Contribution of Raloxifene and Calcium and Vitamin D₃ Supplementation to the Increase of the Degree of Mineralization of Bone in Postmenopausal Women

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Raloxifene has been shown to increase bone mineral density and reduce the risk of vertebral fracture in postmenopausal women with osteoporosis. In this study, we report the results of the first prospective longitudinal study to evaluate the mean degree of mineralization of bone (MDMB) in a group of patients enrolled in the Multiple Outcomes of Raloxifene Evaluation trial. Patients were randomly assigned to one of three treatment groups: placebo (n = 24), raloxifene 60 mg/d (RLX60; n = 22), or raloxifene 120 mg/d (RLX120; n = 18). All patients received daily calcium (500 mg) and vitamin D₃ (400–600 IU) supplementation for the duration of the study. Iliac crest biopsies were taken at baseline and after 2 yr of treatment.

Quantitative microradiography was used to analyze the biopsy specimens and revealed a statistically significant (P < 0.05) mean percentage increase in total MDMB of 7.0, 5.3, and 5% for RLX60-, RLX120-, and placebo-treated patients, respectively, compared with baseline. Raloxifene treatment was found to shift the distribution of total bone mineral to higher values of MDMB (RLX60, 29%; RLX120, 8%) with greater heterogeneity, compared with placebo. The profile of MDMB resembled that of healthy premenopausal women and that observed after antiresorptive treatment (5, 6).

We now report data from the first longitudinal study of paired biopsies demonstrating the effect of an antiresorptive therapy on MDMB. The goal of this study was to investigate the effect of raloxifene treatment on the degree of mineralization of bone tissue (MDMB) by increasing the time of secondary mineralization. Until now, the only evidence of the effect of an antiresorptive therapy on MDMB was cross-sectional data from studies with alendronate. Analysis of biopsies after 2–3 yr of alendronate treatment showed a reduction in bone turnover and an increase in mineralization of alendronate-treated bone, compared with placebo, with higher and more homogeneous mineralization of osteons or trabecular packets (2).

We now report data from the first longitudinal study of paired biopsies demonstrating the effect of an antiresorptive therapy on MDMB. The goal of this study was to investigate the effect of raloxifene treatment on the degree of mineralization of bone tissue in biopsy specimens taken before and after 2 yr of raloxifene plus calcium and vitamin D₃, or calcium and vitamin D₃-supplemented placebo treatment. Although raloxifene induces a smaller increase in BMD compared with bisphosphonates, it reduces the relative risk of vertebral fracture to a level comparable to that observed with alendronate (7–9). It was hypothesized that raloxifene treatment could restore the MDMB to levels that more closely resembled those of healthy premenopausal women and that this improvement in bone quality may therefore contribute to the reduction in fracture risk observed after treatment with raloxifene (10–13).

Subjects and Methods

Experimental subjects

The patients who participated in this biopsy study were women enrolled in the Multiple Outcomes of Raloxifene Evaluation (MORE) trial (8). This placebo-controlled, double-blind, multicenter trial enrolled 7705 postmenopausal women with osteoporosis with either BMD at least –2.5 s below the young adult mean, or at least two radiographically apparent moderate vertebral fractures (≥25% vertebral height loss) (8, 16, 17).

The exclusion criteria for this study have previously been reported (8). Briefly, women were excluded from the study if they had experienced a bone metabolic disease other than osteoporosis; had a history of or suspected breast cancer; had abnormal postmenopausal uterine bleeding or endometrial cancer; had a history of cancer other than skin cancer in the previous 5 yr; used an androgen, calcitonin, or bisphosphonate within the previous 6 months; taken oral estrogen within the last 2 months; had experienced a thromboembolic event within the last

Abbreviations: ANCOVA, Analysis of covariance; BMD, bone mineral density; BSU, basic structural unit; FWHM, full width at half maximum; MDMB, mean degree of mineralization of bone (tissue); MORE, Multiple Outcomes of Raloxifene Evaluation; RLX60, raloxifene 60 mg/d; RLX120, raloxifene 120 mg/d.

