0013-7227/03/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 88(3):1150–1156

doi: 10.1210/jc.2002-021988

Effects of Intermittent Parathyroid Hormone Administration on Bone Mineralization Density in Iliac Crest Biopsies from Patients with Osteoporosis: A Paired Study before and after Treatment

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Anabolic effects of PTH have been observed at several skeletal sites in humans by dual x-ray absorptiometry without differentiating between an actual increase in bone volume and an increase in mineral content within already established bone. The present study addressed this issue by evaluating the bone mineralization density distribution of iliac crest bone biopsies before and after PTH treatment for 18–36 months in men and women with osteoporosis using quantitative backscattered electron imaging. In cortical bone, pairwise comparison of the two biopsies before and after treatment revealed a reduction in the typical calcium concentration in men (−3.32%; $P = 0.02$, by paired t test), but no change in women, and the heterogeneity of mineralization increased in both males and females [+18.80% ($P = 0.09$) and +18.14% ($P = 0.005$), respectively]. In cancellous bone, there was no change in the typical calcium concentration, but there was a greater heterogeneity of mineralization in both men and women [+19.65% ($P = 0.02$) and +21.59% ($P = 0.056$), respectively] due to newly formed bone matrix. Small angle x-ray scattering performed on a subgroup of subjects revealed normal collagen/mineral structure. The findings confirm the observations that PTH stimulates skeletal remodeling, resulting in an increased percentage of newly formed bone matrix of lower mineral density. (*J Clin Endocrinol Metab* 88: 1150–1156, 2003)

ALBRIGHT (1) ORIGINALLY demonstrated that parathyroid extract was anabolic to the skeletons of rodents. These findings were confirmed in humans in a series of small, mostly uncontrolled studies performed about 20 yr ago with the modest amounts of human PTH-(1–34) that could be synthesized (2). Early biopsy data suggested large increases in cancellous bone of the iliac crest. Recent data have shown much more modest increases in cancellous bone on biopsy (3, 4). However, large increases in bone mineral density (BMD), particularly in the spine have been observed using dual energy x-ray absorptiometry (DXA) (5–7). Smaller, but still impressive, increases in BMD by DXA have been observed with agents acting to suppress bone remodeling, particularly the bisphosphonates. A recent study of mineralization density has suggested that most, if not all, of the increase observed using DXA can be explained by an increased degree of mineralization and not by an increased amount of bone tissue (8, 9). The discrepancy results because DXA cannot differentiate between bone that has become more highly mineralized and a greater amount of normally mineralized bone.

Although PTH stimulates remodeling, it is not clear whether the discrepancy between the modest changes seen in biopsies and the greater increases observed with DXA are related to alterations in mineral distribution. To examine this issue we have examined iliac crest biopsies from patients before and after administration of daily sc PTH [human (h) PTH-(1–34)] using quantitative backscattered electron imaging to evaluate calcium distribution within the bone. In a subgroup of subjects we also performed scanning small angle x-ray scattering to examine mineral particle size and alignment. The combination of these two quantitative techniques allows evaluation of alterations in the quality of the bone composite material and differentiation of these changes from alterations in the true mass of tissue.

Abbreviations: BE, Backscattered electron; BMD, bone mineral density; BMDD, bone mineralization density distribution; CaPeak, typical calcium concentration in the sample; CaWidth, width of the distribution; CLSM, confocal laser scanning microscopy; DXA, dual energy x-ray absorptiometry; FWHM, full width at half maximum; hPTH, human PTH; HRT, hormone replacement therapy; Md. BFR/BS, mineralized bone formation rate/bone surface; qBEI, quantitative backscattered electron imaging; scanning-SAXS, scanning small angle x-ray scattering.

Materials and Methods

Samples

Twenty-six iliac crest bone biopsies were obtained from 13 patients with osteoporosis, before and after treatment with hPTH-(1–34), as previously described (3). Seven were men with idiopathic osteoporosis (mean age, 49 yr) treated for 18 months with 25 μg/d hPTH-(1–34) by sc self-administered injection. Six were women with postmenopausal osteoporosis, treated with the same dose of PTH for 18–36 months. All women were receiving standard doses of hormone replacement therapy

(HRT) before and during PTH treatment, and men found to be testosterone deficient were also treated with replacement doses of testosterone before and during treatment with hPTH-(1–34). Before biopsy all patients were double-labeled with tetracycline in a 3:12:3 d sequence (3). For the first biopsy, tetracycline hydrochloride (Sumycin, 250 mg, four times per day, orally; Apothecon, Princeton, NJ) was used to produce the first label, and demeclocycline hydrochloride (Declomycin, 150 mg, four times per day, orally; Lederle Pharmaceutical, Pearl River, NY) was used to produce the second label. The labels produced by each tetracycline differ in color under UV light. For the second biopsy, the order of tetracyclines was reversed to aid distinction between the two sets of labels. Full details of the clinical trials have been published previously (5).

