CLINICAL STUDY

Modifications of bone material properties in postmenopausal osteoporotic women long-term treated with alendronate

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Abstract

Objective: Given recent concern about long-term safety of bisphosphonate (BP) therapy, the effects of long-term alendronate (ALN) therapy on intrinsic bone properties were studied among postmenopausal osteoporotic (PMOP) women.

Design and methods: Transiliac bone biopsies were obtained from 32 outpatient clinic PMOP women treated with oral ALN for 6.4 ± 2.0 years. Variables reflecting bone mineralization were measured both at tissue level using quantitative microradiography and at crystal level by Fourier transform infrared microspectroscopy. Bone microhardness was investigated by Vickers indentation tests. Results were compared with those from 22 age-matched untreated PMOP women.

Results: Long-term treatment with ALN was associated with a 84% ($P < 0.001$) lower remodeling activity compared with untreated PMOP women, leading to an increased degree of mineralization in both cortical and trabecular bone (+9 and +6%, respectively, $P < 0.05$). Despite a more mature and more mineralized bone matrix, after treatment, cortical and trabecular microhardness and crystallinity were lower than that measured in untreated patients. None of the variables reflecting material properties were significantly correlated to the duration of the treatment.

Conclusion: Increased degree of mineralization associated with lower crystallinity and microhardness in ALN long-term-treated PMOP women suggests that ALN could alter the quality of bone matrix. The study also suggested that after 3 years of treatment, the changes in material properties are not dependent on the duration of the treatment. Further studies are requested to assess the short-term (< 3 years) effects of BPs on bone intrinsic properties.

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Introduction

Bisphosphonates (BPs) are widely used in the treatment of postmenopausal osteoporosis (PMOP) as effective anti-resorptive agents. They reduce the risk of vertebral (1) and non-vertebral fractures (2). This effect was associated with an increase in bone mineral density (BMD), but BMD moderately reduces in fracture risk (3). The effects of BPs on bone tissue properties remain unclear. Bone strength is explained by numerous determinants reflecting both extrinsic (bone mass and microarchitecture) and intrinsic (mineral and collagen quality, mineralization and accumulation of microdamage) properties of bone matrix (4–6). Bone quality also depends on bone remodeling activity. The decrease in bone remodeling activity prolongs the lifetime of bone structural units (BSUs), so it increases the duration of secondary mineralization, leading to an increase in the degree of mineralization of bone (DMB), which is a major determinant of bone strength (4, 7). Thus, after 3 years of treatment with alendronate (ALN) (7–9) or risedronate (10), a significant increase in the DMB has

been observed, often associated with a decrease in the heterogeneity of the mineralization.

It has been suggested that oversuppression of both targeted and non-targeted bone remodeling could impair bone quality. In a canine model, the prolonged use of high doses of BPs led to an accumulation of bone microdamage with a decrease in some of the bone mechanical variables (11, 12). In this animal model, alterations in collagen cross-linking and isomerization have also been described (13). However, an increase in bone microdamage in osteoporotic patients receiving oral BPs has not yet been established (14). To date, only few data on the effect of long-term BP therapies on bone intrinsic properties have been reported. The cases of atypical subtrochanteric femoral fractures in OP patients receiving long-term therapy with ALN have been reported (15, 16). In some instances, prolonged BP therapies were also associated with rare cases of adynamic bone and delayed fracture healing (17). BPs may also impair both cortical and cancellous bone elasticity during therapies shorter or longer than 3 years (18). The cases of osteonecrosis of the jaw

have been described in patients receiving oral BP therapies (19) but essentially in the context of high cumulative doses for the treatment of multiple myeloma or bone metastasis.

At the bone tissue level, the analysis of iliac crest biopsies from a subgroup of the FIT long-term extension (FLEX) trial (comparing 10 years ALN and 5 years ALN+5 years placebo groups) showed no accumulation of osteoid and no impaired mineralization. After 10 years of ALN therapy, the mineralization level was not greater than that after 5 years, and its heterogeneity was not decreased (20).

This study aims to highlight the effects of long-term ALN therapies on intrinsic determinants of bone quality. The DMB, mineral characteristics, and the Vickers microhardness (Hv) were assessed in transiliac bone biopsies from 32 outpatient clinic PMOP women on long-term ALN therapy (21). In a cross-sectional study, we have compared these women with untreated PMOP women.

