Alendronate reduces osteoclast precursors in osteoporosis

Alendronate reduces osteoclast precursors in osteoporosis

Patrizia D'Amelio1, Anastasia Grimaldi1, Maria Angela Cristofaro1, Marco Ravazzoli1, Pia Anna Molinatti1, Gian Piero Pescarmona2-3, Giancarlo Isaia 1.

1 Department of Surgical and Medical Disciplines Section of Gerontology, University of Torino 2 Center for Experimental Research and Medical Studies (CERMS)- Hospital San Giovanni Battista -Torino, 3 Department of Genetics, Biology and Biochemistry -University of Torino – Italy.

Running title: Alendronate inhibits osteoclastogenesis.

Disclosure of conflicts of interest: no disclosure

Corresponding author: D'Amelio Patrizia MD, PhD

Department of Surgical and Medical Disciplines Section of Gerontology,

Corso Bramante 88/90, 10126 Torino, Italy.

Tel: +390116335533-Fax: +390116636033, e-mail: patrizia.damelio@unito.it

Mini abstract

This study evaluates the effect of alendronate on osteoclastogenesis, cytokine production and bone resorption in postmenopausal women. We suggest that alendronate acts on mature bone resorbing osteoclast after 3 months of therapy; whereas after one year it reduces osteoclast formation through the reduction of osteoclast precursors and RANKL.

Abstract

Purpose: The target cells of bisphosphonates are osteoclasts, but it is still unclear which steps of osteoclast formation and activity are more sensitive to these drugs. The present study evaluates the effect of *in vivo* alendronate on osteoclastogenesis, cytokine production and bone resorption in postmenopausal women.

Methods: The study was conducted on 35 osteoporotic women; 15 patients were pre-treated with alendronate 70 mg/week while 20 were treated with calcium 1 g/day and vitamin D 800 UI/day, after three months 30 patients received alendonate 70/mg, vitamin D 2800 UI/week and calcium 1 g/day for 12 months (combined therapy), while 5 patients continued with calcium 1 g/day and vitamin D 800 UI/day. The following parameters were assessed before and after therapy: changes in bone resorption marker, circulating osteoclast precursors, osteoclasts formation by peripheral blood mononuclear cells cultures, their viability and variations in cytokines production.

Results: After 3 months of alendronate there is no significant reduction in the number of osteoclast precursors, osteoclast formation and viability and in the level of cytokines, whereas there was a significant reduction of bone resorption marker. One year therapy with alendronate plus calcium and vitamin D progressively reduces osteoclast precursors, osteoclast formation and serum RANKL; no effect was seen with calcium and vitamin D.

Conclusions: We suggest that alendronate acts mainly on mature bone resorbing osteoclast in the short period; whereas one year combined therapy reduces osteoclast formation through the reduction of osteoclast precursors and RANKL.

Key words: alendronate; osteoclast; osteoporosis; RANKL; osteoclast precursors

INTRODUCTION

Bisphosphonates (BPs) have a chemical structure similar to that of inorganic pyrophosphate and have a high affinity for the bone matrix; they are widely used in the treatment of diseases with increased bone resorption. Their mechanism of action depends on their chemical structure, namely on the presence of one or more amine groups. Non-nitrogen-containing bisphosphonates, such as clodronate, etidronate and tiludronate, act by inducing osteoclast (OC) apoptosis through their intracellular accumulation in cytotoxic non-hydrolysable ATP analogues, whereas nitrogen-contaning BPs pamidronate, alendronate, ibandronate, zoledronate and risedronate act as isoprenoid diphosphate lipid analogues and inhibit farnesyl pyrophosphate synthase, an enzyme in the mevalonate pathway [1].

Inhibitions of this pathway prevent the synthesis of the isoprenoid lipids essential for the farnesylation and geranylation of small GTPase signaling proteins and inhibit OC action. BPs have well known cellular effects that cause OC retraction, condensation, and cellular fragmentation and induce apoptosis, both *in vitro* [2-4] and *in vivo* [5]. BPs also inhibit OC recruitment and differentiation [6-8], their attachment to the bone surface [9, 10], and ruffled border formation [11], which is essential for bone resorption. It has been suggested that BPs acts directly on OC through the internalization by the cell itself, but also through the effect on OC recruitment by non-active precursors and through the inhibition of pro-osteoclastogenic cytokines [12, 13]. The role of BPs in reducing pro-osteoclastogenic cytokines is still debated since some studies show an increase in the levels of pro-osteoclastogenic cytokines [14-16], while others show no effect or reduction of these cytokines [12, 17-20]. Some studies suggest that BPs may also affect osteoblast, inducing the release of a factor that inhibits OC activity or formation [21, 22].

Although it is still unclear which steps of OC formation and activity are more sensitive to BPs, we have previously suggested that a three months oral therapy with a nitrogen-containing BPs (risedronate) is able to considerably reduce OC precursors recruitment, bone resorption and production of TNF alpha and RANKL [12].

The aim of the present study is to evaluate the short and long term effect of alendronate on osteoclastogenesis, cytokines production and bone turnover in postmenopausal osteoporosis.

METHODS

Patients and markers of bone turnover.

The study was approved by the "Clinical Study Review Committee" of the Azienda Sanitaria Ospedaliero Universitaria San Giovanni Battista of Torino and all the patients signed an informed consent statement prior to recruitment.

Thirty-five women with postmenopausal osteoporosis were enrolled. Subjects taking calcium and vitamin D, thyroid hormones, corticosteroids, estrogen, BPs, 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 WHO criteria [23]. Bone mineral density was measured by double-emission X-ray absorptiometry with a Hologic QDR 4500 (Hologic Inc.). Secondary osteoporosis was ruled out by means of medical history, physical examination and blood tests: serum calcium and phosphorus, bone alkaline phosphatase, serum protein electrophoresis and 25-OH vitamin D. Serum CTX (measured with an α-Cross Laps ® RIA from Osteometer BioteTech A/S) was used as marker of bone resorption.

Treatment

Patients were randomly assigned to treatment with 70 mg/week alendronate (Fosamax ®, kindly provided by Merck Sharp & Dohme SpA Italy) without calcium and vitamin D supplement (15 subjects) or with calcium 1 g/day and vitamin D 800 UI/day (Cacit vitD3®, kindly provided by Procter & Gamble) (20 subjects) per os for 3 months (pre-treatment). After three months, 30 patients received alendronate 70 mg/week plus vitamin D 2800 UI/week (Fosavance®, kindly provided by Merck Sharp & Dohme SpA Italy) plus calcium 1 g/day (Cacit 1000®, kindly provided by Procter & Gamble) this treatment will be referred to as "combined therapy", while 5 patients continued calcium 1 g/day and vitamin D 800 UI/day, the first five patients randomized to pre-treatment with calcium and vitamin D were designed to continue this therapy for the entire study period (Fig.1).

Blood was drawn from an antecubital vein after an overnight fast of 10 or more hours before and after pre-treatment and after 3, 9 and 12 months of combined therapy or calcium and vitamin D alone. All measurements were performed from a single blood sample at a single time point per patient.

At the end of the study BMD was measured at spine and femoral neck.

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 [24]. 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% CO_2 atmosphere.

Osteoclast formation.

Cells were fed every 3 days; the medium was recovered at each change, pooled and frozen at -80°C. On the 21st day, the cultures 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 (VNR) (Becton Dickinson & Co). 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 treatment.

