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Risedronate reduces osteoclast precursors and cytokine production in postmenopausal osteoporotic women.

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Running title: risedronate inhibits osteoclast formation.

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ABSTRACT

This paper studies the effect of oral risedronate on osteoclast precursors, osteoclast formation and cytokine production in 25 osteoporotic women. Risedronate is effective in reducing the number of osteoclast precursors, their formation, vitality and activity and the level of RANKL and TNF alpha in cultures.

Introduction: Biphosphonates inhibit bone resorption by acting against osteoclasts. Some in vitro studies suggest that they induce osteoclast apoptosis, others that they exert an effect on the production of pro-osteoclastogenic cytokines. The effect of risedronate on osteoclastogenesis by peripheral blood mononuclear cells (PBMC) in postmenopausal osteoporosis has not been previously investigated. This paper examines the influence of risedronate on the formation of osteoclast precursors and cytokine production within the compass of osteoclastogenesis in osteoporosis.

Methods: The study was conducted on 38 osteoporotic women; 25 patients were treated with risedronate 5 mg/day while 13 were treated with calcium 1 g/day and vitamin D 800 UI/day. The following parameters were assessed: changes in bone turnover, circulating osteoclast precursors, formation of osteoclasts in PBMC cultures, their activity and vitality and variations in the production of pro-osteoclastogenic cytokines before and after therapy.

Results: After 3 months of risedronate there is a significant reduction in the number and the degree of differentiation of osteoclast precursors, osteoclast formation, vitality and activity and in the level of RANKL and TNF in cultures and of TNF and OPG in serum, while in the group treated with calcium and vitamin D there were no significant changes.

Conclusion: Our data show that risedronate is effective in lowering the number of circulating osteoclast precursors, their formation, vitality and activity in cultures and in reducing the level of pro-osteoclastogenic cytokines in culture supernatants and in serum.

Keywords: risedronate; bisphosphonates; osteoclast; osteoclast precursor; cytokines.
INTRODUCTION

Biphosphonates inhibit bone resorption by acting against osteoclasts. They have a high affinity for the bone matrix and are currently drugs of choice in the management of bone disorders, such as Paget’s disease, osteoporosis and bone metastases (1-3). Their mechanism of action depends on the presence of one or more amine groups. Non-nitrogen-containing biphosphonates, such as clodronate, etidronate and tiludronate, are analogues of endogenous pyrophosphate and act by inducing osteoclast apoptosis through their intracellular accumulation in cytotoxic non-hydrolysable ATP analogues, whereas nitrogen-containing biphosphonates pamidronate, alendronate, ibandronate, zoledronate and risedronate act as isoprenoid diphosphate lipid analogues and inhibit farnesyl pyrophosphate synthase, an enzyme in the mevalonate pathway (4).

Inhibition of this pathway impedes the synthesis of the isoprenoid lipids essential for the farnesylation and geranylation of small GTPase signaling proteins. Bone resorption by osteoclasts is inhibited and their apoptosis is induced (5).

In vitro studies have clearly shown that biphosphonates induce apoptosis when added to osteoclast cultures (6-9), whereas others suggest that they reduce the ability of osteoclasts to adhere to the mineralized matrix (10). Some studies suggest that biphosphonates antiresorptive action is due to inhibition of the activity of mature osteoclasts as well as of the formation of osteoclasts from the bone marrow precursors (11,12), while other studies suggest that biphosphonates do not act on osteoclasts precursors (13,14). The relative contribution of these mechanisms to the action of biphosphonates in vivo is not known and may not be the same for every individual compound. The effect of biphosphonates on osteoclast formation can be indirect, involving the production of pro or anti osteoclastogenic factors. There are conflicting data on the effect of biphosphonates on cytokine production, for example, it has been shown that biphosphonates interact with osteogenic cells (15,16) in particular Vitte et al. (16) reported that they induce the secretion of
an inhibitor of osteoclast formation by a direct action on osteoblast-like cells. As regards to
the level of pro-osteoclastogenic cytokines, several studies indicate that biphosphonates
enhances IL-6 and TNF alpha \(^{17-20}\), whereas others found no effect or simply a long-term
reduction of these cytokines \(^{21-24}\). A recent study \(^{25}\) shows a significant increase in the
serum levels of OPG in patients treated with oral amino-biphosphonates, while levels of
serum RANKL were unchanged throughout the treatment period.

