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Reduction of Cortical Bone Turnover and Erosion Depth After 2 and 3 Years of Denosumab: Iliac Bone Histomorphometry in the FREEDOM Trial

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ABSTRACT

Denosumab, a RANKL inhibitor, reduced the risk of vertebral, hip, and nonvertebral fractures in the Fracture REduction Evaluation of Denosumab in Osteoporosis every 6 Months (FREEDOM) trial of postmenopausal women with osteoporosis compared with placebo. Previous bone histomorphometric analysis in FREEDOM showed decreased bone resorption and turnover in cancellous bone after 2 and 3 years. The purpose of the present study was to evaluate the effects of denosumab compared with placebo in the cortical compartment from transiliac bone biopsies obtained during FREEDOM. A total of 112 specimens were evaluable for cortical histomorphometry, including 67 obtained at month 24 (37 placebo, 30 denosumab) and 45 at month 36 (25 placebo, 20 denosumab). Eroded surface, osteoclast surface, erosion depth, and wall thickness were measured on the endocortical surface. Cortical thickness and cortical porosity were also measured. Dynamic parameters of bone formation were assessed for endocortical, periosteal, and intracortical envelopes. Endocortical osteoclast surface, eroded surface, and mean and maximum erosion depth were significantly lower in the denosumab group versus placebo at months 24 and 36 ($p < 0.0001$ to $p = 0.04$). Endocortical wall thickness and intracortical measures (cortical porosity and cortical thickness) were not different between the two groups. Dynamic parameters were low with tetracycline labels in cortical bone observed in 13 (43%) and 10 (50%) of denosumab biopsies at months 24 and 36, respectively, reflecting a marked decrease in bone turnover. In conclusion, our data reveal the mechanism of action of denosumab on cortical bone: inhibition of osteoclastic resorption and reduced activation of new remodeling sites. In addition, reduced endocortical erosion depth with no change of wall thickness may contribute to increased bone strength by reducing the bone loss and fragility associated with deep resorption cavities and may likely contribute to the greater BMD gain with denosumab than with other antiresorptive agents. © 2018 The Authors. *Journal of Bone and Mineral Research* Published by Wiley Periodicals Inc.

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Introduction

Denosumab is a fully human monoclonal antibody with a high affinity for RANKL, an essential factor for osteoclast differentiation and activity.[1–3] Denosumab binds and reversibly inhibits the activity of human RANKL and, therefore, osteoclast-mediated bone resorption. In contrast to bisphosphonates, which are incorporated into bone and taken up by osteoclasts during the resorption phase, denosumab directly acts on osteoclast precursors to inhibit osteoclast differentiation. In previous clinical trials, denosumab decreases bone turnover, increases bone mineral density[4–6] and reduces the risk of new vertebral, hip and nonvertebral fractures[7] after 2 or 3 years. A progressive increase in BMD with a low fracture incidence is confirmed after 10 years of treatment.[8] Histomorphometric studies performed on transiliac bone biopsies in women with postmenopausal osteoporosis have shown a marked reduction in bone remodeling, both resorption and formation, in cancellous bone after 2 and 3 years of denosumab.[9] This decreased cancellous bone turnover is maintained after an extension of the treatment up to 5 and 10 years.[10,11] The analysis of bone biopsies obtained approximately 2 years after

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discontinuation of denosumab treatment confirmed that these effects are reversible,[12] which is consistent with the known mechanism of action of denosumab.

The trabecular compartment of bone biopsies has represented the focus of histomorphometry evaluation for the published literature and regulatory requirements as part of evaluation of a new molecular entity. Static and dynamic incidences of bone remodeling at the tissue level in cortical bone has not received as much attention, despite the fact that cortical bone comprises 80% of the skeletal mass. In the evaluation of denosumab as a therapeutic agent for osteoporosis, cortical BMD and thickness assessed by high-resolution peripheral quantitative computed tomography (HR-pQCT) at the tibia and radius increase with denosumab treatment compared with placebo.[13] In addition, there was evidence of a reduction in cortical porosity in bone biopsies as assessed by micro–CT (μCT) at month 24, though not month 36.[9] Similarly, in the cynomolgus monkey, there was evidence of a decrease in cortical porosity with treatment.[14] Given the suggestion of an effect of therapy in the cortical compartment in both preclinical and clinical models, the present study evaluated the effects of denosumab at the tissue level in periosteal, intracortical, and endocortical compartments in bone biopsies obtained after 2 and 3 years of treatment in the Fracture REduction Evaluation of Denosumab in Osteoporosis every 6 Months (FREEDOM) trial.