OCs were always identified by the same operator as previously described [24], and the mean of 3 wells for each subject in stimulated or unstimulated condition was calculated. The PBMC cultures were performed at basal level, after pre-treatment and after 12 months of combined therapy.

The ability of OC to resorb bone in vitro was evaluated at the end of the culture period by removing the cells with 14% sodium hypochlorite after counting; a Nikon Coolpix digital camera attached to an inverted microscope was used to photograph the surface of each well [12].

Flow cytometry

Three-color flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson & Co). The effect of therapy on OC 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 cells CD14+/CD11b+/VNR- were considered as early OC precursors [25, 26]; triple-positive (CD14+/CD11b+/VNR+) cells were considered OC precursors according to previous literature [27-32].

In order to evaluate if alendronate acts also on PBMCs other than OC precursors we evaluated PBMCs subpopulation at baseline and after therapy in the first 5 patients randomized to alendronate. Briefly PBMCs were stained with FITC conjugated anti-CD19, peridinin chlorophyll protein (PerCP) conjugated anti-CD 3, and APC conjugated anti-CD14 mAb, or with the corresponding isotype control, followed by incubation at 4°C for 30 min. Cells were classified as B (CD19+), T (CD3+) lymphocytes and monocytes (CD 14+) according to their Forward Scatter (FSC) and Side Scatter (SSC).

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. Flow cytometry was performed at basal level after pre-treatment and after 3, 9 and 12 months of combined therapy.

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 therapy. The levels of total s-RANKL (Apotech; Apotech Corporation & Immunodiagnostik) were measured in the serum. Each measurement was performed in duplicate for each patient. Serum cytokines were measured at basal level, after pre-treatment and after 3 and 12 months of combined therapy; supernatants cytokines were measured at basal level, after pre-treatment and after 12 months of combined therapy.

Cell viability

Differences in cell viability were assessed by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyltetrazolium bromide (MTT) assay, before and after 12 months of combined therapy in four patients randomly chosen from each group. 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 10, 21 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% CO_2 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.

In the group treated with calcium and vitamin D for 15 months only OC precursors and serum cytokines were evaluated (Fig.1).

Statistics

To test for the normal distribution of each analyzed parameter a Curtosis' test was carried out, baseline patient's characteristics; BMD, OC in cultures, PBMCs' subpopulations and cell viability were normally distributed whereas the others were not.

To exclude possible selection bias, baseline characteristics were compared among the three groups of treatment by means of one way ANOVA. Student's paired t test was used to compare OC number, PBMCs' subpopulations and cell viability at baseline and after therapy at different time points. BMD was compared between baseline and end of the study by Student's paired T test. A Wilcoxon's test was used to compare CTX, number of circulating precursors and cytokine levels at baseline and after therapy at different time points The SPSS 15.0 software package was used to process the data with $p<0.05$ as the significance cut-off.

RESULTS

The three groups of subjects do not significantly differ for baseline characteristics (Table 1).

Combined therapy reduces osteoclast formation.

In order to evaluate the effect of alendronate *in vivo* on *in vitro* OC formation, PBMC cultures were performed with and without the addition of M-CSF and RANKL at different time points. After three months of therapy, alendronate, as well as calcium plus vitamin D, does not significantly reduce OC formation both with and without the addition of M-CSF and RANKL, whereas after 12 months of combined therapy there was a significant reduction in OC formation and activity (Fig. 2). We do not observe a significant increase of OC formation after the addition of M-CSF and RANKL.

On the contrary, serum CTX, a bone resorption marker, decreases already after three months of therapy in patients treated with alendronate, instead it was not reduced by calcium and vitamin D alone (Fig.3).

Combined therapy reduces circulating osteoclast precursors.

In order to evaluate a possible effect of alendronate on bone marrow output of circulating OC precursors we analysed the presence of OC precursors in PBMC at baseline and after therapy. After three months there was no significant decrease in the OC precursors in patients treated with alendronate or with calcium plus vitamin D, instead after 9 and 12 months of combined therapy a significant reduction of the OC precursors was observed, in patients treated with calcium and vitamin D alone there was no reduction of OC precursors at any time point. We also observe a reduction in the percentage of CD14+/CD11b+/VNR- cells after 12 months of combined therapy, these cells have been identified as early OC precursors according to previous literature [25, 26] (Fig.4).

These data suggest a short term effect of alendronate on mature bone resorbing OCs rather than on OC precursors.

In order to evaluate a possible effect of alendronate on PBMCs other than OC precursors, we evaluated the percentage of B (CD19+), T (CD3+) lymphocytes and monocytes (CD14) at basal level, after pre-treatment with alendronate and after 12 months of combined therapy; we found no significant influence of alendronate on PBMCs other then OC precursors (supplemental figure).

It seems important to underline the high specificity of alendronate towards OC precursors as these cells fell by more than 90% and early OC precursors by more than 10% after 12 months of combined therapy while there is no difference after treatment in the total amount of CD14+ cells (general monocytes population). This finding suggests that alendronate acts selectively against OC precursors without affecting the other monocytic lineage cells.

Combined therapy reduces serum RANKL.

In order to evaluate if combined therapy reduced osteoclastogenesis by influencing the cytokines production *in vitro* and *in vivo,* the levels of TNF alpha, RANKL and OPG were measured in culture supernatants and in serum. An increase in TNF alpha level was found in supernatants after 3 months of alendronate, instead there was no difference in the levels of free-RANKL and OPG (Fig.5A). Serum total RANKL and serum OPG were significantly reduced after 3 and 12 months of combined therapy, whereas therapy had no effect on the serum level of TNF alpha (Fig.5 B).

These data suggest an early pro-inflammatory effect of alendronate, that does not persist in the long term and that combined therapy is able to reduce the *in vivo* RANKL production, thus reducing OC formation and activity, these data are reinforced by the positive correlation between serum RANKL and CTX (R=0.46, p=0.011). The reduction of serum OPG may reflect the reduction of bone turnover.

Combined therapy does not reduce PBMC viability.

To asses whether the reduction in OCs formation after one year therapy was due to a reduction in PBMC viability, an MTT test was performed on days 10, 21 and 30 of culture in the presence or absence of growth factors. This test showed that PBMC viability was not significantly reduced after alendronate nor after calcium and vitamin D or combined therapy, both in stimulated and in unstimulated conditions, at any time point (data not shown).

Clinical outcome

We observed a significant increase in BMD at the lumbar spine (0.72±0.017 g/cm^2 at baseline vs 0.75±0.017 g/cm^2 end of the study, p=0.004) and a non significant increase at the femoral neck (0.43±0.17 g/cm^2 at baseline vs 0.60±0.014 g/cm^2 after 15 months of therapy, p=NS); in patients treated with calcium and vitamin D alone there was a non significant reduction of BMD at both sites (data not shown).

DISCUSSION

Although BPs are widely used in the treatment of bone diseases such as osteoporosis, Paget's disease and myeloma, a clear picture on which cells are targeted by these drugs is not available; in particular some studies suggested that mature OC are the only target of BPs [7, 8], whereas other studies suggested that also OC precursors [12, 33, 34] and osteoblasts [22] could be influenced by BPs. A recent publication by Coxon FP et al. [35] demonstrated that BPs uptake by non-resorbing cells can be increased when these cells are cultured in the presence of resorbing OCs. Our group and other authors have previously shown that OC precursors in peripheral blood are increased in diseases characterized by increased bone resorption [32,

36-39] and are decreased after anti-resorptive therapy [12]. It has also been shown that OC formation depends mainly on the amount of precursors recruited and on the balance between pro- and anti-osteoclastogenic cytokines among which the most studied are RANKL, TNF alpha and OPG [36, 40, 41].

