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Perspective Article

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Effects of bisphosphonates on matrix mineralization

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Bone strength is determined not only by the volume of bone tissue and the microarchitectural organization of this bone, but also by the degree of mineralization of bone matrix. The mineralization process consists of a primary deposition of mineral substance on the calcification front, followed by a slow and progressive increase of the mineral deposition named secondary mineralization. In osteoporosis, there is a negative imbalance between bone resorption and bone formation, resulting in bone loss, and microarchitectural deterioration of the trabecular network. Therapeutic agents for osteoporosis could increase bone strength by three separate, but interrelated effects on bone tissue: 1) the prevention of bone loss and thus the preservation of bone microarchitecture, 2) an increase in the volume of bone matrix, and 3) an increase in the degree of mineralization to a level similar to that seen in healthy premenopausal women, through a prolongation of the duration of secondary mineralization. Therefore the use of antiresorptive agents that reduce bone turnover, as bisphosphonates, provide a rational approach to treatment of osteoporosis.

Extensive phase III clinical trials have shown that osteoporotic women treated orally with alendronate (ALN) for 3 years or more had substantial increases in bone mineral density (BMD) of approximately 10% at the spine together with reductions of about 50% in the incidence of vertebral fractures. Since a marked reduction in activation frequency was evidenced in the transiliac biopsies taken after treatment with ALN compared to placebo (PLA), without detectable increase in cancellous bone volume, it was hypothesized that the increase in BMD and the reduction in the incidence of fragility fractures were due, in a substantial part, to an increase in the degree of mineralization of bone (DMB). The mean DMB was measured by quantitative microradiography on transiliac bone biopsies taken from 53 postmenopausal osteoporotic women who had been treated with ALN (10 mg/day) during 2 (9 patients) or 3 years (16 patients) or with PLA (15 and 13 patients, respectively). In the same patients, BMD values were obtained by dual-energy X-ray absorptiometry on lumbar spine at the beginning and end of treatment. Histomorphometric parameters and activation frequency of new remodeling units were also measured on the biopsies.

After 2 years of ALN, mean DMB in compact bone was 9.3% ($p=0.0035$) and in cancellous bone was 7.3% ($p=0.0009$) higher, respectively, versus PLA. After 3 years of ALN, mean DMB in compact bone was 11.6% ($p=0.0002$) and in cancellous bone was 11.4% ($p=0.0001$) higher, respectively, versus PLA. After 2 and 3 years of ALN and compared to the corresponding PLA, the distribution of the DMB clearly showed a shift towards the highest mineralization values and a decrease of the number of bone structure units having low values of mineralization. The between group differences in mean DMB were similar to those of BMD at the lumbar spine level (+8.7% after 2 years +9.6% after 3 years, respectively), suggesting that mean DMB augmentation probably accounts for the major part of the increase in BMD seen with ALN.

These results support our model that the reduction in the activation frequency caused by the antiresorptive effect of ALN is followed by a prolonged secondary mineralization which increases the percentage of bone structure units having reached a maximum degree of secondary mineralization and, through this mechanism, mean DMB. That these effects contribute to improved bone strength is demonstrated by the reduction in fracture incidence previously demonstrated in these patients. In conclusion, quantitative microradiography gives access to the mineral dimension of bone tissue which has been insufficiently taken into account until now as an important determinant of bone strength and quality of bone.

Introduction

The bone mineral substance assumes two main functions, a biomechanical one (rigidity of the bones) and a metabolic one (reservoir for many ions, control of mineral homeostasis). Mature bone tissue is composed of 60-70% of mineral

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substance and 30-40% of organic matrix, mainly (85-90%) type I collagen fibrils. Mineral substance is crystallized as non-stoechiometric carbonated apatite ionic crystals of small size and extended specific surface (100-200 m^2/g). Bone mineral is metabolically active, various and numerous interactions between ions from the extracellular fluid and ions constituting apatite crystals, are thus possible. From its initial formation to growth, maturation and dissolution, apatite crystals interact with the water from the bone matrix. Since crystals do not grow if ions do not diffuse from milieu, the degree of mineralization does not progress when the water content is too low. Consequently, mineralization is rarely complete and stops at about 90-95% of the expected maximum level. Substances such as tetracyclines, polyphosphates, bisphosphonates and strontium can also be incorporated with high affinity.

