

Alendronate Increases Degree and Uniformity of Mineralization in Cancellous Bone and Decreases the Porosity in Cortical Bone of Osteoporotic Women*

P. ROSCHGER,¹ S. RINNERTHALER,^{1,2} J. YATES,³ G. A. RODAN,³ P. FRATZL,² and K. KLAUSHOFER¹

¹Ludwig Boltzmann Institute of Osteology, Fourth Medical Department, Hanusch Krankenhaus and Unfallkrankenhaus Meidling, Vienna, Austria

²Erich Schmid Institute of Materials Science, Austrian Academy of Sciences and University of Leoben, Leoben, Austria

³Merck Research Laboratories, Rahway, NJ, USA

The strength of bone is correlated with bone mass but is also influenced significantly by other factors such as structural properties of the matrix (e.g., collagen mutations) and the mineral. Changes at all levels of this organization could contribute to fracture risk. We investigated the effects of alendronate (Aln) treatment on the density of mineralization and the ultrastructure of the mineral/collagen composite, size and habitus of mineral particles in iliac cancellous bone, as well as on the porosity of iliac cortical bone from postmenopausal osteoporotic women. Twenty-four transiliac bone biopsies from Phase III Aln (10 mg/day) trials (placebo and Aln after 2 and 3 years of treatment, n = 6 per group) were studied. The mineral structure was investigated by quantitative backscattered electron imaging (qBEI) and by scanning small-angle X-ray scattering (scanning-SAXS). qBEI histograms reflect the bone mineralization density distribution (BMDD), whereas SAXS patterns characterize the size and arrangement of the mineral particles in bone. We found that: (i) the relative calcium content of osteoporotic bone was significantly lower than that of data-base controls; (ii) mineralization was significantly higher and more uniform after Aln treatment; (iii) size and habitus of the mineral particles was not different between placebo and Aln-treated groups; and (iv) the porosity of cortical bone was reduced significantly by Aln treatment. We conclude that Aln treatment increases the degree and uniformity of bone matrix mineralization without affecting the size and habitus of the mineral crystals. It also decreases the porosity of the corticalis. Together these effects may contribute to the observed reduction in fractures. (Bone 29:185–191; 2001) © 2001 by Elsevier Science Inc. All rights reserved.

Key Words: Bone mineralization density distribution (BMDD); Quantitative backscattered electron imaging (qBEI); Scanning small-angle X-ray scattering (scanning-SAXS); Cortical porosity; Postmenopausal osteoporosis; Alendronate.

Address for correspondence and reprints: Dr. Klaus Klaushofer, Ludwig Boltzmann Institute of Osteology, 4th Medical Department, Hanusch Hospital, Heinrich Collin-Strasse 30, A-1140 Vienna, Austria. E-mail: klaus.klaushofer@univie.ac.at

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Introduction

Convincing evidence exists in the literature that the nitrogen-containing bisphosphonate, alendronate (Aln), inhibits bone resorption, increases bone mass, and reduces the risk of osteoporotic fractures. This was shown in large clinical trials with postmenopausal osteoporotic women with and without prevalent fractures^{1,19,22,23,28} and in patients with glucocorticoid-induced osteoporosis.³⁶ Other metabolic and dysplastic bone diseases are currently being investigated with regard to antifracture effectiveness of Aln. Both preclinical and clinical studies performed with histological and biomechanical methods have revealed normal quality of bone formed during treatment with Aln.^{7,24,27} In Phase III alendronate studies, transiliac biopsies were obtained from a subset of patients after 2 and 3 years of treatment for quantitative assessment of the effects on bone turnover (measurement of osteoid thickness and mineral apposition rate). These studies showed that Aln can decrease the rate of bone resorption with subsequent decreases in the rate of bone formation reflecting the filling of the remodeling space that was also evidenced by early rapid gains in bone mineral density (BMD; i.e., estimate of total bone mineral content derived from dual-energy X-ray absorptiometry). After approximately 6 months of continuous treatment, a new steady state of bone turnover was attained, although further, small increases in BMD were observed. Dynamic measurement of mineralization rate has provided indirect evidence that Aln does not impair bone mineralization.^{7,24}

