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Microcrack Frequency and Bone Remodeling in Postmenopausal Osteoporotic Women on Long-Term Bisphosphonates: A Bone Biopsy Study

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ABSTRACT: We sought whether microdamage could rise in postmenopausal osteoporotic women on long-term bisphosphonates, as suggested by recent animal studies. We found few microcracks in iliac bone biopsies, despite a marked reduction in bone turnover.

Introduction: Animal studies suggest that bisphosphonates (BPs) could increase microdamage frequency in a dose-dependent manner, caused by excessively suppressed bone turnover. However, there is limited data in humans receiving BP therapeutic doses for >3 yr.
Materials and Methods: We measured microcrack frequency and histomorphometry parameters on transiliac bone biopsies in 50 postmenopausal osteoporotic women (mean age = 68 yr) who had received BP therapy (3 on intravenous pamidronate, 37 on oral alendronate, and 10 on oral risedronate) for at least 3 yr (mean treatment duration = 6.5 yr). We compared these results with transiliac bone biopsies obtained from 12 cadavers. We used bulk staining with green calcein as a fluorochrome. The microcracks were quantified in three 100-μm-thick sections using optic microscopy and were confirmed by laser confocal microscopy. Microcrack frequency (number of microcracks/mm^2 of bone tissue) was compared between treated women and controls using nonparametric tests. We also explored predictors of microcrack frequency, including age, duration of BP therapy, and activation frequency.
Results: Among treated women, cancellous bone microcrack frequency was low (mean, 0.13 microcracks/mm^2) and did not differ significantly from that observed in controls (0.05 microcracks/mm^2; $p = 0.59$). Of note, 54% of the treated women and 58% of the controls had no observable microcracks. There was no association between microcrack frequency and the duration of BP therapy (for microcracks/mm^2 and duration, Spearman $r = 0.04$, $p = 0.80$) and between patients' ages and the number of microcracks (Spearman $r = -0.09$, $p = 0.61$). Although bone remodeling parameters were suppressed in treated women, we found no relationship between microcrack density and activation frequency (Spearman $r = -0.003$, $p = 0.99$). Also, microcrack frequency was not increased in women with prevalent vertebral fracture compared with those without fractures.
Conclusions: Among postmenopausal osteoporotic women on long-term BPs, microcrack frequency in the iliac bone is low, despite a marked reduction of bone turnover.
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Key words: osteoporosis, microcracks, bisphosphonates, alendronate, risedronate

INTRODUCTION

Bisphosphonates (BPs) have been the most widely used drugs in the treatment of postmenopausal osteoporosis in the past 10 yr. They halve vertebral fracture risk[1–5] and reduce nonvertebral fracture risk by ~20–30%.[6,7] The increase in BMD, however, does not seem to be the main mechanism of action of BPs, because the relationship between BMD rise and fracture risk reduction remains modest.[8,9] In contrast, the increase in the mean degree of mineralization and the reduction of bone turnover seem to be stronger predictors of antifracture effectiveness.[10–12]

In recent years, however, some concern has emerged regarding the influence of the deeply prolonged inhibition of bone turnover by long-term BP therapy. For example, a few patients on long-term oral alendronate for the treatment of postmenopausal osteoporosis sustained spontaneous nonvertebral fractures, some of them with delayed healing and histological evidence of a marked reduction of bone turnover.[13] It has been argued that osteonecrosis of the jaws—encountered essentially in cancer patients receiving high

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doses of intravenous pamidronate or zoledronic acid—might be related to excessive suppression of bone turnover.[14] Thus, bone remodeling oversuppression may cause detrimental changes in bone, including a reduction in microdamage repair.

