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Research Article

The effects of denosumab on osteoclast precursors in postmenopausal women: a possible explanation for the overshoot phenomenon after discontinuation

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Abstract

Upon denosumab discontinuation, an observed overshoot phenomenon in bone turnover may occur, potentially leading to a reduction in bone mineral density and the occurrence of vertebral fractures. Several theories have been proposed to explain this phenomenon, one of which is that osteoclast precursors might be accumulating during treatment. Our aim was to study the effects of denosumab on osteoclast precursors in postmenopausal women. This cross-sectional observational study included 30 postmenopausal women with osteopenia or osteoporosis, divided into 2 groups: 15 treated with denosumab (mean duration 4 years, range 6 months-9 years) and 15 treatment-naïve controls. Peripheral blood mononuclear cells were isolated from whole blood and were stained for CD14, MCSFR, CD11b, and TNFRII. Osteoclast precursors (CD14+/MCSFR+, CD14+/CD11b + OR CD14+/TNFRII+) were identified with fluorescent activated cell sorting. The proportion of osteoclasts was determined by calculating their percentage of the total cell population in each whole blood sample. To confirm the expected suppression of bone turnover in the subjects treated with denosumab, we measured serum PINP, CTX, and TRACP5b. Denosumab-treated patients exhibited a significantly higher count of CD14+/CD11b + osteoclast precursors compared with controls (median 4% vs 0.75%, p=.011). There was no correlation with the duration of treatment. Bone turnover markers were significantly lower in the group treated with denosumab than controls. Our findings indicate an increase in osteoclast precursors, which could explain the overshoot phenomenon observed after discontinuing denosumab.

Keywords: osteoporosis, denosumab, osteoclast precursors, overshoot, rebound

Lay Summary

Denosumab is an anti-osteoporotic medication that has proven effective in preventing bone loss and reducing the incidence of fractures. When it is discontinued, there is a risk of overshoot in markers of bone turnover, which may lead to a reduction in bone mineral density and new vertebral fractures. While we suspect the mechanism underlying this phenomenon, we do not know the process in any detail in terms of the cells involved, particularly the role of the progenitors of bone cells that break down bone tissue (osteoclast precursors). We found that in postmenopausal women treated with denosumab, there is an increase in one of the populations of osteoclast precursors. On denosumab discontinuation, accumulated cells can mature into osteoclasts, which remove bone and lead to the overshoot of bone turnover and decreased bone density.

Introduction

Denosumab, an anti-osteoporotic medication, is a fully human monoclonal immunoglobulin G2 (IgG2) antibody that binds and inhibits the receptor activator of nuclear factor kappa-B ligand (RANKL), the principal regulator of osteoclastic bone resorption.[1] This medication has proven effective in preventing bone loss and reducing the incidence of fractures.[2] Long-term treatment with denosumab has demonstrated sustained increases in bone mineral density (BMD) over a decade, without evidence of a plateau. In the extended phase of the FREEDOM trial, patients treated with denosumab for up to 10 years showed significant BMD gains from baseline by 21.7% at the lumbar spine and 9.2% at the total hip.[3]

However, discontinuation of denosumab is associated with a decrease in BMD, accompanied by a rebound increase in bone turnover markers (BTMs) to levels exceeding baseline.[4] Moreover, several reports have described cases of multiple vertebral fractures upon discontinuation.[5–8] The best available data are from a post hoc analysis of the FREEDOM and FREEDOM Extension Trial, where 1475 patients discontinuing treatment were studied. The results revealed an increase in the fracture risk following denosumab cessation, returning to levels similar to those observed in untreated patients, translating to an increased fracture risk. Moreover, the rate of multiple vertebral fractures was higher in patients who discontinued treatment [4.2 (2.8–5.7] vs 3.2 (1.4–5.1)

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per 100 participant-years].[9] The risk appears to increase with longer treatment duration, especially in patients treated for more than 3 years.[10] Notably, a single injection of denosumab does not seem to cause any overshoot effect.[11] Other risk factors for multiple vertebral fractures include greater gains in BMD at the hip during treatment and also greater loss after treatment, prevalent vertebral fractures and longer duration off therapy.[12]

Several theories have been proposed to explain this phenomenon.[13] One possible mechanism involves the role of osteoclast precursors (OCPs). OCPs are generated in the bone marrow from hematopoietic stem cells.[14] These cells circulate into the blood stream and migrate into tissues, where they differentiate into osteoclasts in response to various factors.[15] Anti-osteoporosis drugs might also play a role. A study in mice receiving anti-RANKL antibody showed a higher number of OCP cells at bone marrow compared with controls not receiving the anti-RANKL antibody.[16] In humans, it has previously been shown that OCPs in peripheral blood are decreased after anti-resorptive therapy.[17] It has also been speculated that the long-term sustained reduction in bone resorption beyond the end of treatment with bisphosphonates may be due to prolonged effects on the circulating OCP cells.[18] In contrast, different effects of denosumab on the precursor cell population could explain the very different offset. However, there are no data on OCPs numbers in humans receiving denosumab compared with controls not taking anti-osteoporosis drugs.

