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Changes in Dkk-1, sclerostin, and RANKL serum levels following discontinuation of long-term denosumab treatment in postmenopausal women☆

A. Fassio[a,*], G. Adami[a,b], C. Benini[a], E. Vantaggiato[a], K.G. Saag[b], A. Giollo[a], I. Lippolis[a], O. Viapiana[a], L. Idolazzi[a], G. Orsolini[a], M. Rossini[a], D. Gatti[a]

[a] Rheumatology Unit, University of Verona, Piazzale A. Scuro, 10, 37134 Verona, Italy
[b] Division of Clinical Immunology and Rheumatology, University of Alabama at Birmingham, 510 20th Street South, Faculty Office Tower 820D, Birmingham, AL 35294, USA

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ABSTRACT

Purpose: The positive effects of denosumab (DMAb) on bone mineral density (BMD) are quickly reversible after its discontinuation. We investigated whether this rebound was associated with dysregulation of the Wnt canonical pathway and/or by the increase in the receptor-activator of nuclear factor-kappa B ligand (RANKL) serum levels.

Methods: The study included patients (n = 15) with postmenopausal osteoporosis to whom DMAb was administered for 78 months and then discontinued. We collected BMD data at baseline/month 0 (M0), M60, M84 (6 months after last DMAb administration, coinciding when the next DMAb dose would typically be due), and after 3 and 12 months of follow-up (FU-M3 and FU-M12, respectively). Serum C-terminal telopeptide of type 1 collagen (CTX-I), Dickkopf-1 (Dkk-1), and sclerostin were measured at M0, M60, M84, FU-M3, and FU-M12. Serum N-terminal propeptide of type 1 procollagen (PINP) and RANKL were dosed at M60, M84, FU-M3, and FU-12.

Results: We found a significant decrease in the T-score at all sites at FU-M12, when compared to M84 (−0.51 ± 0.91 at the lumbar spine; −0.72 ± 0.33 at the total hip; and −0.42 ± 0.27 at the femoral neck, $p < 0.05$). After DMAb discontinuation (M84 vs FU M12) CTX-I, PINP increased already at FU-M3 (+0.921 ± 0.482 ng/mL, +126.60 ± 30.36 ng/mL, respectively, $p < 0.01$), RANKL increased at FU-M12 (+0.041 ± 0.062 ng/mL, $p < 0.05$), while Dkk-1 and sclerostin decreased at FU-M12 (−10.90 ± 11.80 and − 13.00 ± 10.52 pmol/L, respectively, $p < 0.01$). No changes in BMD or any of the markers were found between M60 and M84.

Conclusions: RANKL serum levels progressively increased after discontinuation of long-term DMAb while Dkk-1 and sclerostin serum levels decreased. The increase in RANKL serum levels supports the hypothesis of a sudden loss of inhibition of the resting osteoclast line after DMAb clearance, with a hyperactivation of these cells. Our results suggest that the changes in serum Wnt inhibitors after DMAb suspension might represent a mere feedback response to the increased bone turnover.

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* Corresponding author at: Policlinico GB Rossi, Piazzale A. Scuro, 37134 Verona, Italy.
E-mail address: angelo.fassio@yahoo.it (A. Fassio).

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Summary

The rebound effect on bone metabolism occurring after denosumab discontinuation is a timely topic. WNT inhibitors and RANKL have been postulated as possible explanations, but without any data supporting this hypothesis. By investigating WNT inhibitors and RANKL serum levels after denosumab suspension, we provide relevant data concerning the physiology of the denosumab's loss-of-effect phase.

1. Introduction

Denosumab (DMAb) is a fully human monoclonal antibody that binds and inhibits the receptor activator of nuclear factor-kappa B ligand (RANKL) and is used in the treatment of postmenopausal osteoporosis [1]. DMAb has been shown to steadily increase bone mineral density (BMD), without a therapeutic plateau, not only at trabecular sites but also in cortical bone throughout 10 years of treatment [2]. An increasing number of case reports [3] and a recent post-hoc analysis [4] have raised concerns related to DMAb discontinuation owing to an increased fracture risk related to an unfavorable rebound in bone resorption following DMAb loss of effect.

Indeed, the gains in BMD have been shown to be lost upon discontinuation after 12 months [5,6], accompanied by a quick increase in bone turnover markers (BTMs) such as serum C-terminal telopeptide of type 1 collagen (CTX-I) and N-terminal propeptide of type 1 procollagen (PINP) [5]. Moreover, fracture incidence after Dmab discontinuation might have even been underestimated thus far due to methodological flaws [4].