Patients and Methods

Study population

Patients were included in the FREEDOM study, which has been previously described in detail (ClincialTrials.gov: http://clinicaltrials.gov/show/NCT00089791).[7,9] Briefly, FREEDOM was a 3-year international, randomized, double-blind, placebo-controlled trial in women with postmenopausal osteoporosis. Patients received either subcutaneous denosumab 60 mg every 6 months or placebo for 3 years. All patients were supplemented with daily calcium (≥1000 mg) and vitamin D (≥400 IU). Ambulatory women with osteoporosis, aged 60 to 90 years, were considered eligible if a *T*-score measured by DXA at the lumbar spine or the total hip was less than −2.5 SD and greater than or equal to −4 SD at both sites. Patients were excluded if they had received oral bisphosphonates for more than 3 years or had taken oral bisphosphonates for more than 3 months and the last dose was within 1 year of enrollment, had history of disease affecting bone metabolism other than osteoporosis, or were intolerant or contraindicated to tetracycline or its derivatives. Women were also excluded if they had used intravenous bisphosphonates, fluoride, or strontium ranelate within the past 5 years or parathyroid hormone or its derivative, corticosteroids, systemic hormone-replacement therapy, selective estrogen-receptor modulators, tibolone, calcitonin, or calcitriol within 6 weeks before the study enrollment.

Bone histomorphometry

A transiliac bone biopsy was performed with a 7.5-mm inner diameter trephine at month 24 and/or month 36 in patients who enrolled in the bone biopsy substudy. Prior to bone biopsy, patients received double tetracycline labeling, 1 g per day tetracycline hydrochloride according to the following schedule: 3 days on; 14 days off; 3 days on. Biopsies were performed within 5 to 14 days after the last dose of tetracycline. The bone biopsy specimens were stored in 70% ethanol then dehydrated and embedded in glycolmetacrylate.[9]

Additionally, for the current analysis, three sets of 8-μm-thick sections were cut 200 μm apart. In each set, sections were stained with modified Goldner's trichrome or Solochrome cyanin R. Some sections were left unstained for the measurement of the tetracycline labels under fluorescence.[15] In contrast to past assessment, an extended search for tetracycline labels was not undertaken.[9] Histomorphometric analysis was performed on endocortical (Ec), intracortical (Ct), and periosteal (Ps) envelopes of three sections (one per set). According to published methods,[16] the endocortical envelope is defined as the area starting immediately beneath the inner cortex and ending at a distance corresponding to a twofold thickness of the largest trabecula originating from the cortices. The endocortical surface is the border between the endocortical and cancellous area. For all the analyses, the investigators were blinded to treatment allocation.

The parameters of bone structure were measured with an automatic image analyzer (Bone V3.5; Explora Nova, La Rochelle, France). The parameters reflecting bone resorption were measured after rebuilding the resorption cavity by using an interactive automatic-image analyzer as described.[17] The wall thickness and the dynamic parameters of bone formation and mineralization were measured by using a semiautomatic image analyzer (Tablet'Measure V1.54; Explora Nova, La Rochelle, France). The calculations and abbreviations of the bone histomorphometric parameters used were as recommended by the ASBMR Histomorphometric Nomenclature Committee.[18] All measured thicknesses (except cortical thickness) were multiplied by $\pi/4$. Intracortical structural parameters were cortical thickness (Ct.Th, μm) and porosity (Ct.Po, %). Bone resorption was assessed at the endocortical surface with measurements of eroded surfaces (Ec-ES/BS, %), osteoclast number (Ec-Oc.N/BS, 1/mm) and surface (Ec-Oc.S/BS, %), mean and maximum erosion depth (Ec-E.De mean and Ec-E.De max, μm) on Goldner-stained sections. The wall thickness on endocortical surface (Ec-W.Th, μm) was measured on Solochrome cyanin R–stained sections, under polarized light. The mineral apposition rate (MAR, μm/day) and the ratio of mineralizing surface to bone surface (MS/BS, % calculated as double plus half of single-labeled surfaces) were measured on unstained sections under ultraviolet light, in the three envelopes. Bone formation rate (BFR/BS [$\mu m^3/\mu m^2$/day] = (MS/BS) × MAR) was calculated. In this analysis, for biopsy specimens missing label in the endocortical and intracortical envelopes, the value of MS/BS was set to zero and the derived parameters, MAR and BFR/BS, were missing values.[19] When only single labels were present, MAR and BFR/BS were considered both as missing values and expressed after imputation of a value of 0.3 μm/day for MAR.[19]