Although there is no doubt about the positive antifracture effectiveness of this drug, the mechanism by which Aln works to achieve this remarkable increase in bone quality is not fully understood. It seems unlikely that the significant but relatively small increase in BMD, measured at all relevant fracture sites, can explain all of the observed reduction in the incidence of fractures. In contrast to other types of treatment (estrogens, vitamin D), bisphosphonates that act selectively on bone, to the extent of our present knowledge, do not have extraskeletal effects that might contribute to decreased fracture risk. Histochemical analyses of iliac crest biopsies have not revealed significant morphological differences vs. placebo-treated controls, except for a decrease of bone turnover. This could be related to site-specific effects, as iliac cancellous bone does not necessarily reflect changes in bone volume fraction, bone turnover, or increased bone formation at other skeletal sites like the spine or hip. However, other mechanisms, such as changes in mineral-

ization, could at least partially explain the documented gain in BMD as well as quality.

In this study we used two methods that allow investigation of the mineralized matrix in routinely prepared bone samples at the micrometer and nanometer range. First, a widely used technique for the study of mineralized tissues, quantitative backscattered electron imaging (qBEI), in the scanning electron microscope (SEM),^{2,4,5} was validated for quantitative measurement of the bone mineralization density distribution (BMDD) in bone biopsies.³² Second, scanning small-angle X-ray scattering (scanning-SAXS)³⁰ provided structural information on the collagen/mineral composite, while also allowing for such investigations at specific topographic locations within the same bone sections. Specifically, SAXS^{11–16} reveals information about the size and orientation of the mineral crystals and permits calculation of structural parameters, which in several examples were shown to be related to the biomechanical quality of the composite.^{13,16} The use of these two techniques for the investigation of bone samples (vertebrae and ribs) from preclinical studies with Aln in minipigs showed that, in the micrometer range (measurements with qBEI), cancellous bone matrix was higher and more uniformly mineralized after Aln treatment,³³ whereas mineral/collagen composite (measurements with scanning-SAXS) was not different from normal controls.¹⁶ The combined application of these two techniques suggests that the higher and more uniform mineralization density paralleled by a normal ultrastructure of the composite could contribute to the improved strength observed in vertebral bone by mechanical testing.

We also investigated the mineral structure of bone biopsies from the Phase III Aln trials to obtain further insight into the effect of this treatment. We found that Aln in postmenopausal osteoporotic women increases the degree and uniformity of mineralization in iliac cancellous bone without affecting the size and shape of mineral crystals. These findings, together with the observed decrease in cortical porosity, may indicate a positive effect of Aln treatment on bone strength.

Materials and Methods

Bone Samples

Transiliac bone biopsies were obtained from postmenopausal osteoporotic women in Phase III Aln studies at the end of either 24 or 36 months of continuous treatment. All biopsies were sent to a single centralized histomorphometry laboratory where the histological assessment of bone turnover was performed.^{7,24} Biopsies were fixed in 70% ethanol, dehydrated in a graded series of ethanol, and embedded in polymethylmethacrylate (PMMA). It was previously shown that this preparation does not influence SAXS results for mineral/collagen composite.¹¹

Twenty-four biopsies (six per group) from Aln-treated (continuous treatment with 10 mg/day) or placebo-treated patients (2 and 3 years of treatment) were sent by P. J. Meunier and G. Boivin from the laboratory in Lyon to Vienna to compare qBEI and scanning-SAXS parameters for iliac trabecular bone between the groups.

Blocks with planoparallel surfaces were prepared by grinding and careful polishing to avoid topographical artifacts. The sectioned bone samples containing cancellous and cortical bone were carbon coated for the scanning electron microscope (SEM) studies. After SEM analyses, 150- μ m-thick sections were prepared from the blocks for scanning-SAXS measurements.

