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ORIGINAL ARTICLE

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Duration-Dependent Increase of Human Bone Matrix Mineralization in Long-Term Bisphosphonate Users with Atypical Femur Fracture

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ABSTRACT

Bisphosphonates (BPs) are the most widely used drugs for the treatment of osteoporosis but prolonged use of BPs might increase the risk of atypical femur fracture (AFF). There are only a few studies that address the bone material quality in patients on long-term BP treatment with or without AFFs. We analyzed 52 trans-iliac bone biopsies from patients on long-term BP therapy with ($n = 26$) and without ($n = 26$) AFF. At the microscopic level, the degree of mineralization of bone (DMB) was assessed on whole bone by X-ray digitized microradiography while microhardness by Vickers microindentation, and bone matrix characteristics by Fourier transform infrared microspectroscopy (FTIRM) (mineral/organic ratio, mineral maturity and crystallinity, and collagen maturity) were measured at random focal areas. The AFF patients were treated longer than non-AFF patients (9.7 $\pm$ 3.3 years versus 7.9 $\pm$ 2.7 years). As expected, bone remodeling was low in both groups, without difference between them. The AFF group had significantly higher DMB in cortical bone (+2.9%, $p = .001$), which remained so after adjusting for treatment duration ($p = .007$), and showed a trend in cancellous bone (+1.6%, $p = .05$). Consistent with higher DMB, heterogeneity index (HI) was lower in the AFF than in the non-AFF group, illustrating lower heterogeneity of mineralization in the AFF group. A significant positive correlation between the duration of treatment and DMB in cortical bone was found in AFF, and not in the non-AFF group. Microhardness and bone matrix characteristics were similar between groups. We conclude that the AFF group had a duration-dependent increase in DMB leading to a significantly higher DMB than the non-AFF. Because BPs have high affinity to bone mineral and lining the walls of the osteocyte lacunae, the accumulation of matrix-bound BPs in AFF could lead to inhibition of the osteocyte cytoskeleton blunting their response to mechanical strains, a hypothesis to be further investigated. © 2021 American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: ATYPICAL FEMUR FRACTURE; BISPHOSPHONATES; BONE HISTOMORPHOMETRY; BONE MATRIX MINERALIZATION; MICROINDENTATION

Introduction

Atypical femur fractures (AFFs) are stress or "insufficiency" fractures, occurring in the subtrochanteric or diaphyseal region of the femur with minimal or no trauma.[1,2] AFFs are different from classically encountered "typical" proximal femur fractures, and the American Society for Bone and Mineral Research (ASBMR) task force has updated in 2014[3] the first definition of AFFs.[4] At least four of the five criteria of AFF are required to meet the case definition. Two of those criteria are the transverse line through the lateral cortex, and the focal cortical thickness at the fracture line. AFFs arise exclusively on the lateral cortex of the subtrochanteric and diaphyseal regions of the femur, regions subjected to high mechanical loads,[5] suggesting that impaired bone matrix quality might predispose to the development of these rare insufficiency fractures.

It has been shown that long-term use of bisphosphonates (BPs), as defined by the ASBMR task force,[6] with reduction in bone remodeling might increase the risk of AFF.[7,8] However,

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the incidence of AFFs remains low in comparison with the number of avoided osteoporotic fractures,[9] and AFFs may also occur in patients with no exposure to BPs or with other anti-osteoporotic drugs with different mechanism of action such as denosumab, odanacatib, or romosozumab.[10–12] The risk of AFF increases with the duration of treatment with BPs,[2,13,14] and a rapid offset of the risk after discontinuing treatment (risk is decreased 70% after 1 year discontinuation) has been reported in one study.[15]

Asians are also at much higher risk for AFF.[9] The exact mechanism for the pathogenesis of AFFs is unknown, but it has been suggested that the lack of microdamage removal due to prolonged suppression of bone remodeling might lead to AFF.[16–18] However, why an extremely limited number of patients treated with BPs develop AFF is still unclear. Few data on the bone material quality between long-term BP users with and without AFF are available. We have previously shown that bone micromechanical properties were compromised in long-term alendronate users without AFF compared to untreated postmenopausal osteoporotic patients, with a decrease in both microhardness and crystallinity, while the degree of mineralization of bone (DMB) was increased.[19] Two studies using in vivo microindentation on mid-tibia failed to show difference in bone material strength index (BMSi) or in indentation distances between long-term BP users with or without AFFs.[20,21] A study using nanoindentation on plastic-embedded proximal femoral bone showed increased hardness and mineralization in long-term BP users with AFFs compared to long-term BP users with or without typical fracture.[22] Another study using the same technique on iliac bone biopsies showed a higher cortical and cancellous plastic deformation resistance in patients with BPs-associated AFF and a severely suppressed bone remodeling compared to untreated osteoporotic patients.[23] However, there is still a lack of knowledge on several other determinants of bone material quality in long-term BP users with and without AFF, particularly, the DMB, micromechanical properties, and mineral and organic characteristics.

The purpose of the present study was to examine the bone material quality in long-term BP users with or without AFF. At the microscopic level, the DMB was assessed on whole bone by X-ray digitized microradiography, whereas microhardness by Vickers microindentation, and bone matrix characteristics were assessed by Fourier transform infrared microspectroscopy (FTIRM) (mineral/organic ratio, mineral maturity and crystallinity, and collagen maturity) were measured at random focal areas. We hypothesized that long-term BP users with AFF, compared to long-term BP users without AFF, have a higher DMB, higher microhardness, and more mature bone matrix characteristics due to a duration-dependent prolonged secondary mineralization despite similar suppression of bone remodeling.