Statistical analysis

Analysis included data from all patients who received at least one dose of investigational product and had one or more biopsies evaluable for cortical bone histomorphometry. Qualitative assessment of labeling status was summarized using frequency and percentage. Continuous cortical bone histomorphometric variables were summarized using median, 25th percentile (Q1), and 75th percentile (Q3). Two-sided Wilcoxon rank sum test was used for all comparisons between two groups without multiplicity adjustment.

Results

The demographic characteristics were similar in the two groups (Table 1). A total of 112 biopsies obtained from 90 patients (45 placebo and 45 denosumab) were evaluable for cortical bone histomorphometry, including 67 obtained at month 24 (37 placebo, 30 denosumab) and 45 at month 36 (25 placebo, 20 denosumab).

Qualitative observations

A tetracycline label search was performed on the unstained sections cut at three different planes, in addition to those previously analyzed.[9] Tetracycline labels (single and/or double labels) were present in 36 (97%) and 24 (96%) biopsies in the placebo group at month 24 and month 36, respectively. In the denosumab group, single and/or double labels were observed in 13 (43%) and 10 (50%) biopsies at month 24 and month 36, respectively, and double labels were present in four (13%) and five (25%) biopsies at month 24 and month 36, respectively. Fourteen bone samples had only one cortex.

Endocortical bone

At month 24, endocortical bone resorption was significantly decreased in the denosumab group as reflected by the decrease in the Ec-ES/BS and Ec-Oc.S/BS when compared to placebo ($p < 0.0001$). At month 36, Ec-ES/BS and Ec-Oc.S/BS remained significantly decreased ($p = 0.044$ and $p = 0.03$, respectively). In addition to a decrease in the extent of eroded surfaces, denosumab significantly reduced the amount of bone resorbed as shown by the decreased erosion depth (Ec-E.De mean and Ec-E.De max), at both month 24 and month 36 (Table 2). Ec-MS/BS was decreased when compared to placebo ($p < 0.0001$), at both month 24 and month 36. Double labels were not found in all biopsies in the denosumab-treated group at month 24 and present in only one biopsy at month 36. Consequently, tetracycline-based parameters could not be calculated, except by imputation for MAR. Ec-W.Th, the amount of bone formed at the individual bone structural unit (BSU), was similar in both groups at each time point.

Intracortical and periosteal bone

Ct.Po and Ct.Th were not different in placebo and denosumab groups. In intracortical bone, the effects of denosumab were similar to those on the endocortex at both month 24 and

Table 1. Demographic Data at Baseline

	Placebo ($n = 45$)	Denosumab ($n = 45$)
Age (years)	70.0 ± 6.3	72.4 ± 5.0
Years since menopause	23.0 ± 8.2	25.2 ± 7.3
Body mass index (kg/m^2)	26.1 ± 4.0	26.2 ± 4.1
Bone mineral density, *T*-score		
Lumbar spine	−2.9 ± 0.5	−2.9 ± 0.5
Total hip	−1.8 ± 0.8	−2.0 ± 0.8
Femoral neck	−2.0 ± 0.7	−2.3 ± 0.7
Serum CTX (ng/mL)	0.5 ± 0.3	0.5 ± 0.2
Serum P1NP (ng/mL)	59.0 ± 38.6	54.0 ± 18.2

Results are mean ± SD.

CTX = C-telopeptide of type 1 collagen; P1NP = procollagen type 1 N-terminal propeptide.