In 2018, two ancillary studies from the FREEDOM trial, involving 12 and 38 patients, respectively, assessed BMD changes after DMAb discontinuation in patients who received the treatment for 10 years [7,8]. Both studies showed a rapid BMD decrease after DMAb loss of effect, with a drop to near baseline levels within one year from discontinuation. Unfortunately, no longitudinal data on BTMs were provided with these two studies.

The canonical Wnt pathway is currently considered the master regulator of bone remodeling. It promotes bone formation and inhibits osteoclastogenesis through its influence on the receptor activator of nuclear factor-kappa B ligand (RANKL)/osteoprotegerin pathway [9]. In addition, the secreted Wnt antagonists sclerostin and Dickkopf-1 (Dkk-1) block Wnt signaling by binding to Wnt coreceptors, such as low-density lipoprotein receptor-related protein 5 (LRP5) and LRP6, and inhibiting Wnt signaling [10].

The aim of this study was to evaluate the changes in serum levels of Dkk-1, sclerostin, RANKL, BTMs, and BMD in the year following DMAb discontinuation to investigate whether the rebound effect discussed above might be explained by major variations in the Wnt inhibitors and/or by an abrupt increase in RANKL serum levels.

2. Materials and methods

2.1. Study design

We conducted a prospective study in the Rheumatology Division of the *Azienda Ospedaliera Universitaria Integrata* of Verona. The study sample included women affected by postmenopausal osteoporosis without previous history of vertebral fractures who discontinued DMAb after 84 months of active treatment (last administration occurring at M78) due to achieving a lumbar spine T-score > -2.5 and/or concerns about the risk of osteonecrosis of the jaw and atypical fractures during long-term treatment. Exclusion criteria at baseline were: systemic inflammatory diseases; active infections; neoplasms; kidney, liver, endocrine, or metabolic bone diseases; corticosteroids; or drugs known to affect bone metabolism. All patients received calcium (1 g/day) and vitamin D (800 IU/day) supplements. None of the patients received treatment with bone acting drugs in the year of follow-up after DMAb discontinuation.

This study was approved by the institutional research committee (approval CE 1876) and was conducted in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all individual participants included in the study.

2.2. Bone mineral density assessment

BMD was assessed in all patients using DXA (QDR Hologic Delphi) at the lumbar spine (L1–L4), total hip, and femoral neck at treatment-baseline/month 0 (M0), follow-up (FU)/baseline (M84, coinciding with DMAB loss of effect), 3 months later (FU-M3), and 12 months later (FU-M12). The coefficient of variation was 1% for spinal and 1.2% for femoral BMD. Measurements are expressed as T-scores (difference in standard deviation (SD) from the mean of a healthy young adult). Vertebral morphometric fracture assessment (Genant's criteria) was performed at every DXA evaluation.

2.3. Biochemical assessment

Aliquots of serum samples for CTX-I, Dkk-1, and sclerostin were collected at M0 and dosed soon after collection. Aliquots of serum samples were also collected at M60, M84, FU-M3, and FU-M12, stored at $-50\,^{\circ}C$, and then assayed for parathyroid hormone (PTH), PINP, CTX-I, RANKL, sclerostin, and Dkk-1. All samples were processed in the Rheumatology Unit laboratory of the University of Verona in aggregate at the end of the study.

The bone turnover markers (PINP and CTX-I) and intact PTH were measured with the IDS-iSYS Multi-Discipline Automated System (Immunodiagnostic System, Boldon, UK) analyzer, based on chemiluminescence technology. The intra-assay coefficients of variation (CVs) measured in our laboratory were 4% for intact PINP (inter-assay CV 6%), 3% for CTX-I (inter-assay CV 7%), and 2.7% for intact PTH (inter-assay CV 5.5%). Serum Dkk-1 and sclerostin were measured by ELISA (Biomedica Medizinprodukte, Vienna, Austria), with a sensitivity of 1.7 pmol/L and 3.2 pmol/L and intra-assay CVs of 7% and 5% (inter-assay CVs 8.2% and 6.9%), respectively. Serum RANKL was measured using ELISA (Biomedica Medizinprodukte, Vienna, Austria) on the fully automated Microplate Analyzer Personal Lab (Adaltis Italia), according to the manufacturer's instructions.