Recentely, it has been shown that the degree of mineralization of bone tissue is an important determinant of bone strength and bone mineral density (BMD) and is influenced by changes in bone remodeling rate (1–4). One would anticipate that the decrease in bone turnover that is observed after antiresorptive treatment (5, 6) would affect the mean degree of mineralization of bone tissue (MDMB) by increasing the time of secondary mineralization. Until now, the only evidence of the effect of an antiresorptive therapy on MDMB was cross-sectional data from studies with alendronate. Analysis of biopsies after 2–3 yr of alendronate treatment showed a reduction in bone turnover and an increase in mineralization of alendronate-treated bone, compared with placebo, with higher and more homogeneous mineralization of osteons or trabecular packets (2).
10 yr; received treatment for an endocrine disorder (other than type 2 diabetes mellitus or hypothyroidism); had renal lithiasis, abnormal hepatic function, or malabsorption; or consumed more than four alcoholic drinks per day.

Two MORE trial centers in the United States and two in Europe recruited patients to participate in the biopsy study. All participants gave written informed consent to participate in both the MORE trial and the biopsy study, and the Institutional Review Board for Research Involving Human Subjects at each study site approved the protocols.

**Study treatment and protocol**

In the MORE study, women were randomly assigned to one of three treatment groups: placebo, raloxifene 60 mg/d (RLX60), or raloxifene 120 mg/d (RLX120). All participants received daily supplements of calcium (500 mg) and vitamin D3 (400–600 IU).

**Iliac crest bone biopsies**

Sixty-five paired (baseline and 24 month) transiliac bone biopsies were obtained from women enrolled in the MORE trial who had consented to the biopsy procedure. The first biopsy for each patient was taken before the start of treatment, and the second biopsy was taken from the contralateral iliac crest after 2 yr of treatment using a trephine system with an 8-mm inner diameter at study sites in the United States, and 7.5-mm inner diameter at European study sites. One biopsy from the placebo group could not be analyzed by microradiography due to the condition of the sample. Therefore, mineralization data from a total of 64 paired biopsy samples will be reported in this study [placebo (n = 24), RLX60 (n = 22), and RLX120 (n = 18)]. Results from the histomorphometric analysis on these same biopsy samples have recently been published (15).

**Quantitative microradiography**

The technique of computerized quantitative contact microradiography (14) was used to measure the MDMB. This method allows the analysis of each basic structural unit (BSU) of bone tissue, osteons in cortical bone and cancellous packets (BSUs corresponding at the cancellous level to the osteons in cortical bone) in cancellous bone, within the limits that are imposed by the thickness of the biopsy sample. Using this computerized microdensitometric method, the quantity of mineral in a unit volume of bone tissue can be determined.

The equipment used to perform the quantitative contact microradiography (2, 14) was a Philips x-ray diffraction unit (PW 1830/40, Limeil Brevannes, Phillips, France), operated at 25 kV and 25 mA and equipped with a PW 2272/20 diffraction tube. A monochromatic x-ray radiation was used. The focus–film distance was 30 cm. An aluminum step wedge reference system was exposed on the same micrograph as the biopsy section to allow for the quantitative evaluation of the absorption of x-rays.

Using a combined contact microradiography-microdensitometry computerized method (2, 14), MDMB was then independently quantified for both cortical and trabecular bone. Using the calibration curve generated from the aluminum reference step wedge, the gray-scale values were converted to degree of mineralization and expressed as grams of mineral per cubic centimeter of bone adjusted for the exact thickness of each part of the biopsy section measured. An advantage of this method is that all available bone tissue can be measured, thus reducing the error associated with selecting only a small sample area for analysis.

**Statistical analysis**

Overall, there were no significant differences in baseline values between the three treatment groups. Pairwise differences between groups were further evaluated using a t test.

To obtain a more unbiased estimate of the means, as well as to improve precision of the estimates, analyses of covariance (ANCOVA) analyses were performed. Specifically, baseline adjusted comparisons of treatment were conducted for both raw measurements and percentage change measurements from baseline to 2 yr. Treatment by site and treatment by baseline interactions were tested at the significance level of 0.05 and were found not to be significant. Therefore, the ANCOVA model was fit with baseline values, therapy, and geographical site. Assumptions for fitting the ANCOVA model, normality of residuals, linearity of baseline and endpoint values, and interaction between baseline and therapy were also evaluated. Differences in least-square means between raloxifene and placebo treatment groups were computed and reported as primary analysis results for both the actual and percentage change analysis.