Table 2. Endocortical Bone Histomorphometric Parameters

	Month 24					Month 36				
	Placebo (N = 37)		Denosumab (N = 30)			Placebo (N = 25)		Denosumab (N = 20)		
Parameter	*n*	Median (Q1, Q3)	*n*	Median (Q1, Q3)	*p*	*n*	Median (Q1, Q3)	*n*	Median (Q1, Q3)	*p*
Ec-W.Th (μm)	37	32.41 (31.16, 33.83)	29	32.39 (31.68, 34.52)	0.52	24	32.11 (30.48, 33.05)	16	31.82 (29.30, 33.41)	0.79
Ec-Oc.S/BS (%)	37	0.26 (0.00, 0.59)	30	0.00 (0.00, 0.00)	**<0.0001**	25	0.14 (0.00, 0.22)	19	0.00 (0.00, 0.00)	**0.030**
Ec-ES/BS (%)	37	4.40 (3.26, 7.60)	30	1.59 (1.01, 2.17)	**<0.0001**	25	3.53 (1.92, 5.32)	19	1.39 (0.67, 3.52)	**0.044**
Ec-E.De max (μm)	37	16.45 (12.88, 21.61)	30	11.43 (9.00, 13.11)	**<0.0001**	25	17.40 (12.13, 21.01)	19	14.04 (6.92, 15.99)	**0.024**
Ec-E.De mean (μm)	37	10.26 (8.32, 13.34)	30	7.10 (6.03, 8.69)	**<0.0001**	25	10.14 (7.39, 12.87)	19	7.91 (4.94, 9.65)	**0.019**
Ec-MAR (μm/day)[a]	35	0.60 (0.54, 0.65)	0	–		22	0.58 (0.51, 0.65)	1	0.58 (0.58, 0.58)	0.94
Ec-MAR (μm/day)[b]	35	0.60 (0.54, 0.65)	1	0.30 (0.30, 0.30)	0.10	22	0.58 (0.51, 0.65)	4	0.30 (0.30, 0.44)	**0.017**
Ec-MS/BS (%)	37	7.40 (3.66, 10.56)	30	0.00 (0.00, 0.00)	**<0.0001**	25	5.17 (3.67, 10.37)	20	0.00 (0.00, 0.00)	**<0.0001**
Ec-BFR/BS (μm^3/μm^2/day)[a]	35	0.044 (0.03, 0.07)	0	–		22	0.04 (0.02, 0.06)	1	0.018 (0.02, 0.02)	0.26
Ec-BFR/BS (μm^3/μm^2/day)[b]	35	0.044 (0.03, 0.07)	1	0.002 (0.00, 0.00)	0.10	22	0.040 (0.02, 0.06)	4	0.001 (0.00, 0.01)	**0.004**

Bold *p* values are significant.

N = number of biopsies analyzed; *n* = number of biopsies with measurements; Q1 = 25th percentile; Q3 = 75th percentile; Ec = endocortical; W.Th = wall thickness; Oc.S = osteoclast surface; BS = bone surface; ES = eroded surface; E.De = erosion depth; max = maximum; MAR = mineral apposition rate; MS = mineralizing surface; BFR = bone formation rate.

[a]Wilcoxon rank sum test without imputation when only single labels were present.

[b]Wilcoxon rank sum test with imputation when only single labels were present.