Materials and methods

Subjects

We have studied 32 outpatient clinic PMOP women treated long-term with ALN (ALN_{LT}, 10 mg daily or 70 mg weekly). The subject disposition and techniques are shown in Fig. 1. In this group, 18 patients sustained at least one osteoporotic fracture (three at the hip and 15 at the spine) before the treatment. Twenty-four patients received their medication for at least 5 years, and the mean duration of BP therapy was 6.4 ± 2.0 years (mean $\pm$ s.d.; range: 3–10 years). Their mean age was 70.4 ± 8.6 years, at least 5 years postmenopause, and their mean bone volume over tissue volume (BV/TV) was $13.40 \pm 4.5\%$. Exclusion criteria were the presence of metabolic bone diseases other than PMOP; the use of any medication other than BPs likely to interfere with skeletal metabolism within the past 6 months; uncontrolled hyperthyroidism; alcohol or drug abuse, current or within the past 5 years; allergy to tetracycline or novocain; hip anatomy preventing from transiliac bone biopsy or absorptiometry scan; and previous bilateral transiliac bone biopsies (21).

Results were compared with those obtained in a group of 22 iliac bone samples taken from untreated PMOP women (mean age = 69.4 ± 5.5 years, mean BV/TV = $17.2 \pm 3.5\%$) with either a BMD at least -2.5 s.d. below the young adult mean or at least two radiographically apparent moderate vertebral fractures ($> 25\%$ vertebral height loss) (22).

Bone samples

Transiliac bone biopsies were performed after patients were double-labeled with demethylchlortetracycline

Figure 1 Description of the groups involved in the study of the effects of alendronate long-term treatment in material properties (Fourier transform infrared microspectroscopy (FTIRM)).

(600 mg/day, 2 days on, 10 days off, 2 days on). The Samples were fixed, dehydrated, and then embedded in methyl methacrylate (MMA). For quantitative microradiography, thick sections (about 150 μ m) were cut from embedded bone samples using a precision diamond wire saw (Well, Escil, Chassieu, France), progressively ground to a thickness of 100 ± 1 μ m, and polished with an alumina suspension (1 μ m). For microhardness tests, embedded blocks were surfaced and polished using a diamond suspension (0.25 μ m). On the residual blocks, 2–5 μ m-thick sections were cut using a Polycut E microtome (Leica, Wetzlar, Germany) after the grinding of indented surface. The cutting plane perpendicular to the Haversian canals of cortical bone was preferred.

Assessment of bone remodeling by histomorphometry

Bone histomorphometry was performed on cancellous bone only, on 8 μ m-thick unstained sections. Parameters were expressed according to the recommended American Society for Bone and Mineral Research (ASBMR) nomenclature (23): mineral apposition rate (MAR in μ m/day) and mineralizing surface/bone surface (MS/BS in %) was calculated as one-half single+double-labeled surfaces. Bone formation rate/BS (BFR/BS in μ m³/ μ m² per day) was calculated as ((MS/BS) $\times$ MAR). The activation frequency (Ac.F defined by the number of new basic multicellular units created per unit of time expressed in #/years) was calculated as ((BFR/wall thickness (W.Th)) $\times$ 365).

The W.Th (in μm), representing the endproduct of the osteoblastic activity in a remodeling site, was measured under polarized light on Solochrome cyanine R-stained 8 μm -thick sections.

Quantitative microradiography

Contact microradiography of 100 μm -thick bone sections was performed using an x-ray diffraction unit PW 1830/40 (Philips, Limeil Brévannes, France) (24). The nickel-filtered copper $K\alpha$ 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 min (Geola, Slavich International Wholesale Office, Vilnius, Lithuania). Acquisition of microradiographs was performed using a digital camera (resolution: 800×600). After calibration using an aluminum reference, the threshold for measured regions of bone tissue was determined, prior to gray-level segmentation. Finally, gray-level values were converted into DMB values (in g mineral/ cm^3) for each pixel (5.64 μm a side). The main reported variables were DMB and the index of heterogeneity of the distribution of DMB values, calculated as the full width at half-maximum (FWHM) on the individual DMB curves (heterogeneity index (HI) in g/cm^3). These variables were separately measured on cortical and cancellous bone.