The aim of the present study was to asses the effect of \textit{in vivo} oral risedronate treatment
on osteoclast formation \textit{in vitro}; we suggest that risedronate reduces osteoclast formation
and activity mainly by the reduction of osteoclast precursors and pro-osteoclastogenic
cytokines production.

**MATERIALS AND METHODS**

\textit{Patients and markers of bone turnover.}

The study was approved by the human study review board of the Azienda Sanitaria
Ospedaliera San Giovanni Battista of Torino and all the patients signed an informed
consent statement prior to their recruitment.

Thirty-eight women with postmenopausal osteoporosis were enrolled. Subjects taking
calcium and vitamin D, thyroid hormones, corticosteroids, estrogen, bisphosphonates,
strontium ranelate, parathyroid hormone and raloxifene were excluded. All subjects had
been in spontaneous menopause for at least one year and were osteoporotic according to
the WHO criteria \(^{26}\). Bone mineral density was measured by double-emission X-ray
absorptiometry with a Hologic QDR 4500 (Hologic Inc.). Secondary osteoporosis was
ruled out in the light of the anamnesis, physical examination and routine examinations:
blood calcium and phosphorus, bone alkaline phosphatase and 25-OH vitamin D. Serum
osteocalcin (bone Gla protein - BGP - measured with a RIA technique - DiaSorin), and
urinary CTX (measured with an \(\alpha\)-Cross Laps \(\text{®} \) RIA from Osteometer BioteTech A/S)
were used as markers of bone metabolism/turnover.
Treatment

Patients were randomly assigned to treatment with 5 mg/day risedronate without supplement with calcium and vitamin D (25 subjects) or with calcium 1 g/day and vitamin D 800 UI/day (13 subjects) per os for 3 months. Blood was drawn from an antecubital vein after an overnight fast of 10 or more hours before and after therapy. All the measurements were done from a single blood sample at a single time point per patient.

Cell isolation and cultures

Peripheral blood mononuclear cells (PBMC) were obtained with the Ficoll-Paque method from 40 ml peripheral blood in lithium heparin as previously described (27). PBMC cultures were performed in triplicate for each subject in 16-well plates (BD Biocoat™ Osteologic™ Bone Cell Culture System, Becton Dickinson & Co. 4 x 10^5 cell/well) using alpha minimal essential medium (α-MEM: Gibco) supplemented with 10% fetal bovine serum (FBS), benzyl penicillin (100 IU/ml) and streptomycin (100 μg/ml) (unstimulated condition), or in the same medium plus M-CSF (25 ng/ml) and RANKL (30 ng/ml) (stimulated condition). RPMI (Gibco UK) was used for cell isolation. All cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. To validate resorption of the hydroxyapatite matrix, cells from 6 patients before and after treatment with risedronate or calcium and vitamin D were also double-plated on dentin slices [2x10^6 cell/slice]. The capacity of osteoclasts to reabsorb bone in vitro was equally detected by measuring resorption pits on dentin slices and the resorption of hydroxyapatite-coated wells (data not shown), as demonstrated also by other studies (10,28-30).

Osteoclast formation and activity

Cells were fed every 3 days. On the 21st day, they were fixed and stained for Tartrate Resistant Acid Phosphatase (TRAP) (Acid Phosphatase, Leukocyte staining kit, Sigma Diagnostics) and stained with an immune technique to express the vitronectin receptor
The supernatants of the entire culture period were pooled and frozen at -80°C. The formation of TRAP+ and VNR+ multinucleated (> 3 nuclei) cells was quantified by counting the stained cells in each well. The count was blind to subject status. Osteoclasts were always identified by the same operator as previously described (27), and the mean per subject of 3 wells in stimulated or unstimulated condition was calculated. In order to evaluate osteoclasts activity cells were removed with 14% sodium hypochlorite after the count and a Nikon Coolpix digital camera attached to an inverted research microscope was used to photograph the surface of each well. Lacunar resorption, determined by examining each micrograph with a dedicated computer image analysis system developed in our lab (patent n Italian Patent Application n TO2006A00565), was expressed as the total percentage of the surface reabsorbed.