Sample preparation

From the polymethylmethacrylate-embedded undecalcified iliac crest bone biopsies, blocks with planoparallel surfaces were prepared by grinding and polishing, and were subsequently coated with carbon for backscattered electron imaging in the scanning electron microscope. For the scanning small angle x-ray scattering (scanning-SAXS) measurements, 200-μm-thick sections were prepared head-on from six of the quantitative backscattered electron imaging (qBEI)-investigated blocks. Finally, confocal laser scanning microscopy (CLSM) was performed on one sample (from a male PTH-treated patient), which was ground to 100 μm thickness. Subsequently, this 100-μm-thick section was coated with carbon, and the CLSM-studied area was analyzed by qBEI.

qBEI

qBEI was used to determine the mineral density distribution of both cortical and cancellous bone from the biopsies. The equipment used was a digital scanning electron microscope (DSM 962, Zeiss, Oberkochen, Germany) equipped with a four-quadrant semiconductor backscattered electron (BE) detector. 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. The cortical and cancellous bone areas were imaged at $\times$50 nominal magnification, corresponding to a pixel resolution of 4 μm/pixel, using a scan speed of 100 sec/frame, resulting in digital calibrated BE images of 512 $\times$ 512 pixels. The total area analyzed from each sample was about 30 mm^2. From the digital images, gray level histograms were deduced, displaying the percentage of bone area occupied by pixels of a certain gray level. The transformation of these into calcium wt% histograms led to a bin width of 0.17 wt% calcium. A technical precision of 0.3% was achieved. Full technical details of the technique and its precision have been published previously (10–12).

In this study two parameters were obtained from the bone mineralization density distributions. The typical calcium concentration in the sample [CaPeak; synonymous to CaMaxFreq used in previous works (9, 12)] represents the peak position in the histogram. The width of the distribution (CaWidth), obtained as the full width at half maximum (FWHM) and synonymous to FWHM in previous works (9, 12), is a measure of the homogeneity of mineralization in the sample.

CSLM

Imaging of the fluorescence labeling of the biopsies was performed using a confocal scanning laser microscope (TCS^{4D}, Leica Corp., Rockleigh, NJ).

Scanning-SAXS

Two hundred-micron-thick sections from six samples (men, before and after PTH treatment) were cut from the blocks and examined by scanning-SAXS. Measurements were performed using an instrument equipped with a 12-kW rotating Cu-anode generator (CuK-α radiation $\lambda = 0.154$ nm, operating at 40 kV/40 mA), an evacuated pinhole camera (sample to detector distance, 1 m), and a two-dimensional, position-sensitive, proportional counter with a spatial resolution of 100 μm (AXS, Karlsuhe, Germany) as described previously (13). The sample was mounted on a sample holder that could be moved automatically with a precision of 2 μm in the plane perpendicular to the beam (diameter, 150 μm). The scanning-SAXS patterns were background-corrected and analyzed for the mean particle thickness parameter, T, and the degree of alignment, ρ, within each sample as previously described (13, 14). For the evaluation of T, bone is considered as a two-phase system consisting of organic matrix and mineral, differing in electron density. By definition, T is $4\phi(1 - \phi)/\sigma$, where ϕ is the volume fraction of the particles, and σ is the surface of the particles per total volume. With particles of typical dimensions a, b, c, $T = 2(1 - \phi)/(1/a + 1/b + 1/c)$, which is close to the thickness for plate-like particles, if ϕ is on the order of 50%. The alignment of the particles within the x-ray beam interaction volume causes different scanning-SAXS patterns, showing a narrow streak for perfect parallel alignment, a spherically shaped pattern for completely random orientation, and an elliptical pattern for all other configurations. Therefore, the shape of the scanning-SAXS pattern can be used to determine the degree of alignment (ρ) of the mineral particles, with $\rho = 0$ indicating no predominant orientation and $\rho > 0$ revealing the percentage of mineral particles that are not randomly aligned within the area of the x-ray beam.

