2010;47(6):1030–8.

57. Svensson RB, Hassenkam T, Hansen P, Peter Magnusson S. Viscoelastic behavior of discrete human collagen fibrils. J Mech Behav Biomed Mater. 2010;3(1):112–5.

58. van der Rijt JA, van der Werf KO, Bennink ML, Dijkstra PJ, Feijen J. Micromechanical testing of individual collagen fibrils. Macromol Biosci. 2006;6(9):697–702.

59. Oyen ML, Ko CC. Examination of local variations in viscous, elastic, and plastic indentation responses in healing bone. J Mater Sci Mater Med. 2007;18(4):623–8.

60. Hengsberger S, Ammann P, Legros B, Rizzoli R, Zysset P. Intrinsic bone tissue properties in adult rat vertebrae: modulation by dietary protein. Bone. 2005;36(1):134–41.

61. Allen MR, Reinwald S, Burr DB. Alendronate reduces bone toughness of ribs without significantly increasing microdamage accumulation in dogs following 3 years of daily treatment. Calcif Tissue Int. 2008; 82(5):354–60.

62. Ziv V, Wagner HD, Weiner S. Microstructure–microhardness relations in parallel-fibered and lamellar bone. Bone. 1996;18(5):417–28.

63. Bone HG, Hosking D, Devogelaer JP, Tucci JR, Emkey RD, Tonino RP, Rodriguez-Portales JA, Downs RW, Gupta J, Santora AC, Liberman UA. Ten years' experience with alendronate for osteoporosis in postmenopausal women. N Engl J Med. 2004;350(12):1189–99.