This study aimed to assess the effect of denosumab on circulating OCPs and BTMs in women with postmenopausal osteoporosis. We hypothesized that the population of OCP cells increases following denosumab administration.

Materials and methods

Study design and population

This cross-sectional pilot study compared 15 denosumab-treated patients with 15 treatment-naïve controls. Participants were recruited from the Metabolic Bone Unit at the Northern General Hospital, Sheffield, United Kingdom. The treatment group consisted of postmenopausal women over 65 years with osteoporosis or osteopenia who had been on denosumab for at least 6 months. Blood was taken 6 months or less before their next denosumab dose. The control group included postmenopausal women with osteoporosis or osteopenia, who had not received prior treatment.

Participants were excluded if they had previously been on bisphosphonates or SERMs, had sustained fractures in the last year, had evidence of bone-related disease (for example, hyperparathyroidism or Paget's disease), or were using oral steroids. We aimed to match the control group to the treatment group by age within the same decade.

The study was conducted under the South Yorkshire and North Derbyshire Musculoskeletal BioBank (SYNDMB) with ethical approval granted by Sheffield Teaching Hospitals (registration number STH 15691).

Biochemistry—blood collection and analysis

Venepuncture was performed in the non-fasting state of each participant. For the peripheral blood mononuclear cells (PBMC) analysis, whole blood was transported to the University of Sheffield Medical School where PBMCs were measured in real-time. The method used to measure the PBMCs was fluorescent activated cell sorting (FACS). Before FACS was performed, the following was carried out to ensure the samples were prepared for analysis.

One hundred microliters of whole blood was dual stained using the following antibodies (each with a respective isotype control): anti-fluorescein isothiocyanate (FITC), Phycoerythrin (PE) conjugated anti-MCSFR, allophycocyanin (APC) conjugated anti-CD11b, and PE conjugated anti-TNFRII. Staining of the whole blood was followed by an incubation period of 45 min at 4 °C. Once incubated, cells were washed with phosphate buffered saline (PBS) and with 1% bovine solution. To isolate the white blood cells, 1 ml of working strength H-lyse was added to the whole blood and mixed vigorously. Red blood cells within the whole blood were lysed during an incubation period of 20 min at room temperature. To separate the red blood cell debris and white blood cells, centrifugation was performed, which resulted in the formation of a white blood cell pellet and a red blood cell/debris supernatant. Supernatant formed from centrifugation was discarded, while the pallet was resuspended in 1 ml of working strength red lysis buffer. After the final wash of the sample, the remaining cells were resuspended with PBS, which was then followed by flow cytometry.[17]

The PBMCs in the sample were identified by measuring their size and granularity. This process was conducted with the FACS Excalibur.[4] Cells expressing cell surface proteins were identified by the software Cell Quest and were displayed on histograms and bivariate dot plots. The OCP cell population was dual-stained and identified from 10,000 PBMCs.[17] It is worth noting that there is no filter on the sample line within the machine itself so it is unlikely that it would filter out any cells.

BTM measurements

The BTMs were measured using the IDS-iSYS automated chemiluminescence immunoassay (Immunodiagnostic Systems, Boldon, United Kingdom). The analyzer measured BTMs in the serum of participants that were acquired through the centrifugation of whole blood at 3000 rpm at 4 °C for 10 min. BTMs measured from these samples were procollagen-1-N-propeptide (PINP), tartrate-resistant acid phosphatase type 5b (TRAcP 5b), and collagen type I cross-linked C telopeptide (CTX). A single measurement was carried out for each of the participants. The inter assay precisions for TRACP-5b, PINP, and CTX were 5.6%, 3.4%, and 4.3%, respectively.

Statistical analysis

Our study is a pilot study, so a power calculation is not applicable. Several papers have been published about identifying an appropriate sample in pilot studies. Connelly[19] advocated that a pilot study sample should be 10% of the sample projected for the larger parent study. Isaac and Michael[20] suggested 10-30 participants. Our study encompasses 15 participants in each group in line with the current literature.