Fourier transform infrared microspectroscopy

Fourier transform infrared microspectroscopy (FTIRM) was performed on 2–5 μm -thick sections to measure mineral characteristics at the crystal level. Infrared spectra were obtained from a total area of $35 \times 35 \mu\text{m}$ (25). Spectra were obtained using 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 done (Fig. 2). The spectra were then curve-fitted using GRAMS/AI Software (Thermo galactic, Salem, NH, USA) to analyze the peaks $\nu_4\text{PO}_4$ ($500\text{--}650 \text{ cm}^{-1}$) and $\nu_1\nu_3\text{PO}_4$ ($900\text{--}1200 \text{ cm}^{-1}$). The mineral crystallinity index was inversely correlated to the FWHM of the 604 cm^{-1} band in the $\nu_4\text{PO}_4$ domain. Mineral crystallinity index corresponded to the crystal size/perfection. The mineral maturity reflecting transformation of immature precursors of the hydrated layer into a mature apatite was calculated as the area ratio 1030 cm^{-1} over 1110 cm^{-1} ($\nu_3\text{PO}_4$). Spectra were obtained from 720 cortical and cancellous BSUs (osteons, packets, and interstitial bone) from ten untreated PMOP and ten ALN_{IT} samples to fit with the initial population distribution as much as possible (Fig. 1).

Figure 2 Typical infrared spectra obtained on bone with the analyzed vibration domains (A) and curve-fitting of the $\nu_1\nu_3 \text{PO}_4$ domain allowing the calculation of the mineral maturity as the area ratio of 1030 cm^{-1} over the 1110 cm^{-1} peaks (B).

Measurement of microhardness

Mean bone tissue microhardness was measured using a Micromet 5104 tester (Buehler, Lake Bluff, IL, USA) (24) equipped with a Vickers indenter corresponding to a square-based diamond indenter. A load of 25 g applied for 10 s was used. Hv, defined as the mean pressure the material will support under load (Hv in kg/mm^2), was computed from $\text{Hv} = 1854.4 \times P/d^2$, where P is the test load in grams and d is the mean length of both the diagonals in μm . A total of 60 random impressions were analyzed for each bone sample, corresponding to 40 measurements in cortical bone and 20 measurements in cancellous bone. The data obtained for each bone sample were expressed as a mean both in cortical and cancellous bone.

Statistical analysis

Analyses were performed using SPSS v16.0 (SPSS, Inc., Chicago, IL, USA) with an alpha risk level defined at 5%. Results were reported as mean $\pm$ s.d. The distribution

Table 1 Dynamic histomorphometric variables measured in untreated postmenopausal women (PMOP) and in PMOP women long-term treated with oral alendronate (ALN_{LT}).

	PMOP	ALN _{LT}
MAR ($\mu\text{m}/\text{day}$)	0.641 (0.096)	0.718 (0.148)
MS/BS (%)	7.408 (4.652)	0.435 (0.615)*
BFR/BS ($\mu\text{m}^3/\mu\text{m}^2$ per day)	0.048 (0.034)	0.004 (0.005)*
Ac.F (#/year)	0.396 (0.255)	0.063 (0.066)*

* $P < 0.0001$ versus PMOP.

of the degree of mineralization and microhardness was tested with the Shapiro–Wilk procedure. Non-parametric tests were used because the distributions were not normal. Means were compared using Mann–Whitney’s *U* test in case of significant results previously obtained using Friedman’s test. Correlations were tested using Spearman’s rho test (r'). FTIRM analyses were performed on subsets of ten samples per analyzed group. Owing to the small number of individuals, medians were tested. Mood’s median test was used for the null hypothesis H_0 : medians of the populations from which two samples are drawn are identical.

Results

Bone remodeling activity

Even after multiple sectioning, no fluorescent labels were found in seven samples. Those samples were not taken into account in the analysis. Using 2D histomorphometry, the MAR was normal and not significantly different between the groups (Table 1). The MS/BS, BFR/BS, and Ac.F were significantly lower in ALN_{LT} group compared with the PMOP group (Table 1). When data were pooled for ALN_{LT} and PMOP groups, DMB and HI were significantly correlated with the Ac.F, with $r' = -0.37$, $P = 0.02$, and $r' = 0.35$, $P = 0.02$ respectively (Fig. 3). Hv was not significantly correlated with the Ac.F ($r' = 0.23$, NS).