Additionally, the function G(x), which gives information on the size distribution, shape, and alignment of mineral particles, was determined. G(x) is obtained from the radial average density of the scanning-SAXS pattern, where x = qT (where q is the length of the scattering vector, and T is the mineral particle thickness parameter) (14). The shape of the G(x) function is characteristic for healthy human adult bone (14) and would be altered if, for example, intervention resulted in mineral abnormalities.

Statistical analysis

The statistical significance of the qBEI parameters was evaluated using a paired *t* test to study the mean intraindividual change caused by PTH. Comparison of the mean values of this study and those of a reference group of healthy individuals [described in a previous study (9)] was performed by ANOVA, followed by Fisher's *post hoc* test (Fisher's protected least significant difference test). SAXS parameters were studied by determination of changes by comparing all data obtained before PTH treatment with posttreatment biopsy data for each of the three patients using *t* test. Correlation of mineralized bone formation rate/bone surface (Md. BFR/BS) with CaPeak and CaWidth was performed using simple linear regression. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA).

Results

We have demonstrated previously that the BMD of the spine increased by 13% over 3 yr in women (5) and by 13.5% over 18 months in men (6) with PTH treatment. Smaller increments were seen in the hip, and standard histomorphometry also showed modest changes in bone volume in the iliac crest (3).

qBEI

Figure 1 shows typical backscattered electron images of cortical (A and B) and cancellous (C and D) bone before and after PTH treatment in a male patient. In cortical bone, the mineralization pattern becomes less homogeneous, a likely consequence of increased Haversian remodeling. In cancellous bone there is increased bone formation, shown here as the darker gray (low mineralization density) areas at the surface of the trabeculae. The corresponding mineralization distribution densities are displayed in Fig. 2. It is readily apparent that the distribution is shifted to the left (lower mineral density) after PTH treatment and that the peak width is increased.

The bone mineralization density distribution parameters were analyzed for alterations due to treatment by paired *t* test (see Fig. 3). Figure 3 summarizes the mean values for CaPeak and CaWidth before and after treatment for male