**Cell viability**

Differences in cell viability before and after treatment were assessed in three patients per group by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PBMC were cultured in 96 well/plates in the presence or absence of M-CSF (25 ng/ml) and RANKL (30 ng/ml). On days 21, 25 and 30, 10 µl of MTT/PBS solution (5mg/ml) were added in each well (containing 100 µl of cells), followed by 4 hours incubation at 37°C in humidified 5% CO₂ atmosphere. The reaction was stopped by the addition of 100 µl of 0.04 N HCl in absolute isopropanol. The plates were read in a microtiter plate reader (Automatic Microtiter Reader, Biorad) using a 570-nm filter. To automatically subtract background noise we used dual wavelength setting of 570 nm and 630 nm according to manufacturer instructions.

**Cytokine measurement**

ELISA kits were used to measure TNFα (Quantikine; R&D System), OPG (Biomedica; Biomedica Medizinprodukte GmbH & Co KGA) and free s-RANKL (Biomedica; Biomedica
Medizinprodukte GmbH & Co KGA) levels in the pooled unstimulated culture supernatants to investigate if they were modified by risedronate. In the serum we measured the levels of total s-RANKL (Apotech; Apotech Corporation & Immunodiagnostik) to exclude possible influence due to a variation in OPG level after therapy, as suggested by Dobnig et al (25).

Each measurement was performed in duplicate for each patient.

**Flow cytometry**

Three-color flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson & Co). The effect of risedronate on osteoclast precursors was evaluated by staining PBMC with fluorescein (FITC) conjugated anti-VNR, phycoerytrin (PE) conjugated anti-CD14 and allophycocyanin (APC) conjugated anti-CD11b mAb, or with the corresponding isotype control, followed by incubation at 4°C for 30 min. The triple-positive (CD14+/CD11b+/VNR+) cells were considered osteoclast precursors according to previous literature (31-36).

Membrane antigens expression was analyzed through CellQuest (Becton Dickinson & Co) software and displayed as bivariate dot plots or histograms. Each plot depicts the results from 10,000 events representing viable cells gated by cell size and granularity.

All the antibodies were purchased from Becton Dickinson & Co.

**Statistics**

To exclude possible selection bias baseline characteristics were compared among the two groups of treatment by means of one way ANOVA. Student's paired t test was used to compare bone turnover markers, number of circulating precursors, osteoclast number and resorption activity, cell viability and cytokine levels at baseline and after therapy. The SPSS 14.0 software package was used to process the data with p < 0.05 as the significance cut-off.
RESULTS

The two treatment groups were not significantly different for baseline characteristic (Tab. 1), this datum ruled out possible selection biases.

*Risedronate significantly reduces osteoclast formation and activity*

The number and the activity of osteoclast formed after the *in vivo* treatment with risedronate were significantly lower in both unstimulated and stimulated condition (mean reductions of 53% and 68% respectively), while there was no significant reduction in calcium and vitamin D treated patients (Fig. 1). These findings suggest that risedronate reduces the PBMC trend to develop into osteoclasts in cultures, both in stimulated and in unstimulated condition. The markers of bone metabolism confirm the experimental data on the higher osteoclasts activity: in fact the bone resorption marker (CTX) was significantly reduced by risedronate (8.02±1.88 at baseline versus 5.84±1.02 after three months of risedronate, p=0.04) and correlates to the number of osteoclasts formed in culture (r= 0.7, p=0.03).