It is likely that bone remodeling targets microdamage in bone to maintain the skeleton's mechanical integrity.[15,16] In canine compact bone, resorption spaces are associated with microcracks,[17,18] and in rats, fatigue loading stimulates intracortical remodeling in areas where microdamage occurs.[19] Sustained fatigue loading leads to the accumulation of microdamage in bone. Microdamage weakens the bone and must be repaired to prevent fracture. Bone remodeling is considered to be targeted toward the repair of fatigue microdamage. Consequently, an excessive reduction of bone turnover may result in inadequate microdamage repair and cause fracture. Dogs receiving high doses of BPs showed increased microdamage and some degree of impairment of mechanical properties,[20–22] but this finding is not universal.[23] Furthermore, dogs on doses of BPs comparable to that of human clinical use showed an increase in microdamage, but their bone stiffness increased and other material properties remained unchanged.[24]

In humans, there are limited data on the level of bone remodeling and on the potential microdamage accumulation for periods of treatment longer than that of clinical trials. This is, however, of clinical importance, because women tend to be treated for more than the 3–4 yr of the clinical trials. Thus, in a study of postmenopausal women who received alendronate for 10 yr,[25] bone turnover remained substantially suppressed compared with pretreatment baseline, with a reduction of ~70% in urinary N-telopeptide of type 1 collagen (NTX). No bone biopsy analysis was performed. In the FLEX trial, some women received 10 yr of treatment with alendronate. Bone remodeling was markedly reduced with no evidence of frozen bone, but microdamage was not assessed.[26] In women who had been on risedronate for 5 yr, a histomorphometric analysis showed a significant bone turnover reduction, but without any evidence of frozen bone.[27] No microcrack assessment was performed. In a recent study in postmenopausal women on alendronate for an average of 5 yr,[28] microdamage accumulation was associated with age, low BMD, and prevalent fracture, but not with alendronate use.

To address this issue, we performed a cross-sectional analysis on transiliac bone biopsies obtained from outpatient clinic postmenopausal women on long-term BPs to assess the level of bone turnover and its relationship to potential microdamage accumulation.

MATERIALS AND METHODS

Subjects

Fifty outpatient clinic postmenopausal osteoporotic women who had been on BPs (intravenous pamidronate 90 mg, four times a year, alendronate 10 mg/d or 70 mg/wk, or risedronate 5 mg/d or 35 mg/wk) were enrolled; 48 of those patients received their medication for at least 3 yr. They were at least 5 yr postmenopause. Exclusion criteria were the presence of metabolic bone diseases other than postmenopausal osteoporosis; the use of any medication other than BPs within the past 6 mo likely to interfere with skeletal homeostasis, such as estrogen, selective estrogen receptor modulators, calcitonin, high-dose glucocorticoids, heparin, or anticonvulsants; uncontrolled hyperthyroidism; any concurrent disease, which in the opinion of the investigator, would prevent the subject from completing the study or would interfere with the outcome measures; alcohol or drug abuse, current or within the past 5 yr; allergy to tetracycline or Novocain; hip anatomy preventing transiliac bone biopsy or absorptiometry scan; and previous bilateral transiliac bone biopsies.

We also performed a transiliac bone biopsy in 12 recently dead cadavers to serve as controls, because we could not obtain bone samples from untreated postmenopausal women. Information on those individuals' medical history was limited. Therefore, we only included bone samples that were made of lamellar bone and excluded other bone conditions determined by histology, including bone metastases and Paget's disease of bone. In addition, histomorphometric parameters in the control group had to be in an expected range for their age to be accepted for the analysis.

Bone histomorphometry

Transiliac bone biopsies were performed after all patients were double-labeled with demethylchlortetracycline (600 mg/d, 2 days on, 10 days off, 2 days on). Briefly, the biopsies were taken with a 7.5-mm-ID Bordier-Meunier trephine and fixed in 80% ethanol and bulk stained for 48 h at room temperature with green calcein (C 0875; Sigma, St Louis, MO, USA) in ethanol solution at a concentration of 0.5 nM. They were rinsed in ethanol solution for 48 h. After dehydration in absolute ethanol, the biopsies were embedded in methylmethacrylate without previous decalcification.[29] Three different planes were cut 300 μm apart. For each one, one 8-μm-thick section was used for classical histomorphometry and one 100 ± 5-μm section served for the microcrack assessment. All sections were cut at very low speed using a Leica Polycut S microtome equipped with a tungsten carbide knife (d profile at 60°). Thus, three sections were used for histomorphometry and three sections for microdamage evaluation.

