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The effect of bisphosphonate treatment on osteoclast precursor cells in postmenopausal women with osteoporosis

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**Abstract**

Bisphosphonates are used to treat bone disease characterised by increased bone resorption by inhibiting the activity of mature osteoclasts, resulting in decreased bone turnover. Bisphosphonates may also reduce the population of osteoclast precursor cells. Our aims were to investigate the effect of bisphosphonates on i) osteoclast precursor cells and ii) circulating cytokine and cytokine receptor in postmenopausal women with osteoporosis compared with healthy premenopausal women. Participants were 62 postmenopausal women (mean age 66) from a 48-week parallel group trial of bisphosphonates. They received ibandronate 150mg/month (n=22), alendronate 70mg/week (n=19) or risedronate 35mg/week (n=21). Fasting blood was collected at baseline, weeks 1 and 48. At baseline, blood was also collected from 25 healthy premenopausal women (mean age 37) to constitute a control group. Peripheral blood mononuclear cells were extracted and stained for CD14, M-CSFR, CD11b and TNFRII receptors. Flow cytometry was used to identify cells expressing CD14+ and M-CSF+ or CD11b+ or TNFRII+. RANKL and OPG were measured to evaluate potential mediation of the bisphosphonate effect. After 48 weeks of treatment, there was a decrease in the percentage of cells expressing M-CSFR and CD11b receptors by 53% and 49% respectively (p<0.01). Cells expressing M-CSFR and CD11b were decreased with ibandronate and risedronate after 48 weeks to the lower part of the premenopausal reference interval. These effects were not significantly different between each of the treatment groups. There was no significant effect on RANKL and OPG throughout the study period. Bisphosphonates inhibit bone resorption in the short-term by direct action on mature osteoclasts. There is also a later effect mediated in part by a reduction in the population of circulating osteoclast precursors.

**Keywords** Bisphosphonates, osteoporosis, osteoclasts, osteoclast precursor cells

## Introduction

Postmenopausal osteoporosis is a progressive, age-related skeletal disorder characterised by increased bone resorption by osteoclasts, reduced bone mineral density (BMD) and increased fracture risk (1). Estrogen deficiency is associated with increased osteoclastogenesis, activation frequency and subsequently bone loss.

Several cytokines, steroids, hormones and prostaglandins are involved in regulating the differentiation, proliferation, activation and survival of osteoclasts (2-4). These include macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor-ƙB ligand (RANKL) and tumour necrosis factor-α (TNF-α) (5-8). Osteoclast precursor cells express cell surface receptors which are specific for these factors. *In-vitro* studies have demonstrated that M-CSF binds to the M-CSF (c-fms) receptor and induces the expression of genes in the osteoclast lineage leading to the differentiation and development of mature osteoclasts and cell survival (3, 9). TNF-α is an inflammatory cytokine that binds to the TNF receptors-1 and -2, stimulates osteoclastogenesis and regulates cell apoptosis (10). In addition osteoclast precursor cells express β2 integrins such as CD11b/Cd18) adhesion molecules that are necessary for cell trafficking and differentiation (11). It been demonstrated in CD11b-deficient mice where there was a decrease in bone mass and increase in osteoclast number (12).

Treatment with bisphosphonates reduces bone resorption as assessed by bone turnover markers (13). In addition, assessments of histomorphometry parameters on bone biopsies have shown that the reduction in turnover is associated with a reduction in the activation frequency (14). Nitrogen bisphosphonates inhibit osteoclasts by attaching to the hydroxyapatite on the bone surface and then are internalised into the mature osteoclasts by endocytosis, inhibiting the activity of FPPS in the mevalonate pathway and promoting apoptosis (15-21). However it is not clear how they reduce the activation frequency. *In vitro* studies have suggested that a possible mechanism is that they do this by reducing the population of osteoclast precursor cells (22, 23). Others have suggested that bisphosphonates act solely and directly on the mature osteoclasts themselves (24) and reduce their ability to attach to the hydroxyapatite (25). A previous study has demonstrated that alendronate reduced the population of osteoclast precursor cells from peripheral blood. These were identified by their expression of CD14/CD11b and were decreased after 12 months of treatment in osteoporotic women (26). In contrast, another study has demonstrated that zoledronate did not reduce the population of osteoclast precursor after 18 months of treatment in osteopenic women, (27)