Materials and Methods

Bone specimens

Twenty-six trans-iliac bone biopsies obtained from outpatient clinic postmenopausal women with osteoporosis (PMOP) treated with BPs (9.7 $\pm$ 3.5 years) who presented with AFF were compared to 26 biopsies from PMOP patients treated with BPs (7.9 $\pm$ 2.7 years), but without an AFF. Of the 26 non-AFF group, 10 came from our laboratory and were part of a study on the potential influence of BPs on the occurrence of microdamage, that was approved by the Ethics Committee of our Hospital as a usual care study (INSERM UMR 1033, Lyon, France), and 16 from the Bone and Mineral Research Laboratory, Detroit, MI, USA. Informed consent was obtained from each subject. The study was approved by the Institutional Review Board of Henry Ford Hospital. Twenty-five women were treated with only alendronate (ALN) and one patient was treated with ALN for 7 years and risedronate for 2 years. Of the 26 AFF group, 10 came from Quebec City and Montreal, Canada, and 16 were from Detroit, MI, USA. The biopsies from Canada were done for clinical purpose, and did not require ethical approval. Twenty-four women were treated with ALN, one with ALN for 3 years followed by pamidronate for 4.5 years, and another with cyclical etidronate for 5 years followed by risedronate for 3 years. The majority were white women with 2 black patients in each group and one Asian patient in the AFF group.

All bone specimens were embedded in methyl methacrylate after fixation and dehydration in alcohol. Embedded bone biopsies were cut into 150-µm-thick sections using a precision diamond wire saw (Escil, Chassieu, France), then were ground into 100 $\pm$ 1-µm-thick sections and finally polished with an alumina suspension (Escil, Chassieu, France) for the measurement of the degree of mineralization of bone by digitized microradiography.[24] Embedded blocks were polished with an alumina suspension for the measurement of microhardness by microindentation.[25] Sections 2-µm-thick were cut from embedded blocks for the analysis of mineral and organic characteristics by FTIRM analysis.[26–29]

BMD

Bone mineral density (BMD) values by dual-energy X-ray absorptiometry (DXA) at femoral neck, total hip, and lumbar spine were retrieved at baseline (pretreatment) and at the time of the bone biopsy in 22 of 26 patients without AFF and 17 of 26 patients with AFF. Almost all BMD values were measured on Hologic (Marlborough, MA, USA) with the exception of two patients without AFF and one patient with AFF from Detroit who were measured on GE Lunar (Madison, WI, USA) at baseline with values converted to Hologic according to Lu and colleagues[30] for femoral neck and total hip BMD, and Hui and colleagues[31] for lumbar spine BMD. DXA measurements were done in each individual academic institution using established technical standards, although on different DXA instruments considering the long delay between baseline and final assessments in most patients.

Histomorphometry

The histomorphometric data have been provided on the two groups, 16 non-AFF and 16 AFF, by the Bone and Mineral Research Laboratory, Henry Ford Health System Detroit, USA, and partly published.[32] As the criteria for measurements were different between the three laboratories (Detroit, Lyon, and Montreal), only the histomorphometric data from the two groups of 16 non-AFF and AFF from Detroit are reported here. All bone histomorphometric variables were designated in accordance with the nomenclature recommended by the ASBMR.[33] Briefly, the static histomorphometric indices were measured in sections stained with the modified toluidine blue method, and the dynamic remodeling indices were measured on unstained sections, using a Bioquant image analysis system (Bioquant Image Analysis Corporation, Nashville, TN, USA) equipped by bright-field and fluorescent microscope. Cortical and cancellous bone volume/total volume (Ct-BV/TV, Cn-BV/TV; %), cortical and

cancellous wall thickness (Ct-W.th, Cn-W.th; μm), endocortical and cancellous bone formation rate (Ec-BFR, Cn-BFR; $\mu m^3/\mu m^2$/year), endocortical and cancellous activation frequency (Ec-Ac.f, Cn-Ac.f; #/year), endocortical and cancellous mineralizing surface (Ec-MS/BS, Cn-MS/BS; %), have been collected from the Henry Ford Health System laboratory database.

Digitized microradiography

The technique of digitized microradiography was used to measure the DMB and its heterogeneity index (HI).[24] Briefly, 100-μm-thick bone sections were analyzed with a tube Microfocus Hamamatsu X-ray system L9421-02 (Hamamatsu Corporation, Bridgewater, NJ, USA). High voltage of 40 kV, current of 50 μA and power of 2 W were used during X-ray exposure. A Photonic science CCD camera FDI VHR 11 M (Photonic Science, Saint Leonards-on-sea, UK) with an active area of 36 × 24 mm (4008 × 2671 pixels) was used as detector. The image digitization step was done with a 12-bit digital image detector (pixel size: 9 μm, object pixel size: 0.83 μm).The DMB measurements by X-ray microradiography are calibrated using hydroxyapatite calibration phantoms (Skyscan; Bruker, Kontich, Belgium) of known densities (0.25 and 0.75 g/cm^3). Using code from the MATLAB program (MathWorks, Natick, MA, USA), the gray levels of images were converted in g/cm^3 of bone with the calibration curve generated from an aluminum-step wedge reference with eight regular steps (99.5% pure foil; Strem Chemicals Inc., Bischheim, France) that was exposed during the same exposure conditions. A threshold of 0.6 g/cm^3 was applied on the images, thus only the bone matrix was measured, and the macropores and micropores were excluded from the measurements of the DMB. Only the periosteocytic lacunae are included in the DMB measurement (and thus can slightly decrease the DMB) because they are thinner than the thickness of the bone sections (100 μm) used for microradiographs. The mean DMB and mean HI (HI = full-width at half-maximum of the curve of distribution) of the DMB were expressed in g mineral/cm^3 and measured in cancellous and cortical bone separately.