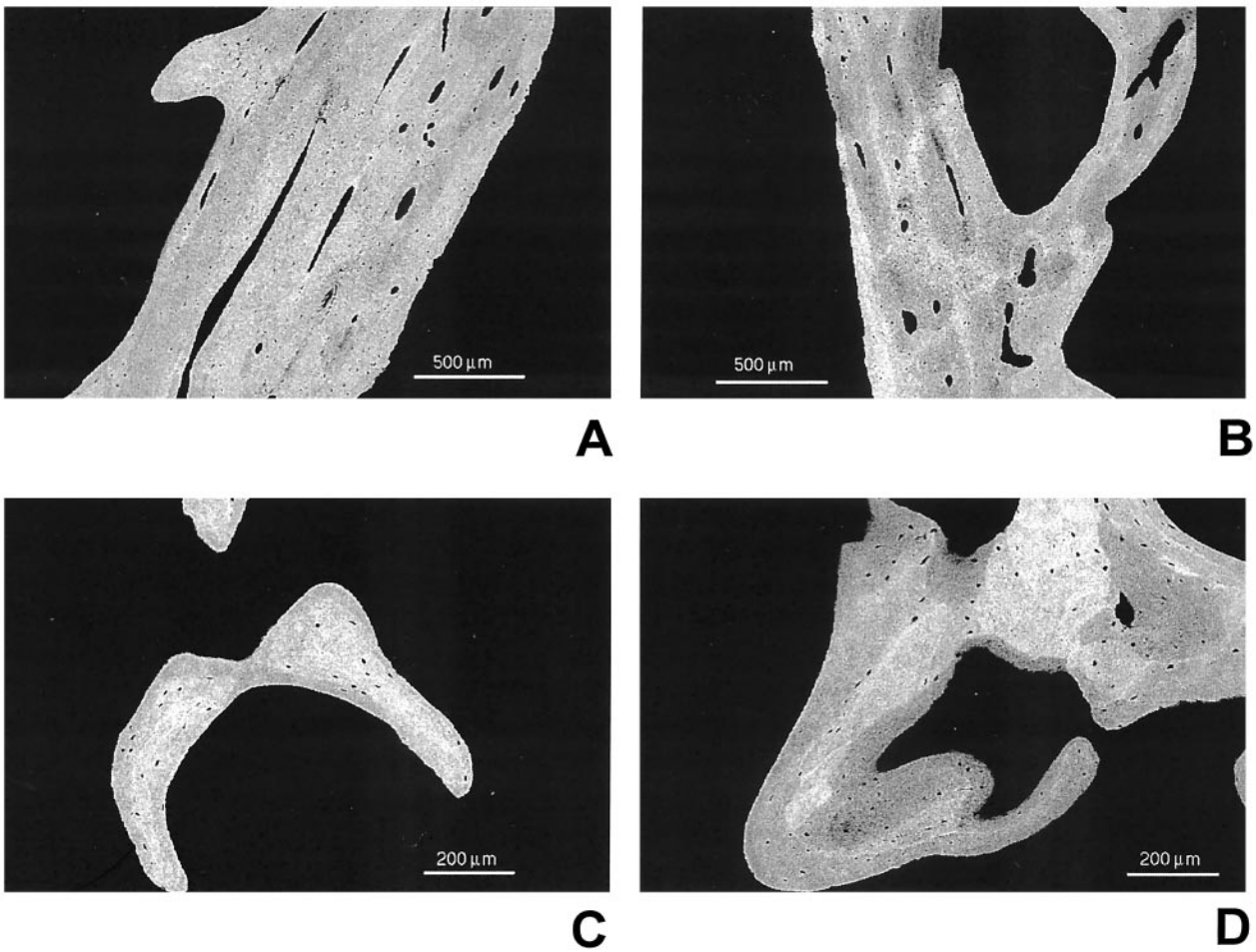

FIG. 1. A typical pair of iliac crest biopsies (from one male patient) taken before (*left column*) and after (*right column*) PTH treatment. A and B, Cortical bone areas; C and D, cancellous bone areas.

(n = 7) and female (n = 6) patients separately. Additionally, we compared the mean value of the parameters for cancellous bone with those of a reference group of healthy individuals published previously (12) (data for the reference group not shown; see Table 1).

The baseline peak calcium concentration in cancellous bone in the male group was significantly lower than that in a reference population (12) ($P = 0.005$; reference population data not shown), in contrast to that in the female group. The peak calcium concentration in cortical bone was lower in men before treatment than in women (−3.1%; $P = 0.041$), but not in cancellous bone. Before treatment, peak width was not different for male or female cancellous bone compared with the reference group, whereas it was increased *vs.* that for the reference group (data not shown) after treatment ($P = 0.028$ and $P = 0.056$ for men and women, respectively).

In cortical bone intraindividual changes were evident in peak calcium concentrations in men, but not in women (−3.32%; $P = 0.02$, by paired t test for men; not significant for women). The increase in heterogeneity of mineralization in cortical bone after treatment was +18.80% ($P = 0.09$ not significant) and +18.14% ($P = 0.005$) for men and women, respectively. In cancellous bone, treatment produced no alteration in the typical calcium concentration, but caused an increase in heterogeneity of mineralization of +19.65% ($P = 0.02$) and +21.59% ($P = 0.056$) for men and women, respectively.

As the bone samples from the patients had been labeled with tetracycline before the biopsies were obtained (3), CLSM could be used to identify the sites of new bone formation. Figure 4 shows the fluorescence labeling (A) and the corresponding backscattered electron image (B) of the same trabecula (male patient, after PTH administration); Fig. 4C gives the overlay of A and B. Comparison of these two images confirmed that very low mineralization density is present at the bone sites, which are forming new bone tissue during PTH treatment. CaPeak within the two labeling lines (18.19 wt%) was approximately 27% lower than CaPeak in adjacent interstitial bone (23.05 wt%).