Descriptive data, including medians and interquartile ranges, were calculated to compare the demographic and OCP data between the treatment and control groups. The Mann-Whitney U-test was used to assess statistical significance of the differences between the 2 groups. We checked for correlations using Person test. All analyses were performed using SPSS version 29 (IBM, SPSS Inc. 2023).

Table 1. Baseline characteristics.

	Control ($n=15$)	Treatment ($n=15$)	p values
Age (years)	71 (65 to 82)	82 (70 to 85)	0.151
BMI (kg/m^2)	25.2 (21 to 30.5)	25.2 (23.7 to 29)	0.820
HIP T-SCORE	–1.8 (–2.3 to –1.4)	–1.8 (–2.9 to –0.7)	0.776
SPINE T-SCORE	–2.1 (–2.9 to –1.4)	–1.7 (–2.4 to –0.3)	0.152

All results are shown as median (Q1, Q3). p values are for Mann-Whitney tests. BMI, body mass index.

Table 2. Bone turnover marker data.

	Control	Treatment	p values
P1NP (ng/ml)	64.3 (49.9 to 90.0)	20.0 (12.7 to 36.1)	**<0.001**
TRAcP 5b (U/L)	4.5 (3.3 to 5.0)	0.6 (0.6 to 2.1)	**<0.001**
CTX (ng/ml)	0.33 (0.21 to 0.46)	0.09 (0.02 to 0.22)	**<0.001**

All results are shown as median (Q1, Q3). p values are for Mann-Whitney tests. Procollagen-1-N-propeptide (PINP), tartrate resistant acid phosphatase type 5b (TRAcP 5b), Collagen Type I Cross-linked C telopeptide (CTX).

Table 3. Data on osteoclast precursors.

	Control	Treatment	p values
CD14+/MCSFR	0.90 (0.4 to 2.0)	1.50 (0.70 to 3.10)	0.561
CD14+/CD11b	0.75 (0.28 to 2.88)	4.00 (2.22 to 19.13)	**0.011**
CD14+/TNFRII	1.10 (0.30 to 2.10)	1.30 (0.30 to 3.30)	0.574

All results are shown as median (Q1, Q3). p values are for Mann-Whitney tests.

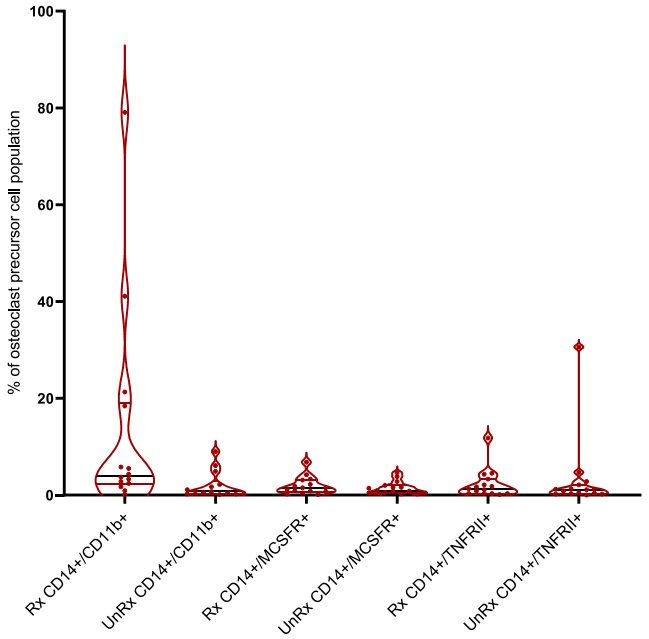

Figure 1. The violin plots show median levels, quartiles, and data distribution of CD14+/CD11b+, CD14+/M-CSFR+, and CD14+/TNFRII+ cells. The population of CD14+/CD11b + cells was significantly higher in the group treated with DMAB (Rx) compared with that in the untreated group (unRX DMAB), $p<.05$ (Mann-Whitney test). There was no difference in population of cells expressing CD14+/M-CSFR+ and CD14+/TNFRII+.

Results

Baseline characteristics

Fifteen postmenopausal (median age: 82 years, interquartile range 70 to 85) women with osteoporosis or osteopenia on denosumab were recruited (33% had osteoporosis, 11 had vertebral fractures).The median duration of treatment was 4 years (range 0.5 to 9 years). Additionally, we recruited 15 age-matched controls (47% had osteoporosis, 1 had vertebral fractures) (Table 1). Baseline characteristics were comparable between the 2 groups (Table 1).