FTIRM analysis

FTIRM was used to assess bone material intrinsic properties as described.[26–29] Briefly, thin bone sections (2-μm thick) were analyzed in transmission mode with a Perkin-Elmer GXII Autoimage Microscope (Norwalk, CT, USA) equipped with a wide band detector (mercury-cadmium-telluride) (7800–400 cm^{-1}). Sixty areas were scanned at 100 μm × 100 μm of spatial resolution (20 areas in each cortex, and 20 areas in cancellous bone) that were randomly chosen. Each spectrum was collected at 2 cm^{-1} resolution, 50 scans by spectrum were performed in the transmission mode, and the contribution from air was subtracted from original spectrum. The raw spectra were then treated by automation in the custom Python software (Python Software Foundation, Fredericksburg, VA, USA) (transformation from transmittance to absorbance values, removal methyl methacrylate (embedding resin) to extract the components of interest from a raw spectrum), a baseline correction method, and curve-fitted with the peak fitting methods.[29] The following variables were determined: the mineral crystallinity (reflecting both crystal size and perfection),[26] and measured as: cryst = 1/full-width at half-maximum of the 604 cm^{-1} peak (apatitic phosphate environment) the mineral to organic ratio (min/org 1184–910 cm^{-1}/1712–1592 cm^{-1}),[34] the mineral maturity (min mat) which is calculated as the area ratio of the apatitic phosphate over non apatitic phosphate (1030/1110 cm^{-1} area ratio) and reflects the age of mineral,[26] the carbonation which is the ratio of the ν_2CO_3 area (862–894 cm^{-1}) to the $\nu_1\nu_3PO_4$ area (910–1184 cm^{-1}) that reflects the incorporation of whole CO_3 ions (type B, type A and labile) into the crystal, and the collagen maturity (coll mat), which is calculated as the ratio of organic matrix bands (1660/1690 cm^{-1} area ratio).[27,28] Results are expressed as mean ± standard deviation (SD) for each bone envelope (cortical or cancellous).

Microhardness testing

A Vickers indenter was used to measure microhardness on polished blocks (Micromet 5104; Buehler, Lake Bluff, IL, USA).[25] Briefly, a test load of 25 g applied for 10 s was used for each indentation. For each sample, 60 indents (20 in each cortical and 20 in cancellous bone) were performed at the tissue level, and randomly chosen. Microindentations were performed in randomly selected areas of the bone surface, separated by at least 500 μm over the whole bone tissue area.

The sizes of the impressions were measured from the direct measurement of diagonal dimensions using manufacturer software (Omnimet HMS v.2.31; Buehler), and the microhardness (Hv) was derived using the following formulae Hv = 1854.4 P/d^2 (where Hv is Vickers microhardness expressed in kg/mm^2, P is test load in kg, and d is the mean length of the two diagonals expressed in mm). Results are expressed as mean ± SD for each bone envelope (cortical or cancellous).

Statistical analyses

Analyses were performed using SPSS® 16.0 (SPSS Inc., Chicago, IL). A *t* test was carried out using SigmaStat (Systat Software Inc., San Jose, CA, USA) for the histomorphometric analysis. Differences between non-AFF and AFF were assessed using nonparametric Mann-Whitney tests and differences in BMD before and after treatment using nonparametric Wilcoxon tests. The associations between DMB, mineral and collagen characteristics, local mechanical properties, and remodeling variables were tested using Spearman's correlations (rho). Adjustments of DMB, mineral characteristics, and collagen characteristics for the duration of treatment were performed using an ordinal regression. A p value <.05 was used to define statistical significance.

Results

Age, BMI, and duration of treatment by BPs

Age and BMI at the time of bone biopsy were not significantly different between non-AFF and AFF. However, the mean duration of treatment was significantly longer in the AFF group than in the non-AFF group (9.7 ± 3.3 years versus 7.9 ± 2.7 years; p = .03; Table 1). The range of the duration of treatment was 2 to 17 years in the AFF group and 3 to 15 years in the non-AFF, respectively.

BMD before and after treatment

Before treatment femoral neck and lumbar spine BMDs were similar in non-AFF and AFF groups, but total hip BMD was higher in the AFF group (p = .03; Fig. 1). After long-term BP treatment, total hip and lumbar spine BMDs (mean % ± SD) significantly

Table 1. Age, BMI, Duration of Treatment, and Histomorphometric Data in Non-AFF and AFF Groups

Parameter	Non-AFF	AFF	*p*
Age, BMI, and duration of treatment, mean ± SD	(*n* = 26)	(*n* = 26)	
Age	68.2 ± 5.9	66.4 ± 5.4	NS
BMI[a]	24.7 ± 5.1	26.8 ± 5.0	NS
Duration of treatment by BPs (years)	7.9 ± 2.7	9.7 ± 3.3	*p* = .03
Histomorphometry	(*n* = 16)	(*n* = 16)	
Cortical			
Ct BV/TV (%)	95.36 ± 1.64	95.50 ± 2.47	NS
Ct-W.th (μm)	41.34 ± 5.35	37.45 ± 5.07	*p* = .04
Ct-BFR ($\mu m^3/\mu m^2$/year)	5.23 ± 6.25	4.69 ± 6.21	NS
Ct-Ac.f (/year)	0.13 ± 0.15	0.14 ± 0.19	NS
Ct-MS/BS (%)	3.34 ± 2.95	3.71 ± 4.09	NS
Cancellous			
Cn BV/TV (%)	16.32 ± 8.47	13.16 ± 4.13	NS
Cn-W.Th (μm)	32.70 ± 5.21	29.00 ± 3.58	*p* = .03
Cn-BFR ($\mu m^3/\mu m^2$/year)	1.63 ± 2.64	1.00 ± 1.86	NS
Cn-Ac.f (/year)	0.05 ± 0.08	0.03 ± 0.06	NS
Cn-MS/BS (%)	1.13 ± 1.46	0.91 ± 1.26	NS

NS = not statistically significant.
[a]BMI: *n* = 22 in non-AFF; *n* = 21 in AFF.