Bone Morphometry

Two-dimensional morphometry was performed on identical sections. At a working distance of 32 mm and a nominal magnification of $\times 12$ backscattered electron images of 1024×1024 pixels (pixel resolution 9 μ m) were taken. The backscattered electron images were converted into binary images (white for mineralized matrix and black for soft tissue and resin). By automatic image analysis (NIH IMAGE 1.52, W. Rasband, National Institutes of Health) the parameters bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), and number of bone features per bone area (N.Bf/B.Ar) were measured.³⁴ In addition, porosity of the cortical bone area was measured.

Quantitative Backscattered Electron Imaging (qBEI)

BMDD was determined using qBEI. This method has been described in detail in previous works.^{32,35}

Briefly, a digital electron microscope (DSM 962, Zeiss, Oberkochen, Germany) equipped with a four quadrant semiconductor BE detector was used. The accelerating voltage of the electron beam was adjusted to 20 kV, the probe current to 110 pA, and the working distance to 15 mm. A $\times 50$ nominal magnification, corresponding to a pixel resolution of 4 μ m/pixel, and a slow scan speed of 100 sec/frame was used. The digital BE image, consisting of 512×512 pixels, was generated by one single frame (five images from the cancellous bone area were taken per bone biopsy and used for evaluation of the BMDD parameters as described in detail in what follows).

The intensity of the backscattered electrons (i.e., one of the signals available in the SEM) is proportional to weight concentration of the mineral in bone^{32,35}. The method uses digital (pixel) images taken from the bone sections (**Figure 1a,b**) reflecting local Ca content within the trabecular areas. From these images, gray-level histograms were deduced (**Figure 1c**) indicating the percentage of mineralized bone area (y axis) occupied by pixels with a certain gray level (bottom x axis). The gray levels were finally transformed into weight percent (wt%) Ca values (top x axis of **Figure 1c**) in two steps (the first step must be done before each imaging run and the second step done once as described elsewhere.³² First, the gray scale was calibrated using the “atomic number contrast” between carbon (C, Z = 6) and aluminum (Al, Z = 13) as reference materials. This was done by adjusting brightness and contrast of the BE detector before BE imaging of the bone areas was started. The gray scale of C was set to a gray-level index of 25 and that of Al to 225. Second, the standardization into wt% Ca of such a calibrated gray scale was performed by measuring the gray value of osteoid (unmineralized collagen) as 0 (exact < 0.17) wt% Ca and pure hydroxyapatite (HA) as 39.86 wt% Ca reference. Because the gray-level value of HA would be beyond the gray level of Al (which is the upper limit of the calibration range), a defined change of brightness was performed for this measurement and the value obtained for HA was subsequently corrected to the original brightness setting. By connecting these two points (osteoid gray level and HA gray level) in a gray level/Ca wt% diagram³² a standardization line transforming BE gray levels into wt% Ca was established. Thus, the gray-level distribution could be interpreted as a bone mineralization density distribution (BMDD). The histogram in **Figure 1c** shows such a BMDD. The area under the curve was normalized to 100%. A histogram bin width of 0.17 wt% Ca was achieved by the aforementioned procedure.

For description and comparison of BMDD histograms six BMDD parameters were introduced.³² Three of these six parameters were employed in this study: Ca_{Mean} , the weighted average Ca concentration of the mineralized bone area; Max_{Freq} , the peak