There are also conflicting in vitro data regarding the effect of bisphosphonates on osteoclastogenic cytokines such as IL-1, TNFα and circulating levels of RANKL and osteoprotegerin (OPG) (28-31)). RANKL is produced by the osteoblasts and binds to the receptor RANK which is located on the osteoclast precursor cells as well as the mature osteoclast (32). This process stimulates the activation of the osteoclast and it may be that high circulating levels of RANKL may lead to increased bone resorption (33). Therefore a possible mechanism of action for bisphosphonates may be to reduce circulating levels of RANKL and/or increase levels of OPG, its soluble decoy receptor, thus reducing the population of osteoclast precursor cells, the activity of mature osteoclasts and hence bone resorption.

The aims of this study were to investigate the effects of ibandronate, alendronate and risedronate on osteoclast precursor cells and levels of circulating cytokines. We hypothesise that nitrogen containing bisphosphonates reduce the population of osteoclast precursor cells and circulating levels of RANKL.

## Subjects and methods

*Subjects*

We conducted a 2-year, open-label, parallel randomised control intervention trial of three orally administered bisphosphonates, at their licensed dose. The overall study design has been fully described (34). We recruited women with postmenopausal osteoporosis, with either (i) a BMD T score $\leq$ -2.5 at the lumbar spine or proximal femur or (ii) a BMD T score $\leq$- 1.0 at the lumbar spine or proximal femur plus a previous fracture sustained during a fall from standing height or less.  A subset of 62 subjects were used for this analysis. The study medications used were (i) ibandronate (Bonviva, Roche, 150 mg once a month) (n = 22), (ii) alendronate (Fosamax, Merck, 70 mg once a week) (n = 19), and (iii) risedronate (Actonel, Warner Chilcott, 35 mg once a week) (n = 21). 25 pre-menopausal women (ages 35 to 40 years) were used in order to generate robust reference intervals for all the measurement variables acquired during the study. All had regular menstrual cycles and did not take any hormonal contraception.

The samples size for this analyses was pre-defined in the study protocol. They were all of the subjects whom had whole blood and serum collected at baseline and then at weeks 1 and 48.

The study was registered with ClinicalTrials.gov (http://clinicaltrials.gov/, number - NCT00666627) and with European Union Drug Regulating Authorities Clinical Trials (EudraCT, number - 2006-004738-33). Itwas approved by the Sheffield Research Ethics Committee and the Medicines and Healthcare Products Regulatory Agency (MHRA), and all participants gave fully informed written consent prior to their participation.  All investigations were carried out in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments, and in accordance with the International Conference on Harmonisation Good Clinical Practice (ICH GCP) guidelines.

### Flow cytometry

100µl of blood were directly stained using the following antibodies and corresponding isotype controls: anti-CD14-flouresceinisothiocyanate (FITC), Phycoerythrin (PE)-conjugated anti-MCSFR, allophycocyanin (APC)-conjugated anti-CD11b and (PE)-conjugated anti-TNFRII.  They were incubated for 45 minutes at 4°C. The cells were washed in 1ml of

phosphate buffered saline (PBS) with 1% foetal bovine serum (FBS). 1ml of working strength H-Lyse buffer was added and mixed vigorously. These were incubated for 20 minutes at room temperature until the red cell lysis process was complete. This step removes the red blood cells, leaving the white nucleated cells. A supernatant was removed following centrifugation and washed using 1ml working strength red lysis buffer. Following the final wash the remaining cells were re-suspended in 500µl of PBS was buffer and flow cytometry was performed.