We evaluated the histomorphometric parameters with either a semiautomatic Tablet Measure (Explora Nova, La Rochelle, France) or an automatic (Bone Morpho, Explora Nova, La Rochelle, France) analyzer. All parameters were expressed according to the recommended American Society for Bone and Mineral Research (ASBMR) nomenclature.[30]

Bone structure: We evaluated the W.Th (μm) of cancellous packets, which represents the endproduct of the osteoblastic activity exerted in a remodeling site, under polarized light on Solochrome cyanin–stained sections.

Assessment of bone remodeling: Dynamic parameters also were evaluated on unstained sections measured under UV light: mineral apposition rate (MAR; μm/d) and mineralizing surface/bone surface (MS/BS; %) calculated as

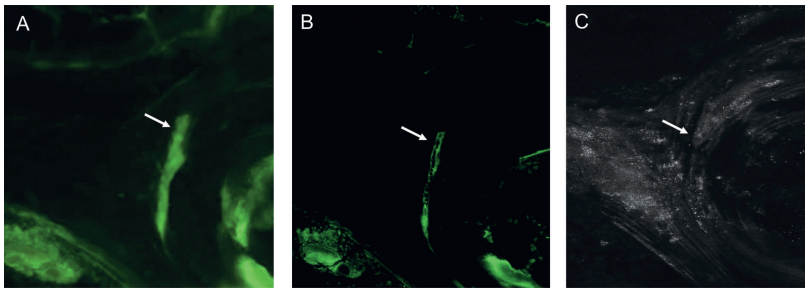

FIG. 1. Example of a trabecular bone microcrack. (A) Fluorescent microscopy of a 100-μm-thick section bulk-stained with calcein observed under UV light. A linear microcrack is visible (arrow; magnification, ×200). (B) Laser confocal microscopy. Virtual 2-μm-thick section observed under fluorescent light. Calcein is visible in the whole microcrack in its lower part and only on the bone surfaces in its upper part (arrow; magnification, ×200). (C) Laser confocal microscopy. Virtual 2-μm-thick section observed under reflected light. The microcrack is visible as a black line between lamellae (magnification, ×200).

one-half single- + double-labeled surfaces. Bone formation rate/bone surface (BFR/BS) was calculated as (MS/BS) × MAR ($\mu m^3/\mu m^2/d$). The activation frequency was calculated as (BFR/W.Th) × 365 and expressed as number per year.

Microdamage evaluation

Before embedding and sectioning, bone samples were bulk stained with green calcein as a fluorochrome. With bulk staining, microcracks that occur after staining, such as those induced by microtome sectioning, are not visible.[31,32] Green calcein was preferred over basic fuchsin, the traditional marker of microdamage, because unlike basic fuchsin, it allows for the simultaneous study of bone formation marked by tetracycline double labels. Furthermore, because this study involves humans, we had no possibility to obtain separate bone biopsies for histomorphometric analyses and microdamage evaluation.

Several fluorochromes have been shown to stain microcracks in bovine[33,34] and ovine bone.[35] For this study, we tested various fluorochromes in water or ethanol solutions, in tetracycline double-labeled ewes iliac crest biopsies, to determine whether both tetracycline double labels and microcracks were visible in the same sample. Some of these biopsies were analyzed using the standard procedure with basic fuchsin and were compared with those with green calcein. Tetracycline double labels were best preserved in green calcein 0.5 nM ethanol concentration, allowing for accurate measurement of bone remodeling parameters on Goldner-stained sections and of marrow cells in toluidine blue– and May-Grunwald–stained sections.

Consistent with results of a previous study showing that the same cracks could be stained by several dyes,[34] all microcracks stained with basic fuchsin were also observed with green calcein. We also tested the precision of microcrack length assessment by performing intraobserver duplicate measurements. We found an excellent reproducibility, with an r^2 of 0.93 for the correlation between the two measurements.