The aim of the present study was to evaluate the effect of short term oral alendronate and one year combined therapy on OCs recruitment, formation and activity. The presence of circulating precursors, the formation and viability of cells produced in PBMC cultures, and cytokine levels in culture medium and serum were evaluated.

Our data confirmed the early effect of oral alendronate on bone resorption *in vivo,* as demonstrated by the reduction of CTX after three months of treatment, whereas there was no effect of three months of alendronate on *in vitro* OC formation in PBMC cultures. After 12 months of combined therapy we observed a significant reduction in OC formation both in the presence and in the absence of M-CSF and RANKL that directly stimulates osteoclastogenesis. We did not observe an increased OC formation after the addition in cultures of M-CSF and RANKL; this finding is in line with previous studies on osteoclastogenesis from PBMCs in various bone lytic diseases (see [42] for review). These observations can suggest that cellular composition of PBMCs from patients with increased bone resorption is sufficient to increase osteoclast formation and activity in vitro. In order to evaluate the mechanism by which therapy reduces OC formation, we examined the OC precursors in peripheral blood: a significant decrease of OC precursors (CD14+/CD11b+/VNR+ cells) in patients treated for at least 9 months with combined therapy was found, whereas early OC precursors (CD14+/CD11b+/VNR- cells) decrease only after 12 months of combined therapy. In the pre-treatment period with alendronate we do not observe a reduction of OC precursors, even though there was a significant decrease in bone resorption; these data could suggest that short term therapy with alendronate acts mainly on the mature bone OC and does not influence the precursors' recruitment, instead OC formation in the long term therapy is reduced also through the decrease of OC precursors. As alendronate, similarly to risedronate [12], acts on circulating OC precursors, we evaluated if this drug has any effects on other types of PBMCs; in particular we analyzed monocytes, B and T cells: our data demonstrated that alendronate acts specifically on OC precursors without affecting other cell types. As regards monocytes it seems important to point out that alendronate acts specifically on monocytes committed towards OC lineage (CD14+/CD11b+/VNR- and CD14+/CD11b+/VNR+) without affecting the general monocytes population (CD14+):

Regarding the effect of alendronate on *in vitro* cytokines production, increased levels of TNF alpha were observed after three months therapy, whereas after one year of combined therapy TNF alpha levels returned

to baseline. These data could indicate an early pro-inflammatory effect of alendronate and confirm previous data from an *in vivo* animal study [43] and *in vitro* studies on human cells [15, 44, 45] suggesting that alendronate can increase TNF production from T cells. However, it is important to point out that a systemic increase in the TNF alpha levels or acute phase reaction were not observed in these patients; moreover the observed increase in TNF alpha was transitory since TNF returned to baseline after one year of therapy.

No effect of alendronate on PBMC ability to produce OPG and RANKL was observed, instead serum levels of RANKL were significantly reduced by combined therapy after 3 and 12 months. This datum could confirm recent *in vitro* data that indicate that zoledronic acid, another BPs, may decrease RANKL levels through the cleavage of trans-membrane RANKL in osteoblast-like cells by up-regulating TACE, an enzyme known to cleave RANKL [46].

Serum OPG levels were significantly reduced by treatment; this datum may provide further evidence that serum OPG concentrations are at least partly reflective of bone turnover [13, 40], and confirms our previous work on risedronate [12]. Our observation is in contrast with a previous work by Dobnig et al [13] that demonstrated an increase in OPG levels after long term treatment with alendronate or risedronate, The present study suggests striking differences in the effect of alendronate with respect to risedronate. In our previous work, we showed that risedronate was able to reduce OC formation from PBMC after only three months by reducing OC precursors, RANKL and TNF production. Alendronate seems to have a different onset of action, reducing bone resorption by targeting mature OC (CTX reduction) rather then OC precursors and cytokines production in the short period; only after at least 9 months of combined therapy a reduction in OC precursors was observed. Moreover, alendronate, differently from risedronate, does not influence PBMC viability. These differences in targeting OC precursors may be clinically relevant and may help to explain the supposed quicker onset of action of risedronate as respect to alendronate [47]. In the authors' opinion, the differences among the two oral BPs could be interpreted through their different affinity for hydroxiapatite [48]; the higher affinity of alendronate could explain its lower availability for non-resorbing cells, as bone marrow precursors, and its different time-dependent action in bone tissue.

Further studies are required to elucidate the mechanism underlying the different action of alendronate and risedronate.

The major limitation of this study is the relative small size of the cohort analyzed, this, together with the high variability of the biological markers could explain some negative finding in the study.

In conclusion we suggest that alendronate is able to reduce the activity of mature OC in the short period and to reduce OC formation in the long period, acting both on OC precursors' recruitment and on RANKL production, without affecting OC precursors' viability.

Acknowledgments: This work was supported by an unconditioned grant from the Merck Sharp & Dohme SpA Italy and a grant from the Fondazione Internazionale Ricerche Medicina Sperimentale (FIRMS) Compagnia San Paolo. M.A. Cristofaro was supported by a fellowship from the Ministry for Education, University and Research (MIUR), P. D'Amelio was supported by a fellowship from the Regione Piemonte. Alendronate and alendronate plus vitamin D were kindly provided by Merck Sharp & Dohme SpA Italy, calcium and vitamin D supplements were kindly provided by Procter & Gamble.

REFERENCES

1 MJ Rogers (2003) New insights into the molecular mechanisms of action of bisphosphonates. Curr Pharm Des 9:2643-2658.

2 HL Benford, NW McGowan, MH Helfrich, ME Nuttall,MJ Rogers (2001) Visualization of bisphosphonate-induced caspase-3 activity in apoptotic osteoclasts in vitro. Bone 28:465-473.

3 H Sudhoff, JY Jung, J Ebmeyer, BT Faddis, H Hildmann,RA Chole (2003) Zoledronic acid inhibits osteoclastogenesis in vitro and in a mouse model of inflammatory osteolysis. Ann Otol Rhinol Laryngol 112:780-786.

4 MJ Rogers, KM Chilton, FP Coxon, J Lawry, MO Smith, S Suri,RG Russell (1996) Bisphosphonates induce apoptosis in mouse macrophage-like cells in vitro by a nitric oxide-independent mechanism. J Bone Miner Res 11:1482-1491.

5 DE Hughes, KR Wright, HL Uy, A Sasaki, T Yoneda, GD Roodman, GR Mundy,BF Boyce (1995) Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo. J Bone Miner Res 10:1478-1487.

6 MG Cecchini, R Felix, H Fleisch,PH Cooper (1987) Effect of bisphosphonates on proliferation and viability of mouse bone marrow-derived macrophages. J Bone Miner Res 2:135-142.

7 PM Boonekamp, LJ van der Wee-Pals, MM van Wijk-van Lennep, CW Thesing,OL Bijvoet (1986) Two modes of action of bisphosphonates on osteoclastic resorption of mineralized matrix. Bone Miner 1:27-39.

8 GA Rodan,AA Reszka (2002) Bisphosphonate mechanism of action. Curr Mol Med 2:571-577.

9 S Colucci, V Minielli, G Zambonin, N Cirulli, G Mori, M Serra, V Patella, A Zambonin Zallone,M Grano (1998) Alendronate reduces adhesion of human osteoclast-like cells to bone and bone protein-coated surfaces. Calcif Tissue Int 63:230-235.