Inorganic pyrophosphate, a compound of the family of the polyphosphates, used industrially for their property of inhibiting calcium carbonate, is present in biological fluids. Inorganic pyrophosphate inhibits both the formation and the dissolution of calcium phosphate *in vitro*. *In vivo*, it prevents ectopic calcification and might be a physiological regulator of mineral deposition and dissolution. The bisphosphonates are analogs of pyrophosphate and are used today primarily in diseases with increased bone resorption and with bone loss (osteoporosis, Paget disease of bone, fibrous dysplasia, for review see Fleisch[1]). Bisphosphonates bind avidly to apatite crystals,mainly on remodeling surfaces, and inhibit their growth, aggregation and dissolution. Bisphosphonates inhibit bone resorption in cell and organ culture, as well as in intact animals. The action of bisphosphonates on bone resorption is not mediated, as thought earlier, by their physicochemical effect on crystal dissolution, but mostly if not entirely through cellular mechanisms. Bisphosphonates decrease bone turnover and therefore bone loss. They decrease the number of osteoclasts by inhibiting the recruitment and activating apoptosis. Furthermore, they inhibit osteoclast activity directly by inducing morphological changes in these cells (*in vitro* and *in vivo*). The nitrogen-bisphosphonates can inhibit the mevalonate pathway and hence the protein prenylation. Other bisphosphonates may be incorporated in ATP-containing compounds. Bisphosphonates can also inhibit osteoclasts by stimulating the secretion of inhibitor(s) of osteoclast recruitment by osteoblast lineage cells. Therefore their target cells may include members of the osteoblastic cell family. It is not yet known which of these mechanisms (direct and indirect) is the more important *in vivo*. Bisphosphonates act specifically on bone, because of their affinity for bone mineral.

It is generally agreed that strength of bones depends on the volume of bone matrix and the microarchitectural distribution of this volume, while the degree of mineralization of bone tissue (DMB) is almost never mentioned as a determinant of bone strength. We now have evidence that DMB strongly influences not only the mechanical resistance of bones but also the bone mineral density (BMD) measured by dual X-ray absorptiometry[2,3]. From microradiographic observations made in the 70s, it was clear that DMB vary over basic structure units (BSU), namely the osteons in cortical bone and the trabecular packets in cancellous bone, the recently deposited ones being much less mineralized than the older ones. The «young» ones appear in dark grey on the microradiographs, the «old» ones are whiter (Figure 1). This heterogeneity in the DMB is explained by the fact that bone formation which follows bone resorption in the remodeling sequence is a multi-step process: following its deposition, the new matrix begins to mineralize after about 5 to 10 days from the time of deposition and the linear rate of this *primary mineralization* can be measured directly *in vivo* using double tetracycline labeling. After full completion of the BSU, a phase of *secondary mineralization* begins. This process consists of a slow and gradual maturation of the mineral component, including an increase in the amount of crystals and/or an augmentation of crystal size toward their maximum dimensions. This secondary mineralization progressively augments the mineral content on bone matrix. This content represents at the end of the primary mineralization only about 50 to 60% of the maximum degree of mineralization obtained at the end of the secondary mineralization phase. Given at high doses for preventing ectopic calcification or ossification, etidronate inhibits primary mineralization and induces rickets and osteomalacia. It is not the case with other bisphosphonates used at doses decreasing bone resorption (alendronate, risedronate...).

In adult bone, the DMB depends on the rate of remodeling[2]. In other words, the biological determinant of mineralization is the rate of turnover. Our model (Figure 2) is based on the impact of changes in bone remodeling rate on the DMB, i.e., on the bone mineral density at the tissue level. Thus, any agent (parathyroid hormone) or event (menopause, ovariectomy) which provokes an augmentation in the «birthrate» or activation frequency of Basic Multicellular Units (BMU), induces a decrease of the «lifespan» of BSU, in other words in the time available for the secondary mineralization. This leads to the fact that new BSU are resorbed before they have fully completed their secondary mineralization, as proven by the presence of a large amount of uncompletely mineralized BSU and a low mean DMB. Conversely, antiresorptive agents (bisphosphonates, estrogen, SERMs) which cause a marked reduction in the «birthrate» of BMU, prolong the «lifespan» of the BSU, allowing a more complete secondary mineralization. This should finally provoke an increase of DMB. This new approach of the determinants of bone strength and BMD and the results of our recent studies, as well as others now in progress, using antiresorptive (alendronate, raloxifene) and forming (Strontium ranelate) agents used in the treatment of osteoporosis, emphasize that bone mineral substance is an important factor to take into account in the pathophysiology of osteoporosis and other bone conditions. It is finally important to rediscover the mineral dimension of bone which has been forgotten for many years.

