Teriparatide increases the maturation of circulating osteoblast precursors.

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Running title: Teriparatide affects osteoblast precursors.

MINI ABSTRACT.

This study shows that teriparatide promotes the circulating osteoblast precursor degree of maturation in patients affected by postmenopausal osteoporosis.

ABSTRACT.

Purpose: Anabolic treatment with teriparatide has proven effective for the therapy of post-menopausal osteoporosis and significantly reduces the risk of non-vertebral fragility fractures. The aim of this study was to investigate the effect of teriparatide on circulating osteoblast precursors.

Methods: We evaluated by flow cytometry and real time PCR the expression of osteoblasts typical markers in peripheral blood mononuclear cells during treatment with teriparatide plus calcium and vitamin D, raloxifene plus calcium and vitamin D or vitamin D alone at various time points. Serum Bone alkaline Phosphatase and Osteocalcin were measured as markers of bone turnover.

Results: Our results show that circulating osteoblast precursors are more numerous and more immature in patients affected by fragility fractures than in osteoporotic patients without fractures. We also show that teriparatide treatment increases the expression of alkaline posphatase and of osteocalcin in osteoblast precursors, thus it increases their degree of maturation.

Conclusions: we suggest that teriparatide acts as anabolic agents also by promoting the maturation of osteoblast precursors.

INTRODUCTION.

The number of osteoblast (OB) precursor cells circulating in the peripheral human blood has been for longer underestimated due to peculiarity of the designed assays employed by researchers [1]. Recent papers reported that circulating OB precursor cells are present in peripheral human blood in significant number, correlate with typical OB markers such as osteocalcin (OC) and alkaline phosphatase (AP) and can form nodules *in vitro* and bone tissue *in viv*o [1, 2]. The role of these cells during skeletal growth and fracture repair has also been proposed by Eghbali-Fatourechi et al [1]. These authors observed that OB precursors were over five times more numerous in the circulation of adolescent boys compared to adult men and that they increased after fracture. Otherwise, we recently demonstrated that, during fracture healing, there is an important individual variation in the amount of OB and osteoclast precursors; hence it is not possible to foresee a role for these cells in the callus formation in humans [3]. Kumagai et al. elegantly demonstrated the homing ability of circulating osteogenic cells during fracture healing by a parabiotic mice model [4]. They showed that these cells are physiologically mobilized to contribute to callus formation and fracture repair in mice. Reduction of the circulating OB precursor pool has been described in postmenopausal osteoporosis [5], whereas our previous study described an increased maturation of these cells during fracture healing [3], however their physiological role in bone metabolism remains to be clarified.

Recent data suggest that postmenopausal osteoporosis may be due not only to an increased osteoclast formation and activity, but also to a reduced OB activity: in particular our and other workgroups demonstrated that OB inhibitors are increased in patients affected by fragility fractures [6, 7].

Over the last years, anabolic treatment with teriparatide [rhPTH(1-34)] or with 1-84 PTH has been proposed as therapy for post-menopausal osteoporosis: these drugs significantly reduce the risk of non-vertebral fragility fractures [8, 9]. Anabolic treatment stimulates both trabecular and cortical bone formation, resulting in the improvement of the bone microarchitecture. These changes represent a reversal of osteoporotic bone loss and may explain the vertebral and non-vertebral skeletal effects, including reduction in fracture rates. Furthermore, in contrast to antiresorptive agents, anabolic treatment preferentially increases bone formation through direct early stimulation of OBs [10]. Previous studies demonstrated that PTH acts directly on OBs by binding the PTH receptor I [11-14]. A recent paper demonstrates that the PTH/PTHrP receptor expression and the cAMP accumulation in response to PTH were increased in accordance with the differentiation of murine OBs [15]. PTH receptor was also found to be expressed in AP positive cells in human bone marrow [16]. PTH signalling pathway involves the activation of genes important for the functions of the mature OBs [17-20], increases the number of OBs, decreases their apoptotic rate and increases their bone forming activity. Some literature data suggest that, among anti-resorptive drugs, the selective estrogen receptor modulator raloxifene has some effects on mature OB and, in particular, is able to stimulate OB activity and enhance bone formation in vitro [21, 22] and in vivo [23].

Nowadays, in literature there are no available data on the possible effects of teriparatide on the bone marrow output of circulating osteoblast-lineage cells, thus the present study aims to evaluate the effect of teriparatide in stimulating bone marrow release of osteoblast-lineage cells as compared to calcium and vitamin D supplements and to raloxifene.

METHODS.