PBMCs were identified according to their size and granularity using the fluorescent-activated cell sorting (FACS)-Calibur (Becton Dickinson & Co) (35). Unstained, isotype control and dual stained cells were identified from $10^4$ PBMCs. Cell surface antigen expression was analysed using CellQuest software (Becton Dickinson & Co). The results were displayed as histograms and bivariate dot plots (Figure 1). The dual positive CD14+/M-CSFR+, CD14+/CD11b+ and CD14+/TNFRII+ cells were identified as being osteoclast precursor cells (35, 36).
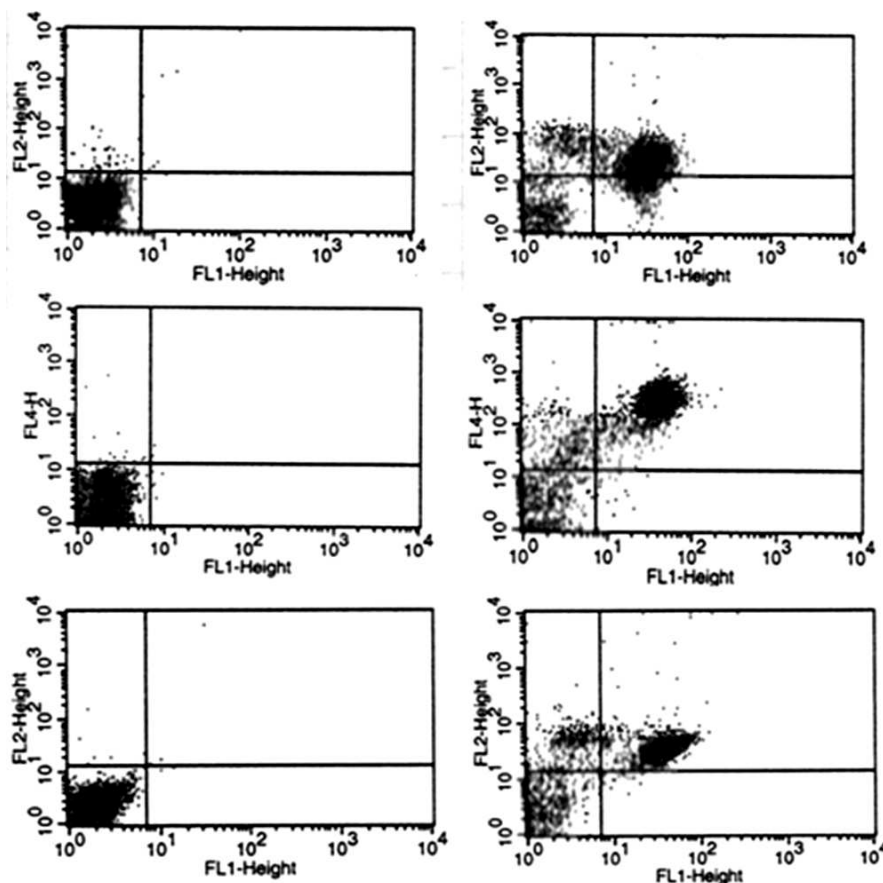


Figure 1: antibody and isotype control stained PBMCs. The number of PBMCs that are positive for and express CD14/MCSFR (top panel), CD14/CD11b (middle panel) and CD14/TNFRII (bottom panel) on their cell surface are shown in the upper right of each quadrant of each dot plot

Bone turnover markers

Carboxy-terminal cross-linking telopeptides of type I collagen (CTX) and N-terminal propeptide of type I collagen (PINP) were measured in serum using the IDS-iSYS multi-disciplined automated chemiluminescence immunoassay (Immunodiagnositics Systems, Boldon United Kingdom). Each measurement was performed in duplicate and the inter-assay coefficient of variations (CV's) were 4% and 5.2% respectively.

Cytokine measurements

Circulating levels of RANKL and OPG were measured in serum using manual sandwich enzyme immunoassays from Biomedica Gruppe (Vienna, Austria) and Biovendor (Czech Republic), respectively. Each measurement was performed in duplicate and the inter-assay CV's were 4.2% and 3.8% respectively.

Statistical analyses

Descriptive data were presented as mean (standard deviation) and the differences between the 2 groups of patients were analysed by two-sample $t$-tests.

Box and whisker plots and a non-parametric Wilcoxon's signed test were used illustrate and compare the percentage of circulating osteoclast precursor cells at baseline and after 1 and 48 weeks of bisphosphonate treatment. The median levels and 25 and 75 percentiles were calculated.

A one-way analysis of variance (ANOVA) was used to compare the change from baseline of levels of circulating cytokines, CTX and PINP between each treatment group after 48 weeks. A Kruskal-Wallis test was used to determine significance. A Spearman correlation was performed to assess the relationship between the change in CD14+/M-CSFR+ and the change in vitamin D levels from baseline to week 48.

For each biochemical test the median levels and 25 and 75 percentiles were calculated for the premenopausal comparator group. The data was analysed using GraphPad Prism 6 software, version 6.05, 2014 and $p<0.05$ was the significance cut-off.