10 A Carano, SL Teitelbaum, JD Konsek, PH Schlesinger,HC Blair (1990) Bisphosphonates directly inhibit the bone resorption activity of isolated avian osteoclasts in vitro. J Clin Invest 85:456-461.

11 M Sato, W Grasser, N Endo, R Akins, H Simmons, DD Thompson, E Golub,GA Rodan (1991) Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. J Clin Invest 88:2095-2105.

12 P D'Amelio, A Grimaldi, S Di Bella, C Tamone, SZ Brianza, MG Ravazzoli, P Bernabei, MA Cristofaro, GP Pescarmona,G Isaia (2008) Risedronate reduces osteoclast precursors and cytokine production in postmenopausal osteoporotic women. J Bone Miner Res 23:373-379.

13 H Dobnig, LC Hofbauer, V Viereck, B Obermayer-Pietsch,A Fahrleitner-Pammer (2006) Changes in the RANK ligand/osteoprotegerin system are correlated to changes in bone mineral density in bisphosphonate-treated osteoporotic patients. Osteoporos Int 17:693-703.

14 A Sauty, M Pecherstorfer, I Zimmer-Roth, P Fioroni, L Juillerat, M Markert, H Ludwig, P Leuenberger, P Burckhardt,D Thiebaud (1996) Interleukin-6 and tumor necrosis factor alpha levels after bisphosphonates treatment in vitro and in patients with malignancy. Bone 18:133-139.

15 RE Hewitt, A Lissina, AE Green, ES Slay, DA Price,AK Sewell (2005) The bisphosphonate acute phase response: rapid and copious production of proinflammatory cytokines by peripheral blood gd T cells in response to aminobisphosphonates is inhibited by statins. Clin Exp Immunol 139:101-111.

16 K Thompson,MJ Rogers (2004) Statins prevent bisphosphonate-induced gamma,delta-T-cell proliferation and activation in vitro. J Bone Miner Res 19:278-288.

17 G Mossetti, D Rendina, G De Filippo, R Viceconti, G Di Domenico, M Cioffi, L Postiglione,V Nunziata (2005) Interleukin-6 and osteoprotegerin systems in Paget's disease of bone: relationship to risedronate treatment. Bone 36:549-554.

18 HA Papadaki, C Tsatsanis, A Christoforidou, N Malliaraki, M Psyllaki, C Pontikoglou, M Miliaki, AN Margioris,GD Eliopoulos (2004) Alendronate reduces serum TNFalpha and IL-1beta, increases neutrophil counts, and improves bone mineral density and bone metabolism indices in patients with chronic idiopathic neutropenia (CIN)-associated osteopenia/osteoporosis. J Bone Miner Metab 22:577-587.

19 D Santini, ME Fratto, B Vincenzi, A La Cesa, C Dianzani,G Tonini (2004) Bisphosphonate effects in cancer and inflammatory diseases: in vitro and in vivo modulation of cytokine activities. BioDrugs 18:269-278.

20 A Gur, A Denli, R Cevik, K Nas, M Karakoc,AJ Sarac (2003) The effects of alendronate and calcitonin on cytokines in postmenopausal osteoporosis: a 6-month randomized and controlled study. Yonsei Med J 44:99-109.

21 M Sahni, HL Guenther, H Fleisch, P Collin,TJ Martin (1993) Bisphosphonates act on rat bone resorption through the mediation of osteoblasts. J Clin Invest 91:2004-2011.

22 C Vitte, H Fleisch,HL Guenther (1996) Bisphosphonates induce osteoblasts to secrete an inhibitor of osteoclast-mediated resorption. Endocrinology 137:2324-2333.

23 JA Kanis (1994) Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. WHO Study Group. Osteoporos Int 4:368-381.

24 P D'Amelio, A Grimaldi, GP Pescarmona, C Tamone, I Roato,G Isaia (2005) Spontaneous osteoclast formation from peripheral blood mononuclear cells in postmenopausal osteoporosis. Faseb J 19:410-412.

25 CT Ritchlin, SA Haas-Smith, P Li, DG Hicks,EM Schwarz (2003) Mechanisms of TNF-alpha- and RANKL-mediated osteoclastogenesis and bone resorption in psoriatic arthritis. J Clin Invest 111:821-831.

26 N Dalbeth, T Smith, B Nicolson, B Clark, K Callon, D Naot, DO Haskard, FM McQueen, IR Reid,J Cornish (2008) Enhanced osteoclastogenesis in patients with tophaceous gout: urate crystals promote osteoclast development through interactions with stromal cells. Arthritis Rheum 58:1854-1865.

27 M Ramnaraine, W Pan,DR Clohisy (2006) Osteoclasts direct bystander killing of cancer cells in vitro. Bone 38:4-12.

28 HM Massey,AM Flanagan (1999) Human osteoclasts derive from CD14-positive monocytes. Br J Haematol 106:167-170.

29 V Shalhoub, G Elliott, L Chiu, R Manoukian, M Kelley, N Hawkins, E Davy, G Shimamoto, J Beck, SA Kaufman, G Van, S Scully, M Qi, M Grisanti, C Dunstan, WJ Boyle,DL Lacey (2000) Characterization of osteoclast precursors in human blood. Br J Haematol 111:501-512.

30 J Faust, DL Lacey, P Hunt, TL Burgess, S Scully, G Van, A Eli, Y Qian,V Shalhoub (1999) Osteoclast markers accumulate on cells developing from human peripheral blood mononuclear precursors. J Cell Biochem 72:67-80.

31 A Matayoshi, C Brown, JF DiPersio, J Haug, Y Abu-Amer, H Liapis, R Kuestner,R Pacifici (1996) Human blood-mobilized hematopoietic precursors differentiate into osteoclasts in the absence of stromal cells. Proc Natl Acad Sci U S A 93:10785-10790.

32 I Roato, M Grano, G Brunetti, S Colucci, A Mussa, O Bertetto,R Ferracini (2005) Mechanisms of spontaneous osteoclastogenesis in cancer with bone involvement. Faseb J 19:228-230.

33 ER van Beek, CW Lowik,SE Papapoulos (1997) Effect of alendronate treatment on the osteoclastogenic potential of bone marrow cells in mice. Bone 20:335-340.

34 ER Van Beek, CW Lowik,SE Papapoulos (2002) Bisphosphonates suppress bone resorption by a direct effect on early osteoclast precursors without affecting the osteoclastogenic capacity of osteogenic cells: the role of protein geranylgeranylation in the action of nitrogen-containing bisphosphonates on osteoclast precursors. Bone 30:64-70.

35 FP Coxon, K Thompson, AJ Roelofs, FH Ebetino,MJ Rogers (2008) Visualizing mineral binding and uptake of bisphosphonate by osteoclasts and non-resorbing cells. Bone 42:848-860.

36 P D'Amelio, A Grimaldi, S Di Bella, SZ Brianza, MA Cristofaro, C Tamone, G Giribaldi, D Ulliers, GP Pescarmona,G Isaia (2008) Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: A key mechanism in osteoporosis. Bone

37 G Brunetti, S Colucci, P Pignataro, M Coricciati, G Mori, N Cirulli, A Zallone, FR Grassi,M Grano (2005) T cells support osteoclastogenesis in an in vitro model derived from human periodontitis patients. J Periodontol 76:1675-1680.