For each MDMB endpoint, predicted response values for each treatment group were computed from the fitted ANCOVA model. The range of baseline values used in the prediction model was formed from the baseline data of all patients in each treatment group. In a secondary analysis, the percentage of patients in each raloxifene group with greater endpoint MDMB was estimated. To compute these proportions, normal density plots were used to approximate the predicted response.

To determine whether there was a shift in the heterogeneity of the distribution of the MDMB at treatment endpoint compared with baseline, the full width at half maximum (FWHM) was calculated (FWHM = 2.35 a) (16).

**Results**

The demographics of the patients who participated in the biopsy study are shown in Table 1. There were no statistically significant differences in baseline characteristics between treatment groups at baseline, including serum levels of 25-OH vitamin D and parathyroid hormone.

**MDMB**

Baseline MDMB is shown in Table 2. At baseline, the RLX120 group was significantly greater than placebo for all

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**TABLE 1. Baseline patient demographics**

|                | Placebo | RLX60 | RLX120 | P<sup>b</sup> |
|----------------|---------|-------|--------|-------------|
| No. of patients| 24      | 22    | 18     |             |
| Age (yr)       | 68.32 (5.34) | 69.36 (5.51) | 66.66 (7.74) | 0.391 |
| No. of years postmenopausal | 19.21 (6.11) | 21.55 (7.40) | 18.11 (8.81) | 0.322 |
| Height (cm)    | 160.90 (6.47) | 161.86 (5.43) | 158.74 (5.60) | 0.247 |
| Weight (kg)    | 66.72 (9.57) | 64.98 (8.81) | 64.51 (7.43) | 0.675 |
| BMI (kg/m<sup>2</sup>) | 25.79 (3.48) | 24.85 (3.60) | 25.61 (2.76) | 0.612 |
| Prevalent vertebral deformity | 7 (29.2%) | 8 (36.4%) | 4 (22.2%) | 0.621 |

**Lumbar spine BMD (g/cm<sup>2</sup>)** | 0.85 (0.13) | 0.85 (0.12) | 0.88 (0.12) | 0.703 |
| **Total hip BMD (g/cm<sup>2</sup>)** | 0.70 (0.08) | 0.69 (0.07) | 0.72 (0.09) | 0.594 |
| **25 OH vitamin D (nmol/liter)** | 79.73 (23.25) | 80.43 (23.65) | 78.61 (31.14) | 0.976 |
| **PTH (pmol/liter)** | 3.06 (1.57) | 3.32 (1.12) | 3.55 (1.10) | 0.476 |

Values are reported as mean (SD). Data adapted from Ref. 15 with permission of the American Society for Bone and Mineral Research.

<sup>a</sup> Patients in placebo and RLX treatment groups received calcium and vitamin D<sub>3</sub> supplementation.

<sup>b</sup> Overall difference between treatment groups.
measured sites of MDMB. Therefore, all subsequent analyses of endpoint values were adjusted to correct for these baseline differences, as described in the statistical analysis methods.

The baseline adjusted endpoint measurements and percentage change in MDMB for each treatment group are also presented in Table 2. There was a statistically significant (\(P < 0.05\)) increase from baseline in cortical, trabecular, and total MDMB for all three treatment groups. After treatment with RLX60, baseline adjusted cortical bone mineralization increased by 7.6%, and trabecular bone mineralization increased by 6.5%, resulting in a total (cortical plus trabecular) mean increase in mineralization of 7.0%, compared with baseline. The mean percentage increase in MDMB for the placebo group was 4.9, 5.4, and 5.0% for cortical, trabecular, and total bone, respectively. The mean percentage change in MDMB (95% confidence interval) after treatment with RLX60, compared with placebo, was 2.7% (−2.8, 8.3), 1.1% (−3.1, 5.3), and 2.1% (−2.9, 7.0) for cortical, trabecular, and total bone, respectively. The statistically significant increases from baseline in MDMB for the RLX60 treatment group were not found to be significantly different from the calcium and vitamin \(D_3\)-supplemented placebo group. In biopsy samples analyzed from patients who had received treatment with RLX120, the percentage change from baseline was numerically larger than the placebo group, with a 5.3% mean percentage increase in total mineralization, but was smaller than that observed in the RLX60 treatment group.

Estimated increases in mineral content were also determined using an ANCOVA model as described in the statistical analysis methods. Endpoint distributions of MDMB and percentage changes were statistically significant for all groups, with a 5.3% mean percentage increase in total mineralization, but was smaller than that observed in the RLX60 treatment group.