Study design

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

A total of 25 women (65 ± 11 years) were enrolled in this study: 15 women affected by postmenopausal osteoporosis without fractures and 10 with fractures. The presence of diseases that influence bone metabolism was ruled out based on medical history and physical examination. All the women had been in spontaneous menopause for at least one year. Bone mineral density (BMD) was measured by double-emission X-ray absorptiometry with a Hologic QDR 4500 (Hologic Inc.) bone densitometer. Secondary osteoporosis was ruled out by means of medical history, physical examination and blood tests (serum calcium and phosphorus, bone AP, serum protein electrophoresis and 25-OH vitamin D).

To be included into the study women have to be after at least 1 year of spontaneous menopause with a BMD T-score value of -2.5 S.D. or less according to the WHO criteria, with or without fractures. Male subjects, patients with mental incapacity to give the informed consent, patients with secondary osteoporosis, or taking calcium and vitamin D, thyroid hormones, corticosteroids, estrogen, bisphosphonates, parathyroid hormone, fluoride, strontium ranelate, and raloxifene in the previous six months or treated with an immunosupressant (e.g. cyclosporine, azathyoprine) within the previous year, subjects with hypersensitivity or serious adverse experience with raloxifene, teriparatide or calcium and vitamin D or with a history of cancer were excluded from the study.

Treatment.

Patients without fractures were randomly assigned to treatment with 60 mg/day raloxifene

(Evista ®, kindly provided by Ely Lilly SpA Italy, 5 patients) plus calcium 1200 mg/day and vitamin D 800 UI/day, or with Teriparatide 20 μg/day (Forsteo ®, kindly provided by Ely Lilly SpA Italy, 5 patients) plus calcium 1200 mg/day and vitamin D 800 UI/day, or with calcium 1200 mg/day and vitamin D 800 UI/day alone (5 patients). Patients with fractures were treated with teriparatide plus calcium and vitamin D. Presence of osteoporotic fracture was defined as the presence of at least a major (hip, vertebral, wrist) fracture due to low-energy trauma. The existence of a fracture was demonstrated by a physician evaluation of X-ray. Calcium and vitamin D supplements (Natecal D3®) were kindly provided by Italfarmaco S.p.A.

Blood was drawn from an antecubital vein after an overnight fast of 10 or more hours at baseline and after 15 days, 1 month, 6, 12 and 18 months of treatment. All measurements were taken from a single sample at a single time point: spine and femoral neck BMD were measured at the end of the study.

Markers of bone turnover:

ELISA kits were used to measure serum bone AP (BAP, Instant ELISA; Bender), and serum osteocalcin (Instant ELISA; Bender) in duplicate according to standard procedures [3].

Effect of treatment on OB circulating precursor

Peripheral blood mononuclear cells (PBMCs) were obtained with the Ficoll-Paque method from 30 ml of blood in lithium heparin, as previously described [24-26]. The serum was stored at -80°C until the measurements were done.

To evaluate the effect of treatment on circulating OB precursors, we measured these cells in the PBMCs at baseline and at different time points after treatment. Briefly, PBMCs were stained with FITC conjugated anti-CD15 antibody (in order to exclude granulocytes that expressed AP, supplied by e-Bioscience) or with the corresponding isotype control and then incubated for 30 min at 4°C. The cells were fixed for 15 min at 4°C with 1 ml cold 0.4%

paraformaldehyde in PBS and then incubated for 15 min at RT with PBS supplemented with 5% saponin (Sigma Aldrich), APC conjugated anti-AP antibody (supplied by R&D System Inc), PE conjugated anti-OC antibody (supplied by R&D System Inc), or with the corresponding isotype control, in accordance to manufacturer instruction. Then cells were washed with PBS supplemented with 0.5% saponin. CD15-/AP+/OC+ cells were regarded as OB precursors according to the literature [1-3]. The degree of maturation of OB precursors was evaluated by the mean fluorescence intensity (MIF) of OC. The MIF express the number of molecule present on each cell [3]. The flow cytometry analysis is shown in the supplemental figure.

To assess the possible effect of teriparatide on stem cell compartment [27], we evaluated the espression of CD34 and AP on PBMCs according to previous studies [2, 28]. Briefly, freshly isolated PBMCs from 5 patients were stained with FITC conjugated anti-CD34 antibody (eBioscience), PE conjugated anti-OC antibody (R&D System Inc) and APC conjugated anti-AP antibody(R&D System Inc), or with the corresponding isotype control, and incubated at 4°C for 30 min; the cells were analyzed at baseline after 15 and 30 days.

Membrane antigen expression was analyzed with CellQuest software (Becton Dickinson & Co), and displayed as bivariate dot plots or histograms. The Mean Fluorescence Intensity (MIF) was also recorded. Each plot depicts the results from 100,000 events representing viable cells gated by cell size and granularity.