Correlations of the two parameters obtained from the bone mineralization density distribution (BMDD) after PTH treatment, peak calcium concentration and peak width, with Md. BFR/BS (nomenclature according to Ref. 15) and osteoid perimeter previously determined using standard histomorphometry (3) showed linear correlations (Fig. 5). CaPeak *vs.* Md. BFR/BS resulted in $R^2 = 0.62$ ($P < 0.05$), and CaWidth *vs.* Md. BFR/BS resulted in $R^2 = 0.97$ ($P < 0.0001$). For this correlation, patients with a mineralizing perimeter lower

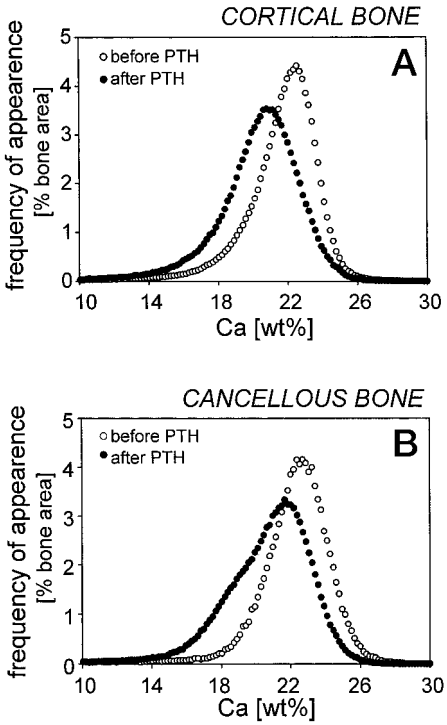

FIG. 2. The BMDD of cortical (A) and cancellous (B) bone from one male patient (see Fig. 1). ○, Before treatment; ●, during PTH treatment. Note the shift toward lower mineralization density and the increased peak width after treatment.

than 1% were excluded. Although the Md. BFR/BS data after treatment were spread within the range of 0.02–0.12 $\mu m^2/\mu m \cdot d$ (Md. BFR/BS data are taken from Ref. 3), the Md. BFR/BS data before treatment lie within a narrow range ($\sim$0.05 $\mu m^2/\mu m \cdot d$). Before treatment, no significant linear correlation of Md. BFR/BS with CaPeak and CaWidth was observed (data not shown). CaPeak and CaWidth *vs.* the osteoid perimeter revealed $R^2 = 0.46$ ($P < 0.05$) and $R^2 = 0.45$ ($P < 0.05$), respectively. For this correlation, one female patient had to be excluded because no osteoid perimeter data were available for her. The same parameters, CaPeak and CaWidth, measured before treatment did not show any significant correlation with the osteoid perimeter (data not shown).

Scanning-SAXS

The mean particle thickness parameter, T, in both cortical and cancellous bone (from three male patients) was approximately 3.2 nm and was not significantly altered due to treatment, although there was a trend toward smaller particle thickness (−0.4% and –2.1% for cortical and cancellous bone, respectively). There was also a nonsignificant trend toward poorer alignment of the particles at both skeletal sites (−4.9% and −8.3% for cortical and cancellous bone, respectively). Thickness and alignment of the particles are not shown in the figure. The shape of the G(x) function was normal before treatment and was not modified by treatment with PTH (see Fig. 6) for any of the three male patients.

Discussion

We have shown in this comparison of paired iliac crest bone biopsies before and after PTH treatment that distinct alterations in the mineralization pattern of iliac crest bone occur in both men and women. qBEI revealed significant effects on the homogeneity of mineralization, but only minor changes in the typical calcium concentration. The heterogeneity of mineralization was markedly increased after PTH. This broadening of the distribution was caused by a higher percentage of lower mineralized bone matrix after PTH treatment. These findings confirm the anabolic effect of PTH and are in perfect agreement with one previous animal study (16). In this work the comparison of the images, which show the fluorescence labeling, on the one hand, and the backscattered electron signal, on the other, confirm that the broadening of the calcium concentration peak observed after treatment is due to the newly formed, but not yet fully mineralized, bone matrix. This is consistent with the observed correlation of CaPeak and CaWidth with Md. BFR/BS and osteoid perimeter.

From the scanning-SAXS investigation we obtained information on the collagen/mineral composite of bone. We found that the bone matrix formed during treatment is normal; thus there is no change in the G(x) function. This finding of normal particles is in contrast to the abnormalities observed after the administration of other anabolic agents, such as fluoride (17, 18). Scanning-SAXS also revealed no significant alterations in particle thickness and alignment after PTH, although a trend toward smaller thickness and lower degree of alignment (neither statistically significant, at least in part due to the small sample size) could be observed after treatment, in agreement with a higher proportion of less mineralized bone suggesting less mature bone (19).