**Results**

*Patient characteristics*

The baseline characteristics for the postmenopausal women with osteoporosis and healthy premenopausal women in the study population are shown in table 1. Overall, mean BMD T-scores at the spine and total hip were lower in postmenopausal women with osteoporosis compared to the premenopausal women.

Table 1 Baseline characteristics of the postmenopausal women with osteoporosis and healthy premenopausal women. The mean and SD are shown.

| Variable | Postmenopausal women with osteoporosis (N = 62) | Healthy premenopausal women (N = 25) |
|---|---|---|
| Age (years) | 65.8 (6.6) | 37.6 (1.8) |
| Height (cm) | 160.7 (5.5) | 166.3 (6.3) |
| Weight (kg) | 68.1 (10.9) | 68.9 (10.3) |
| BMI (kg/m$^2$) | 26.4 (4.1) | 25.0 (4.2) |
| Lumbar spine BMD T-score | -2.2 (0.8) | 0.5 (1.1) |
| Total hip BMD T-score | -1.3 (0.8) | 0.6 (1.0) |
| 25 OH D ng/ml | 22.9 (10.5) | 19.0 (8.1) |

*The effect of bisphosphonates on osteoclast precursor cells*

The effects of bisphosphonates on the population of osteoclast precursor cell are shown in figure 2. At baseline there was no significant difference in the median percentage of osteoclast precursor cells expressing CD14$^+$/M-CSFR$^+$, CD14$^+$/CD11b$^+$ and CD14$^+$/TNFRII between postmenopausal women with osteoporosis and premenopausal women. After 1 week of treatment there was no significant change in the percentage of osteoclast precursor cells expressing CD14$^+$/M-CSFR$^+$, CD14$^+$/CD11b$^+$ and CD14$^+$/TNFRII. After 48 weeks of

treatment there was a significant reduction in the percentage of CD14$^+$/M-CSFR$^+$ and CD14$^+$/CD11b$^+$ osteoclast precursor cells, $p<0.001$. There was no difference in the percentage of CD14$^+$/TNFRII$^+$ osteoclast precursor cells. There was no correlation between the change in CD14+/M-CSFR+ and the change in vitamin D levels from baseline to week 48.
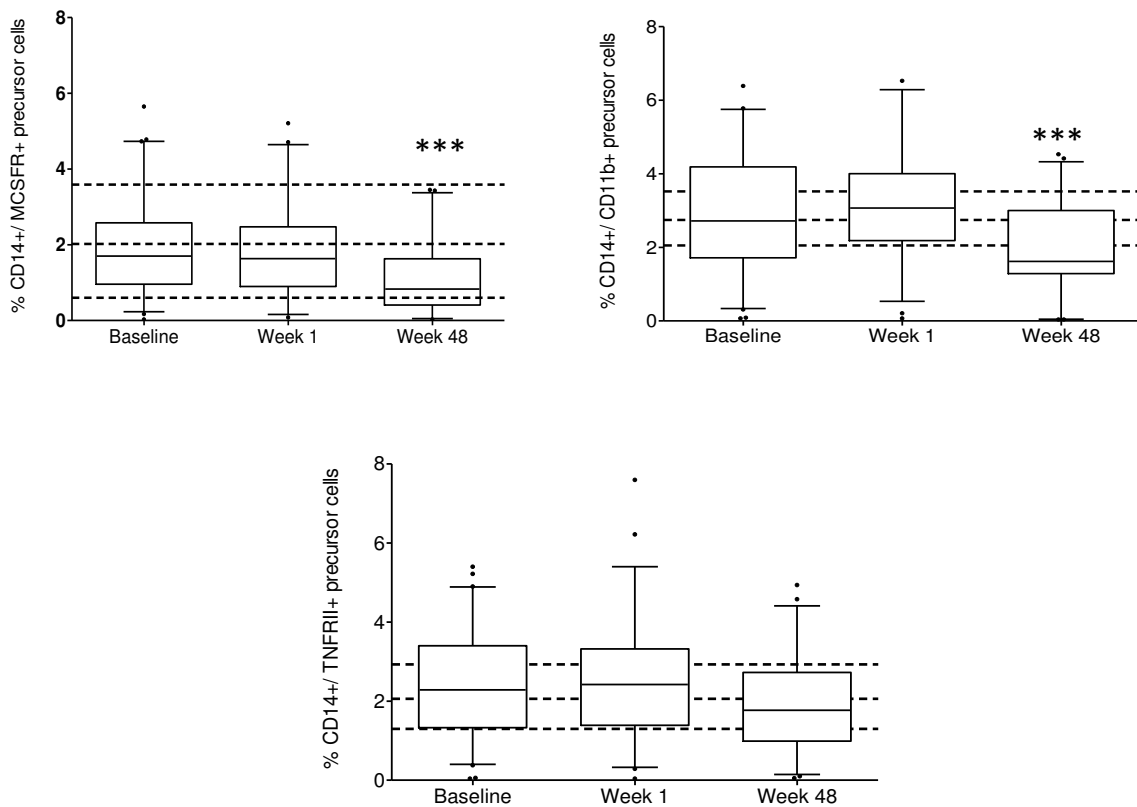