*In vivo administration of risedronate significantly reduces cell viability in vitro.*

To asses whether the reduction in osteoclasts formation and activity was due to a reduction in PBMC viability we performed an MTT test on days 21, 25 and 30 of culture. This test shows that PBMC viability was significantly reduced after risedronate both in stimulated and in unstimulated condition at any time: the optical density (OD) was significantly reduced from 0.92±0.16 to 0.24±0.02 (p=0.002) on average, while in the controls there was no difference in cell viability after three months of treatment with calcium and vitamin D. PBMC viability after risedronate was significantly higher in the stimulated condition on days 21 and 25: OD day 21= 0.18±0.05 unstimulated vs 0.27±0.05 stimulated, p=0.03, OD day 25= 0.25±0.04 unstimulated vs 0.31±0.02 stimulated, p=0.04. These data could suggest that risedronate reduces PBMC viability decreasing cytokines production.
**Risedronate reduces pro-osteoclastogenic cytokines**

To investigate whether risedronate acts on the PBMC cytokines production we measured the levels of TNFα, free s-RANKL and OPG in the culture supernatants. The levels of the pro-osteoclastogenic cytokines TNFα and free s-RANKL were significantly reduced in PBMC cultures from risedronate treated patients, OPG levels were also lower, though not significantly (Table 2). These data confirm the hypothesis that risedronate could reduce PBMC viability through the reduction of cytokines production. To investigate a possible systemic effect of risedronate on cytokines production we also measured the levels TNFα, total s-RANKL and OPG in the serum: we observe a significant reduction in both TNF α and OPG levels, while total s-RANKL remains unchanged (Table 3). The reduction of TNF and OPG confirms the data obtained in the cellular cultures, while the datum on s-RANKL does not.

**Risedronate reduces osteoclast precursors**

To establish whether risedronate reduces osteoclastogenesis through the selective reduction of osteoclast precursors we assessed the numbers of osteoclast precursors in the peripheral blood. Circulating osteoclast precursors (CD14+, CD11b+ and VNR+) fell by more than a third after risedronate, while there is no difference after treatment in the total amount of CD14 positive cells. This finding suggests that risedronate acts selectively against osteoclast precursors without affecting the other monocytic lineage cells. The mean fluorescence intensity (MFI) of CD11b was also notably reduced (Fig. 2). As far as MFI depicts the number of CD molecules expressed on the cell surface, this finding suggests that risedronate selectively reduces the more committed osteoclast precursors. The number of osteoclast precursors and the MFI do not change after calcium and vitamin D treatment (Fig 2).
DISCUSSION

It has been demonstrated that circulating osteoclast precursors exist primarily within the monocytic fraction of peripheral blood, (31-36) and their presence in the circulation serves both as a reservoir for replenishing pre-osteoclast populations in the bone marrow as needed and as a potentially abundant source of pre-osteoclasts that can be recruited into bone or joint tissue in response to reparative or pathological signals. In contrast to the beneficial nature of pre-osteoclast recruitment during normal bone remodelling or fracture repair, excessive pre-osteoclast recruitment in pathological conditions causes significant bone loss in many skeletal disorders characterized by increased osteoclast formation and activity (37-39). We have shown that mature and active osteoclasts can be differentiated from PBMC cultures and that their activity is significantly greater in patients with postmenopausal osteoporosis compared with healthy controls, and that enhanced osteoclast formation is largely dependent on the greater ability of PBMC from such patients to produce pro-osteoclastogenic cytokines (TNF alpha and RANKL) (27). The aim of our study was to evaluate the effect of oral risedronate treatment on osteoclasts recruitment, formation and activity. Account was taken of the presence of circulating precursors, activity and vitality of cells produced in cultures, and cytokine levels in the culture medium and in the serum.

There is as yet no unequivocal explanation of the effect of risedronate on the presence of preosteoclasts in the circulation, though there are data on the induction of osteoclast apoptosis by biphosphonates in vivo and in vitro, and on the effect of biphosphonates on preosteoclasts on bone surface (13,14). It has been demonstrated that biphosphonates can activate T cells, thus inducing proinflammatory cytokines release in the early period of treatment (17-20), whereas other studies suggested that this release is reduced after long-term therapy (21-25). It is interesting to point out that none of the above mentioned study
analyzed the cytokines production by PBMC *ex vivo* before and after treatment with biphosphonates.

The new feature of the present study is the *in vitro* evaluation of *in vivo* management by means of relatively simple methods that could be routinely applied to assess patient response and compliance to treatment.