It is interesting to note that the calcium concentration in the biopsies before treatment was greater in women than in men, possibly due to the HRT of the women. There seems also to be a different response to PTH in the mineralization pattern for women and men in that their bone calcium concentrations did not change to the same degree. One explanation could again be the antiresorptive effect of HRT in the women. Another explanation for the differences between the responses of male and female skeletons may simply be the longer interval of treatment and thus the more mature status of mineralization in the skeletons of the treated women. When the mineralization patterns were compared with those of a reference group of healthy individuals from a previous work (9, 12), we found lower mineralization in men in this study. This lower mineralization is consistent with that in the placebo-treated group of a previous study (9) and could be an indication of lower mineralization in osteoporosis. However, a larger group of healthy patients (in this work n = 20 for a reference group) as well as osteoporotic patients is needed to clarify whether osteoporotic bone is less mineralized generally.

The shift of the BMDD toward lower mineralization density and the peak broadening are in contrast to other treatments of osteoporosis that inhibit bone resorption, such as bisphosphonates. Alendronate has been reported to increase BMD and homogeneity (8, 9, 20). It is interesting that these

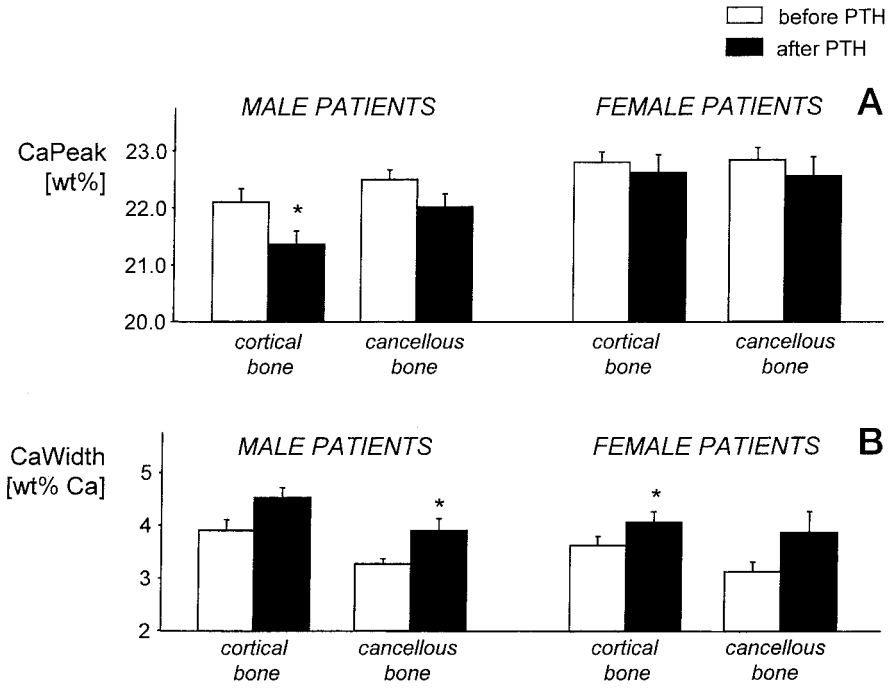

FIG. 3. CaPeak (A) and CaWidth (B) before (□) and after (■) PTH treatment. The *bars* represent the mean for all male (n = 7) and female (n = 6) patients (mean ± SE) of cortical and cancellous bone. *, Significant difference due to treatment ($P < 0.05$, by paired t test).

TABLE 1. Comparison of the measured BMDD-parameters (of cancellous bone) before and after treatment with the data from cancellous bone of a group of healthy, adult individuals from a previous study (12)

			Difference to healthy control group (from Ref. 12)	*P*
Male patients	CaPeak	Before PTH	−2.39%	0.005
		After PTH	−4.47%	0.0001
Female patients	CaPeak	Before PTH	−0.92%	n.s.
		After PTH	−2.17%	0.052 (n.s.)
Male patients	CaWidth	Before PTH	−3.32%	n.s.
		After PTH	+15.38%	0.028
Female patients	CaWidth	Before PTH	−6.64%	n.s.
		After PTH	+14.00%	0.056 (n.s.)

P values show the result from ANOVA test. n.s., Not significantly different from control group.