Table 3. Cortical Bone Histomorphometric Parameters

	Month 24					Month 36				
	Placebo (N = 37)		Denosumab (N = 30)			Placebo (N = 25)		Denosumab (N = 20)		
Parameter	*n*	Median (Q1, Q3)	*n*	Median (Q1, Q3)	*p*	*n*	Median (Q1, Q3)	*n*	Median (Q1, Q3)	*p*
Intracortical bone										
Ct.Th (μm)	37	749.3 (572.0, 904.9)	30	753.6 (521.3, 1022.0)	0.85	25	689.7 (478.7, 920.4)	19	605.8 (491.0, 799.3)	0.57
Ct.Po (%)	37	6.43 (4.56, 9.34)	30	5.90 (3.83, 8.12)	0.16	25	5.42 (3.77, 7.42)	19	6.37 (4.39, 7.88)	0.52
Ct-MAR (μm/day)[a]	34	0.62 (0.54, 0.72)	3	0.59 (0.54, 0.60)	0.45	22	0.60 (0.54, 0.64)	3	0.65 (0.55, 0.67)	0.48
Ct-MAR (μm/day)[b]	36	0.61 (0.53, 0.71)	6	0.42 (0.30, 0.59)	**0.03**	23	0.59 (0.52, 0.64)	3	0.65 (0.55, 0.67)	0.42
Ct-MS/BS (%)	37	18.22 (6.50, 29.79)	30	0.00 (0.00, 0.00)	<**0.0001**	25	14.06 (7.04, 23.86)	20	0.00 (0.00,0.36)	<**0.0001**
Ct-BFR/BS ($\mu m^3/\mu m^2$/day)[a]	34	0.115 (0.05, 0.19)	3	0.008 (0.00, 0.01)	**0.006**	22	0.105 (0.06, 0.16)	3	0.047 (0.01, 0.13)	0.26
Ct-BFR/BS ($\mu m^3/\mu m^2$/day)[b]	36	0.108 (0.04, 0.18)	6	0.006 (0.00, 0.01)	**0.0003**	23	0.101 (0.05, 0.16)	3	0.047 (0.01, 0.13)	0.30
Periosteal bone										
Ps-MAR (μm/day)[a]	12	0.59 (0.49, 0.65)	1	0.61 (061, 0.61)	0.89	6	0.54 (0.52, 0.61)	1	0.82 (0.82, 0.82)	0.21
Ps-MAR (μm/day)[b]	22	0.42 (0.30, 0.59)	8	0.30 (0.30, 0.30)	**0.07**	15	0.30 (0.30, 0.54)	6	0.30 (0.30, 0.30)	0.52
Ps-MS/BS (%)	36	0.54 (0.00, 2.55)	30	0.00 (0.00, 0.29)	**0.006**	25	0.40 (0, 1.71)	20	0.00 (0.00, 0.23)	**0.041**
Ps-BFR/BS ($\mu m^3/\mu m^2$/day)[a]	12	0.015 (0.01, 0.03)	1	0.003 (0.00, 0.00)	0.14	6	0.013 (0.01, 0.03)	1	0.03 (0.03, 0.03)	0.45
Ps-BFR/BS ($\mu m^3/\mu m^2$/day)[b]	22	0.008 (0.00, 0.02)	8	0.005 (0.00, 0.01)	0.31	15	0.005 (0.00, 0.01)	6	0.003 (0.00, 0.01)	0.51

Bold *p* values are significant.

N = number of biopsies analyzed; *n* = number of biopsies with measurements; Q1 = 25th percentile; Q3 = 75th percentile; Ct = cortical; Th = thickness; Po = porosity; MAR = mineral apposition rate; MS = mineralizing surface; BS = bone surface; BFR = bone formation rate; Ps = periosteal.

[a]Wilcoxon rank sum test without imputation when only single labels were present.

[b]Wilcoxon rank sum test with imputation when only single labels were present.

month 36 (Table 3). Ct-MAR and consequently Ct-BFR/BS could be calculated in only three biopsies in the denosumab group, double labels being absent in the other biopsies.

The bone turnover rate was low in the periosteal envelope as shown by the low values of Ps-MS/BS and Ps-BFR/BS in placebo-treated samples and trended lower after denosumab treatment (Table 3).

Discussion

Consistent with what was observed in cancellous bone,[9] denosumab induced a marked reduction of resorption and formation in cortical bone, reflecting decreased bone remodeling on the endocortical surfaces and in intracortical bone. The absence of any tetracycline labels in 54% of the biopsies and of double labels in 82% of the biopsies in the denosumab group at months 24 or 36 reflected a markedly reduced bone turnover in endocortical, intracortical, and periosteal envelopes after 2 and 3 years. This observation confirmed the extensive label search that previously reported the absence of any cortical label in 43% of these biopsies.[9] However, in the present study, the absence of tetracycline labels in the analyzed slides did not mean that denosumab totally suppresses bone remodeling throughout the entire skeleton. It has been previously shown, in the same population, that bone formation markers were similar in patients with unlabeled biopsies to those with single and/or double labels.[9] The new information in this assessment was the quantification of the decrease in eroded and osteoclast surfaces as well as the erosion depth, which reflects the decreased osteoclast activity in endocortical bone. Decreased erosion depth may contribute to a reduction of bone loss on endocortical bone surfaces. Consistent with observations in cancellous bone,[9] endocortical wall thickness—a reflection of the amount of bone formed at each remodeling site—was unchanged. These observations suggest that at the BMU level, denosumab reduced the volume of bone resorbed and the resorption cavities were fully re-filled with new bone. Of note, erosion depth was measured independently of the stage of the resorption, including erosion cavities not totally achieved[17]; hence, the erosion depth was likely underestimated in both the denosumab and placebo groups, and thus bone balance at the BMU level could not be calculated. These effects observed at the BSU level may result in an increase in Ct.Th, but no significant effect on Ct.Th could be evidenced. As cortical bone represents 80% of the skeletal mass, small changes at the cortical level may have substantial effects.