2.4. Statistical analysis

All statistical analyses were performed with the SPSS software, Version 22 (SPSS, Inc., Chicago, IL, USA).

The paired sample *t*-test was used to test T-scores and serum marker values for significant differences between the observations at the various time points. Linear regression was used to test the absolute changes of the markers from M84 to FU-M12. Two-sided p-values of 0.05 or less were considered significant. Data are presented as mean ± SD.

3. Results

The study sample consisted of 15 patients, with a mean age of 76.8 ± 5.7 years, mean body weight of 60.5 ± 8.2 kg, and mean height of 157.3 ± 5.8 cm.

Comparing the BMD T-scores of treatment-baseline (M0) with those of M84, we found a significant increase in the lumbar spine T-score (1.58 ± 0.93; $p < 0.01$) and total hip T-score (0.47 ± 0.68; $p < 0.05$), with a trend toward a significant increase in the femoral

Table 1
Absolute values of BTMs, PTH, Wnt inhibitors, RANKL, and BMD at treatment baseline (M0), M84 (coinciding with DMAb loss of effect), and after 3 and 12 months of follow-up. Data reported as mean ± SD.

	Treatment baseline (M0)	FU-baseline (M84)	FU-M3	FU-M12
PINP ng/mL	NA	28.13 ± 11.76	154.73 ± 56.80 [a]	152.26 ± 61.29 [a]
CTX-I ng/mL	0.758 ± 0.428	0.144 ± 0.132 [c]	1.066 ± 0.50 [a]	0.891 ± 0.123 [a]
PTH pg/mL	NA	36.8 ± 20.5	19.2 ± 13.6 [a]	28.3 ± 10.6
Dkk-1 pmol/L	58.68 ± 27.31	44.37 ± 16.94 [c]	42.79 ± 17.52 [c]	33.47 ± 13.13 [a, c]
Sclerostin pmol/L	27.21 ± 10.94	48.22 ± 13.17 [c]	44.80 ± 14.89 [c]	35.22 ± 10.55 [a, c]
RANKL ng/mL	NA	0.050 ± 0.047	0.064 ± 0.046	0.092 ± 0.063 [b]
Lumbar spine T-score	−3.20 ± 0.37	−1.62 ± 0.89 [c]	−1.90 ± 0.86 [a, c]	−2.13 ± 1.02 [b, c]
Total hip T-score	−1.68 ± 0.58	−1.21 ± 0.86 [c]	−1.33 ± 0.84 [a]	−1.93 ± 0.69 [a]
Femoral neck T-score	−2.24 ± 0.48	−1.82 ± 0.84 (p = 0.05 vs. M0)	−1.93 ± 0.82	−2.24 ± 0.77 [a]

PINP: N-terminal propeptide of type 1 procollagen; CTX-I: C-terminal telopeptide of type 1 collagen; PTH: parathyroid hormone; RANKL: receptor activator of nuclear factor-kappa B ligand.
[a] $p < 0.01$ vs. FU-baseline (M84).
[b] $p < 0.05$ vs. FU-baseline (M84).
[c] $p < 0.05$ vs. treatment baseline (M0).

neck T-score (0.42 ± 0.79; p = 0.05). At FU-M3, when compared to M84, we found a statistically significant decrease in the T-scores at the lumbar spine and total hip (−0.28 ± 0.33 at the lumbar spine and − 0.12 ± 0.14 at the total hip, respectively, $p < 0.01$ for all observations), while the decrease at the femoral neck was not statistically significant. At FU-M12, when compared to M84, we found a statistically significant decrease in the T-scores at all sites (−0.51 ± 0.91 at the lumbar spine, $p < 0.01$; −0.72 ± 0.33 at the total hip, $p < 0.01$; and −0.42 ± 0.27 at the femoral neck, $p < 0.05$).

No significant changes for any of the assessed parameters (either biochemical or BMD) were found between M60 and M84 (data not shown).

Absolute values for CTX-I, Dkk-1, sclerostin, and BMD (expressed as T-scores) at treatment baseline (M0) and absolute values for PINP, CTX-I, PTH, Dkk-1, sclerostin, RANKL, and BMD (expressed as T-scores) at FU-baseline (M84), FU-M3, and FU-M12 are presented in Table 1. The absolute serum levels of Dkk-1 and sclerostin at all time points are also depicted in Fig. 1, while the absolute serum levels of RANKL at all time points are depicted in Fig. 2a.