Figure 2: Box and whisker plots representing the percentage of CD14+ cells which are positive for M-CSFR, CD11b and TNFR-II at baseline and after bisphosphonate treatment (N=62). The dashed lines represent the median levels and 25 and 75 percentiles for premenopausal women (N=25).

The effects of ibandronate, alendronate and risedronate on the population of osteoclast precursor cells are shown in figure 3 and table 2. After 1 week there was no significant change in the percentage CD14$^+$/M-CSFR$^+$, CD14$^+$/CD11b$^+$ and CD14$^+$/TNFRII$^+$ cells with any of the bisphosphonates. After 48 weeks of treatment with ibandronate there was a significant decrease from baseline in the population of cells expressing CD14$^+$/M-CSFR$^+$ by 65%, $p<0.05$. There was a decrease with alendronate treatment in the population of cells expressing CD14$^+$/CD11b$^+$ by 40% but this was not statistically significant. There was a significant decrease with risedronate treatment in the population of cells expressing CD14$^+$/M-CSFR$^+$ and CD14$^+$/CD11b$^+$ by 61% and 54%, $p<0.01$, respectively. These effects

were not significantly different between each of the treatment groups. None of the bisphosphonates had an effect on the population of cells expressing CD14+/TNFRII+.
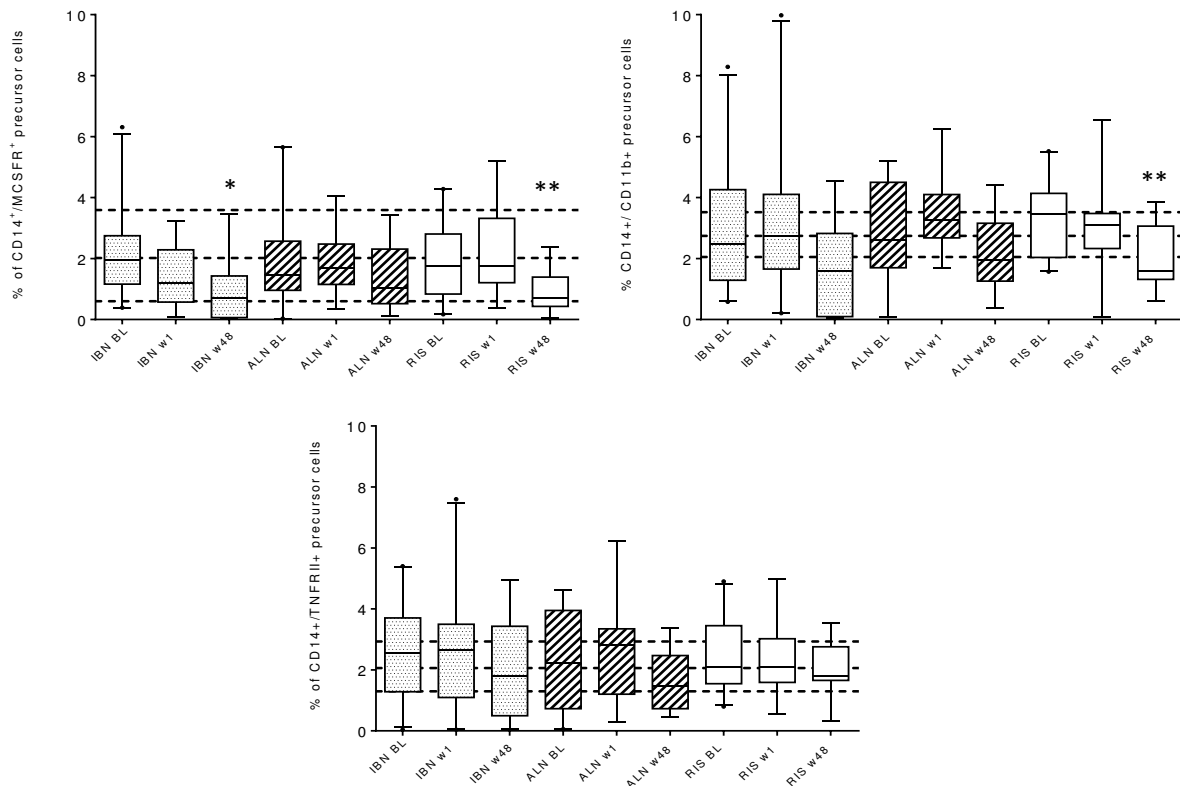