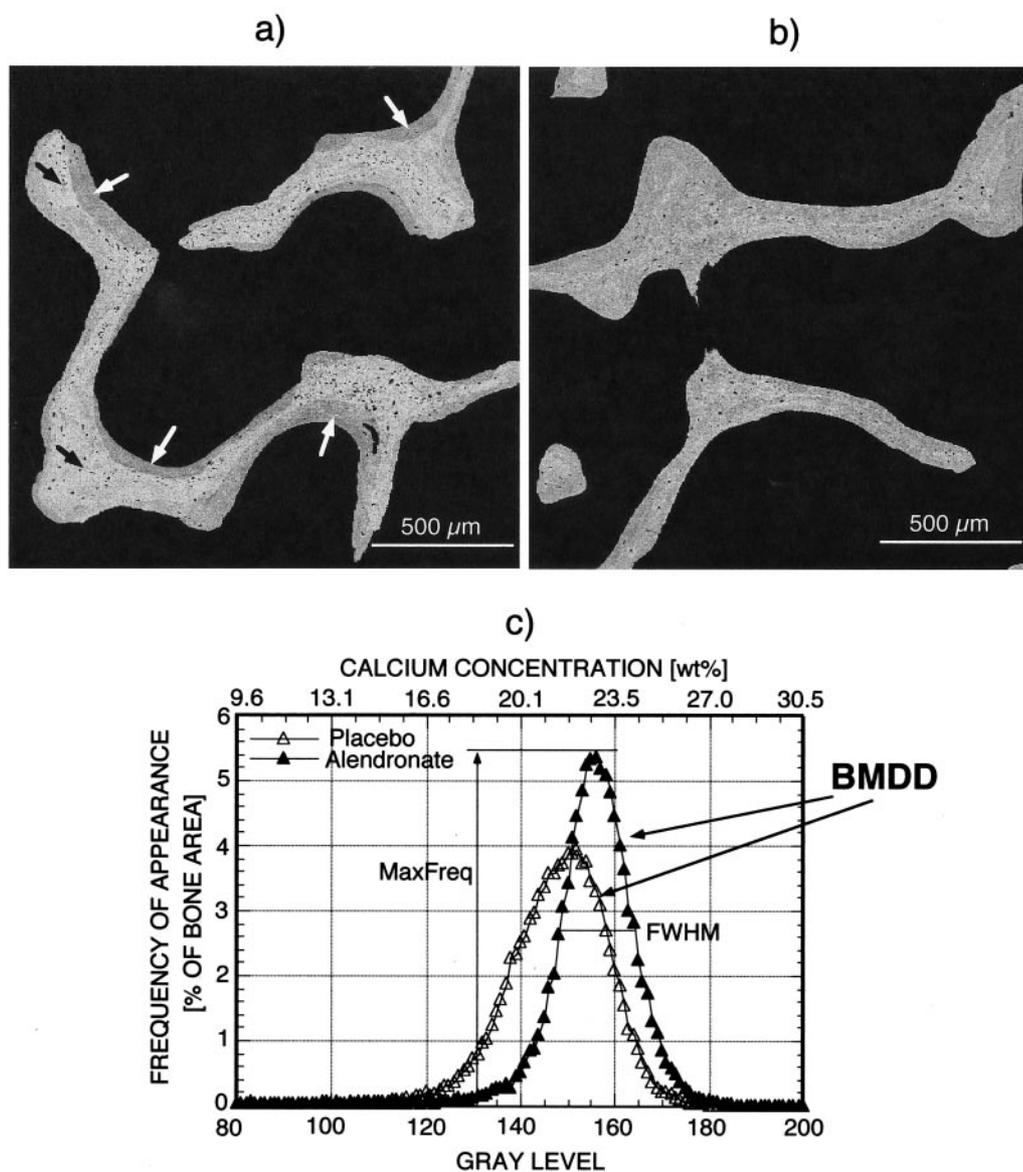


Figure 1. Typical BE images of iliac cancellous bone from one placebo-treated woman (a) and one Aln-treated woman (b) after 2 years of treatment. Different gray levels reflect different Ca content. Black arrows: high degree of mineralization; white arrows: low degree of mineralization. (c) Corresponding BMDD histograms. The example shows a 35.3% increase of Max_{Freq} and 29.4% reduction of FWHM between Aln treatment and placebo. BMDD parameters: Max_{Freq} , peak height; FWHM, full-width half-maximum.

height of the BMDD showing the maximal portion of bone area having an identical Ca concentration; and FWHM, the peak width (full-width half-maximum) of the BMDD histogram reflecting the uniformity of mineralization (Figure 1c). The technical precision for Ca_{Mean} (standard deviation of repeated measurements) of 0.3% could be achieved using this method.