38 BJ Olivier, T Schoenmaker, RE Mebius, V Everts, CJ Mulder, KM van Nieuwkerk, TJ de Vries,SW van der Merwe (2008) Increased osteoclast formation and activity by peripheral blood mononuclear cells in chronic liver disease patients with osteopenia. Hepatology 47:259-267.

39 ST Tjoa, TJ de Vries, T Schoenmaker, A Kelder, BG Loos,V Everts (2008) Formation of osteoclast-like cells from peripheral blood of periodontitis patients occurs without supplementation of macrophage colony-stimulating factor. J Clin Periodontol 35:568-575.

40 LC Hofbauer, CA Kuhne,V Viereck (2004) The OPG/RANKL/RANK system in metabolic bone diseases. J Musculoskelet Neuronal Interact 4:268-275.

41 I Roato, G Brunetti, E Gorassini, M Grano, S Colucci, L Bonello, L Buffoni, R Manfredi, E Ruffini, D Ottaviani, L Ciuffreda, A Mussa,R Ferracini (2006) IL-7 up-regulates TNF-alpha-dependent osteoclastogenesis in patients affected by solid tumor. PLoS ONE 1:e124.

42 TJ De Vries, Everts V (2009) Osteoclast Formation from Peripheral Blood of Patients with Bone-lytic Diseases

43 X Deng, Z Yu, H Funayama, K Yamaguchi, T Sasano, S Sugawara,Y Endo (2007) Histidine decarboxylase-stimulating and inflammatory effects of alendronate in mice: involvement of mevalonate pathway, TNFalpha, macrophages, and T-cells. Int Immunopharmacol 7:152-161.

44 P Pietschmann, P Stohlawetz, S Brosch, G Steiner, JS Smolen,M Peterlik (1998) The effect of alendronate on cytokine production, adhesion molecule expression, and transendothelial migration of human peripheral blood mononuclear cells. Calcif Tissue Int 63:325-330.

45 A Toyras, J Ollikainen, M Taskinen,J Monkkonen (2003) Inhibition of mevalonate pathway is involved in alendronate-induced cell growth inhibition, but not in cytokine secretion from macrophages in vitro. Eur J Pharm Sci 19:223-230.

46 B Pan, AN Farrugia, LB To, DM Findlay, J Green, K Lynch,AC Zannettino (2004) The nitrogen-containing bisphosphonate, zoledronic acid, influences RANKL expression in human osteoblast-like cells by activating TNF-alpha converting enzyme (TACE). J Bone Miner Res 19:147-154.

47 RG Russell, NB Watts, FH Ebetino,MJ Rogers (2008) Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos Int 19:733-759.

48 RG Russell, Z Xia, JE Dunford, U Oppermann, A Kwaasi, PA Hulley, KL Kavanagh, JT Triffitt, MW Lundy, RJ Phipps, BL Barnett, FP Coxon, MJ Rogers, NB Watts,FH Ebetino (2007) Bisphosphonates: an update on mechanisms of action and how these relate to clinical efficacy. Ann N Y Acad Sci 1117:209-257.

FIGURE LEGENDS

Figure 1. Diagram of the methodology of the study: times of sample collection and analyses performed at each point are indicated. The grey box indicates the pre-treatment period.

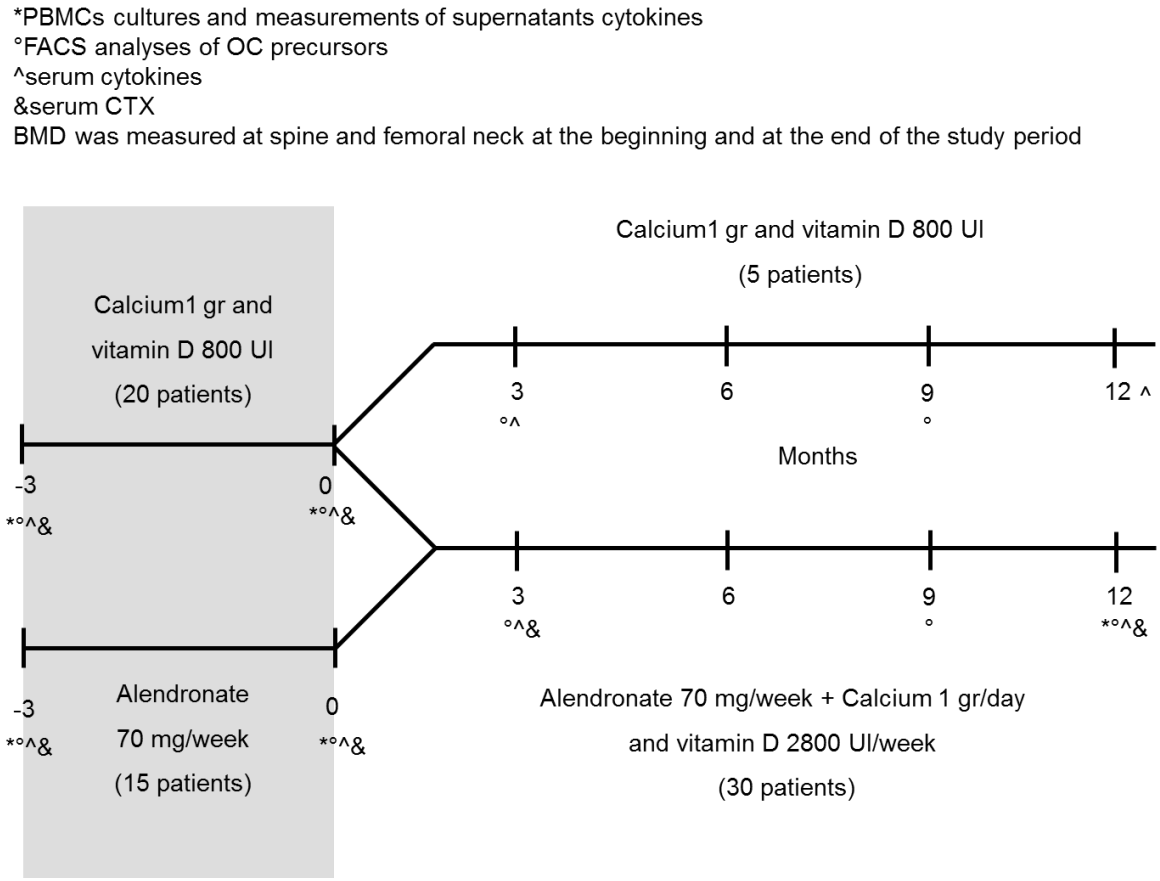
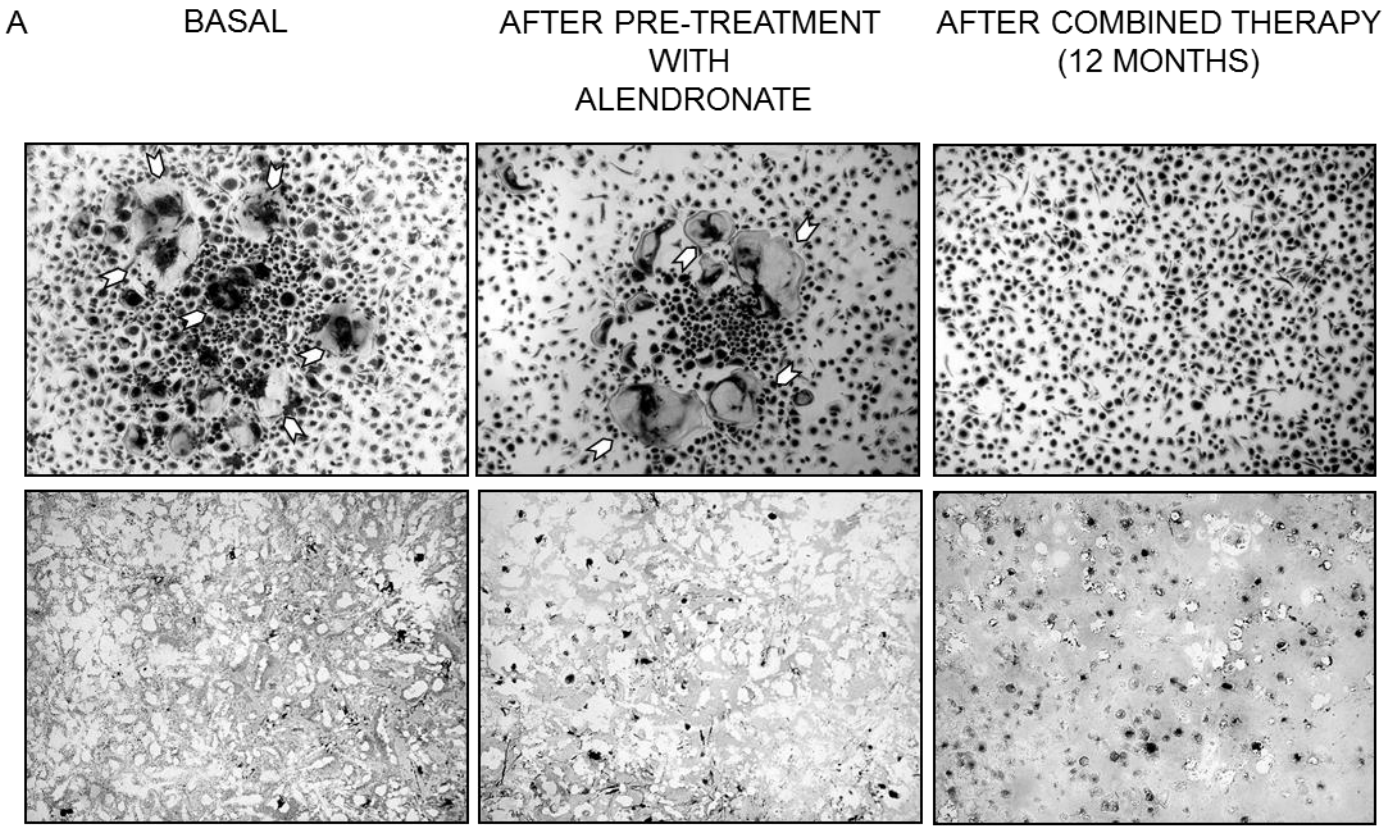