Microdamage was assessed in the human biopsies from three 100-μm sections per subject to minimize the probability of crackless samples, using the entire cortical and cancellous areas (the peripheral area, at 0.8 mm of the edge, was excluded to avoid counting cracks potentially induced by the biopsy procedure). Microdamage was observed with photon microscopy under fluorescent light and confirmed with laser confocal microscopy (LSC TCS SP2; Leica, Wetzlar, Germany) using a krypton/argon gas laser (excitation = 495 nm; emission = 540 nm). Microcracks were measured at ×200 magnification. Stained microcracks were defined by sharp edges, some depth in the field, permeation of stain into microcrack walls, and their relative size (smaller than vessels and greater than canaliculi). Microcrack frequency in cancellous and cortical bone was expressed as the number of microcracks per millimeter squared of bone tissue (or bone area). We also examined microcrack length (μm), and microdamage morphology was categorized as linear, cross-hatched, or diffuse microcracks.

Figure 1 provides a representative example of a microcrack.

Statistical analysis

Because the distribution was not normal, nonparametric tests were used to compare microcrack frequency in treated women and controls. We also explored predictors of microcrack frequency, including the duration of BP therapy, age, and the Ac.f. Data were fitted with the lowess smoother function, and a Spearman rank correlation test was applied if no clear relationship emerged from the smoothed data. Nonparametric tests were also used for comparison of histomorphometry variables between the control and treated groups.

A precise a priori sample size calculation was not possible because there were no data on the amount and statistical distribution of microcracks in transiliac bone specimens in osteoporotic women treated with BPs. Data have been published in untreated women for various cadaveric bones: femoral and tibial diaphyses, cancellous bone from the femoral head and neck and vertebrae, but it was unclear whether we could extrapolate to the iliac bone, because microcrack density is different in various bones.[36–40] Therefore, we used a sample size that allowed adequate estimates of histomorphometric parameters.

Table 1. Patient and Control Characteristics*

	Patients (n = 50)	*Controls (n = 12)*
Age†	68.6 (9.1)	84 (8.7)
Hip BMD	0.656 (0.111)	NA
Duration of treatment (yr)‡	6.5 (3, 12)	NA
Type of bisphosphonate, *n* (%)		
Alendronate	37 (74)	NA
Risedronate	10 (20)	NA
Pamidronate	3 (6)	NA
Body mass index (kg/m^2)	24 (4)	NA

* Mean (SD).
‡ Mean (range)
† $p < 0.001$, for the difference between patients and controls.
NA, not applicable.

Table 2. Remodeling Parameters in Women Treated With Bisphosphonates

Parameters	*All*	*Alendronate*	*Risedronate*
Trabecular bone			
MAR (μm/d)	0.72 (0.15)	0.72 (0.16)	0.69 (0.06)*
MS/BS (%)	0.526 (0.767)	0.435 (0.615)	0.924 (1.165)*
BFR/BS (μm^3/μm^2/d)	0.004 (0.005)	0.004 (0.005)	0.007 (0.007)*
Ac.f (/yr)	0.06 (0.07)	0.06 (0.06)	0.09 (0.09)*

Values are mean (SD).
* $p > 0.05$, Wilcoxon rank sum test.

RESULTS

Patients and controls characteristics are displayed in Table 1. Controls were significantly older than treated women (84 versus 68.6 yr; $p < 0.001$).

Histomorphometry

In 33% of the treated women, double labeling was not sufficiently visible to be measured accurately, without evidence of increased osteoid tissue. The mean MAR was normal in other patients, at 0.72 ± 0.15 μm/d. Bone remodeling was substantially suppressed in the treated group, with a mean Ac.f = 0.06 ± 0.07/yr, in contrast to a normal value in our laboratory of 0.53 ± 0.39/yr.[41] Values of bone remodeling parameters are shown in Table 2. There was no significant difference between the Ac.f in women on alendronate (mean = 0.056/yr) and on risedronate (mean = 0.085/yr, $p = 0.69$). Similarly, the BFR did not differ significantly between patients treated with alendronate (mean BFR = 0.004 μm^3/μm^2/d) and those receiving risedronate (mean BFR = 0.007 μm^3/μm^2/d, $p = 0.69$).

Microdamage evaluation

Fifty-four percent of treated women and 58% of controls had no visible trabecular bone microcracks. Among treated women, cancellous bone microcrack frequency of bone tissue was low, with a mean crack density of 0.13 microcracks/mm^2, which did not differ significantly from that observed in controls (0.05 microcracks/mm^2, $p = 0.59$; Fig. 2). Only one cross-hatched microcrack and one area of diffuse microcracks were observed. The mean cancellous bone microcrack length was 120 μm and did not differ between treated women and controls (Fig. 2).