Gene expression in OB cells.

To characterize the expression of OB genes in OC+ cells, we isolated these cells from buffy coats obtained from 5 healthy donors from the blood bank. Briefly, PBMCs were incubated with normal donkey serum 10% (Jackson ImmunoResearch) and human IgG 10% (FcR Blocking Reagent, Miltenyi Biotec) at RT for 30 minutes in order to block non-specific binding.

Cells were stained with PE conjugated anti-OC antibody (R&D System Inc) and then processed with Easy Sep PE selection kit (supplied by StemCell Technology) as described [1]. The cells were processed for RNA extraction and a real time PCR was carried on.

Expression level of OB genes in OC+ cells were measured performing real time RT-PCR assay on RUNX-2, Osterix, OC and alkaline Phosphatase (TNAP). In order to evaluate whether teriparatide affect these genes, their expression was analyzed also in PBMCs from treated patients at each time point.

Total cellular RNA was extracted using TRIzol reagent (Ambion, Huntingdon, UK), chloroform extraction, and subsequent isopropanol precipitation according to the manufacturer's protocol. 1 µg of total RNA was reverse transcripted to single-stranded cDNA using the Promega ImProm-II™Reverse Transcription System (Promega). cDNA was stored at -20 °C until use. Real time RT-PCR was performed with IQ SYBR Green Supermix (BIORAD), gene expression was quantified by using β-Actin amplification as housekeeping control and $2^{-\Delta\Delta Ct}$ method for the quantitative analysis. The primers used are reported in the supplemental table 1. All the genes were quantified by considering signals under 33 Ct.

Statistical Analysis

The normal distribution of each parameter was determined by kurtosis; all except OC MIF were normally distributed. One way ANOVA was used to compare baseline characteristics among patients with and without fractures, OC MIF was compared by the Mann-Whitney U test; the Wilcoxon test was used to compare OC MIF values at baseline and after treatment, whereas the other parameters were compared by means of Student's paired T test. As patients with fractures were older than patients without fractures, OB precursors amongst these cohorts were compared after weighting cases for age with the specific function of the statistical software used. Correlations between parameters were performed by means of Spearman's coefficient or with partial correlation after correction for age. The IBM SPSS 17.0 software package was used to process the data with $p<0.05$ as the significance cut-off.

RESULTS.

Baseline features of patients are reported in the Table 1 according to treatment group.

Circulating OB precursors are more immature in fractured than in non fractured patients.

Patients with fractures were older (77±7 years±SD vs 58±4 years±SD, p=0.000) and with longer postmenopausal period (28±12 years±SD vs 9±3 years±SD, p=0.000) than patients without fractures, hence OB precursors were compared between the two groups after weighting cases for age.

OC + and OC+/AP+ cells are increased in patients exhibiting fractures (Fig. 1A and B) while OC MIF was higher in patients without fractures (Fig. 1C) These data suggest that OB precursors are more immature in patients affected by severe osteoporosis. According with other studies [3, 5], this may account for a reduced homing ability in the skeleton and hence an increase in the number of circulating cells.

Teriparatide increases the maturation of circulating OB precursors.

The effect of teriparatide on OB precursor cells was not significantly different in patients with and without fractures, hence the results for the two groups where pooled and analyzed together.

After teriparatide treatment, OB precursor cells do not show any increase over the time (Fig. 2 A), whereas increased MIF OC and MIF AP values (Fig 2B and C) indicates upregulation of both OC and AP leading to an increased maturation of OB precursor cell. In order to evaluate whether the increase in the degree of maturation accounts for an increased typical OB gene expression, we performed a quantitative RT-PCR experiments programmed on patients' PBMCs analyzing the expression of TNAP, OC, RUNX2 and Osterix at each time point. The expression of these genes was not significantly affected by treatment in patients' PBMCs (data not shown); otherwise their expression were about two folds higher in OC+ cells than

in OC- cells, obtained from heathy donors' buffy coats (Fig.3),This finding might be explained by the low number of OC+ cells in the patients' PBMCs.

By contrast, bone formation markers increased in all patients during treatment (Fig.4A and B). There were no correlations between these markers and OB precursors or between the increase in the degree of maturation of these cells and the increase in bone formation at any time point.

The increase in stem cells compartment does not account for the increase in AP MIF.