Our findings show that risedronate inhibits osteoclastogenesis by PBMC in postmenopausal osteoporosis, in both unstimulated and stimulated condition after only three months of treatment. We also suggest that risedronate inhibits the *in vitro* formation of osteoclasts by reducing the number and the degree of differentiation of osteoclast precursors, and also by reducing their half-life and by inhibiting the production of pro-osteoclastogenic cytokines. We suggest that risedronate is able to reduce osteoclasts recruitment from peripheral blood and that this could be due to the reduction in cytokines production. There is a growing body of evidence that biphosphonates may also act on osteoblastic bone lineage cells. It has been suggested that biphosphonates require osteoblasts for the mediation of bone resorption (40,41) and the possible modulation of the secretion of osteoblast-derived soluble factor(s) (16,25); this could be a possible mechanism to explain the reduction of osteoclasts recruitment from peripheral blood.

We observe a reduction in OPG levels in serum, this datum confirms other reports on the early effects of biphosphonates on OPG production (25,42). Early reduction in OPG serum levels provides still more evidence that serum OPG concentrations are at least partly reflective of bone turnover (25,42). In the present study, we detect a significant reduction in free sRANKL levels during biphosphonates treatment; this report confirms recent *in vitro* data that indicate that zoledronic acid may decrease RANKL levels through the cleavage of trans-membrane RANKL in osteoblast-like cells by the up-regulation of TACE, which is a known enzyme capable of cleaving RANKL (43).

We have also observed a higher reduction in the PBMC half-life in absence of growth
factors, even without the addition of biphosphonates in culture, therefore we hypothesize that this effect is attributable to either reduction of the number of cells able to become osteoclasts \textit{in vitro}, or to induction of a pro-apoptotic pattern in these cells, as suggested in the literature \cite{44,45}. However the half-life and number of osteoclast \textit{in vitro} are greater after treatment in the wells supplemented with growth factors: this suggests that decreased cytokine production \textit{in vitro} is one of the determinants of the reduction of osteoclasts formation.

Elucidation of the mechanism whereby risedronate \textit{in vivo} alters PBMC-induced osteoclastogenesis \textit{in vitro} will require further studies focusing on both the induction of a pro-apoptotic pattern in mature osteoclasts and pre-osteoclasts by risedronate and its effect on cytokines production.
REFERENCES


TABLE 1. CHARACTERISTICS OF RISEDRONATE AND CALCIUM VITAMIN D TREATED PATIENTS AT BASELINE.

<table>
<thead>
<tr>
<th></th>
<th>Risedronate (25 patients)</th>
<th>Calcium vitamin D (13 patients)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>64.08±53.14</td>
<td>62.33 ± 5.79</td>
<td>N.S.</td>
</tr>
<tr>
<td>Postmenopausal period (yrs)</td>
<td>16.58 ± 6.27</td>
<td>14.58 ± 13.57</td>
<td>N.S.</td>
</tr>
<tr>
<td>BMI</td>
<td>22.99 ± 2.69</td>
<td>23.33 ± 2.67</td>
<td>N.S.</td>
</tr>
<tr>
<td>Bone alcaline phosphatase (ng/ml)</td>
<td>15±4</td>
<td>16.22 ± 7.31</td>
<td>N.S.</td>
</tr>
<tr>
<td>Parathyroid hormone (pg/ml)</td>
<td>26.5±9.4</td>
<td>28.08 ± 16.15</td>
<td>N.S.</td>
</tr>
<tr>
<td>Osteocalcin (ng/ml)</td>
<td>5.6±4</td>
<td>5.14±4</td>
<td>NS</td>
</tr>
<tr>
<td>Serum calcium (mEq/l)</td>
<td>4.8±0.2</td>
<td>4.86 ± 0.25</td>
<td>N.S.</td>
</tr>
<tr>
<td>Serum phosphorus (mMol/l)</td>
<td>1.17±0.1</td>
<td>1.05 ± 0.16</td>
<td>N.S.</td>
</tr>
<tr>
<td>CTX (µg/L)</td>
<td>8.02±1.88</td>
<td>8.17±2.56</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
### TABLE 2. CYTOKINES PRODUCTION BY PBMC CULTURES IN RISEDRONATE AND CALCIUM VITAMIN D TREATED PATIENTS AT BASELINE AND AFTER THERAPY.