Scanning Small-angle X-ray Scattering (Scanning-SAXS)

The scanning SAXS method³⁰ and related data evaluation procedures¹⁵ have been described previously. The instrument used was equipped with a 12 kW rotating anode generator operated with Cu K α radiation (X-ray wavelength $\lambda = 0.154$ nm), an evacuated pinhole camera (sample-to-detector distance 1 m) and a two-dimensional position-sensitive proportional counter (Bruker AXS, Karlsruhe, Germany). This technique measures the

intensity of the X-rays scattered around the primary X-ray beam. The SAXS patterns obtained were evaluated for average mineral thickness (T) and for mineral shape parameter (η), which depends on the shape and arrangement of mineral crystals within the collagenous bone matrix.¹⁵ In each specimen, about 50 points were analyzed.

Statistical Analysis

qBEI and scanning SAXS measurements were carried out in a blinded manner and correspondence to the four treatment groups was disclosed only after all values of Ca_{Mean} , Max_{Freq} , FWHM, T, and η had been determined. For statistical analysis, Student's *t*-test and analysis of variance (ANOVA; STATVIEW 4.5, Abacus Concepts, Inc., Berkeley, CA) were used.

Results

In this study, the mineralized matrix of iliac cancellous bone from 24 biopsies of Aln- or placebo-treated postmenopausal osteoporotic women (6 per group after 2 and 3 years of treatment) was investigated by qBEI in the micrometer range and scanning-SAXS in the nanometer range. In addition, histomorphometric analysis of mineralized cancellous bone tissue and determination of cortical bone porosity were performed.

Figure 1 shows typical BE images of cancellous bone from placebo-treated (Figure 1a) or Aln-treated (Figure 1b) women after 2 years of treatment. The different gray levels of the images reflect the local Ca content in the bone matrix. Lighter gray represents higher Ca concentrations and darker gray represents lower Ca concentrations. Most strikingly, local variation (arrows) in Ca content was much higher in the placebo-treated than in the Aln-treated women. This obvious effect was quantified by BMDD histograms (Figure 1c) obtained from the corresponding digital BE images (Figure 1a, b). The two histograms differed clearly in peak height (Max_{Freq}) and peak width (FWHM). Max_{Freq} was 35.3% higher and FWHM 29.4% lower in the case of Aln compared with placebo. In addition, a small shift in peak to a higher degree of mineralization (5.1%) can be seen in this example (Figure 1) for the Aln-treated patients.

With all the BMDD data derived from the 24 biopsies statistical analysis was performed. The difference, as shown in Figure 1 for a single pair of data, was confirmed to be statistically significant. Max_{Freq} and FWHM were altered significantly ($p < 0.001$) by Aln treatment after 2 and 3 years of treatment (Figure 2a, b). Max_{Freq} was increased by 22% and 14%, respectively, and FWHM was reduced by 18% and 12% (comparison of 2 year and 3-year-Aln-treated groups showed no significant difference). These results indicate that, after Aln treatment, the trabeculae are significantly more uniformly mineralized. A weak but significant ($p < 0.05$) increase in uniformity, as shown by differences in Max_{Freq} and FWHM, was also measured for the placebo groups between 2 and 3 years, possibly due to the continued use of a calcium and vitamin D supplement. For mean calcium content (Ca_{Mean}) there was no significant difference between the placebo groups after 2 and 3 years. A slight but significant increase (+3%, $p < 0.01$) in Ca_{Mean} however, was measured after 3 years of Aln treatment when compared with placebo (Figure 2c).

In addition, the BMDD parameters from the placebo- and Aln-treated osteoporotic women (pooled 2 and 3 years, $n = 12$ per group) were compared with BMDD parameters from a control group ($n = 20$) of bone-healthy individuals (13 women, 7 men, ages 30–85 years) previously assessed.³² No significant differences between osteoporotic placebo-treated patients and bone-healthy controls were found for Max_{Freq} and FWHM (Figure 3a, b). Again, Aln treatment significantly ($p < 0.001$) increased the uniformity of mineralization when compared with placebo or controls (Figure 3a,b). Interestingly, Ca_{Mean} was significantly ($p < 0.0001$) decreased in the pooled placebo group vs. the bone-healthy controls (Figure 3c).