It has been reported that teriparatide is able to increase stem cells release from bone marrow into the circulation (see [27] for a review), hence we evaluated if the early increase in AP MIF could be due to an increase in stem cells. To address this issue, we performed FACS analyses on 5 patients with fractures treated with teriparatide to evaluate the expression of CD34, AP and OC in the monocyte compartment as previously described [2, 28], the baseline characteristic of these patients were comparable to those of the overall teriparatide treated group. We found no increase in CD15-/CD34+/AP+ and CD15-/CD34+/AP- cell number or in CD34 MIF despite an increase in AP MIF, during the first month of treatment (data not shown). These data confirms that the effect of teriparatide is direct on OB precursor cells, whereas we found no effect on stem cell compartment.

Calcium plus vitamin D and raloxifene treatment do not influence bone formation markers and circulating OB precursors.

In the cohort of patients treated with calcium and vitamin D, no significant variations in bone formation markers (Fig. 5A and B), in the percentage nor in the maturation of OB precursors (Fig. 5C and D) were observed.

Also, in the group treated with Raloxifene, we did not observe variations neither in bone formation markers (Fig. 6A and B) nor in the level of circulating OB precursors or their degree

of maturation (Fig. 6C and D).

DISCUSSION

It is well known that intermittent treatment with teriparatide is able to reduce fracture risk in postmenopausal osteoporosis acting as a bone anabolic agent [9], inducing new bone formation and improving bone microarchitecture [29]. Teriparatide promotes bone formation by increasing the number of OBs through multiple effects, including activation of quiescent lining cells [30], increased OB proliferation [31, 32] differentiation [31, 33], and attenuation of pre-OB and OB apoptosis [10, 34, 35]. However, the specific contribution of each of these effects to the overall anabolic activity of teriparatide remains controversial. A recent study by Rubin MR et al. [36] suggests that PTH 1-84 stimulates bone formation by increasing the number and the degree of maturation of circulating osteogenic cells in patients affected by hypoparathyroidism. Today there are no evidences on the possible effect of teriparatide on circulating OB precursors in osteoporosis.

In this study we showed that OB precursor cells are increased in patients affected by severe form of osteoporosis complicated with fractures and that in these patients the degree of maturation of the precursors is lower than in osteoporotic patients without fragility fractures. The OC MIF reduction accounts for more immature OB precursors in the circulation of patients with severe osteoporosis and possibly a reduction of their recruitment in bone [3, 5] ,. The observation of an increased number of circulating OB precursors with low expression of OC corroborates the previous data from Pirro et al [5].

After teriparatide therapy, we observed an early increase in the MIF of AP, after only 30 days, whereas the increase in the OC MIF was observed after 6 months of therapy. To investigate whether the early increase in AP was due to a generic increased release of stem cells from the bone marrow, we evaluated CD34 expression. Our data show that the observed increased AP MIF is not due to a generic increase in stem cell compartment following

teriparatide treatment, as previously suggested [27].

We did not detect an increase in the circulating OB precursor cells, but we observed an increased maturation rate of these cells as shown by an increase in the MIF of AP and of OC. In the recent study by Rubin et al [36] an increase in the number and in the maturation of OB precursors in patients with hypoparathyroidism treated with 1-84 PTH has been demonstrated. Our study confirms Rubin's observation of increased maturation of OB precursors after treatment, but doesn't show increased OB precursors number. This last result may be due to the different kind of population enrolled (osteoporotic patients with normal parathyroid function as respect to patients with hypoparathyroidism) and for the treatment used (1-34 PTH vs 1-84 PTH). These differences may account for the different finding of increased OB precursors in Rubin's model; on the other hand our study confirms Rubin's observation of increased maturation of OB precursors following treatment [36]. As regards the two control groups, we do not observe significant variation in any of the considered parameters in calcium and vitamin D group nor in the raloxifene group, thus confirming the effect seen in the teriparatide treated patients.

In order to address the question whether the observed increased expression of OC in OB precursor is accompanied by a general increase in OB gene expression, we performed a quantitative RT-PCR. In the whole PBMCs we did not find an up-regulation of OB gene expression profile following teriparatide treatment. Otherwise, PCR confirmed that OC+ cells in the circulation expressed from 1.5 to 2.0 fold increase in OB genes as respect to OC- cells. These data further confirm that OC+ cells may be interpreted as early OB precursors. The lack of a general increase in OB genes within the PBMCs may be due to the lower and variable number of these cells in peripheral blood.

The increase in bone formation markers in the serum of patients treated with teriparatide is in

line with literature data [8] and is due to the increased bone apposition by OBs present on the bone surface. In our study, the increase in these markers confirms that the drug works and also warrants patient's compliance to treatment.

Despite some previous data in literature that suggest an effect of raloxifene on bone formation [21-23], we did not observe any significant effect of this treatment on bone formation markers or on circulating OB precursors.