Figure 2. A. Effect of therapy on osteoclasts formation and activity: Osteoclasts (indicated with white arrows) in a light micrograph (10 X) after TRAP staining before (upper left panel) and after *in vivo* treatment with alendronate (3 months) or with combined therapy (12 months upper right panel), the lower panels shows the pit formation assays performed on hydroxiapatite coated wells after cells removal. The micrographs refer to osteoclasts generated in PBMC cultures without stimulus after 21 days of culture.

B. Graphs showing the number of osteoclasts (OC) in unstimulated (without M-CSF and RANKL, left) and stimulated (with M-CSF and RANKL added, right) conditions, in PBMC cultures from osteoporotic women pre-treated with alendronate (continuous line) or calcium and vitamin D (hatched line) for three months and with combined therapy for 12 months. The symbols show the mean and SE for all patients, p significant values calculated with paired Student's T test are indicated (the hatched lines refers to the group pre-treated with calcium and vitamin D). The grey boxes show the three months pre-treatment period.


A
BASAL
AFTER PRE-TREATMENT WITH ALENDRONATE
AFTER COMBINED THERAPY (12 MONTHS)

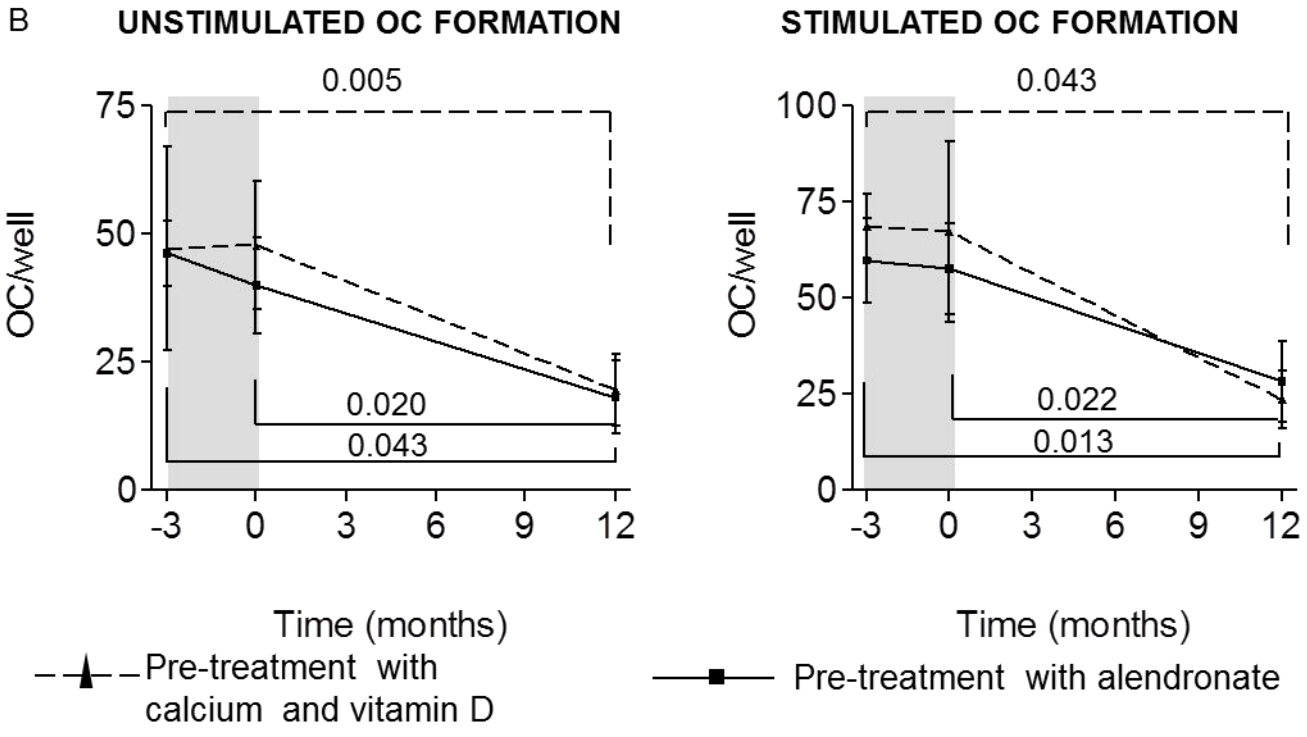

B
UNSTIMULATED OC FORMATION
STIMULATED OC FORMATION
0.005
0.020
0.043
0.043
0.022
0.013
OC/well
Time (months)
Pre-treatment with calcium and vitamin D
Pre-treatment with alendronate

Figure 3. Effect of therapy on *in vivo* bone resorption: graph showing the level of serum CTX in osteoporotic women pre-treated with alendronate (continuous line) or calcium and vitamin D (hatched line) for three months and with combined therapy for 12 months. The symbols show the mean and SE for all patients, p significant values calculated with Wilcoxon's test are indicated (the hatched lines refers to the group pre-treated with calcium and vitamin D). The grey box shows the three months pre-treatment period.