The probability that a patient treated with RLX60 will have an increase in mineral content of cortical, trabecular, and total bone was estimated to be 36, 16, and 29%, respectively, compared with placebo. Using the same model, the probability that RLX 120 treatment would increase cortical and total bone mineralization was 18 and 8%, respectively, compared with placebo. However, in RLX120-treated patients, the degree of trabecular mineralization shifted to the left, corresponding to a 6% chance that patients in this treatment group will have a decrease in trabecular MDMB, compared with placebo (Fig. 2).

The homogeneity of the MDMB was also evaluated in each treatment group (Table 3). From the curves of the distribution of MDMB at baseline and endpoint (raw values of MDMB not adjusted for baseline differences), the full width of the distribution at half maximum was calculated and used as an index of homogeneity (18). The distribution curves for the calcium and vitamin \(D_3\)-supplemented placebo group are shown in Fig. 3. The narrowing width of the peak at half maximum from baseline to endpoint can be seen in these figures. This reduction in half maximum peak width corresponds to an increase in mineral homogeneity in the placebo group. The raloxifene treatment groups did not show this trend and were found to have a similar degree of mineral heterogeneity at baseline and endpoint (Table 3).

### Discussion

In this study, quantitative microradiographic analysis of paired iliac crest biopsies from patients treated with raloxifene for 2 yr revealed a statistically significant increase in MDMB from baseline and a modest increase in MDMB compared with calcium and vitamin \(D_3\)-supplemented placebo-treated patients that did not reach significance. Because patients in all treatment groups received daily supplements of both calcium and vitamin \(D_3\), we could also evaluate, for the first time, the effect of dietary supplementation on MDMB. This is the first study of bone mineralization to report data on the MDMB from a longitudinal study with paired biopsies from a randomized placebo-controlled osteoporosis treatment trial (8). In this analysis, the availability of baseline biopsy samples permitted the adjustment for observed differences in baseline mineralization, producing a more accurate estimate of the improvement of bone tissue mineral
content, compared with previously published studies that did not have a baseline biopsy (2).

The observed increase in mineral content is a result of the ability of raloxifene to decrease bone turnover, therefore extending the duration of secondary mineralization of bone BSUs. By increasing the time of secondary mineralization, new bone is able to achieve a higher degree of mineralization, which has been shown to improve the biomechanical properties of bone (3, 19), and is likely to contribute to the reduction in fracture risk observed after treatment with raloxifene. Increased skeletal mineral affects several biomechanical properties of bone, including an increase in stiffness and a decrease in ultimate displacement (20). Increasing mineral to an appropriate level may improve structural rigidity, but too much mineral may lead to an increase in brittleness (20).

The relationship between the degree of mineralization and biomechanical properties has not been delineated in women with postmenopausal osteoporosis. There is, however, animal data suggesting that mineralization is related to the stiffness (directly) and toughness (inversely) over a wide range of values seen in different types of bone. Early studies with alendronate in ovariectomized baboons (19) and minipigs (21) revealed the ability of an antiresorptive therapy to improve bone mineralization (22, 23). Histomorphometry performed on the iliac crest after treatment with alendronate (19) was shown to decrease indices of bone formation, including activation frequency, in ovariectomized baboons compared with control animals. Bone strength was also increased. The techniques of small-angle x-ray scattering (21, 24, 25) and quantitative backscattered electron imaging (21, 26, 27) have also been used to determine the mineral content of minipig ribs after treatment with alendronate or sodium fluoride (21, 23). Both studies showed that there was a more homogeneous distribution of mineralized bone matrix after

![Fig. 1](https://academic.oup.com/jcem/article-abstract/88/9/4199/2845727)  
![Fig. 2](https://academic.oup.com/jcem/article-abstract/88/9/4199/2845727)
alendronate treatment, compared with either sodium fluoride or vehicle control. It has been reported that long-term treatment of dogs with high doses of bisphosphonates can result in bone brittleness, microdamage accumulation, and an increase in the homogeneity of the tissue matrix, which may lead to a reduction of the biomechanical competence of bone (5, 28). However, measurements of MDMB were not performed in the dog study.