In contrast to the significant difference found in the BMDD, the crystal thickness, shape, and arrangement, as determined by the scanning SAXS parameters, T and η , did not vary significantly between Aln and placebo groups (data not shown). This result shows that the characteristics of the nanostructure of the mineral/collagen composite visible by the SAXS technique (size and shape of the mineral particles) were not changed after Aln treatment.

Some data with regard to trabecular and cortical microarchitecture were obtained by automated histomorphometric analyses, which showed no significant differences for BV/TV, Tb.Th, or connectivity (N.Bf/B.Ar) between treatment and placebo groups

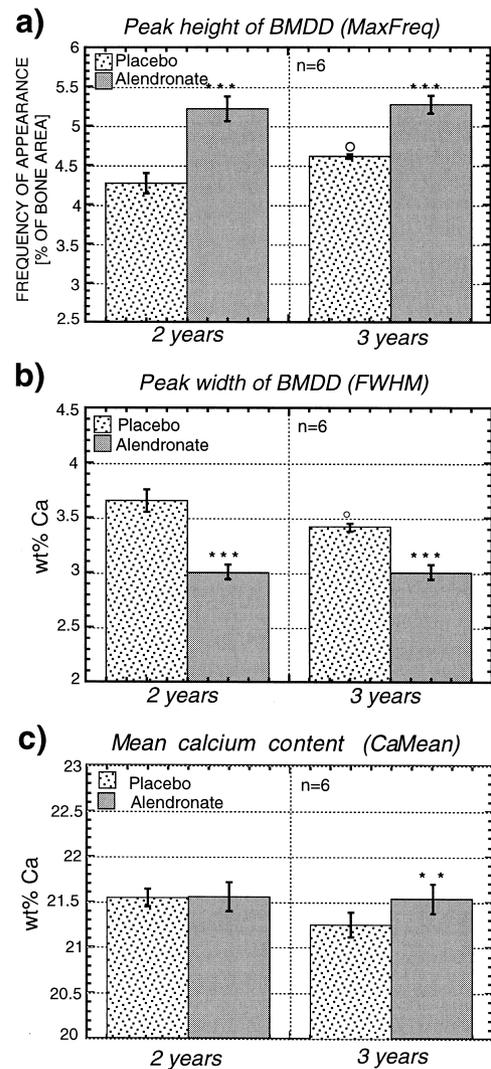


Figure 2. Comparison of BMDD parameters Max_{Freq} (a); FWHM (b); and Ca_{Mean} (c) between placebo-treated and Aln-treated groups after 2 and 3 years (** $p < 0.01$, *** $p < 0.001$ placebo vs. Aln; ° $p < 0.05$ placebo at 2 years vs. placebo at 3 years). Error bars = SEM.

(data not shown). Remarkably, however, analysis of cortical porosity resulted in a highly significant ($p < 0.01$) reduction (–46%) in porosity in the Aln-treated group compared with the placebo-treated group (Figure 4).

Discussion

The biomechanical quality of bone is highly correlated with bone mass, which is a good predictor of fracture risk. However, bone strength is also influenced by factors independent of mass. In fact, the outstanding mechanical properties of bone are due to its hierarchical organization and optimization at all levels, from the macroscopic anatomical dimension to the micrometer and nanometer range.⁸ Relevant parameters include shape and geometry, cortical and trabecular architecture, mineralization density, material properties of the organic matrix, and damage state. Hence, it is clear that bone remodeling, which may alter some of these factors, can affect bone quality.⁹