Figure 3: Box and whisker plots representing the percentage of CD14+ cells which are positive for M-CSFR, CD11b and TNFR-II at baseline and after ibandronate (N=22), alendronate (N=19) and risedronate (N=21) treatment. The dashed lines represent the median levels and 25 and 75 percentiles for premenopausal women (N=25). *p<0.05, **p<0.01 and ***p<0.001.

Table 2: Median levels (25%-75% percentiles) of CD14+ cells which are positive for M-CSFR, CD11b, TNFR-II and bone turnover markers at each time point. *p<0.05, **p<0.01 and ***p<0.001 change from baseline.

| Variable | Premenopausal Limits | Baseline | | | Week 48 | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | IBN | ALN | RIS | IBN | ALN | RIS |
| %CD14+/MCSFR+ | 2.0 (0.6-3.6) | 2.0 (1.2-2.8) | 1.5(1.0-2.6) | 1.8(0.8-2.8) | 0.7 (0.1-1.4)* | 1.0(0.5-2.3) | 0.7(0.4-1.4)** |
| %CD14+/CD11b+ | 2.7 (2.1-3.5) | 2.5(1.3-4.3) | 2.6(1.7-4.5) | 3.5(2.0-4.1) | 1.5(0.1-2.8) | 2.0(1.3-3.2) | 1.6(1.3-3.1)** |
| %CD14+/TNFRII+ | 2.1 (1.3-2.9) | 2.6(1.3-3.7) | 2.2(0.7-4.0) | 2.1(1.5-3.5) | 1.8(0.5-3.4) | 1.5(0.7-2.5) | 1.8(1.7-2.8) |
| CTX ng/ml | 0.33 (0.27-0.44) | 0.59(0.5-0.9) | 0.73(0.6-0.9) | 0.69(0.4-0.9) | 0.12(0.1-0.3)*** | 0.10(0.1-0.9)*** | 0.12(0.1-0.4)*** |
| PINP ng/ml | 25.5 (36.2- 46.0) | 48.8(34.8-61.8) | 54.3(44.4-67.1) | 45.5(37.9-52.0) | 15.5(10.0-17.8)*** | 14.1(11.5-21.3)*** | 19.8(11.4-32.0)*** |

*The effect of bisphosphonates on circulating cytokines and bone turnover markers*

At baseline median levels of circulating serum RANKL and OPG were 0.07 pmol/l and 5.2pmol/l respectively. There were no significant changes in RANKL and OPG after 48 weeks of bisphosphonate treatment, (Figure 3). There were significant decreases in CTX after one week of treatment with each of the bisphosphates, $p<0.001$. There were significant decreases in PINP at week 48 with each of the bisphosphonates, $p<0.001$, (Table 2). Spearman's correlation analysis showed that changes in CTX and in PINP levels did not correlate with the changes in the $CD14^+/M\text{-}CSFR^+$ and $CD14^+/CD11b^+$ osteoclast precursor cell populations.

**Discussion**

Bisphosphonates are widely used to treat postmenopausal osteoporosis and some studies suggest that their effects are exerted only on mature and active osteoclasts, (22, 23). Here we have shown that nitrogen containing bisphosphonates significantly reduced the population of osteoclast precursor cells as assessed by their expression of cell surface antigens. There was no difference in effect between bisphosphonates.