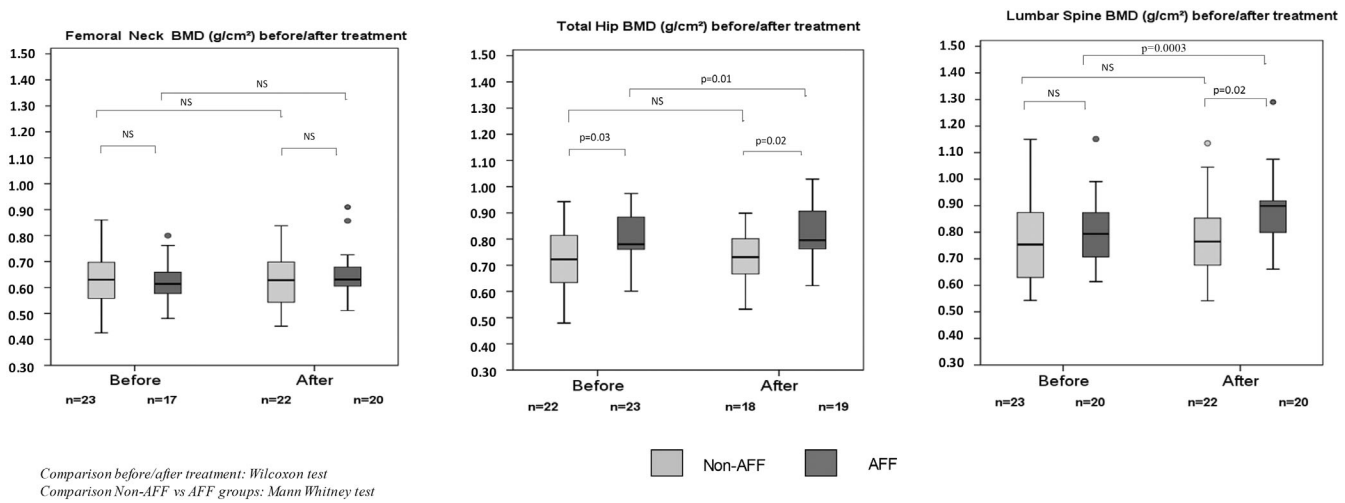

Fig 1. BMD measured by DXA before and after treatment in non-AFF and AFF groups, at femoral neck, total hip, and lumbar spine. BMD = bone mineral density.

increased from baseline in AFF (total hip: 3.95 ± 5.6%; *p* = .01; lumbar spine 9.41 ± 5.98%; *p* = .0003) but remained unchanged in the non-AFF group (Fig. 1). The BMD increase at total hip in AFF remained significant after adjustment for the duration of treatment (*p* = .03).

Bone histology and histomorphometry

All biopsies were evaluated for histology and showed a normal lamellar bone texture, with no evidence of pathological findings such as osteomalacia, woven bone, or marrow fibrosis. No significant differences were found in static variables (Ct-BV/TV and Cn-BV/TV) except in Ct-W.Th and Cn-W.Th, which were lower in the AFF compared to the non-AFF group (*p* ≤ .04). Dynamic variables of bone remodeling as reflected by BFR, Ac.f and MS/BS were not significantly different between the groups in either cortical or cancellous bone (Table 1).

DMB

The distribution of the mineralization is illustrated on microradiographs of iliac bone samples from patients with or without AFF (Fig. 2C-D). In the AFF group, we found significantly higher DMB in cortical bone (+2.9%, *p* = .001) than in non-AFF and a trend toward a similar change in cancellous bone (+1.6%, *p* = .05, Fig. 2A) After adjusting for the duration of BP treatment, the DMB was still significantly higher in cortical bone (*p* = .007) but not in cancellous bone. Although we found a decrease in HI in cortical (trend, *p* = .05) and cancellous (*p* < .007, Fig. 2B) bone in the AFF group when compared to non-AFF, the