DMB and its heterogeneity

The DMB measured in cancellous bone was significantly greater after long-term ALN treatment compared with untreated PMOP women (Table 2). The cancellous HI was significantly lower after ALN treatment versus PMOP (-18% , $P = 0.043$ ALN_{LT}; Table 2). In cortical bone, the DMB also increased in ALN-treated women (Table 2), whereas the HI was not as low as observed in cancellous bone. When measured in cortical bone, neither DMB nor HI was significantly correlated with the duration of the treatment ($r' = 0.15$ and -0.14 NS respectively). This was also true in cancellous bone ($r' = 0.21$ and 0.10 , NS).

Mineral characteristics measured by FTIRM

In cortical bone, mineral maturity was significantly greater in the ALN_{LT} group than in the PMOP group, both in osteonal and interstitial bone ($+14$ and $+16\%$, $P < 0.05$, respectively; Table 3). These results were systematically observed in cancellous bone ($+6$ and $+7\%$, $P < 0.05$, for bone packets and interstitial bone respectively). In each analyzed bone compartment of treated bone, FWHM of the peak at 604 cm^{-1} was significantly higher (from 2 to 4%, $P < 0.05$) than that measured in untreated women (Table 3). This indicated that in long-term-treated patients, crystallinity index was significantly lower than that in untreated PMOP women. Mean Hv (cortical+cancellous bone) was significantly and inversely correlated ($r' = -0.50$, $P = 0.03$) with the mean FWHM 604 cm^{-1} , i.e. mean of all measurements performed per sample (Fig. 4). Thus, microhardness increases with the size/perfection of crystals at tissue level.

Bone microhardness

The Hv measured in the ALN_{LT} group (Table 2) was significantly lower than that in the PMOP women. This result was observed both in cancellous (-7.8% , $P = 0.0006$) and cortical (-7.5% , $P = 0.0004$) bone.

Figure 3 Significant correlations were observed between the activity of bone remodeling assessed by 2D histomorphometry (activation frequency (Ac.F)) and degree of mineralization of bone (DMB, A) and its heterogeneity index (HI, B) obtained using quantitative microradiography among the whole population.

Table 2 DMB, HI, and microhardness (Hv) measured in iliac bone tissue from PMOP and ALN_{LT}. Values are reported as mean (s.d.).

Subjects	Cortical bone			Cancellous bone		
	DMB (g/cm ³)	HI (g/cm ³)	Hv (kg/mm ²)	DMB (g/cm ³)	HI (g/cm ³)	Hv (kg/mm ²)
PMOP	1.033 (0.082)	0.249 (0.065)	48.773 (2.657)	1.077 (0.081)	0.244 (0.070)	49.302 (2.538)
ALN _{LT}	1.123 (0.069)*	0.211 (0.058) [†]	45.139 (4.211)*	1.140 (0.062)*	0.200 (0.050)*	45.435 (4.651)*

**P* < 0.05 and [†]*P* = 0.07 versus PMOP.

Hv values were not significantly correlated with the duration of the treatment period ($r' = -0.21$ and -0.12 , NS, for cancellous and cortical bone respectively).

Discussion

This study is the first report of the effects on bone mineralization, mineral characteristics, and microhardness of a long-term treatment with ALN in outpatient clinic PMOP women. The DMB measured in PMOP women treated for 6.4 years with ALN was significantly greater than that measured in untreated PMOP women in cancellous and cortical bone. This study also showed significant correlations between variables of bone remodeling measured by 2D histomorphometry and the DMB and its HI, as observed with other methods (26). This correlation can be explained by the fact that the increase in DMB was linked to a profound decrease in remodeling activity (e.g. Ac.F was up to 80% lesser in ALN_{LT} group compared with untreated PMOP women). This was confirmed by the decrease in HI. The increase in DMB observed during ALN treatment is due to a higher proportion of bone matrix with greater mineralization. That is, it had a greater duration of secondary mineralization due to the depressed remodeling activity (7–9). This phenomenon has also been described with several other inhibitors of bone resorption used in the treatment of PMOP such as other BPs and selective estrogen receptor modulators (22, 26). Interestingly, changes in microhardness were not correlated with Ac.F, as opposed to the increase in mineralization, suggesting that the decrease in hardness is independent of the slowdown in remodeling activity.