At FU-M3, when compared to M84, we found a statistically significant increase in CTX-I and PINP (+0.921 ± 0.482 ng/mL and +126.60 ± 30.36 ng/mL, respectively, $p < 0.01$ for all observations). No significant change was found for Dkk-1, sclerostin, or RANKL, while PTH decreased significantly (−17.6 ± 14.05 pg/mL, $p < 0.01$).

At FU-M12, when compared to M84, we found a statistically significant increase in CTX-I, PINP, and RANKL (+0.846 ± 0.590 ng/mL, $p < 0.01$; +124.6 ± 62.72 ng/mL, $p < 0.01$; and +0.041 ± 0.062 ng/mL, $p < 0.05$, respectively), while Dkk-1 and sclerostin decreased (−10.90 ± 11.80 and −13.00 ± 10.52 pmol/L, respectively, $p < 0.01$ for all observations). In contrast, PTH returned to levels comparable to M84.

A strong positive correlation was observed between the lumbar spine BMD gains from baseline to M84 (expresses as T-score gains) and the changes in RANKL from M84 to FU-M12 ($R^2 = 0.53$, p = 0.002) (Fig. 2b).

A negative correlation of moderate strength between the changes of

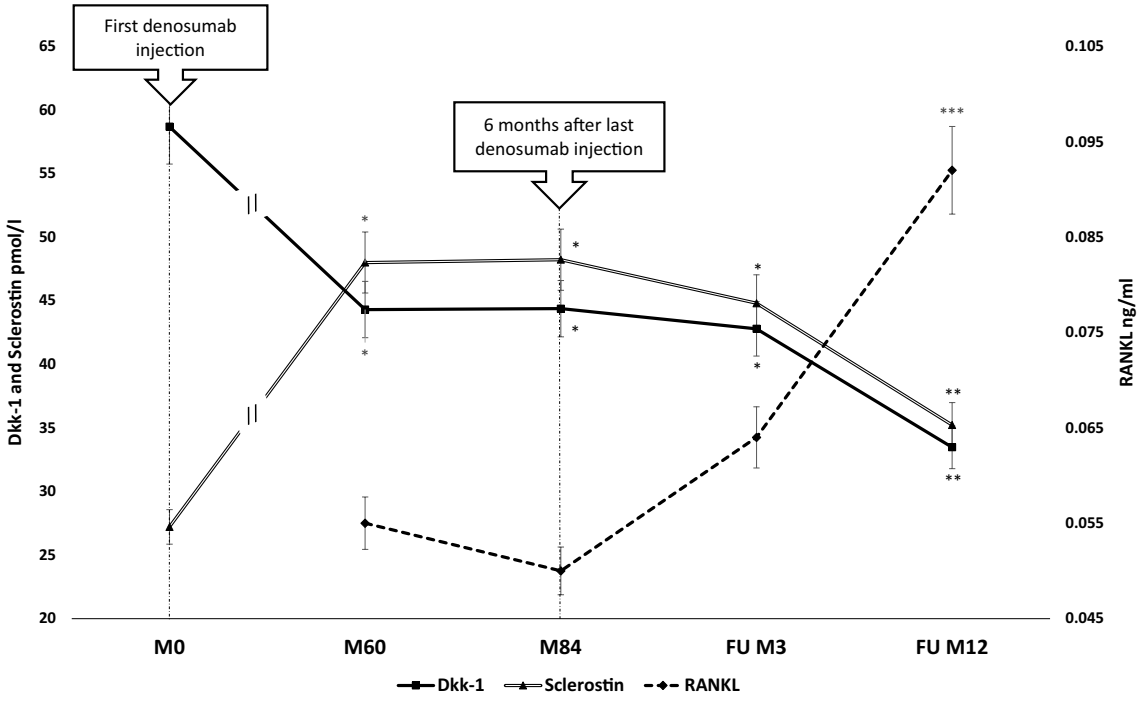

Fig. 1. Absolute values of Dickkopf-1 (Dkk-1), Sclerostin and Receptor Activator of Nuclear factor Kappa B Ligand (RANKL) before denosumab administration and following discontinuation. *$p < 0.01$ vs M0; **$p < 0.01$ vs M84; ***$p < 0.05$ vs M84.