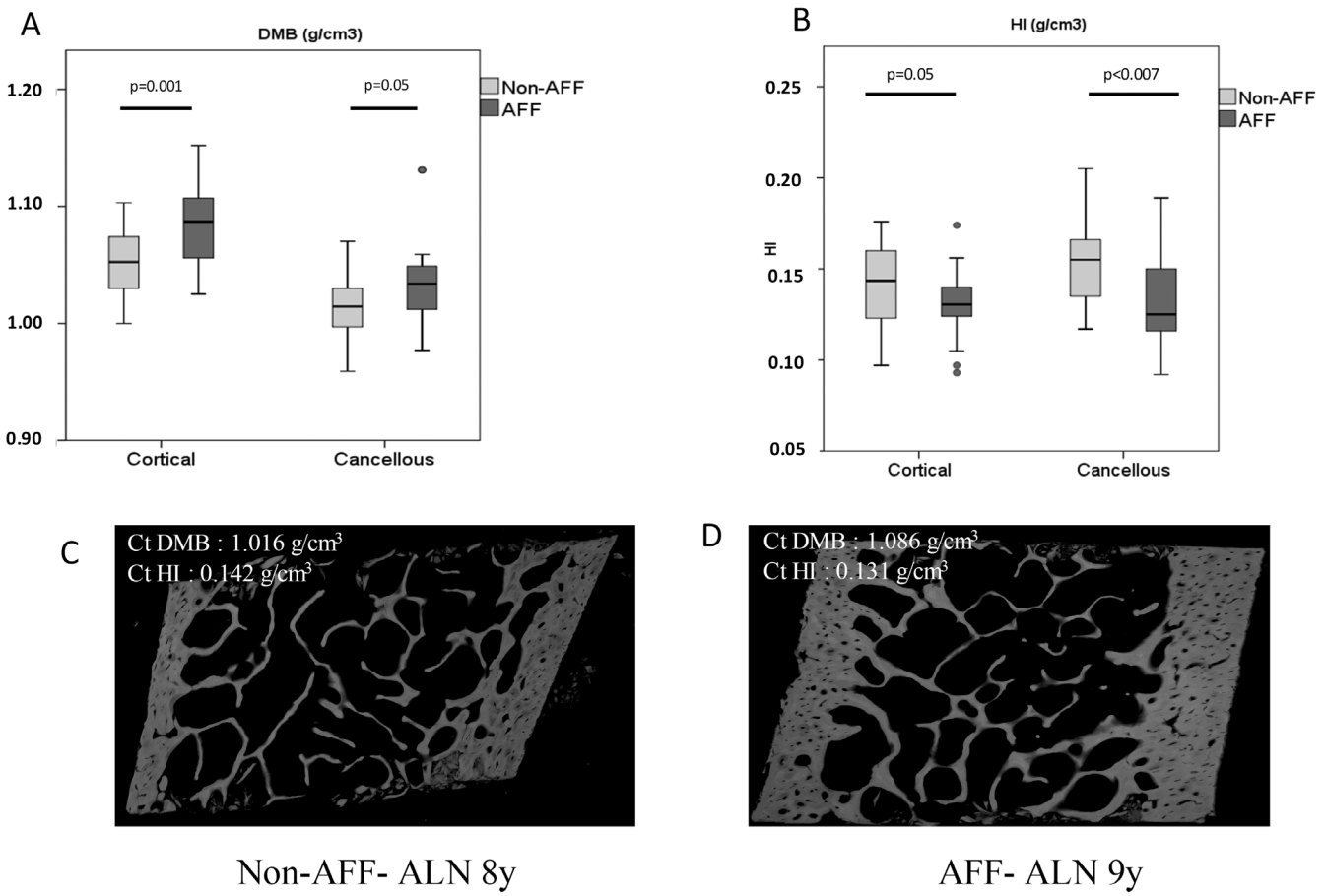

Fig 2. (*A*) DMB (g/cm^3) and (*B*) HI (g/cm^3) in non-AFF and AFF groups. DMB values were significantly higher and HI lower in AFF group compared to non-AFF in cortical and cancellous bone. (*C*,*D*) X-ray digitized microradiographs of 100-µm-thick section of iliac bone from (*C*) non-AFF (woman treated 8 years with alendronate) and (*D*) AFF (woman treated 9 years with alendronate). DMB = degree of mineralization of bone; HI = heterogeneity index.

difference between the groups disappeared after adjusting for the duration of BP treatment in cortical bone but persisted in cancellous bone (p = .09 and p = .02, respectively). No adjustments for age were needed because mean age and distribution were similar.

Mineral and organic characteristics

FTIRM analysis of iliac bone samples showed no significant differences in mineral/matrix ratio, crystallinity, mineral maturity, carbonation, or collagen maturity between non-AFF and AFF groups when assessed in both cortical and cancellous bone tissues (Table 2).

Microhardness

No significant difference was found in microhardness between non-AFF and AFF groups, neither in cortical nor in cancellous bone tissues (Table 2).

Correlations between the duration of treatment and DMB, microhardness, and mineral/organic characteristics

Correlations between the duration of BPs treatment and DMB, crystallinity and microhardness, separately in non-AFF and AFF groups, were tested. A significant positive correlation (rho = 0.468; p < .02) between the duration of treatment and DMB was only shown in cortical bone from the AFF group (Fig. 3A, right). No other correlation was found between the duration of treatment and the other variables, microhardness, and mineral/organic characteristics (Fig. 3B-D).

Discussion

The purpose of the present study was to compare bone material quality in trans-iliac bone biopsies from patients with and without AFF after long-term BP therapy. Both groups had histomorphometrically confirmed low remodeling. As we hypothesized, DMB was higher in cortical (borderline in cancellous bone) and HI lower in cancellous (borderline in cortical bone), in the AFF compared to the non-AFF group. However, the higher DMB in AFF was not associated with a significantly lower bone remodeling. Because patients with AFF were treated for significantly longer duration than non-AFF patients (2 additional years on average), this higher DMB could potentially be explained by a longer duration of treatment. However, after adjustment for the duration of treatment, the DMB remained significantly higher in the AFF group than in the non-AFF group. When

Table 2. Bone Material Properties in Non-AFF and AFF Groups

Property	Non-AFF (n = 26)	AFF (n = 26)	p
Microindentation			
Cortical microhardness (Ct-Hv)	51.2 ± 3.6	52.8 ± 3.0	NS
Cancellous microhardness (Cn-Hv)	53.5 ± 3.0	54.9 ± 2.8	NS
FTIRM analysis			
Cortical			
Ct-Min/org ratio	4.8 ± 0.3	4.9 ± 0.3	NS
Ct-Crystallinity	0.038 ± 0.001	0.038 ± 0.001	NS
Ct-Mineral maturity	1.9 ± 0.6	2.0 ± 0.5	NS
Ct-Carbonation	0.0070 ± 0.0005	0.0071 ± 0.0006	NS
Ct- Collagen maturity	4.4 ± 0.6	4.5 ± 0.6	NS
Cancellous			
Cn-Min/org ratio	4.8 ± 0.4	4.9 ± 0.4	NS
Cn-Crystallinity	0.038 ± 0.001	0.038 ± 0.002	NS
Cn-Mineral maturity	1.7 ± 0.4	1.7 ± 0.4	NS
Cn-Carbonation	0.0072 ± 0.0006	0.0073 ± 0.0009	NS
Cn- Collagen maturity	4.0 ± 0.6	4.2 ± 0.7	NS

NS = not statistically significant.

correlated and analyzed separately, the AFF group had a duration-dependent increase in DMB, not found in the non-AFF group. As expected, we also found a lower HI in the AFF group compared to the non-AFF, indicating a lower heterogeneity of bone mineralization in AFF. The BMD measurements are consistent with our material property results, confirming a significant gain of bone mineral density in the AFF group after treatment, at both lumbar spine and total hip, that was not observed in non-AFF patients. Also, this higher BMD in AFF-group persisted at the total hip after adjustment for the duration of treatment, as observed for DMB. Microhardness and others mineral and organic characteristics were not significantly different between the groups. As AFFs, by definition, occur in femur that is composed mainly of compact bone, the discussion will now focus mostly on cortical bone.