Compared with data previously published following a 3-year treatment (7), even if long-term ALN treatment maintained DMB at a higher level than untreated PMOP women, it seemed that it was not time dependent. Indeed, after 3 years of ALN therapy, the reported mean DMB values were 1.19 ± 0.016 g/cm³ in both cortical and trabecular bone while our ALN_{LT} group presented 6 and 4% lower values in cortical and trabecular bone respectively. Results concerning BMD and biochemical markers of bone remodeling in the FLEX trial showed that a 10-year treatment with ALN (10 mg/day) had been related to a higher BMD in total hip and other skeletal sites and lower levels of bone turnover markers (27, 28). Moreover, bone mineralization, measured by quantitative backscattered electron imaging, did not significantly differ between the 5-year ALN group followed by 5-year placebo and the 10-year ALN groups (20). This suggested no progressive increase in the degree of mineralization with continued therapy and the reaching of a new equilibrium situation. This is in accordance with a previous study showing that between 5 and 10 years of treatment with ALN (FLEX trial), the increase in the degree of mineralization plateaued while heterogeneity increased (20). This is consistent with a rapid decrease in biochemical markers of bone turnover observed during the first months of treatment and the decrease in mineralization heterogeneity up to 5 years of therapy (9, 29, 30). Thereafter, levels of biochemical markers of bone turnover remained lower at the same level over 10 years of treatment, i.e. the continuation of the therapy was not associated with a more marked inhibition of bone turnover (28). Similar trends have also been reported with risedronate in a triple biopsy study (at baseline and after 3 and 5 years of treatment). Risedronate was

Table 3 Mineral characteristics measured by FTIRM measured in cortical and cancellous bone. Crystallinity index is inversely proportional to the FWHM of the peak at 604 cm⁻¹. Values are reported as median (range).

FTIRM variables	Cortical bone		Cancellous bone	
	Osteonal bone	Interstitial bone	Bone packets	Interstitial bone
Mineral maturity				
PMOP	1.618 (1.384–2.027)	1.891 (1.744–2.127)	1.559 (1.252–1.716)	1.674 (1.508–2.053)
ALN _{LT}	1.852 (1.646–1.986)*	2.189 (1.942–2.331)*	1.655 (1.265–1.887)*	1.795 (1.469–2.271)*
FWHM 604 cm ⁻¹				
PMOP	25.391 (23.596–26.889)	24.422 (23.695–26.343)	25.197 (24.597–26.296)	24.422 (23.164–25.496)
ALN _{LT}	25.864 (25.113–26.929)*	25.296 (24.779–26.019)*	25.790 (24.282–27.314)*	25.329 (24.161–26.181)*

**P* < 0.05 versus PMOP.

Figure 4 Mean microhardness (Hv pooled for cortical and cancellous bone) was significantly and inversely correlated with the FWHM of the peak at 604 cm^{-1} . As the FWHM 604 cm^{-1} is inversely proportional to the crystallinity index, this correlation implies that Hv increases at tissue level with the size/perfection of crystals.

associated with a significant increase in mineralization after 3 years toward premenopausal values and no further increase after 5 years of treatment (10, 31). Taken together, these results are consistent with the fact that this study detected no association between duration of treatment and the outcome variables.

Regarding the upper tails of histograms of DMB values, there were no signs of hypermineralization compared with untreated women from a separate study ($n=30$, mean age $\pm$ S.D. (range): 49 ± 21 (20–93) years) (24, 32). Indeed, the highest represented DMB class was $1.45\text{--}1.50\text{ g/cm}^3$ in the two groups and their frequencies of observation were 0.1 and 0.003% in ALN_{LT} and untreated control women respectively. Furthermore, mean DMB and HI values measured in ALN_{LT} were in the range of values obtained from untreated control women (24, 32). The increase in mean DMB observed after a long-term ALN treatment was due to a highest frequency of upper values of DMB.

A positive correlation between hardness and bone mineralization has previously been observed at both the tissue and the BSU levels (24). Thus, the lower hardness values in cortical and cancellous bone of ALN_{LT} group compared with untreated PMOP group was an unexpected result. DMB and hardness have also been measured in a small group of PMOP women long-term treated with oral risendronate ($n=13$, age $=63 \pm 10$ years, duration of the treatment $=5 \pm 1$ years). In this group, DMB, HI, and Hv measured for total bone (cortical+cancellous) were 1.101 ± 0.061 , $0.196 \pm 0.063\text{ g/cm}^3$, and $46.522 \pm 2.253\text{ kg/mm}^2$ respectively. Values were not different from those obtained in ALN_{LT} patients, suggesting that the decrease in hardness is not specific to ALN (33). However, this group of biopsies was no longer available for FTIRM analyses. Thus, if the decrease in hardness seems to be not exclusive to ALN, this study cannot raise the potential differences in the mechanism leading to a lower hardness between ALN and risendronate.