Microdamage was rare in cortical bone, because only three treated women had at least one observable microcrack, and no cortical bone microcrack was visible among controls.

We found no relationship between microcrack density and age in treated women (Spearman $r = -0.09$, $p = 0.61$) and in controls (Spearman $r = -0.29$, $p = 0.36$; Fig. 3). There was no association between microcrack frequency and the duration of BP therapy (Spearman $r = 0.04$, $p = 0.80$; Fig. 4). In addition, there was no significant association between microcrack frequency and the Ac.f in treated women (Spearman $r = -0.003$, $p = 0.99$; Fig. 5). Microcrack frequency was not increased in women with prevalent vertebral fractures compared with those without fractures, nor in those with prevalent nonvertebral fractures compared with those without fractures (data not shown). There were too few events to perform meaningful statistics regarding the association between microdamage and incident fractures.

DISCUSSION

We found that more than one half of the women on BPs and the untreated controls had no visible microcracks. Among those with visible microdamage, the microcrack density was low and was not related to age, duration of BP therapy, or degree of bone turnover suppression.

In our study, the degree of bone turnover suppression did not differ significantly between alendronate and risedronate. After an initial reduction, the magnitude of which depends on the potency of each compound, bone resorption—as assessed with biochemical markers—remains stable during BP therapy for postmenopausal osteoporosis.[42] After discontinuation of the BP, bone resorption tends to increase, but its behavior varies with different drugs. With alendronate, bone resorption remains substantially diminished when the treatment is stopped, compared with baseline or placebo levels, but tends to return toward pretreatment levels after 5 yr of treatment discontinuation.[43] With risedronate, bone resorption tends to resume more rapidly and returns to levels comparable to that of placebo within 1 yr.[44] One year after a 4-mg injection of zoledronic acid, bone resorption is still deeply reduced.[45] The maintenance of the drug effect after its discontinuation may be favorable because of improved cost-effectiveness and could be useful in patients with partial adherence to oral therapy.

Long-term profound inhibition of bone resorption, however, might be associated with bone adverse effects, because it could prevent microdamage repair. Indeed, in a dog model, animals receiving high doses of alendronate or risedronate showed a substantial increase in microcracks frequency in ribs, which was also proportional to the reduction in Ac.f.[20] This was not associated, however, with impaired stiffness or bone strength, but only to decreased toughness. Increased microdamage in lumbar vertebrae was also ob-

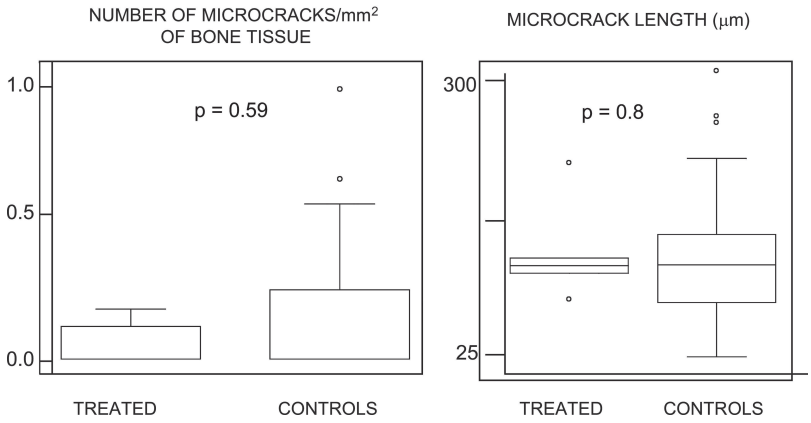

FIG. 2. Cancellous bone microcrack density and length among treated women and controls. The microcrack density did not differ between treated women and untreated controls. Microcrack length was similar in treated women and in untreated controls.