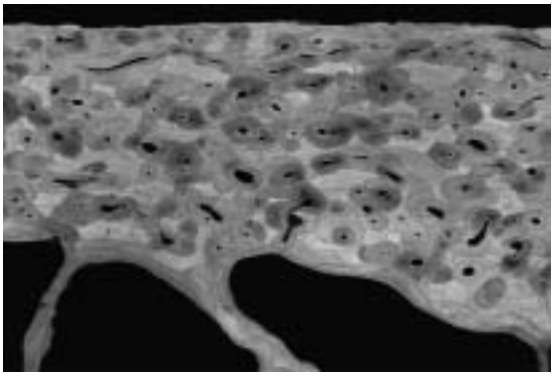

Figure 1: Microradiograph of a section of an iliac bone sample from a control man aged 86 years. The degree of mineralization of bone is heterogeneously distributed with the recently deposited BSU being much less mineralized than the older ones. The «young» BSU appear in dark grey on the microradiograph and the «old» ones are almost white.

Materials and methods

DMB can be measured at the histological level by means of a computerized quantitative contact microradiographic method[2,4,5]. This technique allows the quantity of mineral substance contained in a unit volume of tissue matrix to be determined. Bone samples were fixed in 80% alcohol, dehydrated in absolute alcohol, then embedded in methyl methacrylate without prior decalcification[6]. Bone samples have been oriented in order to cut the osteons perpendicularly to the Haversian canals in at least one of the cortices (compact bone). Thick sections were cut from embedded bone samples, progressively ground to a thickness of 100±1μm, and polished. Cleaned with ultrasound, bone sections were then microradiographed. Contact microradiography was performed using a X-ray diffraction unit PW 1830/40 equipped with a diffraction tube PW 2273/20 (Philips, Limeuil Brévannes, France). The nickel-filtered copper Kα radiation was used under 25 kV and 25 mA. A Geola high-resolution film (VRP-M green sensitive) was exposed for 20 minutes (Slavich International Wholesale Office, Vilnius, Lithuania). For quantitative evaluation of the X-ray absorption by the bone section, a reference system composed of aluminum was exposed on each microradiograph[4,5]. The DMB was quantified using a new combined contact microradiography microdensitometry computerized method[2,3,5] described briefly as follows. A custom-developed software was used for the automatic analysis of gray levels of microradiographs with Visiolab 1000® (Biocom, France), a true color image processing workstation operating under Microsoft Windows®. The image of the microradiograph was captured through a microscope using a 3-CCD color camera. Each field analyzed was divided into 4,350 square measurement units (about 100 pixels each). After calibration using the aluminum reference step-wedge, the measured area was automatically selected and the gray levels were measured from the computer generated map indicating the spatial distribution of the "measurement units" (Figure 3). Data were converted from gray-level values in the degree of mineralization with the construction of a calibration curve based on the measurements obtained on the aluminum step-wedge. The DMB was finally expressed in g mineral/cm^3 bone and results were measured separately in compact, cancellous and total bone (compact + cancellous) tissues.