If the increase in DMB and its homogenization after BPs are now well documented, there is a lack of data concerning hardness or elastic modulus measured at the tissue level after either a short- or a long-term administration with either ALN or other BPs. These data were obtained exclusively in animal models. In ovariectomized (OVX) rats treated with pamidronate (0.6 mg/kg , 5 day per month) for 16 weeks, a significant decrease in the indentation modulus was found, while hardness was not changed compared with OVX receiving the vehicle (34). In dogs receiving ALN (6 mg daily) for 23 weeks, there was no modification of microhardness (35). Conversely, during a prolonged treatment of 1 year with ALN (0.2 mg/kg per day), canine cancellous vertebral bone was harder than that in control group, but the modulus was not changed (36). Our observation could be explained by changes in tissue mineral quality.

In this study, we found a significant increase in mineral maturity and a decrease in crystallinity. As recently published, the mineral crystallinity index (crystals size/perfection) has to be separated from the mineral maturity (progressive transformation of non-apatitic into poorly then crystallized apatite) (25, 37, 38). Mineral maturity is related to the ratio between apatitic over non-apatitic phosphates and gives indication on the evolution of the maturation of phosphate environments in bone mineral. Indeed, mineral maturity is strongly linked with the 'age' of the bone matrix. The mineral maturity measured on the $1030/1110\text{ cm}^{-1}$ ratio is comparable to the $1030/1020\text{ cm}^{-1}$ ratio used by other authors (8) and good correlations were observed between the two variables ($r^2=0.86$, $P<0.0001$) (25). After 3 years of ALN, a slight but not significant increase in $1030/1020\text{ cm}^{-1}$ ratio was observed, showing a trend in the increase in age of bone mineral consistent with BP treatment (8). Consistently, after a long-term treatment with ALN, we also found an increase in $1030/1110\text{ cm}^{-1}$ ratio in agreement with the effect of BPs. The ratio $1030/1020\text{ cm}^{-1}$ has been called mineral maturity/crystallinity due to the good correlation in normal bone with absolute crystallinity measured by X-ray diffraction. Crystallinity reflects both a combination of the relative sizes of the crystal as well as the extent to which the atoms in the lattice are ordered. We also found this correlation in normal bone between mineral maturity and crystallinity index. However, this was not always the case. For example, in skeletal fluorosis compared with normal bone, mineral exhibits a lower maturity (stimulation of osteoblasts by fluoride ions) and a higher crystallinity (substitution OH^- by F^-). It has been recently shown that the FWHM of 604 cm^{-1} band was a good indicator of the state of bone crystallinity (25).

In the current study, an impairment of crystallinity was observed after a long-term ALN treatment while the mineral maturity was higher than that in untreated PMOP women. This dissociation between crystallinity

and the age of mineral has already been reported in women treated with zoledronate for 3 years (39). In the latter study, mineral apposition rate was significantly increased. Thus, for a bone of same age, crystals were younger in zoledronate than in placebo groups, explaining the lower crystallinity (39). Our study did not reveal any changes in mineralization apposition rate between PMOP and ALN_{LT} groups, limiting this hypothesis in ALN case.

Vibrational spectroscopic methods do not allow to distinguish whether the perfection of the apatite crystalline domain and/or their size/strain were influenced by long-term ALN. Furthermore, the decrease in crystal size being unlikely, a decrease in mineral perfection (organization of atoms within apatite lattice), seems more probable and could be related to the adsorption of ALN on bone apatite. Some studies have suggested a direct implication of ALN in crystal structure changes. Indeed, from the observation that small differences in BP structure could lead to great changes in mineral affinity, it has been suggested that the 3D configuration of nitrogen containing BP and its spatial orientation may play an important role (40). This was evaluated using a 3D numerical model that simulated chemical links between BPs and apatite surfaces. This work showed that the P-C-P (common structure of BPs) bridge binds ALN to apatite on a Ca (Ca I site) but also that the nitrogen atom could create a N-H-O hydrogen bond with an OH coordinated to another Ca (Ca II site) (41). Such a binding in two sites related to Ca I and Ca II sites could directly influence apatite crystal properties as its perfection (40). Furthermore, the binding of ALN to apatite leads to modification of both the zeta potential and interfacial tension (42). The description of the shape and the kinetics of the growth of crystals are sensitive to the electric environment (42, 43). ALN is thus able to influence the crystallinity of apatite by various mechanisms.