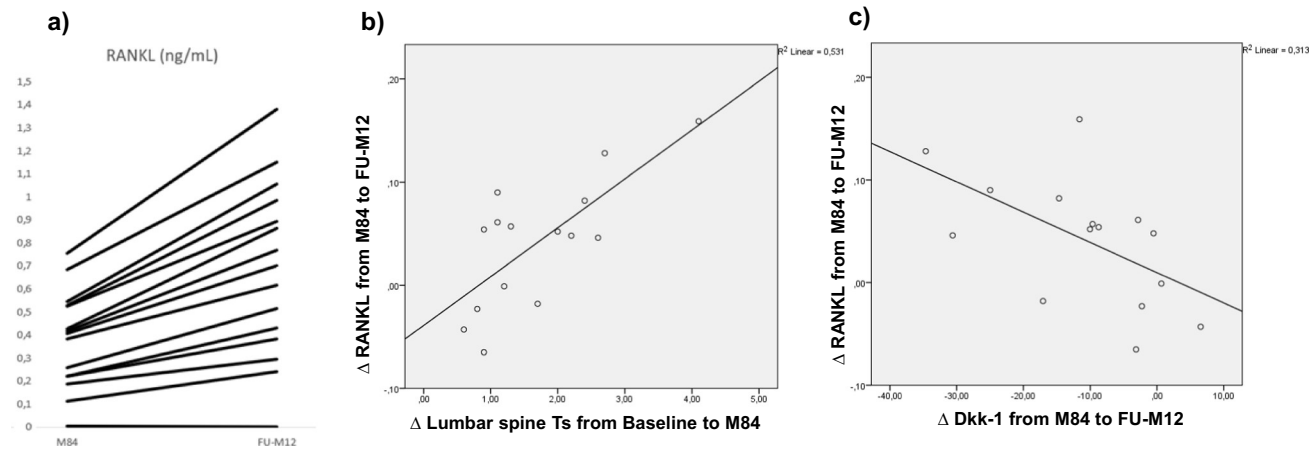

Fig. 2. a) M84 to FU-M12 changes in RANKL for each patient. b) Linear correlation between RANKL absolute changes and lumbar spine T-scores from M84 to FU-M12. c) Linear correlation between Dkk-1 and RANKL absolute changes from M84 to FU-M12.

Dkk-1 and RANKL from M84 to FU-M12 was observed ($R^2 = 0.31$, $p = 0.03$) (Fig. 2c). A positive correlation was also found between the changes in RANKL and CTX-I from M84 to FU-M3 ($R^2 = 0.33$, $p = 0.024$) and between RANKL and CTX-I from M84 to FU-M12 ($R^2 = 0.38$, $p = 0.013$). No correlation was found between the changes in RANKL and PINP. No further significant correlation between the changes in serum markers and/or BMD was found.

A single incidental morphometric fracture assessed by DXA was reported at FU-M12.

4. Discussion

In this study, we investigated the changes in Dkk-1, sclerostin, and RANKL serum levels in the year following DMAb discontinuation to determine whether the rebound effect on bone metabolism following DMAb loss of effect was associated with the dysregulation of Wnt inhibitors and/or by an abrupt increase in RANKL expression, as previously hypothesized [11]. We found, to our knowledge, for the first time in the literature, that 12 months after DMAb loss of effect, Dkk-1 and sclerostin decreased, while RANKL increased. Furthermore, we have provided complete data on Dkk-1 and sclerostin changes from the baseline of treatment with DMAb to its discontinuation and during the subsequent 12 months. Consistent with previous reports [5–8], we confirmed the quick decrease of BMD values at all sites after DMAb loss of effect, along with a relevant and expected increase in BTMs. In our study, after the drug discontinuation, the BMD loss at the spine was roughly 1/3 of the gain that had been accumulated during the 7 years of DMAb, while the BMD loss at the hip appeared to be even greater than the increase occurred during the treatment. This is unexpected in comparison with other studies [7,8] but might be explained by the limited sample size.

In previous studies, after the initiation of an antiresorptive treatment such as bisphosphonates [12,13] or DMAb [14], sclerostin has been shown to increase, while after 6 months of DMAb treatment Dkk-1 decreased [14]. We now show that in the late phase of DMAb treatment (M60 to M84), no relevant fluctuations in Dkk-1, sclerostin, and BTMs serum levels seem to occur. Overall, these findings suggest a possible plateauing of the changes in Dkk-1 and sclerostin (and BTMs) after many years of treatment. Furthermore, they support a hypothesis that the changes observed were caused by suspension of the DMAb and did not occur by chance.