These reports following bisphosphonate treatment are consistent with the fact that increasing bone mineral content above 65% reduces the toughness of bone and will inhibit the bone from resisting the propagation of microdamage (5, 29). Mineral density homogeneity also negatively impacts the quality of bone tissue. Less energy is required for a microcrack to propagate through a bone with homogeneous tissue mineral density (30, 31). The improvement in MDMB induced by raloxifene treatment reported here is not believed to reach levels that could impair some of the biomechanical properties of bone (5, 20, 28, 32), and the data presented in this study suggest no significant change in the heterogeneous mineral distribution after raloxifene treatment, compared with placebo. The lower suppression of bone remodeling observed with raloxifene compared with bisphosphonates (5, 6), combined with a more heterogeneous mineral distribution and the moderate improvement of the degree of mineralization of bone, is anticipated to prevent microdamage accumulation and the progression of microcracks in bone. At this time, microdamage accumulation has not been observed after 2 yr of raloxifene treatment in an in vivo monkey model (28).

The moderate improvement in total MDMB observed after raloxifene treatment may be due in part to the ability of calcium and vitamin D3 to stimulate bone mineralization. The MDMB and the distribution of the DMB observed after 2 yr of placebo treatment in this study are similar to those measured in a study of 43 control patients (MDMB = 1.09 ± 0.11 g/cm³) and a subset of 20 premenopausal women (MDMB = 1.08 ± 0.12 g/cm³) (14). In addition, values obtained after 2 and 3 yr of placebo treatment in another study (2) were also similar to premenopausal levels. The antiresorptive effect of raloxifene treatment appears to allow more complete mineralization of bone due to prolonged secondary mineralization of newly formed BSUs, as shown by histomorphometry analysis (15). The restoration of skeletal mineral balance as a result of extending the time of secondary mineralization, due to the antiresorptive action of raloxifene, is supported by an observed decrease in biochemical markers of bone turnover after raloxifene treatment (9, 15).

The bone tissue mineralization increases reported in this study are consistent with the recently published histomorphometry data from the same biopsy samples (15). Treatment with raloxifene, a mild to moderate antiresorptive agent, maintained healthy bone structure, with no evidence of woven bone resulting from abnormalities in collagen structure, or osteomalacia, which would occur due to an impairment of primary mineralization. Data trends were
observed for formation period, activation frequency, and total bone volume; however, they were not found to be significantly different at endpoint for either raloxifene treatment group, compared with placebo. In addition, although not significantly different from placebo-treated patients, both mineralizing surface and bone formation rate/bone volume were within the range for healthy premenopausal women (10–13). The finding that there was no significant increase in total bone volume for either the RLX60 or RLX120 treatment group, compared with placebo, suggests that improved mineralization of newly formed bone tissue, as assessed by quantitative microradiography, results in an increase in bone strength and subsequent fracture protection.

This study is limited by the number of paired biopsy samples available for analysis and was not powered to detect significant differences in MDMB between treatment groups. However, this analysis of 64 biopsy pairs is the most rigorous compared with published data from cross-sectional studies (2). All patients in the biopsy study were at least 80% compliant with treatment. Compared with baseline, we were able to observe statistically significant increases in MDMB after treatment with raloxifene or calcium and vitamin D₃. The increase in MDMB in the raloxifene treatment groups was numerically larger than the placebo group but did not reach statistical significance. In addition, constraints associated with the analysis of iliac crest biopsies, which include small bone sample size, and the limited analysis of cortical bone also affected these analyses. Additional analyses using the technique of Fourier transform infrared imaging are planned to further support the observed trend of mineral density heterogeneity reported in this study and to determine mineral crystallinity and collagen maturity.

In conclusion, this study has shown that in the absence of large changes in BMD, treatment with raloxifene or with calcium and vitamin D₃ results in a moderate increase in MDMB and a preserved heterogeneity of mineral distribution. This may contribute to a decrease in fracture risk by improving the quality of the bone. It is probable that for antiresorptive therapies in general, increased MDMB in combination with BMD may in part explain the observed decrease in fracture risk. Additional analyses will be required to determine the extent to which indices of bone quality (including level of bone turnover, microdamage, degree of mineralization, collagen matrix properties, crystal size and distribution, and microarchitecture) (33) may be improved by antiresorptive therapies, such as raloxifene, and will assist in further defining the mechanism of action of this class of molecules.

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