The main limitation of the study is the small size of the observed cohort, neverteless our results suggest that the increased maturation of OB precursors is one of the anabolic effect of teriparatide.

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Teriparatide and Raloxifene were kindly provided by Ely Lilly, Calcium and vitamin D supplements were kindly provided by Italfarmaco SpA.

Conflict of interest: no disclosure.

Table 1. Mean and standard deviation of baseline features of patients divided according to treatment. All the patients received calcium and vitamin D supplements. Parameters normally distributed were compared by one way ANOVA, OC MIF values were compared by means of Kruskal-Wallis test as it has a non Gaussian distribution. P values refer to differences between fractured and non fractured patients, whereas there were no significant differences in non fractured subjects, randomized to different treatment.

	TERIPARATIDE		**RALOXIFENE**	**CALCIUM AND VITAMIN D**	**p**
	Fractured (10)	Non fractured (5)	Non fractured (5)	Non fractured (5)	
Age (years)	77±7	60±4	58±5	59±3	**0.001**
Menopausal period (years)	28±12	9±5	9±3	9±4	**0.017**
Body mass index	22.6±2.2	22.9±1.8	22±1.3	23.5±2.1	NS
Lumbar BMD (g/cm2)	0.671±0.08	0.699±0.08	0.718±0.07	0.761±0.25	NS
Femoral neck BMD (g/cm2)	0.520±0.07	0.563±0.1	0.551±0.07	0.555±0.1	NS
BAP (UI/L)	29.9±7.5	26.7±6.62	27.1±1.27	27.07±1.8	NS
BGP (ng/mL)	3.92±1.84	4.39±1.84	4.2±1.34	4.3±1.93	NS
OC+ cells	12.21±2.7	8.41±2.6	7.5±1.8	7.52±1.9	**0.000**
OC+/AP+ cells	1.36±0.5	0.44±0.17	0.36±0.17	0.33±0.17	**0.000**
MIF OC	56.93±9.99	85.8±6.12	84.9±10	83.82±8.55	**0.000**
MIF AP	77.82±2.3	38.79±2.81	39.2±15.2	38.1±16.1	NS

FIGURE LEGENDS.

Figure 1. Osteoblast precursors numbers are higher and more immature in the circulation of patients with fragility fractures than in patients without fractures.

A. Box and whisker plot shows the percentage of OC+ cells in the PBMCs of osteoporotic patients with (10) and without fragility fractures (15).

B. Box and whisker plot shows the percentage of OC+/AP+ cells in the PBMCs of osteoporotic patients with (10) and without (15) fragility fractures.

C. Box and whisker plot shows the Mean Fluorescence Intensity (MIF) of OC in the PBMCs of osteoporotic patients with (10) and without (15) fragility fractures.

Each Box represents the 25th to 75th percentiles. Lines outside the boxes represent the minimum and maximum values. Lines inside the boxes represent the medians calculated for all the data set. p values were calculated by ANOVA (A,B); or with the Mann-Whitney U test (C).