All BTMs (PINP, CTX, TRAcP 5b) were significantly lower in denosumab-treated patients compared with the control group (Table 2).

With respect to the OCPs, the population of CD14+/CD11b + cells were significantly higher in the denosumab-treated group compared with the control group (p=.011) (Table 3, Figure 1). There was no difference in the populations of cells expressing CD14+/M-CSFR+ and CD14+/TNFRII+ (Table 3, Figure 1).

There was no correlation between the levels of CD14+/CD11b and the duration of denosumab treatment (r -0.144, p value >0.05). To minimize any potential confounding effect of treatment duration, a sensitivity analysis was performed excluding 2 patients who had received only 1 injection of denosumab. The results remained consistent after this exclusion. Lastly, a sensitivity analysis was performed to compare patients on denosumab treatment for longer than 3 years to the ones on treatment for fewer years. Again, no statistical difference was found between the levels of CD14+/CD11b, although patients on shorter duration had higher results (median 5.8 in those on shorted duration vs 2.8, p value >0.05). Moreover, no statistically significant correlation was identified between the levels of CD14+/CD11b and BTMs or between age and levels of CD14+/CD11b + in women taking denosumab (r values: TRAcP 0.094, CTX 0.288, PINP –0.202, all p values >0.05).

Discussion

This study showed that denosumab treatment in postmenopausal women with osteoporosis and osteopenia leads to a significant increase in CD14+/CD11b + OCPs just before the next dose of denosumab, while BTMs were still suppressed. This was shown by the significant reduction in BTMs (CTX, PINP, and TRAcP) in the denosumab-treated group. This finding suggests that denosumab may inhibit the differentiation of these precursors into mature osteoclasts, leading to their accumulation. This blockade in differentiation could explain the overshoot phenomenon in bone turnover seen upon denosumab discontinuation.[4] These findings provide valuable insights into the cellular mechanisms underlying the rebound increase in bone turnover following denosumab discontinuation and suggest that targeting the accumulation of OCPs could be a strategy to mitigate this adverse effect.

Our findings align with several other studies. Anastasilakis et al.[21] reported that denosumab-treated women who discontinued treatment had downregulated microRNAs associated with osteoclastogenesis and osteoclast activity (miR-503 and miR-222-2), especially those with vertebral fractures. These women also had higher RANK mRNA levels. In our study, women on denosumab had more vertebral fractures than controls. In silico studies added OCPs in their model and suggested that accumulation of OCPs was essential to predict the experimentally observed rapid rise in osteoclast numbers and the associated increase in bone resorption.[22] Moreover, another in silico study predicted significantly higher OCP

numbers after 3 years of denosumab treatment compared with placebo (p=.003).[23] However, not all studies have shown similar results; for instance, Kong et al.[24] recruited 11 patients in the denosumab group and 12 in the zoledronate group and reported no change in OCPs after denosumab or zoledronate treatment, although they did observe that old age and suppressed bone turnover were associated with increased OCPs in denosumab-treated individuals. There were some similarities and differences in the study of Kong et al.[24] which could explain the different findings. Their participants were approximately 10 years younger. Moreover, the study had a short duration (48 weeks). This timeframe may not have been sufficient to observe the overshoot phenomenon, which typically occurs later. In the context of the zoledronate arm, our research on oral bisphosphonates in 62 women with osteoporosis, showed a clear reduction in CD14+/CD11b + cells (p<.001).[17] The magnitude of reduction is comparable with Kong's 38% decrease at 48 weeks. However, their result may not have reached significance due to the limited number of participants. The strengths of their study include the prospective analysis of denosumab and the use of DAPI staining as well as VNR staining. They checked for vitronectin receptor cells, i.e. VNR- or VNR+ representing early and late OCPs, respectively, and saw no differences with denosumab.