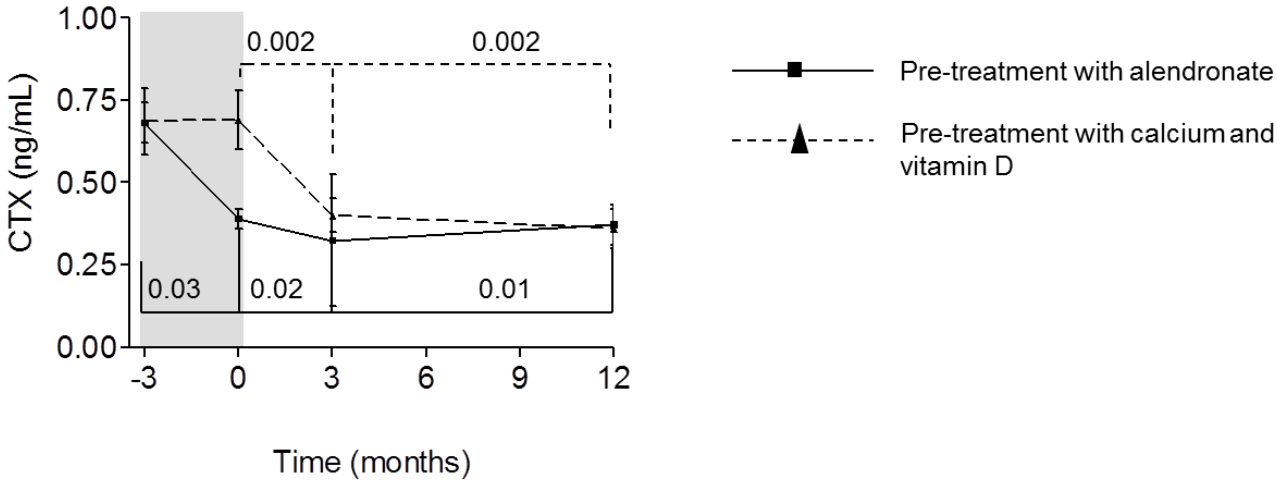


Figure 4. Effects of therapy on circulating osteoclast (OC) precursors: FACS analysis of circulating OC precursors from PBMC of osteoporotic women labelled with FITC-conjugated anti-VNR, PE-conjugated anti-CD14 and APC-conjugated anti-CD11b mAbs before and after alendronate and calcium plus vitamin D *in vivo* treatment.

A. Dot plots represent VNR+ and CD11b+ cells gated on CD14+ cells (OC precursors). Percentages of positive cells are indicated.

B. Graphs represent the percentage of CD14+ CD11b+ VNR+ cells (OC precursors) before and after therapy. The symbols show the mean and SE for all patients, p significant values calculated with Wilcoxon's test are indicated.

C. Graphs represent the percentage of CD14+/CD11b+/VNR- cells (early OC precursors) before and after therapy. The symbols show the mean and SE for all patients, p significant values calculated with Wilcoxon's test are indicated.

The grey boxes show the three months pre-treatment period.