Increased depth of resorption cavities lead to trabecular perforation and in cortical bone to endosteal porosity and conversion to a trabecular-like structure.[20] Deeper erosion cavities may also cause exaggerated reduction in bone strength via stress-rising effects that may contribute to skeletal fragility.[21] In the current study, decreased erosion depth with an unchanged W.Th after denosumab suggests a less negative BMU balance and the potential for association with improved bone strength. Translation to clinical outcomes demonstrated that denosumab was associated with reduction of hip and non vertebral fracture risk reported in the FREEDOM trial.[7] In contrast to denosumab, bisphosphonates such as alendronate inhibit bone resorption, but no significant diminution of the cancellous erosion depth is observed.[22] This probably contributed to the greater increase in BMD with denosumab than alendronate.[13,23] In contrast to alendronate, which reduces bone resorption after osteoclasts resorb bone tissue containing bisphosphonate, denosumab rapidly inhibits osteoclast differentiation. Fewer newly eroded sites appear with denosumab, which, associated with the re-filling of the preexisting resorption cavities and the increased matrix mineralization,[11] results in a greater gain in BMD.[13,23] An increase in cortical thickness with denosumab treatment has been observed in the distal tibia and radius assessed by HR-pQCT.[13] The increase in tibial BMD was detected earlier at cortical than cancellous sites. In the present study, we did not see a decrease in cortical porosity, which is in contrast to the transient observation at year 2 in the earlier study of the same biopsy samples from the FREEDOM study[9] and in proximal femur analyzed by QCT.[24] The measurement of Ct.Po may have been influenced by the methods using 2D or 3D imaging with different resolutions.[25] Histomorphometry allows the detection of small pores with a diameter of about 5 μm when the pores detected by μCT depend on the resolution used, which was 27 μm in the earlier assessment.[9] In addition, we cannot exclude a decrease in the pore diameter. Specifically, Zebaze and colleagues[26] raised the hypothesis that after denosumab injection, the osteoclast activity is stopped in the existing cavities, which are slowly refilled by the osteoblasts. Before the next injection, new remodeling sites appear, which are rapidly stopped with the subsequent dose, thereby explaining a progressive decrease in the pore diameter. Finally, reproducible identification of the transition from cortical to cancellous bone is difficult from 3D to 2D methods.[27]

The study has a number of limitations, including a relatively small sample size and the absence of baseline biopsies. In addition, erosion depth was measured independently of the stage of resorption and no data were available on final erosion depth, which precluded calculation of bone balance at the BMU level.

In conclusion, denosumab inhibits osteoclast formation and activity in cortical bone as reflected by the reduced erosion depth and surface. In addition to a lesser extent of osteoclastic erosion surface, reduced erosion depth, which is a novel finding for denosumab, may contribute to increased bone strength by reducing endocortical bone loss and the fragility associated with the stress-rising effects of deep resorption cavities. The present study confirms that denosumab strongly reduces cortical bone turnover but without change in the volume of bone formed at the individual BSU level, which may contribute to the BMD gain during denosumab treatment and consequently the reduced fracture risk observed in FREEDOM trial.

Disclosures

PC has received travel grants from Amgen Inc. and UCB. DWD has received grant and research support and served on speakers bureaus for Amgen Inc. and Eli Lilly, and has served as a consultant for Amgen Inc., Eli Lilly, Radius, Tarsa, and Ascendis. JPR has received travel grants from Amgen Inc. SH, RBW, and AW are employees of and own stock and/or stock options in Amgen Inc. RC has received grants and research support from Merck, Chugai-Roche, and Amgen Inc., has served as a consultant for Amgen Inc., Sandoz, Radius, UCB, and Janssen, and has received speaking fees from Pfizer, Abbvie, BMS, MSD, Lilly, and Amgen Inc. NPM has no conflicts of interest.

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Authors' roles: PC and RC had full access to the data and developed the initial and subsequent drafts of the manuscript. PC, SH, and RC conducted the study. PC, RC, NPM, JPR, SH, and RBW interpreted the data. AW performed the analyses according to prespecified statistical analysis plan. All authors revised and approved the final version of the manuscript and the decision to submit the manuscript for publication.

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