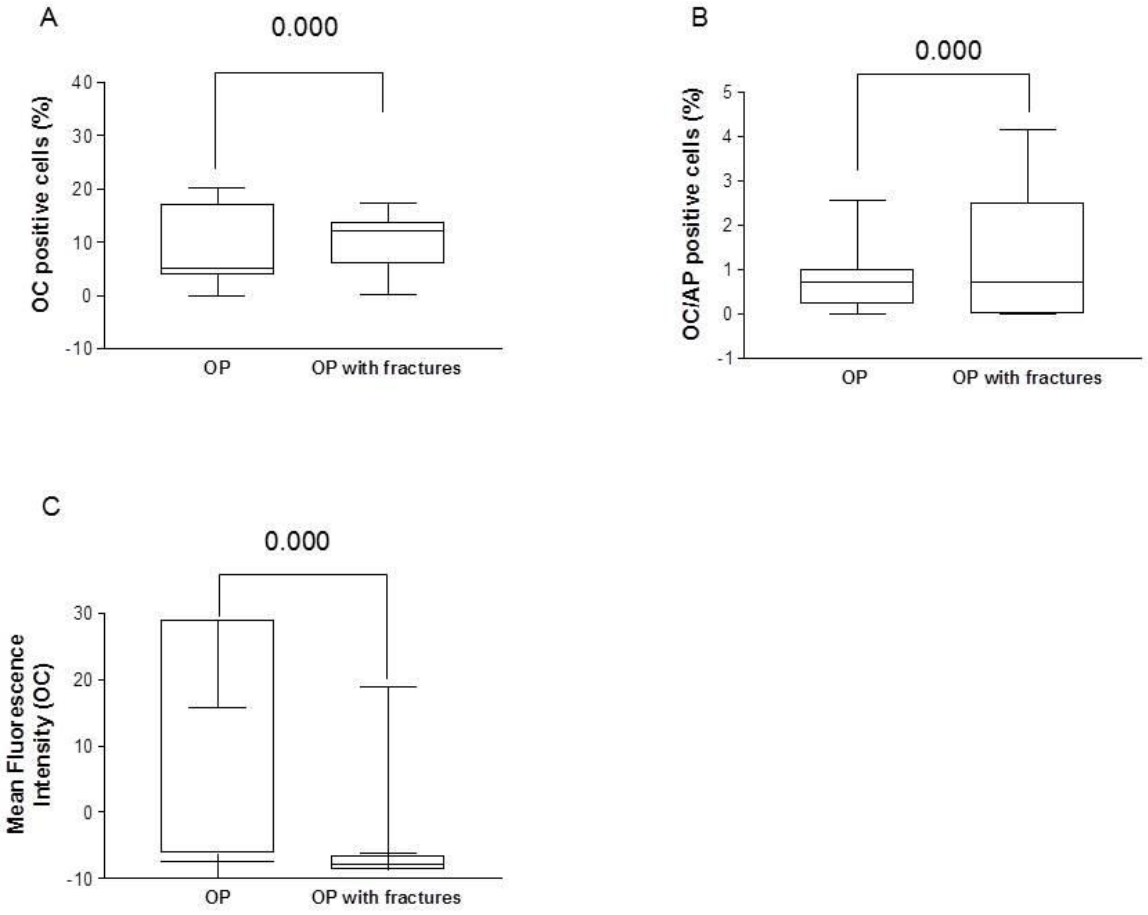


Figure 2. Teriparatide increases osteoblast precursors maturation, but not their number in the circulation.

A. Graph shows the variation during treatment of OC+ and OC+/AP+ cells in the PBMCs of osteoporotic patients.

B. Graph shows the OC MIF in the PBMCs of osteoporotic patients during treatment.

C. Graph shows the AP MIF in the PBMCs of osteoporotic patients during treatment.

Symbols represent mean and standard deviations calculated on 15 patients, p values were calculated with Students paired T test (A and C) or with the Wilcoxon test (C).