Control values

To be used as a control group, iliac bone samples were taken at necropsy from 43 subjects (30 women aged 48.4±3.7 years and 13 men aged 66.0±4.4 years) who died suddenly, showing no apparent bone disease. In terms of mean values, distribution and evolution with age, the DMB was not significantly different between the genders[5]. Thus, the control group was studied as a whole, i.e., 43 persons (aged 53.7±3.2 years; range: 20 to 93 years). In this control group, the mean DMB (± SEM) was 1.082±0.017 g mineral/cm^3 in compact bone (227,344 measurements), 1.099±0.018 g mineral/cm^3 in cancellous bone (69,379 measurements), and 1.087±0.017 g mineral/cm^3 in total bone (compact + cancellous: 296,723 measurements). In these three structures of bone, the distributions of DMB are similar (Figure 4) and the DMB did not change significantly with age, even if the DMB varied from one subject to another at a similar age (Figure 5). The distribution of the normal DMB strongly suggests that the oldest BSU has a mean DMB 2-fold higher than that of the youngest BSU (end of primary mineralization). This demonstrates the amplitude of the secondary mineralization, a phase during which as much mineral can be deposited during the primary mineralization. The main parameters used in the description of the mineralization of bone are the mean DMB and the distribution of DMB. From the curve showing this distribution (Figure 6), the highest frequent DMB value (DMB freq. max.) and the width at half-maximum, an index reflecting the homogeneity of DMB, are also measured. Concerning this latter, the more the width is small, the more the distribution of DMB is homogeneous and conversely. For example, in cancellous bone tissue, DMB is almost 2-fold more heterogeneous in human calcaneums than in human iliac samples (Boivin et al., unpublished results). The control values are necessary for interpreting the changes in the measurements reflecting DMB observed in bone conditions untreated or treated[3,5].

Bisphosphonates and degree of mineralization

Age-related changes in bone are characterized by a loss of bone with trabecular thinning more marked in females than in males. The amount of bone formed in each remodeling unit decreases. After the menopause, the activation frequency and the resorption are augmented. Consequently, the risk of trabecular perforation is increased. This results in the loss of the trabecular connectivity which is one of the determi-

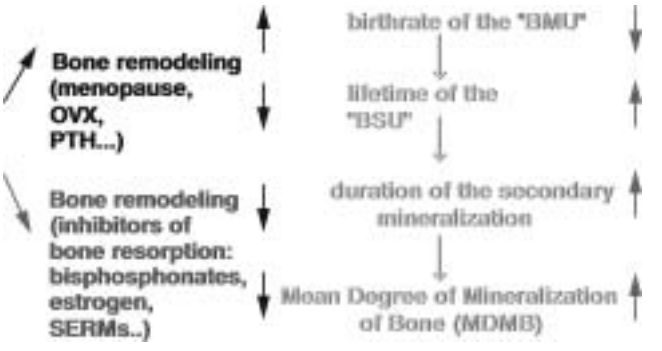

Figure 2: Working model of the potential mechanisms by which events or compounds act on bone remodeling activity and, consequently modify the degree of mineralization of bone. The dark arrows follow the sequence corresponding to an increased bone remodeling activity, and the grey arrows illustrate the inverse sequence when bone remodeling activity is decreased.

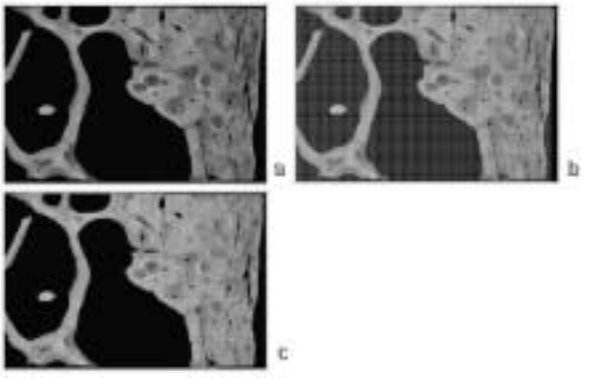

Figure 3: Illustration of the main steps allowing measurement of the degree of mineralization of bone. (a) microradiograph image, (b) the field is divided into 4,350 measurement units (squares of 100 pixels each), (c) calibration with an aluminum step-wedge and selection of the units containing only calcified bone tissue.

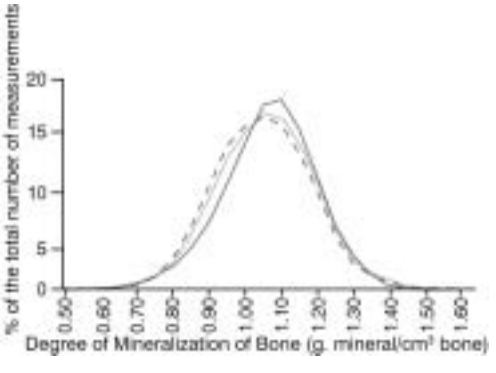

Figure 4: Distribution of the degree of mineralization of bone measured in compact (dotted line), cancellous (large line) and total bone (thin line) tissues in 43 iliac bone samples from human control subjects.