Another proposed mechanism for the increase in osteoclast numbers following denosumab discontinuation is the "osteomorph" theory. According to this theory, osteoclasts that have completed the resorption process, might experience fission into daughter inactive cells called osteomorphs. Once RANK is no longer inhibited, these RANK-positive cells could refuse into active osteoclasts (osteoclast recycling).[25] However, a mouse study by Fu et al.[26], did not show significant accumulation of osteoclast progenitors in the bone marrow microenvironment, or a significant increase in osteomorph markers (there was a non-statistically significant trend). Instead, their study suggested that a decrease in osteoprotegerin (OPG, natural antagonist of RANK), linked with the loss of osteoblasts and newly formed osteocytes, might be a key mechanism. It is worth mentioning that whether such cells exist in humans remains unknown.[13] Further supporting the osteoclast recycling theory, a recent study used a 2-week treatment regime with osteoprotegerin-Fc fusion protein (OPG: Fc), a reversible inhibitor of RANK, to resemble denosumab treatment in mice. After 2 weeks of treatment, there was a complete absence of osteoclasts by histomorphometry, but, by week 11, the numbers were significantly higher in mice treated with OPG:Fc than in controls. Serum TRAP, a marker of osteoclast activity was the first to elevate following treatment discontinuation. The study also observed an increase in RANKL locally and systemically even before TRAP elevation, suggesting that OCP accumulation and osteoclast recycling may explain the overshoot phenomenon after denosumab discontinuation.[27] Similar results were found in human studies, where increased levels of RANKL were evident 6 months after the last denosumab injection. This could stimulate an increase in the number of active osteoclasts, consistent with the rise in TRAcP found in this study.[28]

The reason for the abundance of CD14+/CD11b + OCPs compared with other subtypes remains unclear. Tsai et al.[14] state that the molecular and cellular properties of human OCPs, which are unique, are not well elucidated. Thus, it is difficult to give detailed information about the differences in the cell populations; we can only speculate that it likely relates to the stage of differentiation. A study involving bisphosphonate treatment for 48 weeks demonstrated a significant reduction in CD14+/CD11b + OCPs, as well as a significant reduction in CD14+/MCSFR+ precursors.[17] Additionally, the study supporting the osteomorph theory found that cells expressing CD11b were involved, which aligns with our findings.[25] Another study that could justify the abundance of CD11b + OCPs involved an in vitro growth of OCPs through the use of Colony Forming Unit-Granulocyte/Macrophage (CFU-GM) colony assays. The study found that 90% of the colonies rich in OCPs expressed CD11b proteins.[15] Moreover, during osteoclastogenesis, one of the first variants of osteoclasts precursors formed after myeloid progenitor differentiation expresses CD11b +.[15] This suggests that CD11b expression is an early marker of OCPs, while other cell surface proteins develop later, which may explain the higher presence of CD11b+ precursors in circulation among denosumab-treated patients.

We were surprised not to find a correlation between the levels of CD14+/CD11b and the duration of treatment. The results remained the same even after we excluded patients that were on treatment only for 6 months. However, the small sample size might not allow to detect significant differences. A future study with more participants and longer duration might give different results.

To the best of our knowledge, this is the first study in humans to evaluate this possible mechanism using treatment naive controls with low bone mass. However, as a pilot study, it is limited by its small sample size. Larger studies are needed to confirm these findings and we are looking into future international collaborations. One further limitation of our study is that we did not measure the levels of RANKL.

While the FACS analyzer was used as part of a research protocol, we recognize its potential for future clinical applications. Although currently limited by logistical challenges—such as the need for immediate sample processing and specialized equipment—there is a promising opportunity to explore this in clinical practice. Automated FACS systems or selective screening in high-risk patients could make it a feasible tool for identifying individuals more prone to overshoot after discontinuation, which would allow for more tailored treatment regimens. As such, the future use of FACS in clinical settings remains to be elucidated in refining therapeutic strategies and improving patient outcomes.

In conclusion, denosumab treatment in postmenopausal women with osteoporosis resulted in a significant increase in CD14+/CD11b + OCPs, by the time the next injection is due. This accumulation may contribute to the overshoot effect in bone turnover observed upon discontinuation. Further research is warranted to explore the mechanisms behind this phenomenon and to develop strategies to mitigate the rebound effect and prevent adverse outcomes in patients discontinuing denosumab therapy.

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Author contributions

Marian Schini (Data curation, Formal analysis, Investigation, Project administration, Software, Supervision, Writing—original draft,

Writing—review & editing), Fatma Gossiel (Formal analysis, Investigation, Methodology, Software, Visualization, Writing—original draft, Writing—review & editing), Tanya Saini (Data curation, Formal analysis, Investigation, Project administration, Writing—original draft), Peter Banda (Data curation, Formal analysis, Investigation, Project administration, Software, Writing—original draft), Rachel Ward (Data curation, Investigation, Writing—review & editing), Tatiane Vilaca (Investigation, Supervision, Writing—review & editing), Richard Eastell (Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing—original draft, Writing—review & editing), and Andreas Fontalis (Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing)

Marian Schini and Fatma Gossiel made equal contributions.

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Conflicts of interest

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Data availability

Data available on request.

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