<table>
<thead>
<tr>
<th></th>
<th>Baseline (25)</th>
<th>After risedronate (25)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free s-RANKL (pg/ml)</td>
<td>7.84±4.12</td>
<td>1.56±0.3</td>
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<tr>
<td>TNF alpha (pg/ml)</td>
<td>35.7±14.14</td>
<td>12.16±7.45</td>
<td>0.01</td>
</tr>
<tr>
<td>OPG (pg/ml)</td>
<td>41.48±20.46</td>
<td>26.95±10.49</td>
<td>NS</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Baseline (13)</th>
<th>After calcium and vitamin D (13)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANKL (pg/ml)</td>
<td>4.5±3</td>
<td>3.92±2.6</td>
<td>NS</td>
</tr>
<tr>
<td>TNF alpha (pg/ml)</td>
<td>121.6±46.1</td>
<td>205.4±122.5</td>
<td>NS</td>
</tr>
<tr>
<td>OPG (pg/ml)</td>
<td>21.85±20</td>
<td>44.5±18.3</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Baseline (25)</td>
<td>After risedronate (25)</td>
<td>p</td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------</td>
<td>------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Total s-RANKL (ng/ml)</td>
<td>325.2±260.4</td>
<td>356.2±304.9</td>
<td>NS</td>
</tr>
<tr>
<td>TNF alpha (pg/ml)</td>
<td>129.1±10.8</td>
<td>113.6±11.1</td>
<td>0.009</td>
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<tr>
<td>OPG (ng/ml)</td>
<td>2.8±1.3</td>
<td>0.9±0.4</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Baseline (13)</td>
<td>After calcium and vitamin D (13)</td>
<td>p</td>
</tr>
<tr>
<td>Total s-RANKL (ng/ml)</td>
<td>258.4±129.1</td>
<td>312.5±121.3</td>
<td>NS</td>
</tr>
<tr>
<td>TNF alpha (pg/ml)</td>
<td>142.7±23.2</td>
<td>143.9±22.8</td>
<td>NS</td>
</tr>
<tr>
<td>OPG (ng/ml)</td>
<td>1.5±0.2</td>
<td>1.2±0.2</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figures

**Figure 1 A. Osteoclasts formation and activity.** Appearance of osteoclasts (indicated with white arrows) in a light micrograph (20 X) after TRAP staining before (upper left panel) and after in vivo treatment with risedronate (upper right panel). Pits formation before (lower left panel) and after in vivo treatment with risedronate (lower right panel). The micrographs refers to osteoclasts generated in PBMC cultures without stimulus after 21 days of culture.

**B.** Histograms showing the number of osteoclasts (OC) and the percentage of hydroxyapatite matrix reabsorbed, in unstimulated (without M-CSF and RANKL, upper) and stimulated (with M-CSF and RANKL added, lower) conditions, in PBMC cultures from osteoporotic women treated with risedronate or calcium and vitamin D for three months. The bars show the mean and SD for all patients.
Figure. 2. Effects of risedronate on circulating osteoclast precursors. FACS analysis of circulating osteoclast precursors from PBMC of osteoprotic women labelled with FITC-conjugated anti-VNR, PE-conjugated anti-CD14 and APC-conjugated anti-CD11b mAbs before and after risedronate in vivo treatment.

A. Histograms with CD14+ cells (monocytes) gated on PBMC, before (left panel) and after (right panel) risedronate treatment.

B. Dot plots represent VNR+ and CD11b+ cells gated on CD14+ cells (osteoclast precursors), as indicated in region R1, (upper right panels: triple positive cells, lower right panels CD14 and CD11b positive cells). Percentages of positive cells are indicated.

C. Histograms represent the mean fluorescence intensity (MFI) of CD11b of CD14+ cells (as indicated in region R1, panel A) before (broken line) and after (unbroken line) treatment. The curve shift shows the reduction of MFI after risedronate treatment. The graph on the left shows the MFI of CD11b before and after treatment with risedronate or with calcium and vitamin D. The bars show the mean and SD for all patients.

D. Histograms showing the percentage of CD14+, CD14+ CD11b+ and Cd14+ CD11b+ VNR+ (osteoclast precursors) cells before and after treatment with risedronate or with calcium and vitamin D. The bars show the mean and SD for all patients.