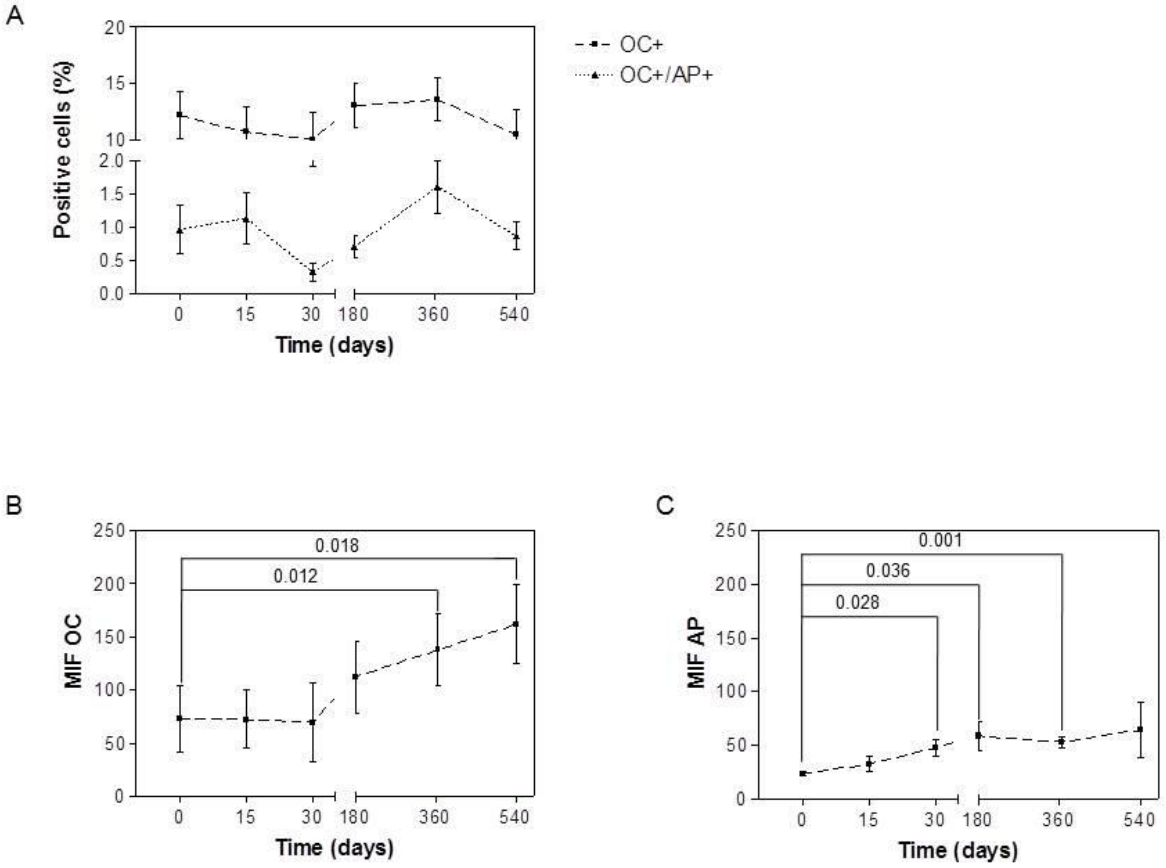


Figure 3. OC+ cells express OB genes. Graph shows the expression of OB genes (TNAP, OC, RUNX-2 and Osterix) measured in real time RT-PCR among OC+ and OC – cells obtained from healthy donors' buffy coats. Bars represent mean and standard deviations calculated on 5 buffy coats, p values were calculated with Student's paired T test.

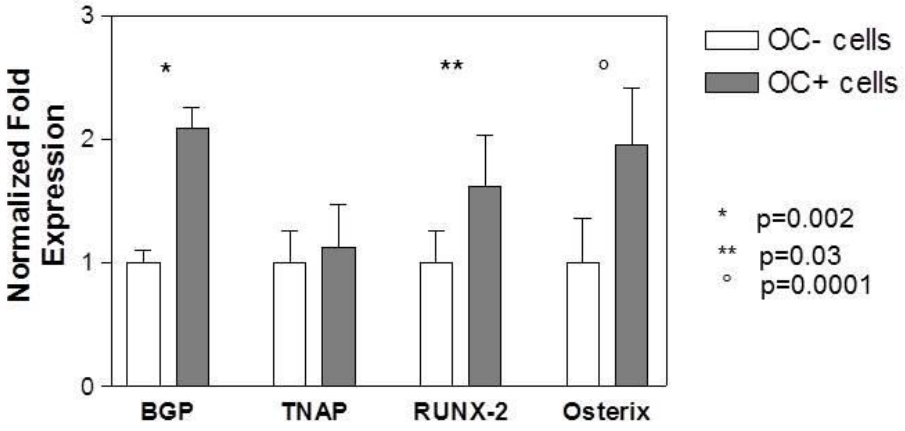


Figure 4. Teriparatide increases bone formation markers.

A. Graph shows the variation during treatment of serum OC in osteoporotic patients.

B. Graph shows the variation during treatment of BAP in serum of osteoporotic patients

Symbols represent mean and standard deviations calculated on 15 patients, p values were calculated with Students paired T test.

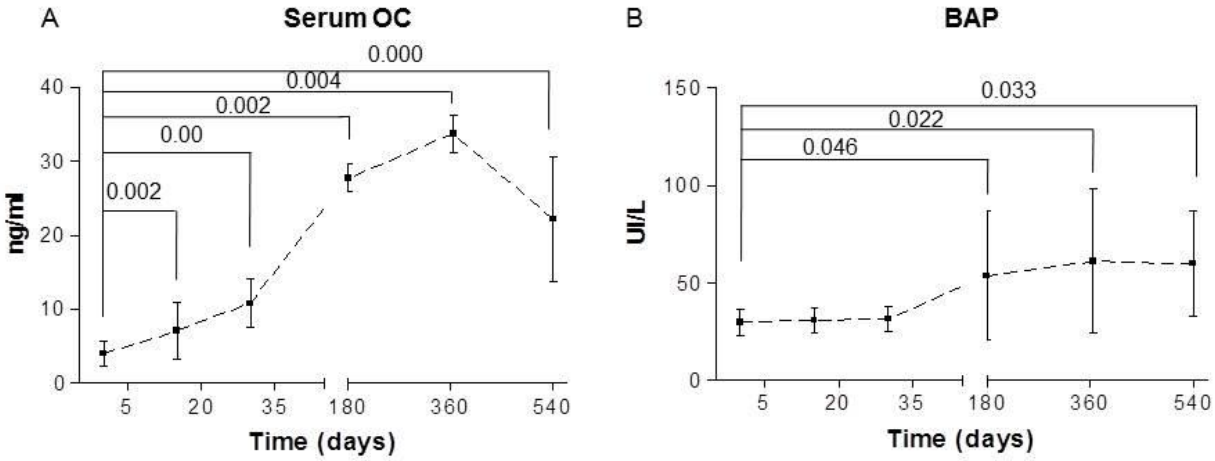


Figure 5. Calcium plus vitamin D does not influence bone formation markers or OB precursors.