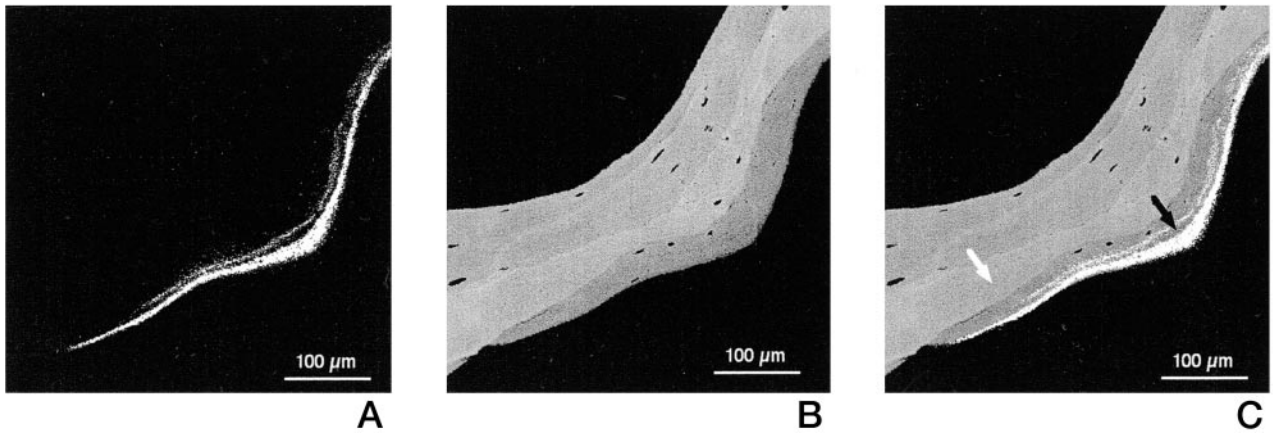

FIG. 4. CLSM image with the fluorescence labeling (A) and the corresponding BE image of the trabecula (B) from one biopsy (male patient, after treatment). C, Overlay of A and B. Within the fluorescence-labeled lines (*black arrow*) CaPeak was 18.19 wt%; CaPeak of the adjacent interstitial bone (*white arrow*) was 23.05 wt%.

diverse effects by PTH and bisphosphonates both result in decreased fracture incidence. Therefore, it is very likely that the main effect of PTH is increased bone formation and subsequently bone volume, which contribute to the improved mechanical competence of PTH-treated bone.

Another question that might be asked is what will happen

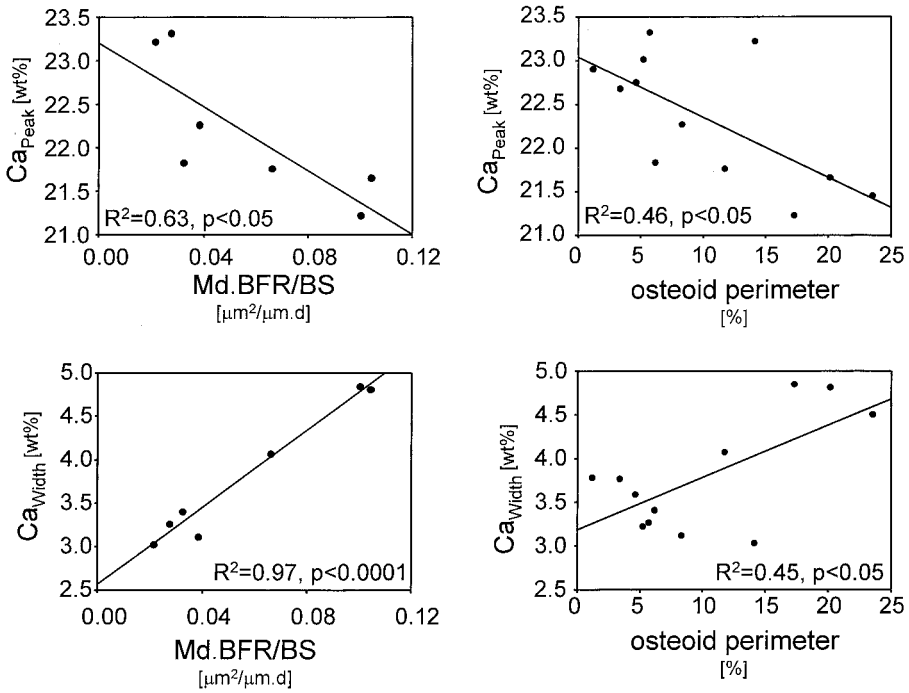

FIG. 5. The linear correlations of Md. BFR/BS and osteoid perimeter with CaPeak and CaWidth in male and female patients after PTH treatment. For the correlations with Md. BFR/BS, patients with a mineralizing perimeter lower than 1% were excluded. For the correlations with the osteoid perimeter, one patient had to be excluded because her osteoid perimeter was not available.