Atypical femur fracture is a rare complication of long-term BP treatment and the pathogenesis is not yet fully elucidated. Concern has arisen that long-term BP use may increase the risk of AFF through several putative mechanisms including a prolonged reduction in bone remodeling and consequently a delayed microcracks repair. Incidence rates of AFF range from 1.8 per 100,000 cases per year with a 2-year BP exposure to 113.1 per 100,000 cases per year with BP exposure from 8 to 9.9 years.[3,35] AFFs also occur in treatment-naïve patients, or in patients treated with osteoporosis medications other than BPs.[1] Thus, a low bone remodeling, universally found with antiresorptive drugs, does not seem to be sufficient, but perhaps necessary, to cause AFFs, as illustrated by the low incidence rate (8 per 100,000 person-years) reported with the prolonged use of denosumab (up to 7 or 10 years), whereas it is a more potent antiresorptive,[36] and in treatment-naive postmenopausal women with osteoporosis. Furthermore, several cases of AFFs were associated with odanacatib, a cathepsin K inhibitor with antiresorptive effect but attenuated reduction of bone formation.[37]

The percentage difference in DMB of cortical bone between non-AFF and AFF (+2.9%), accounts for approximately one-third of the difference of DMB after 2 years of ALN compared with a placebo group as previously shown by our group (+9.3%, p = .004).[38] The increase in DMB after 2 years of ALN was close to the increase in BMD assessed by DXA at the lumbar spine (+8.7% at 2 years), suggesting that the improved bone strength after ALN was mainly explained by the increase in DMB. It results from the completion of mineralization of bone structural units (BSUs) by extension of the secondary mineralization subsequent to the reduction in activation frequency. BMD measured by DXA depends on both bone mass and bone tissue degree of mineralization. Consistent with the higher DMB in AFF group after treatment, we also found higher BMD in AFF at total hip and lumbar spine, despite no difference in baseline BMD between non AFF and AFF groups, except at the total hip. After treatment, total hip and lumbar spine BMDs were significantly increased from baseline in AFF but remained unchanged in non-AFF. This might be explained in part by BMD measurement inaccuracies due to the long period between the 2 measurements (up to 17 years) and changes in DXA instruments. It is likely that BMDs increased in both groups but due to these measurement inaccuracies, only the higher increases in BMDs in AFF group were significant. This increase in BMD in the AFF group could favor a continuous adsorption of bisphosphonates on bone mineral matrix and potential interaction with osteocytes entrapped in the bone matrix.

The most notable finding was an unexpected duration-dependent increase in DMB found only in the AFF group, because DMB should not increase further once the plateau of mineralization is reached, which usually occurs after about 3 years of treatment.[38,39] Indeed, in non-AFF, DMB no longer increases once a new equilibrium of bone turnover is reached after 2 to 3 years of BPs (first increases and then stabilizes) whereas in AFF it continues to increase. Present values of bone mineralization were compared to historical values obtained in patients treated with denosumab (DMAb; FREEDOM study [Fracture Reduction Evaluation of Denosumab in Osteoporosis Every 6 Months])[40] known to have a greater antiresorptive effect than BPs. Values obtained with DMAb at 2 to 3 years of treatment were higher than those obtained in AFF group treated 10 years on average (Supplementary Fig. S1). The DMB under DMAb continued to increase until 5 years in FREEDOM Extension and reached a plateau from 5 to 10 years.[40] This is consistent with the lower values of HI with DMAb than BPs. Thus, the plateau of mineralization was reached later with DMAb than with BPs