It is of interest that the effects on osteoclast precursor cells may only occur in the long term. This is in contrast to the immediate effect on bone resorption as shown by the short term reduction in CTX after 1 week of treatment. The absence of a short term effect of bisphosphonates on osteoclast precursor cells suggests that the initial short term reduction in bone resorption is a result of bisphosphonates exerting their effect on mature, resorbing osteoclasts rather than the osteoclast precursor cells. The long term sustained reduction in bone resorption may be due to the bisphosphonates exerting their effect on both the osteoclast precursor cells and the mature osteoclasts. However in this subset of participants the only long term time point assessed was at week 48. Therefore one particular limitation is not knowing exactly when the bisphosphonates begin exerting their effect on the osteoclast precursor cell population. The data is supported by others (26), who also demonstrated that alendronate significantly reduced CTX after 3 months of treatment but the reduction in osteoclast precursor cells occurred after 12 months. It may be therefore be that the effect on osteoclast precursor cells occurs somewhere between 3 and 12 of treatment.

Treatment with vitamin D did not have an effect on the osteoclast precursor cells. Previously, *in vivo* experiments have shown that bone marrow cells from mice treated with vitamin D had a reduction in osteoclast progenitor cell population compared to vehicle treated OVX-mice (37). Another study investigated the effects of 1,25-(OH)2D and 25-(OH) D on osteoclast differentiation in human monocyte cell cultures in the presence on M-CSF and RANKL (38). Neither 1,25-(OH)2D and 25-(OH) D had any significant effects on osteoclast differentiation.

There was no significant difference in the population of osteoclast precursor cells in postmenopausal osteoporosis before treatment and premenopausal women. In comparison levels of bone turnover markers are significantly higher suggesting that the proportion of osteoclasts in maintained but their activity is increased with age. In contrast, D'Amelio P et al, 2008 (39) demonstrated that postmenopausal women had an increased number of osteoclast precursor cells compared to premenopausal women. This may be due to

increased production of RANKL and TNF by monocytes and T-cells and therefore the upregulation of osteoclastogenesis.

In *in vitro* models RANKL and OPG have been shown to regulate osteoclastogenesis. The reduction in the population of osteoclast precursor cells with bisphosphonate treatment may be mediated by circulating levels of RANKL. However our data demonstrated that bisphosphonate treatments had no direct effect on circulating levels of RANKL and OPG. These findings are comparable to other clinical studies (40-43) and inconsistent with others (44, 45). Dundar et al 2009 demonstrated that in postmenopausal women with osteoporosis receiving 35mg/week of oral risedronate there was a significant decrease in levels of RANKL and an increase in levels of OPG after 3 and 6 months of treatment (44). In contrast and in a similar study design, Dobnig et al 2006 demonstrated that women receiving either 10mg of alendronate or 5mg of risedronate daily showed no effect on RANKL but OPG increased after 6 and 12 months of treatment, (46). Another clinical trial in which postmenopausal women with osteoporosis were receiving 35mg or risedronate, demonstrated that there was no effect on OPG but a gradual decrease in RANKL, also occurring in the osteopenic control group receiving no treatment, (40). The discrepancies may be due to the different populations and diseases studied and to the limitations of the ELISA's themselves.

**Conclusion**

Nitrogen-containing bisphosphonate reduce the population of osteoclast precursor cells in the long term and bone turnover in the short term. Both of these mechanisms may reduce the activation frequency of bone remodelling by having a direct and indirect effect on mature osteoclasts. These findings further suggest that bisphosphonates act selectively and directly on the osteoclast precursor cells to reduce their population without influencing levels of circulating cytokines. The differences between bisphosphonates targeting osteoclast precursors may help explain and be related to their magnitude of effect on bone turnover markers.

**Conflicts of interest**

Miss Gossiel, Mr Hoyle and Dr Naylor have no disclosures. Dr N Peel has received speaker's honoraria and funding to attend educational events from Warner Chilcott, Lilly, Servier, Merck, Roche, GSK and Prostrakan and consultancy fees from Internis Pharma and Lilly. Dr Walsh has received speaker's honoraria from Lilly and the donation of drug and placebo from Prostrakan. Professor McCloskey has received speaker's honoraria and/or research funding and/or advisory board funding from Warner Chilcott, Merck, Amgen, GSK, Bayer, Consilient Healthcare, Hologic, Lilly, Novartis, Pfizer, Servier, Wyeth and Roche. Professor Eastell has received grant funding from Warner Chilcott and the National Institute for Health research (NIHR) and consultancy funding from Warner Chilcott, Roche, Immunodiagnostic Systems and Merck.

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