Osteoporotic fractures have been correlated with a decrease in

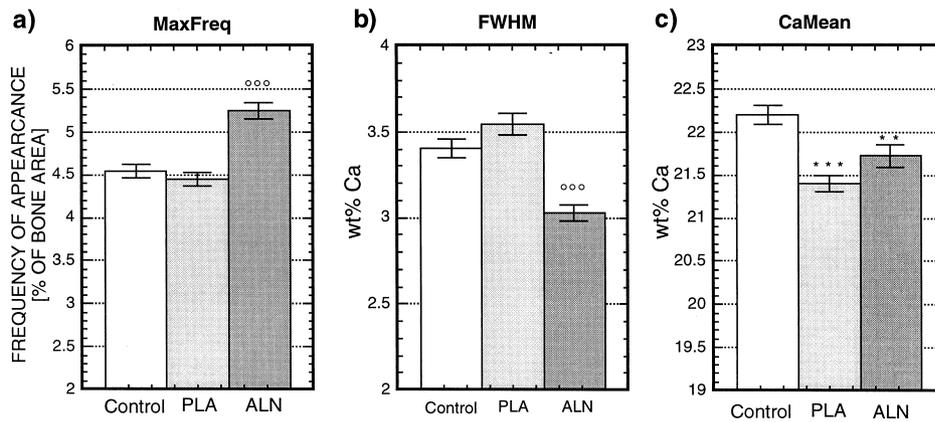


Figure 3. Comparison of BMDD parameters Max_{Freq} (a), FWHM (b), and Ca_{Mean} (c) between our previously published³² control group from bone-healthy individuals ($n = 20$) and pooled placebo-treated ($n = 12$) or pooled Aln-treated ($n = 12$) groups (** $p < 0.01$, *** $p < 0.001$ control vs. placebo or Aln; ooo $p < 0.001$ placebo vs. Aln).

BMD but cannot be fully explained by this decrease.^{9,10,18} Part of the uncertainty in this correlation is that differences in BMD do not account for differences in trabecular connectivity. Moreover, BMD may also be affected by the degree of mineralization of the individual trabeculae and of the corticalis.^{25,32} The variation of the mineral distribution at this level of organization can be quantified in the form of a BMDD by qBEI.^{32,35} Reduced values of Ca content are found, for example, in areas of newly formed bone at the surface of trabeculae or in osteons of compact bone. Recently, it was reported that BMDD in normal human spongiosa is remarkably constant, as shown by measurements of interindividual changes, different skeletal sites (iliac crest, vertebral body, femoral neck and head, or patella), and age (5-97 years).³¹

The main finding from this study is that the distribution of mineral within individual trabeculae was more uniform after 2 or 3 years of continuous Aln treatment. This observation is in precise agreement with that of a preclinical trial in minipigs where cancellous bone matrix was found to be more uniformly mineralized in correlation with reduced bone turnover.³³ In addition, we also observed a slight increase in average mineral density in the trabecular bone tissue after 3 years of treatment. These findings, increased uniformity and average degree of mineralization, are also consistent with the results of a recent study that used a microradiographic technique.³ We speculate

that, as a consequence of the decrease in bone turnover, a greater proportion of matrix (via secondary mineralization) reaches higher levels of calcium content. However, we found no evidence of hypermineralized matrix areas nor disturbances at the ultrastructural level, as discussed later. It is our interpretation that this increase in uniformity accounts for at least part of the increase in BMD values (about 6% in the spine and trochanter) found by dual-energy X-ray absorptiometry in these patients.

A second observation that there was decreased cortical porosity after Aln treatment presumably due to decreased remodeling activity within the osteons. Because the cortical shell provides most of the bending stiffness of bones, reduction in porosity could have a profound effect on the antifracture effectiveness of Aln. Indeed, porosity decreased from 7.5% in the placebo group to about 4% after Aln treatment, which suggests a considerable increase in stiffness. The corresponding increase in cortical bone volume (3.5%) may have also contributed to the observed increase in BMD values noted earlier. Thus, the combination of increased bone volume (including reduced cortical porosity) and increased mineralization density within the bone matrix in the trabeculae may provide an explanation for the BMD changes after the first 6-12 months of treatment.