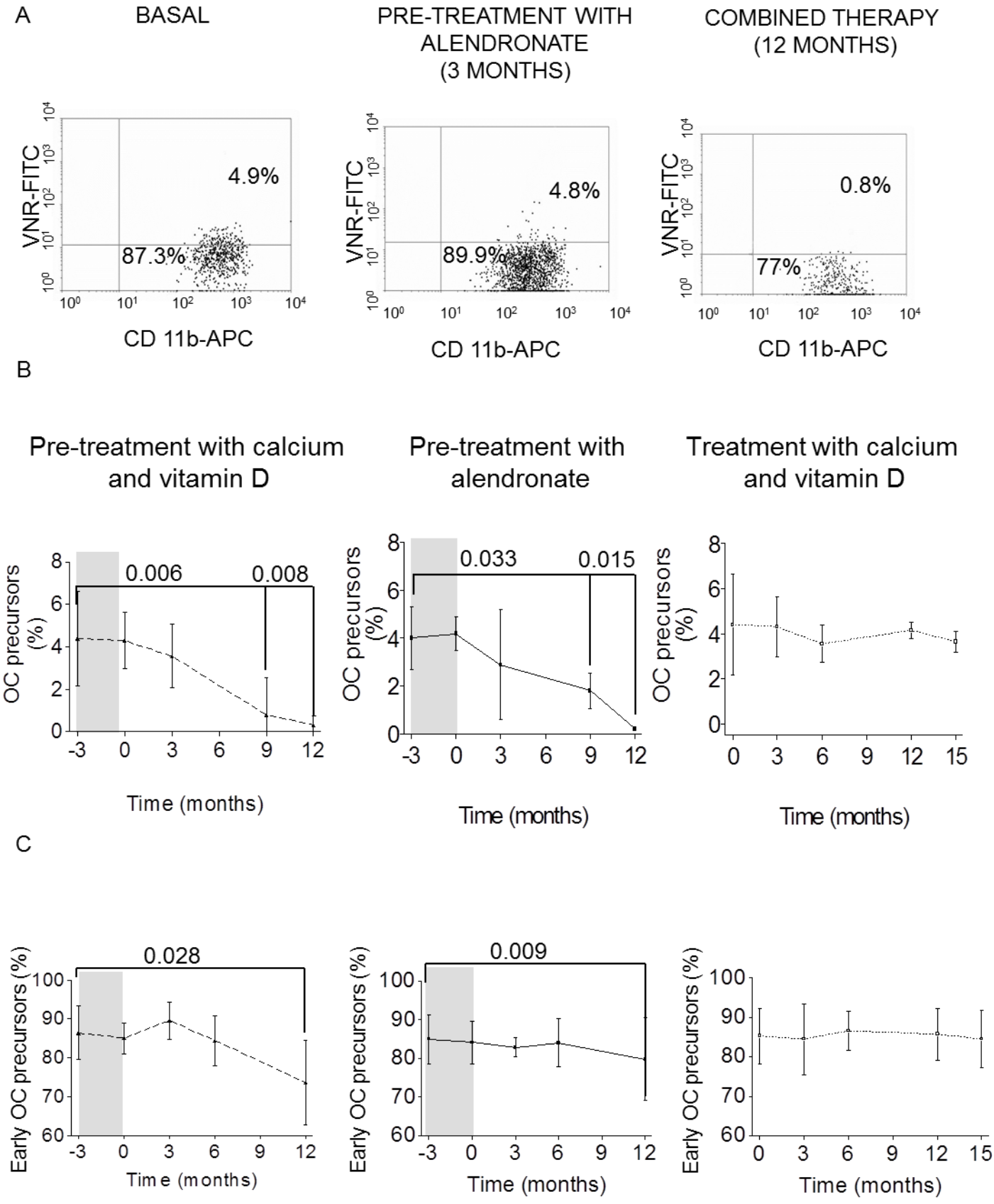


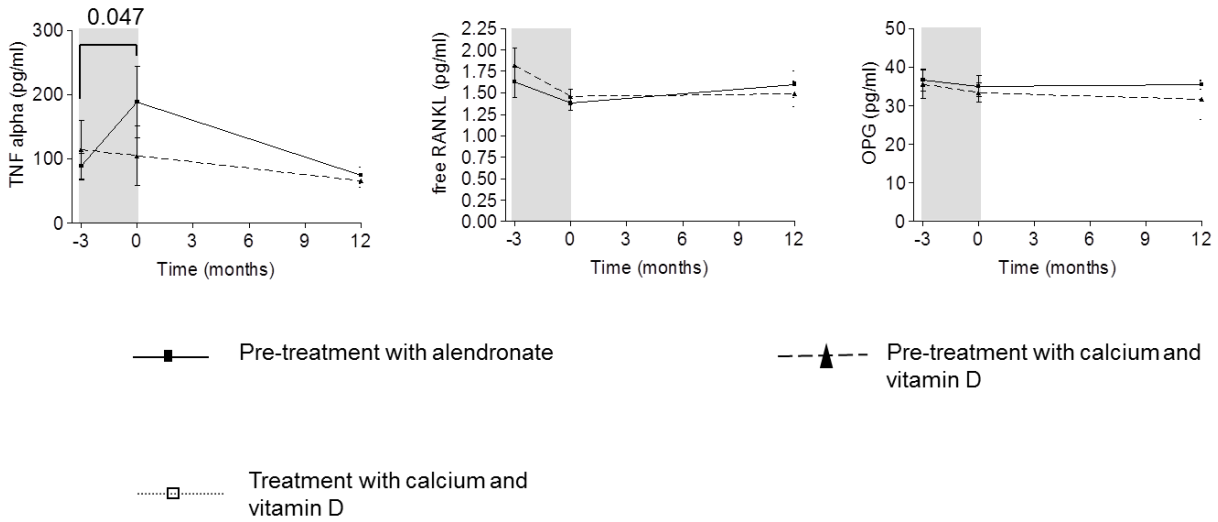
Figure 5. Effects of therapy on cytokines production.

A. In vitro cytokines production measured in the supernatants of PBMC cultures in patients pre-treated with calcium and vitamin D (hatched line) or with alendronate (continuous line).

B. In vivo cytokines production measured in the serum of patients pre-treated with calcium and vitamin D (hatched line), with alendronate (continuous line) or with calcium and vitamin D during the entire study period (dotted line).

The symbols show the mean and SE for all patients, p significant values calculated with Wilcoxon's test are indicated, the grey boxes show the three months pre-treatment period.

A. CYTOKINES IN THE PBMC CULTURE SUPERNATANTS



Supplemental figure. Effects of therapy on PBMCs: FACS analysis of PBMCs' subpopulation labelled with APC-conjugated anti-CD14 (monocytes), FITC-conjugated anti-CD19 (B lymphocytes) and PerCP-conjugated anti-CD3 (T lymphocytes) mAbs before and after alendronate (3 months) or with combined therapy (12 months),

A. Dot plots represent CD14+ cells gated on monocytes (upper panels), CD19+ cells gated on lympocytes (middle panels) and CD3+ cells gated on lympocytes (lower panels)..

B. Graph represents the percentage of CD14+, CD19+ and CD3+ cells before and after therapy. The bars show the mean and SE for 5 patients.

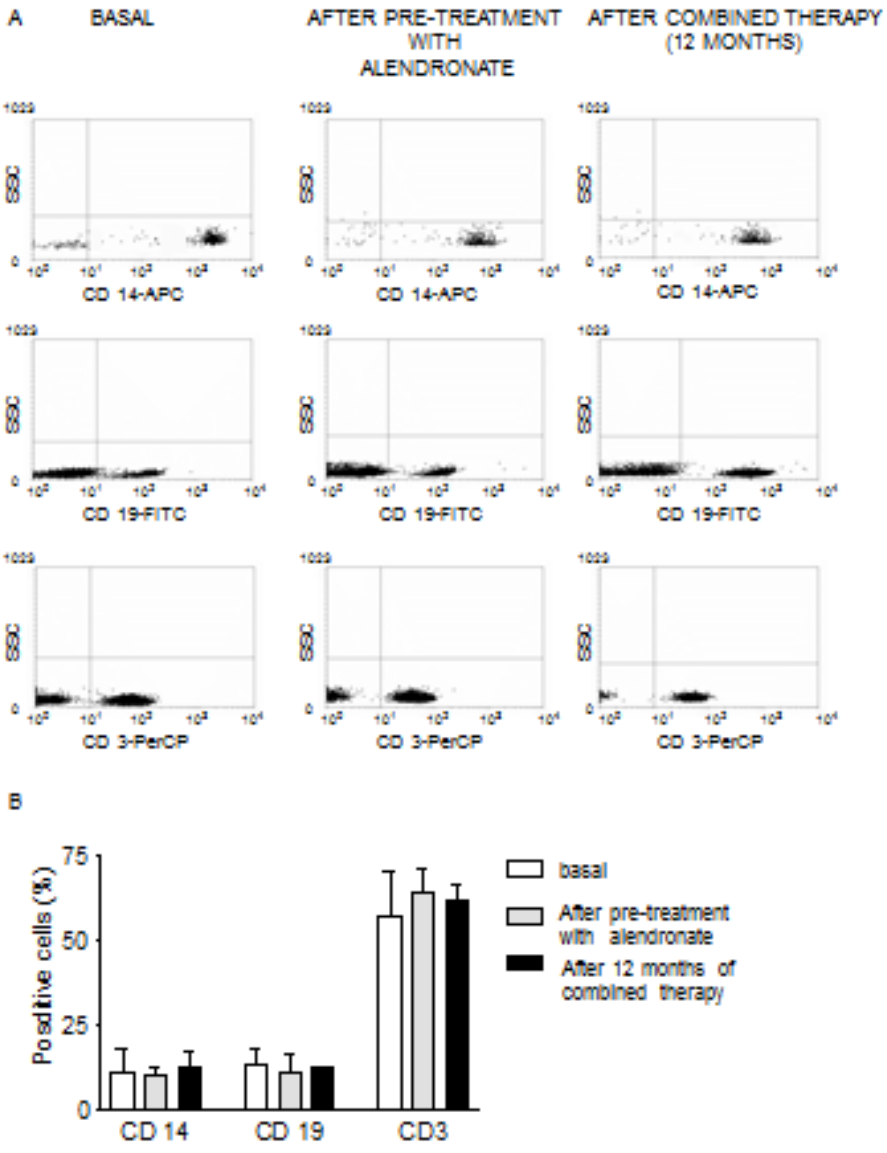


Table 1. Baseline characteristics of the three groups of patients. Means and SD are shown; p values were calculated by one-way ANOVA and were not significant. The references values are indicated in square brackets.

	Pretreatment with		
	Alendronate (15 patients)	**Calcium and Vitamin D (15 patients)**	**Calcium and Vitamin D (5 patients)**
Age (yrs)	64±6	63±6	64±5
Postmenopausal period (yrs)	13±6	14±8	14±7
B.M.I.	23.±1.21	24.31±0.87	23.5±
BAP (UI/L) [<21 UI/L]	14.98±4.8	14.14±4.93	14.2±
PTH (pg/ml)	28.58±12.04	27.52±12.48	27.48±
Blood calcium (mEq/L) [4.4-5.2 mEql/L]	4.89±0.15	4.87±0.24	4.88±
Serum phosphate (mMol/L) [0.65-2.10 mMol/L]	1.18±0,16	1.16±0.16	1.14±
25 OH vitamin D (ng/mL) [advisable>30 ng/mL]	30.48±2.3	31.63±2.25	32.8±