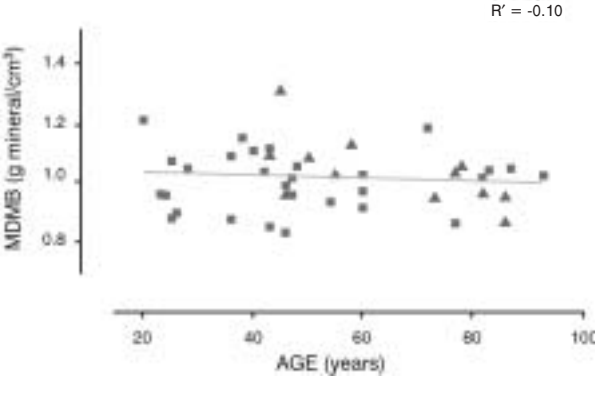

Figure 5: Evolution with age of the mean degree of mineralization of bone (MDMB) measured in 43 iliac bone samples taken from 30 women (squares) and 13 men (triangles).

nants of the mechanical strength of bone. Bone histomorphometry is also used for evaluating the histological positive and side-effects of treatment on bone diseases. The use of bone histomorphometry in both human trials and experimental studies in animals are required for the evaluation of the effects on the quality of bone of antiosteoporotic drugs and to understand the mechanisms of action of these compounds at the bone tissue level. Recently, the effects of alendronate have been evaluated by histomorphometric analysis of iliac biopsies from patients with postmenopausal osteoporosis.

In osteoporosis, the negative imbalance between bone resorption and bone formation is accelerated by the increase in the activation frequency of new remodeling units induced by the menopause. This imbalance persists until the end of life and shortens the duration of the secondary mineralization of BSU[2]. Osteoporosis treatment should not only prevent the loss of bone tissue and not interfere with apatite and avoid bone mineral changes at the crystal level, but it should also increase the mechanical resistance of bone and thus

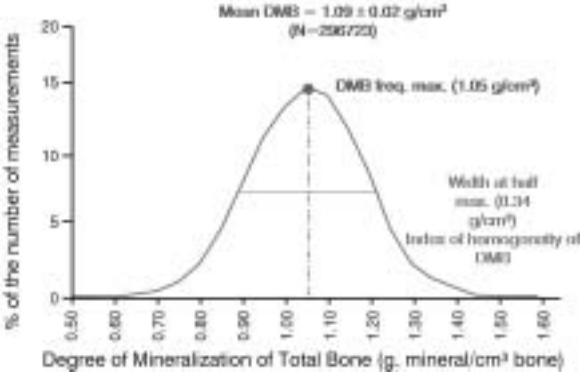

Figure 6: Distribution of the degree of mineralization of bone measured in total (compact + cancellous) bone tissue in 43 iliac bone samples from human control subjects. Main parameters measured and calculated and describing the degree of mineralization of bone (N corresponds to the total number of measurements performed in bone).

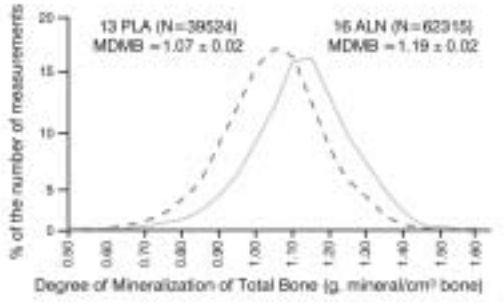

Figure 7: Distribution of the values of degree of mineralization in total (compact + cancellous) bone from patients treated with placebo (dotted line) or alendronate (intact line) during 3 years. After alendronate, the shift of the degree of mineralization towards the highest values associated with a decrease of the low values, is clearly observable (N corresponds to the total number of measurements performed in bone).