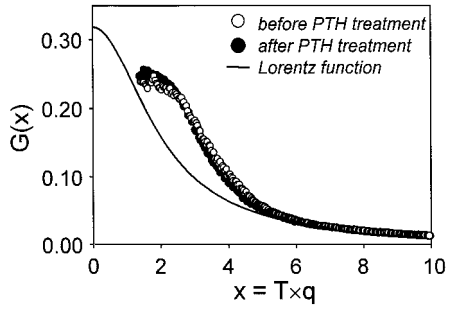

FIG. 6. The G(x) function obtained from scanning-SAXS reveals the size distribution, shape, and mineral particle alignment. Typical G(x) for trabecular bone from one patient before (○) and after (●) PTH treatment. The *solid line* reveals the Lorentz function, which corresponds to a broad (exponentially decreasing) size distribution of the mineral particles as found in disturbed mineralization, *e.g.* in fluorosis (17).

upon withdrawal of PTH therapy. Although it is still not clear, the data presented here suggest that there may be differences in response depending upon the duration of PTH treatment. Early discontinuation might result in continued increases in BMD measured by DXA as the mineral matures. Later withdrawal might result in a lesser change or stability or even loss of bone mass. One recent study reported that BMD could be maintained 1 yr after treatment with PTH for 3 yr in women receiving HRT (21, 22). One other study in which PTH was used only for 1 yr showed increases in BMD after PTH withdrawal enhanced by bisphosphonate treatment, as might be expected (23). These observations are consistent with the possibility that bone matrix formed during PTH treatment matures during prolonged secondary mineralization. Therefore, the lower calcium concentrations seen here during PTH treatment would be expected to be transient.

The observed change in BMDD confirms many clinical observations, showing that intermittent PTH stimulates bone formation, which results in deposition of new bone and increased bone volume (5–7, 22, 24, 25). However, the reported discrepancy in DXA (significant increase in BMD) and the more modest changes in histomorphometry measurements (3, 4) cannot be explained by the results of this study. In contrast, the significant increase in BMD could be underestimated due to the lower mineralization as found by qBEI. One possible explanation could be the difference in the way the measurements are performed by these two approaches. In DXA, exactly the same bone region (thus, the same vertebra, for instance) was measured before and after treatment, whereas histomorphometrical analysis was performed on two biopsies containing different bone regions from each patient, thus contributing to a greater uncertainty in the comparison of the measured parameters. Another possibility is that there may be an interaction between the stimulation of new bone formation by PTH and mechanical stress, such that the PTH effect is magnified in loaded parts of the skeleton.

From the point of material properties, the newly formed matrix during PTH administration has been now shown by qBEI and SAXS to reveal no abnormalities in its micro- or nanostructure. Consequently, we can speculate that the finite state of mineralization of the formed bone matrix depends on the overall rate of bone turnover, which is to some extent coupled to mechanical loading by the adaptation to the mechanical stress pattern. Thus, physiological mechanical usage together with antiresorptive-like estrogens and bisphosphonates should probably support the normalization of bone turnover and the optimization of trabecular architecture and BMDD. These considerations therefore suggest a triple combination therapy for postmenopausal and idiopathic male osteoporosis: normalization of calcium metabolism and bone

turnover with calcium, vitamin D, and antiresorptives; stimulation of bone formation with intermittent PTH; and consequently controlled mechanical loading. Long-term observations of PTH-treated patients at the level of biopsies would help us to clarify the further development and mineralization of the matrix newly formed during PTH treatment.

Acknowledgments

We gratefully acknowledge Doris Scherer (Erich Schmid Institute of Materials Science, Leoben, Austria) for assistance with the SAXS measurements, and Gerda Dinst (Ludwig Boltzmann Institute of Osteology, Vienna, Austria) for preparation of the specimens for the SAXS measurements.

Received July 22, 2002. Accepted November 18, 2002.

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