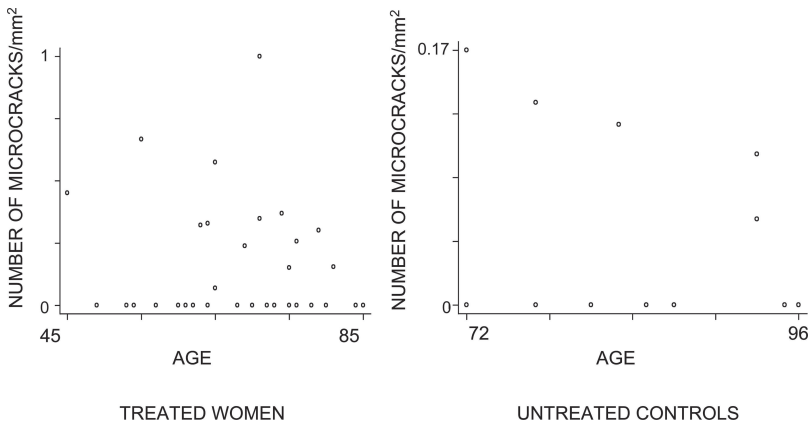

FIG. 3. Relationship between microcrack frequency and age. Microcrack frequency was not influenced by age, either in treated women or in untreated controls.

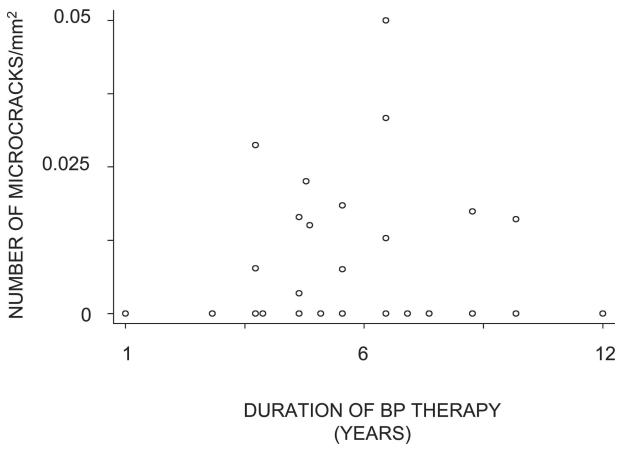

FIG. 4. Relationship between microcrack frequency and duration of bisphosphonate treatment Microcrack frequency was not modified by the duration of bisphosphonate treatment.

served in beagle dogs receiving the same high doses of alendronate or risedronate, but these animals showed improved lumbar compressive strength.[21] Similar results were observed in beagle dogs on high doses of incadronate.[22] In dogs taking doses of risedronate or alendronate comparable to that of postmenopausal women, microcrack frequency increased, proportionally to the degree of reduction in Ac.f, yet this was associated with improvement in vertebral bone stiffness.[24] In postmenopausal women treated with long-term BPs for osteoporosis, we found few microcracks, and their frequency was not related to the decreased bone turnover. Microcrack frequency was not greater in treated women than in untreated controls. In prior studies in animals treated with high doses of BPs,[21] microcracks were increased at different skeletal sites, including the ilium, the femoral neck, and vertebrae, and bone turnover was similarly diminished at those different sites. Thus, if the effect of BPs is similar in human versus canine bone, one may assume that our findings in the human ilium could be extrapolated to clinically relevant skeletal sites such as the vertebrae.

In the only study examining the occurrence of microdamage in humans treated with BPs,[28] no difference in microcrack density between women on 5 yr of alendronate and treatment-naïve women was observed. The microdamage frequency, however, was higher than in our study, both in treated and untreated women. The reasons for this discrepancy remain unclear, but may be related to the technical differences in microcrack staining and/or differences in the studied populations. Adherence to therapy, severity of osteoporosis, and comorbidities are also likely to be different. In addition, there were significant differences in crack density between the two centers performing the biopsies, whereas, in our study, the biopsy technique was standardized between centers, and 80% of the samples were ob-