Index of homogeneity of Iliac DMB
Width at half max. (g/cm³)

	Compact	Cancellous	Total
Human Controls	0.34	0.28	0.34
Placebo 2 yrs	0.38	0.28	0.33
Alendronate 2 yrs	0.34	0.25	0.29
Placebo 3 yrs	0.31	0.26	0.28
Alendronate 3 yrs	0.26	0.24	0.26

Figure 8: Index of homogeneity of the degree of mineralization of bone calculated in postmenopausal osteoporotic patients treated with either placebo or alendronate for 2 and 3 years.

protect the skeleton against new fractures. Osteoporosis is characterized by an absolute decrease in the amount of bone to an extent such that fragility fractures may occur after minimal trauma. In a recent study[3], mean DMB was measured by quantitative microradiography on transiliac bone biopsies taken from 53 postmenopausal osteoporotic women who had been treated with alendronate (ALN, 10mg/day) during 2 (9 patients) or 3 years (16 patients) or with a placebo (PLA, 15 and 13 patients, respectively). In the same patients, BMD values were obtained by dual X-ray absorptiometry at lumbar spine level. Histomorphometric parameters and activation frequency of new remodeling units were also measured on the iliac biopsies[7]. After 2 years of ALN, mean DMB in total bone was 7.5% ($p=0.0026$) higher versus PLA. After 3 years of ALN, mean DMB in total bone was 10.7% ($p=0.0001$) higher versus PLA. After 2 and 3 years of ALN and compared to the corresponding PLA, the distribution of the DMB in total bone clearly showed a shift towards the highest mineralization values concomitantly with a decrease of the number of bone structure units having low values of mineralization (Figure 7). The between group differences in mean DMB were similar to those of BMD at the lumbar spine level (+8.7% after 2 years and +9.6% after 3 years, respectively), suggesting that mean DMB augmentation probably accounts for the major part of the increase in BMD seen with ALN. In the 53 patients, the index of homogeneity has been measured (Figure 8). After 2 and 3 years of ALN, this index was smaller than after PLA, in compact, cancellous and total bone. This illustrates the fact that ALN allowing the secondary mineralization to be more completed, the homogeneity of the mineralization is better.

Similar results have been reported in patients suffering from steroid-induced osteoporosis and treated with ALN. Treatments with other bisphosphonates as risedronate[8] and zoledronate[9] also revealed decreases of vertebral fractures and augmentation of BMD without significant changes of bone mass and microachitecture but marked reduction of activation frequency. Even if DMB at tissue level was not yet measured in the bone biopsies of the corresponding patients, the explanation could be similar to the one of the effects of ALN.

Another antiresorptive agent (SERM = Raloxifene) has also been tested[10,11] even if its action on the decrease of remodeling rate is less potent than ALN. Raloxifene induces a mild increase of BMD, a moderate decrease of the biochemical markers of bone turnover and decreases the risk of vertebral fractures in postmenopausal women. In the absence of significant changes in bone mass and microarchitecture, the modifications of BMD could be the reflect of even mild modifications in the DMB (Boivin et al., unpublished results).

Finally, Strontium ranelate, a new orally effective and safe treatment of vertebral osteoporosis, has been shown to decrease vertebral fractures and increase BMD[12]. The unique mechanism of action of Strontium ranelate is to decrease bone resorption and increase bone formation. The effects at the bone tissue level are now investigated in animals[13,14] and in man[15].

Conclusion

These results support our model that the reduction in the activation frequency caused by the antiresorptive effect of bisphosphonates is followed by a prolonged secondary mineralization which increases the percentage of BSU, having reached a high degree of secondary mineralization and, through this mechanism, increases mean DMB. That these effects contribute to improved bone strength is demonstrated by the reduction in fracture incidence previously demonstrated in these patients. In a patient suffering from osteopetrosis, characterized by the absence of an active osteoclastic resorption, we have had the opportunity to illustrate the evolution of all bone mineral towards the highest values of DMB. This showed that, in the absence of osteo-

clastic resorption, the remodeling rate was extremely low and consequently a great part, if not all, bone mineral tissue has time to complete its secondary mineralization, and thus, presented a uniformly distributed high DMB. However, after ALN treament, the mean DMB was always lower than the one found in osteopetrosis.

Does a loss of heterogeneity in the DMB in different BSUs induce a partial loss of elastic properties of bone? This would require measurements in bone biopsies taken after a very long-term treatment (more than 3 years) with ALN. Furthermore, it would be of great interest to correlate DMB with biomechanical data obtained at the tissue level.

In conclusion, changes in bone remodeling activity directly influence the degree of mineralization of bone. This may explain the modifications in fracture incidence, the increase in bone mineral density and in bone strength without necessary changes of the bone matrix volume and bone microarchitecture.

Acknowledgments

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