It is noteworthy that the mean calcium content in bone from a control group of healthy individuals investigated previously³² (13 women, 7 men, ages 30-85 years) was significantly greater than in the placebo group studied herein. First, the BMDDs indicate that bone from the placebo group after 2 and 3 years was significantly less mineralized (Ca_{Mean} , $p < 0.0001$) when compared with bone from our control group.³² This observation is difficult to interpret because the patient group was quite different, but it may indicate that the mineralization density within individual trabeculae may be negatively affected in postmenopausal osteoporosis. The differences found are highly statistically significant, emphasizing the need for further studies. Material density measurements using the Archimedes principle, as reported by others,²¹ have shown a similar trend and confirm our results. Indeed, this could imply a reduction of tissue quality occurring in osteoporosis, although microarchitectural deterioration remains the major structural defect.

It is generally assumed that antiresorptive treatment normalizes bone remodeling at a new steady state but cannot significantly alter the microarchitectural damage. The present study suggests that it may, however, affect bones in several different ways. First, amount of mineral per unit volume of trabecular bone tissue is slightly increased and the distribution of the

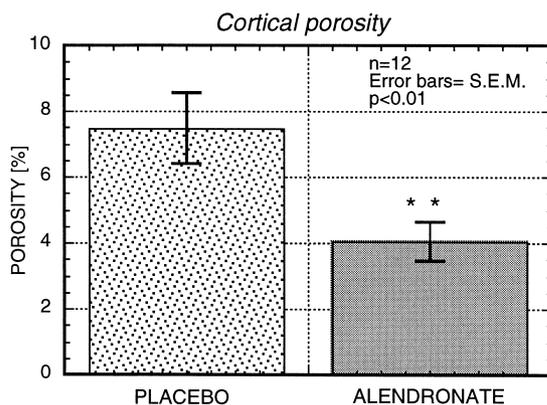


Figure 4. Cortical porosity measured by automated morphometry in bone from Aln- and placebo-treated patients (pooled data from the 2 and 3 year treatment groups).

mineral is more uniform. Second, decreased porosity is found in cortical bone after Aln treatment. Although this last effect clearly enhances the stiffness of bones and, consequently, helps to prevent, for instance, compression fractures, the mechanical consequence of a change in mineralization density is more difficult to evaluate. Mineral is hard and brittle whereas collagen is soft and ductile, and only an ideal composition of mineral/collagen composite guarantees the outstanding mechanical properties for mineralized matrix. It is presently not known what the ideal mineralization density should be. From sclerosing and dysplastic bone diseases we do know that higher mineralization is often accompanied by increased brittleness. Lower or highly inhomogeneous mineralization patterns, such as in osteomalacia, for instance, also weaken bone tissue. Changes after Aln treatment, as found in the present study, are significant but still very small when compared with these severe pathological cases, and therefore not thought to impair bone quality. Moreover, no Aln-induced changes were found by SAXS with regard to size or shape of the mineral crystals. This is entirely consistent with a recent study using minipigs.¹²

As noted earlier, healthy bone is optimized at all levels of its hierarchical organization by mechanical forces. Consequently, an ideal structure from the nanometer to the macroscopic range is a prerequisite for optimal function. We now know several examples where alterations at different levels of the hierarchical structure cause a reduction of bone quality and increased fracture risk. For example, the main defect in osteoporosis is deterioration of the trabecular microarchitecture, whereas disturbed mineralization in osteomalacia affects the next-lower level— (the bone tissue itself). Osteogenesis imperfecta originates from a defect at the molecular level but results in disorders at higher levels,²⁹ including the mineral/collagen composite^{12,17,26} and mineralization density of tissue.^{6,17,20} Finally, changes in the collagen/mineral composite, as shown for fluoride treatment in studies of animals and humans,^{14,16} must be considered major structural alterations underlying the increased risk of fractures.

The nitrogen-containing bisphosphonate, alendronate, inhibits osteoclastic bone resorption, reduces bone turnover, decreases cortical porosity, and increases uniformity of mineralization, most likely reflecting optimization at this level of organization. However, it does not increase mineralization density excessively or negatively affect the mineral/collagen composite. Improved mechanical quality of the mineralized matrix caused by structural changes such as a lower cortical porosity, together with a positive bone balance, may be the major contributing factors to antifracture effectiveness.

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