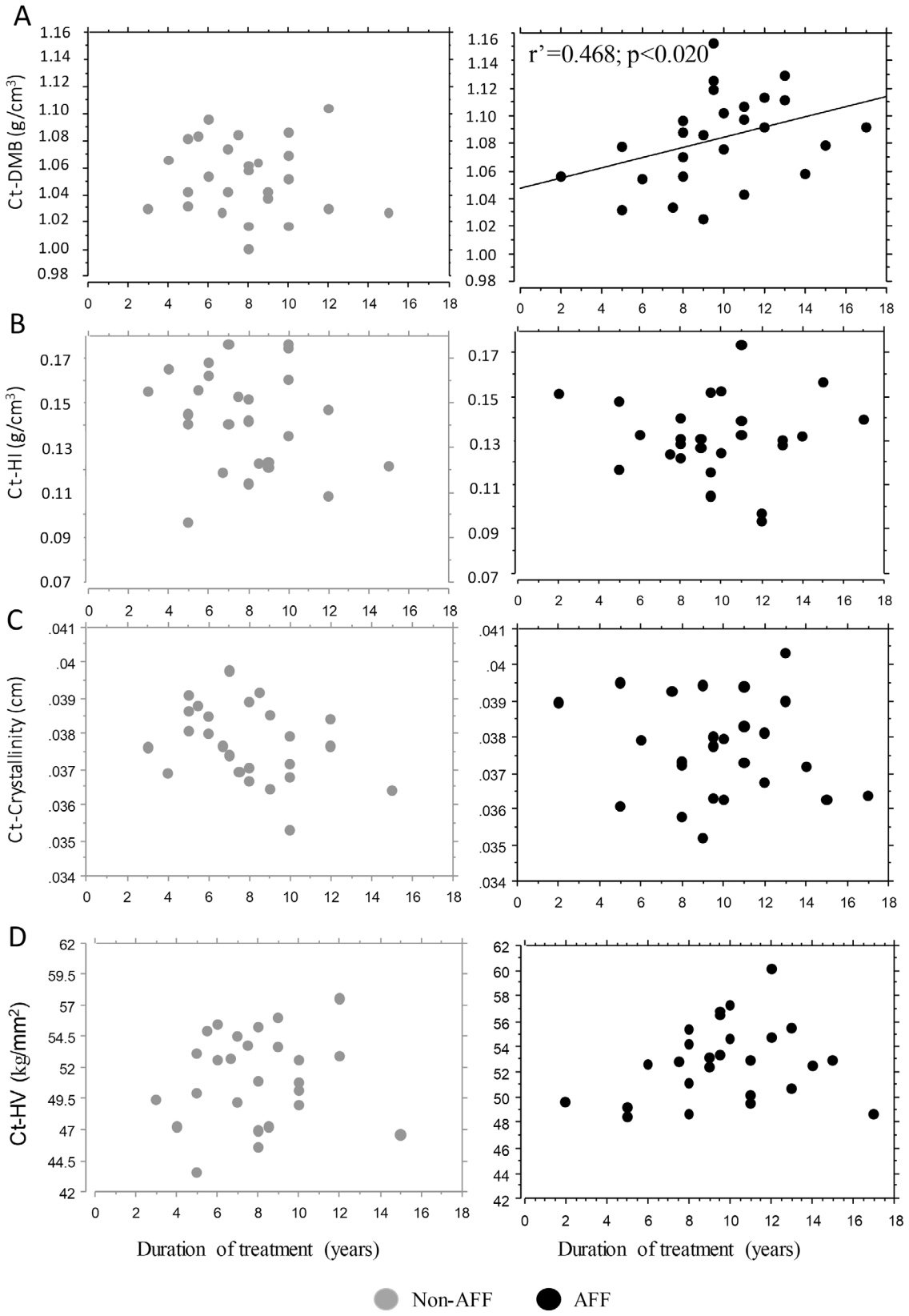

Fig 3. Spearman's correlations between duration of treatment with bisphosphonates and (*A*) Ct DMB, (*B*) Ct-HI, (*C*) Ct-crystallinity, and (*D*) Ct-microhardness, separately in non-AFF group (left panels) and in AFF group (right panels). A statistically significant positive correlation was only found in AFF group between Ct-DMB and duration of treatment. Ct = cortical.

(5 versus 3 years of treatment; Supplementary Fig. S1). Despite a higher DMB with DMAb, and a much smaller and shorter clinical use, the incidence rate of AFFs with DMAb is lower than with oral bisphosphonate (8 vs 113.1/100,000 cases per year) and is not duration-dependent. Thus, it appears that the impact of BPs is more dependent on duration of adsorption to bone matrix than the duration or potency of their antiresorptive effect (and thus of the magnitude of bone remodeling suppression). Several studies showed that long-term users of BPs (≥5 years) have increased AFF risk than short-term users of BPs, supporting this reasoning.[41]

Microhardness was not different between non-AFF and AFF groups, whereas higher values were expected in the AFF group as DMB was increased. Indeed, DMB and microhardness often evolve in the same way, as previously shown in control or untreated osteoporotic bone,[25] but not always, especially in BPs-treated osteoporotic bone.[19] By histomorphometry, although there was no difference in dynamic bone turnover variables between AFF and non-AFF, both cortical and cancellous W.Th were significantly lower ($p = .04$, $p = .03$, respectively) in AFF than in non-AFF, meaning that the interstitial bone volume was higher in AFF. This could explain why a higher DMB was found in AFF than non-AFF by X-ray microradiography (measured on the entire whole-bone section), whereas no differences were detected by microhardness focal measurements. However, the values of microhardness in non-AFF and AFF groups were within physiological range and similar to values found in healthy women after menopause.[42] Vickers microindentation performed in the present study was not able to differentiate the respective contributions related to elastic and plastic parts, as with nanoindentation or instrumented indentation. Thus, the percentage of increase in the DMB in AFF group compared to non-AFF (+2.9%) might be too low to produce a significant increase in microhardness values that could be measured by Vickers microindentation. A study performed by nanoindentation on a subset of the bone biopsies used in the present study (14 biopsies in each group from Detroit) showed that elastic modulus was higher in cortical bone with AFF than without AFF ($E^{AFF} = 16.18 \pm 0.12$ GPa versus $E^{Non\text{-}AFF} = 15.83 \pm 0.11$ GPa, $p = .0448$), and that plastic energy (damage) was greater in AFF than in non-AFF.[43] Thus, impact of the small increase in DMB on elastic modulus was highlighted with nanoindentation.

No differences were reported between both groups in variables measured by FTIRM, indicating that there were no differences in crystal size, mineral maturity, mineral/organic ratio, carbonation, or collagen maturity between AFF and non-AFF groups. A higher mineral/organic ratio in AFF group was expected as a higher DMB (absolute value of mineralization) was observed in this group. As for microhardness, FTIRM measurements were assessed as focal measurements whereas DMB consider the entire bone section. Furthermore, mineral/organic ratio is a relative value of mineralization (organic content may simultaneously increase). Because no difference in bone remodeling activity was found between both groups, the absence of difference in the FTIRM variables is not surprising, because the variables measured have likely reached their "plateau". Crystallinity was decreased in long-term alendronate users (6.4 ± 2.0 years), compared with untreated PMOP women, and this was associated with a decrease of the microhardness.[39] These two variables were not modified in non-AFF and AFF groups, likely because patients in both groups were treated with BPs. However, as for microhardness data, the values of bone intrinsic variables measured by FTIRM in non-AFF and AFF groups were within physiological range and similar to values found in healthy women after menopause.[42] Other mechanisms involved in bone fragility and not assessed in this study might also have a role in the genesis of AFF. First, the accumulation of microcracks has been suggested to increase with the duration of BPs therapy.[44] Nonetheless, we have shown that the microcrack frequency in the iliac bone of long-term BPs users was low, despite the marked reduction in bone remodeling, compared to controls (iliac bone from cadavers).[45] It should be noted that femoral stress fractures initiate in the lateral cortex (area with high tensile stresses), and are affected by the femoral geometry (greater lateral curvature and varus alignment in AFF).[46] Thus, as the mechanical strain is high is this area, the microcrack frequency is perhaps much higher than in iliac bone where the mechanical load is low. Second, another significant factor in bone fragility is the presence of advanced glycation end products (AGEs), known to reduce the capacity of bone matrix to dissipate energy.[47] AGEs exponentially increases with age and with senescence of tissues.[48] Because the AFF group was treated longer on average than non-AFF, and bone remodeling being consequently decreased longer, bone matrix from AFF likely contains more AGEs than non-AFF. However, this needs to be confirmed by additional studies.