While the strength of bone is highly related to the amount of mineral (4), there are published results showing that changes in microhardness might be induced by modifications of crystal properties such as its perfection. It has been shown in human femur that crystallinity measured by Raman spectroscopy explained 6.7–48.3% of the variability of monotonic mechanical properties and 11.3–63.5% in fatigue properties (44). Recently, we have also shown that during physiological mineralization of bone tissue, the kinetics of the mineral crystallinity index was slower than that of the mineral maturity or mineralization index, and microhardness (37). In the latter study, it was observed that during the mineralization of an osteon, microhardness was increased by 60% while the crystallinity was increased by 8%. This could explain why in this study, a decrease of 8% in microhardness could be related to a change of only 2% in mineral crystallinity. These results allow to speculate that changes in crystallinity could explain, at least partially,

the decrease in bone microhardness. This hypothesis is reinforced by the finding of a significant correlation between mean Hv and mean crystallinity index, showing that the higher mean crystal size/perfection is, harder is the bone tissue. However, this correlation has to be considered with cautions as the design of the methodologies did not allow to test this correlation directly in the same regions of interests.

The effect of ALN on mineral characteristics is probably only a part of the mechanism leading to a decrease in microhardness. This is supported in this study by the scatter plot showing the correlation between hardness and crystallinity. Indeed, the overlap of the values of crystallinity while the values of hardness were higher in the untreated group implied that the role of crystallinity in local mechanical properties is different between the groups. The differences might be explained by changes in organic matrix properties. The DMB explains about 60% of the variability of bone microhardness, the remaining being partially explained by the hardness inherent to organic matrix (24). In addition, many works have highlighted modifications of organic matrix associated with the use of BPs. In beagle dogs, high doses of both risedronate and ALN were followed by an alteration of the collagen cross-linking and isomerization, i.e. an index of collagen maturity (13). In human recently deposited bone matrix, after 3 and 5 years of risedronate, the ratio of pyridinium (non-reducible cross-link) to dehydrodihydroxylysinonorleucine (reducible cross-link), reflecting the maturation of collagenic matrix, was significantly lower compared with baseline (45).

Our study has some limitations. The comparisons were cross-sectional, with samples obtained from different studies, but all the measurements of variables were performed similarly. In ALN_{LT} group, patients were treated from 3 to 10 years and this did not allow to conclude on the precise effect of the duration of the treatment. The fact that none of the variables of bone matrix quality correlated with the duration of treatment in the present study does not exclude such associations in shorter therapies (<3 years). Furthermore, we had limited data concerning the demographic profiles of our groups, and the long-term-treated group was composed of women recruited during medical consultations and few data concerning the compliance were available. However, the calculated reduction of Ac.F by up to 80% in cancellous bone was an argument for a good compliance to the medication as it was of the same magnitude of the decrease previously observed with a 3-year ALN treatment (46). MS/BS and BFR/BS were also significantly reduced. Furthermore, we had no information on the vitamin D status of the patients, which is a factor known to increase the DMB (22, 31). The fact that MAR was not different among the groups also validated our interpretation. The last limitation is the fact that the design of the study initially planned at tissue level did not permit to test the impact of the

highlighted changes in whole bone strength. Among the patients of the treated group, no complications and no low-energy fractures were reported. This suggests that the decrease in hardness and crystallinity in most of the cases is insufficient to decrease whole bone strength.

To conclude, among PMOP women from outpatient clinics, a prolonged treatment with oral ALN maintained the DMB at a level higher than that in PMOP women, with no sign of hypermineralization despite markedly suppressed bone turnover. Long-term treatment was also associated with an increase in mineral maturity consistent with anti-resorptive effect, while both bone microhardness and bone mineral crystallinity were decreased. This could lead to a decrease in bone intrinsic quality. The study also suggests with caution that the decrease in local mechanical properties may not be specific to ALN or to the duration (>3 years) of the treatment. Further studies need to be done to test these results in bone samples taken from patients who sustained femoral atypical fracture. Such an approach would help clarifying to what extent impairments in material and micromechanical properties may influence the whole bone resistance to fracture.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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