The decrease in sclerostin was expected. Presently, our new data also show that after this early increase, the serum levels of sclerostin may stabilize later. It is then logical to expect a reversal of the trend following discontinuation of the drug.

Conversely, different considerations might be appropriate for Dkk-1. As for sclerostin, no late fluctuation for Dkk-1 was found during DMAb treatment. Given the tendency for sclerostin to return to baseline levels after DMAb discontinuation, similarly a concurrent increase in Dkk-1 should be expected. Furthermore, also in animal models, when the expression of one Wnt inhibitors is impaired, the second often show a compensatory shift in the opposite direction [15], while our data show a decrease also for Dkk-1. One hypothesis to explain this inconsistency is that in the DMAb loss of effect phase, the changes in both sclerostin and Dkk-1 may not be “prime movers” for the abrupt decrease in BMD but merely a counter-regulative response to the overshoot in bone resorption and consequent bone loss.

Currently, the mechanism underlying this phenomenon remains unclear. One possible explanation is the presence of an increased pool of dormant osteoclast precursors [11] and the lack of balance of RANKL/osteoprotegerin after DMAb clearance [16]; however, these hypotheses remain untested.

We also evaluated the changes in RANKL serum levels after DMAb discontinuation and showed an increase in this marker, which reached statistical significance only after 12 months from the DMAb loss of effect. We believe this finding may support the hypothesis of the presence of a growing pool of dormant osteoclast precursors, which may also explain the rapid increase in bone resorption. Nevertheless, the delay of the increase in RANKL serum levels remains difficult to explain, and further studies are warranted to investigate whether, for instance, it might be explained by the early sequestration of the ligand by a large pool of receptor-expressing precursors or by the kinetics of the membrane bound-to-soluble ratio of RANKL.

Interestingly, we also found a strong correlation between the gains in terms of lumbar spine BMD during the treatment course and the RANKL overshoot seen 12 months after its discontinuation. This data suggest that BMD gains might be a strong predictor of the increase in RANKL serum levels after DMAb discontinuation. In turn, the correlation between the increases in CTX-I (but not PINP) and RANKL observed both at FU-M3 and FU-M12 suggest that the increase in RANKL is associated with the increase in bone resorption.

On the contrary, dysregulation of Wnt inhibitors initially seemed to be a promising mechanism for the BMD impairment after DMAb discontinuation; nevertheless, our data suggest that this pathway is probably not the major regulator of bone response to its discontinuation. The changes in Dkk-1 and sclerostin might simply be a negative-feedback response to the abrupt increase in bone resorption. The negative correlation that we found between the increase of RANKL and the decrease in Dkk-1 might provide a further clue to support this hypothesis.

Finally, we also observed a decrease in PTH serum levels after DMAb loss of effect. This phenomena could be expected since is known

that DMAb treatment is associated with an increase in compensatory PTH [17,18] in order to maintain blood calcium homeostasis. Accordingly, the discontinuation of DMAb treatment may lead to shifts in PTH in the opposite direction.

The main strength of our study is the availability of complete data on BMD, BTMs, and serum Wnt inhibitors, as well as the evaluation of RANKL. To our knowledge, this is the first time that data on serum RANKL, Dkk-1, and sclerostin changes after DMAb discontinuation have been reported. The main limitations are the small sample size, the absence of a control group, and lack of complete serum marker data starting from the treatment baseline. Thus, our results do not yet allow conclusive considerations. However, they may guide the future research agenda dealing with the consequences of DMAb suspension.

In conclusion, while dysregulation of the Wnt inhibitors initially appeared to be a promising metabolic explanation for the decline in BMD after DMAb suspension, our results do not support this explanation and better attribute the Wnt inhibitor changes to a mere feedback response. The hypothesis of a sudden and uncontrolled loss of inhibition of the osteoclast line after DMAb clearance still stands even though the increase in serum RANKL levels only partially justifies such a phenomenon.

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Authors' roles

Study design and execution: AF, OV, DG. Data collection: EV, CB, LI, IL, GO, GA, AG.

Data analysis: AF, DG. Data interpretation: AF, OV, DG, MR, KS. Drafting manuscript: AF. Revising manuscript content: All. Approving final version of manuscript: All. AF and DG take responsibility for the integrity of the data analysis.

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