The main result of this study is that the duration-dependent increase in DMB after long-term BP therapy leads to a significantly higher DMB in AFF than in non-AFF patients. However, all the values were within the physiological range, for microhardness and others variables assessed by FTIRM, indicating an absence of deleterious effect of BPs on bone characteristics assessed in this study. Numerous recent studies highlighted the role of osteocytes in the local regulation of mineral homeostasis by sensing the mineralization levels in the surrounding matrix, while the mechanisms are still unknown.[49,50] Recently, in a mouse model, it has been shown that a deletion of receptor tyrosine kinase ligand Ephrin-B2 in osteocytes led to osteocyte autophagy and to a higher and more rapid secondary mineralization and matrix maturation than wild-type mice. While the primary mineralization was normally initiated, a brittle bone phenotype was observed, making the Ephrin-B2 as a potential candidate for the regulation of secondary mineralization and limitation of mineral accumulation.[51,52] Both elevated exocytic matrix vesicles production and autophagy were observed in those osteocytes deficient in Ephrin B2. Thus, EphrinB2 suppresses autophagic processes and limits matrix vesicle release via RhoA-ROCK signaling.

Furthermore, alendronate inhibits farnesyl pyrophosphate synthase, an enzyme in the mevalonate pathway needed to produce geranylgeranyl groups, and affects the osteoblast actin cytoskeleton (and also osteoclasts) through impairing the activation of RhoA.[53]

In osteoblasts, autophagy is involved in mineralization and bone homeostasis through the autophagic vacuoles used as vehicles in osteoblasts to secrete apatite crystals, and an autophagy deficiency reduces the mineralization capacity.[54] Zoledronic acid has been shown to induce autophagy in breast cancer cells.[55] Considering the strong binding affinity of BPs for hydroxyapatite,[56] their presence around the osteocyte lacunae and around the lacuna-canalicular osteocyte network,[57] their long-term accumulation in AFF might disrupt the ability of osteocytes to regulate the mineral homeostasis during the secondary mineralization and sense the mechanical strains.

Our study has several limitations. First, the sample size is small although noticeable for this rare condition, and duration of therapy was longer in the AFF group. Second, bone biopsies were not obtained at the site of AFF with potential differences in bone material quality between non-weight-bearing and weight-bearing bones. Third, others intrinsic bone properties of women with AFF might differ even before therapy from those without AFF, which might not be captured by our retrospective design.

In conclusion, the AFF group had a BP therapy duration-dependent increase in DMB leading to a significantly higher DMB than the non-AFF mainly in cortical bone, which persisted even after adjustment for the duration of treatment. Considering the high affinity of BPs to bone mineral, and their lining along the walls of the osteocyte lacunae, the accumulation of matrix bound BPs in AFF could lead to inhibition of the osteocyte cytoskeleton, blunting their response to mechanical strains. This hypothesis remains to be further investigated.

Disclosures

The authors have no direct conflicts of interest on the study on AFF patients. However, RDC had received grants for non-AFF patients. DF, SNM, and GB report grants from AMGEN, outside the submitted work. LM reports personal fees from Amgen, personal fees from Sanofi, personal fees from Eli Lilly, personal fees from Abbvie, personal fees from Pfizer, outside the submitted work. LGSM reports personal fees from Amgen, personal fees from Eli Lilly, personal fees from AstraZeneca, outside the submitted work. PC reports grants from Amgen, UCB Pharma, outside the submitted work. SDR, SQ, and SR have nothing to disclose. RDC reports grants and personal fees from Amgen, grants and personal fees from UCB, personal fees from Lilly, during the conduct of the study; grants and personal fees from Chugai, personal fees from Amolyt, personal fees from Inventiva, grants and personal fees from MSD, personal fees from Pfizer, personal fees from Abbvie, grants and personal fees from Chugai, personal fees from Sanofi, personal fees from BMS, grants from Fresenius-Kiabi, personal fees from Novartis, personal fees from Janssen, outside the submitted work. JPB reports grants and personal fees from Amgen, grants from Mereo BioPharma, grants from Radius Health, grants and personal fees from Servier, outside the submitted work.

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Authors' roles: Study design: DF, LGSM, JPB, and GB. Study conduct: DF, SR, GB. Data collection: DF, SR, LGSM, LM, SM, SQ, RC, SDR, JPB, and GB. Data analysis: DF, SR, LGSM, LM, SM, SQ, SDR, JPB, and GB. Data interpretation: DF, SR, LGSM, LM, SM, SQ, PC, RC, SDR, JPB, and GB. Drafting manuscript: DF and JPB. Revising manuscript content: all authors. Approving final version of manuscript: all authors. DF takes the responsibility for the integrity of the data analysis.

Author contributions: DF: Conceptualization; formal analysis; investigation; methodology; project administration; resources; supervision; validation; visualization; writing-original draft; writing-review and editing. SR: Investigation; validation; writing-review and editing. LGSM: Conceptualization; investigation; methodology; resources; validation; writing-review and editing. LM: Investigation; resources; validation; writing-review and editing. SM: Investigation; resources; validation; writing-review and editing. SQ: Investigation; resources; validation; writing-review and editing. PC: Validation; writing-review and editing. RC: Resources; validation; writing-review and editing. SDR: Funding acquisition; investigation; methodology; resources; validation; writing-review and editing. JPB: Conceptualization; investigation; resources; supervision; validation; visualization; writing-original draft; writing-review and editing. GB: Conceptualization; investigation; resources; validation; writing-review and editing.

PEER REVIEW

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