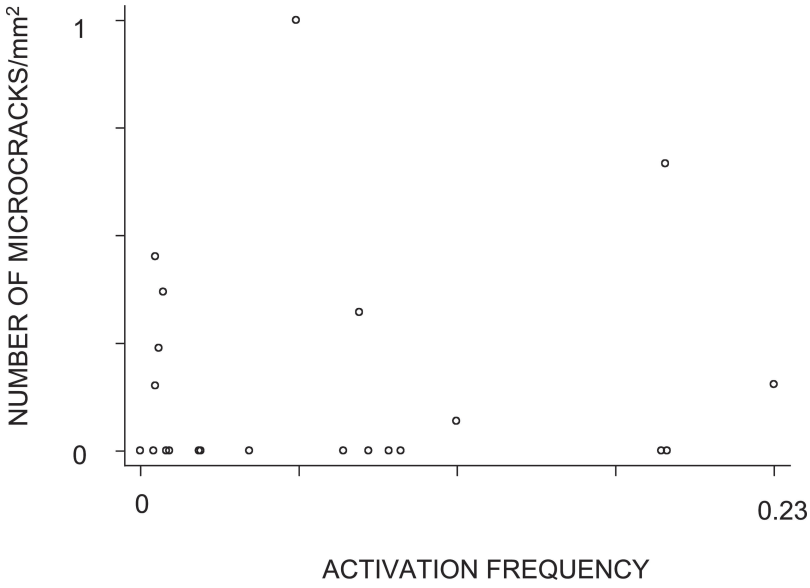

FIG. 5. Microcrack frequency and bone turnover suppression. Microcrack frequency was not influenced by the degree of bone turnover suppression, as assessed by the activation frequency.

tained by the same operator (RC). This might explain an absolute difference in crack density between centers in the previous study,[28] because we know that the transiliac bone biopsy itself can induce microdamage in the peripheral area of the bone sample. In our study, the peripheral area of sections (0.8 mm from the edge) was excluded from the analysis to avoid counting microcracks induced by the biopsy itself.

It has been shown in a computerized model of bone remodeling that microdamage would increase linearly if the activation frequency were completely suppressed but that this microdamage accumulation would be limited if remodeling were incompletely suppressed.[46] In addition, consistent with the evolution of BMD in clinical practice that tends to plateau after several years of BP therapy, the BV/TV would increase by a greater magnitude in the first phase of BP treatment and would level off after several years. An initial increased microdamage caused by lowered activation frequency and damage removal could trigger a secondary increase in activation frequency, leading to a new equilibrium, implying that microdamage could accumulate during the early months or years of treatment and then plateau or decrease. These concepts have been confirmed in a recent study showing that the microdamage accumulation in beagle dogs on BPs occurs at the beginning of treatment, but is self-limiting over time.[47] Because the antifracture effect of BPs in clinical trials seems to be of greater magnitude during the first year of treatment, it is unlikely that this potential initial small increase in microdamage is of clinical relevance.

Our study has some limitations. The sample size is adequate to detect a sizable difference between the control and the treated groups but does not allow capture of small differences of limited or no clinical relevance. Also, the limited number of available cadaveric samples hampered our estimation of microcrack frequency in untreated individuals. With the 0.13 microcracks/mm^2 (SD = 0.22) in the treated group and 0.05 microcracks/mm^2 (SD = 0.07) in the control group that we found, with $\alpha = 0.05$ and $1 - \beta = 0.8$, one would need 20 controls and 80 treated patients to detect a doubling in microcrack frequency. In previous animal or human studies on microcracks, the sample size was generally comparable to or smaller than ours.[24,28,40,47] The area of iliac bone where bone biopsies are performed is not affected by fragility fractures, and it is not mechanically loaded like the spine or the femoral neck and therefore may not be the best anatomical site to evaluate BP-induced microcracks. Additionally, the absolute number of microcracks seems to be greater in other bone sites, such as the femoral neck and the vertebrae. There are, however, some animal data suggesting that the variation in microcracks density in the iliac bone parallels that of the spine,[21] but human data are lacking. If this observation can be extrapolated to humans, microdamage is probably not increased at the spine of women on BPs.

In conclusion, among postmenopausal women on long-term BPs, microcrack frequency in the iliac bone is low, despite a marked reduction in bone turnover. This is reassuring for the clinical use of oral BPs in the treatment of postmenopausal osteoporosis.

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