A. Graph shows the variation during treatment of serum OC in of osteoporotic patients.

B. Graph shows the variation during treatment of BAP in serum of osteoporotic patients.

C. Graph shows the variation during treatment of OC+ and OC+/AP+ cells in the PBMCs of osteoporotic patients.

D. Graph shows the OC and AP MIF in the PBMCs of osteoporotic patients during treatment.

Symbols represent mean and standard deviations calculated on 5 patients, p values were calculated with Students paired T test (A,B,C) or with the Wilcoxon test (D).

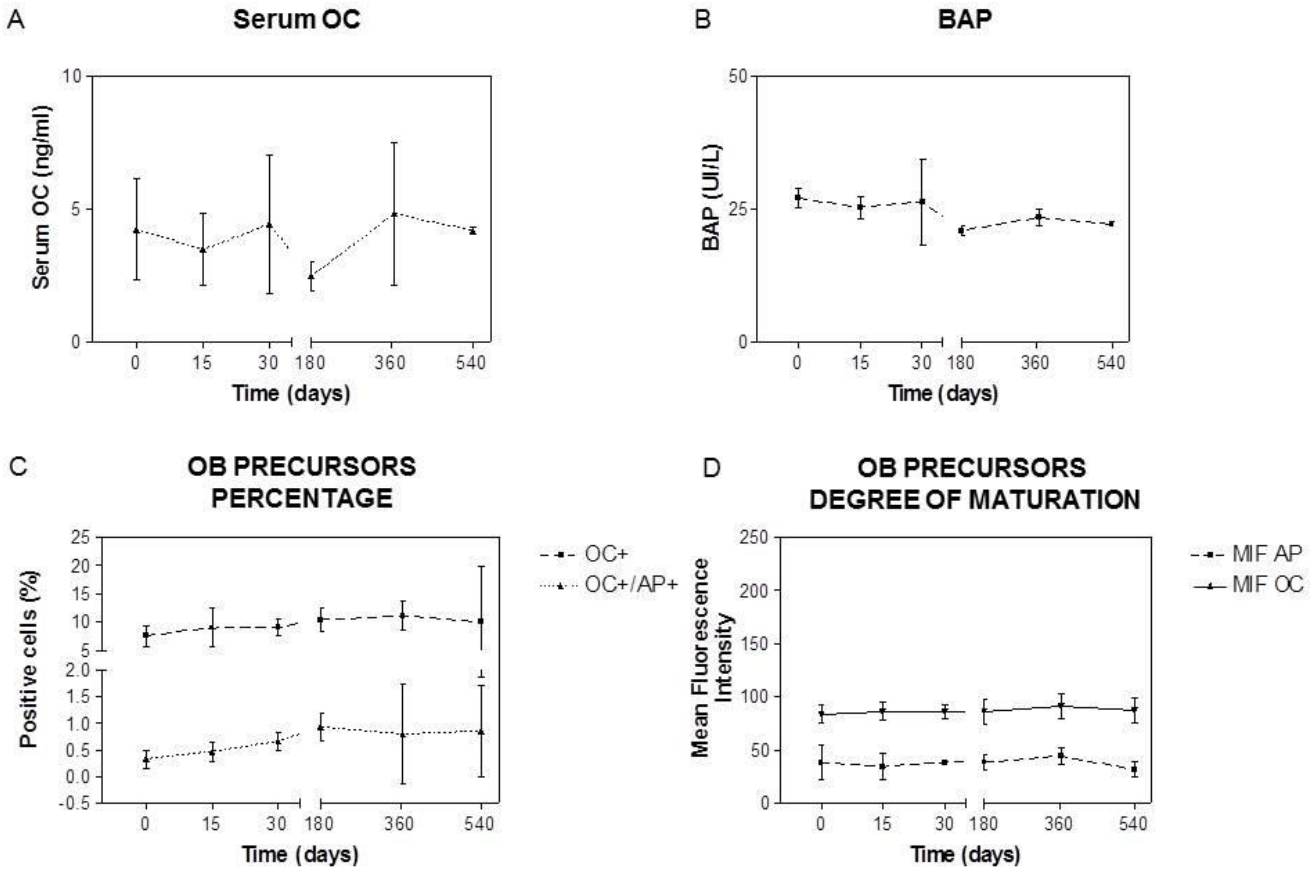


Figure 6. Raloxifene does not influence bone formation markers or OB precursors.

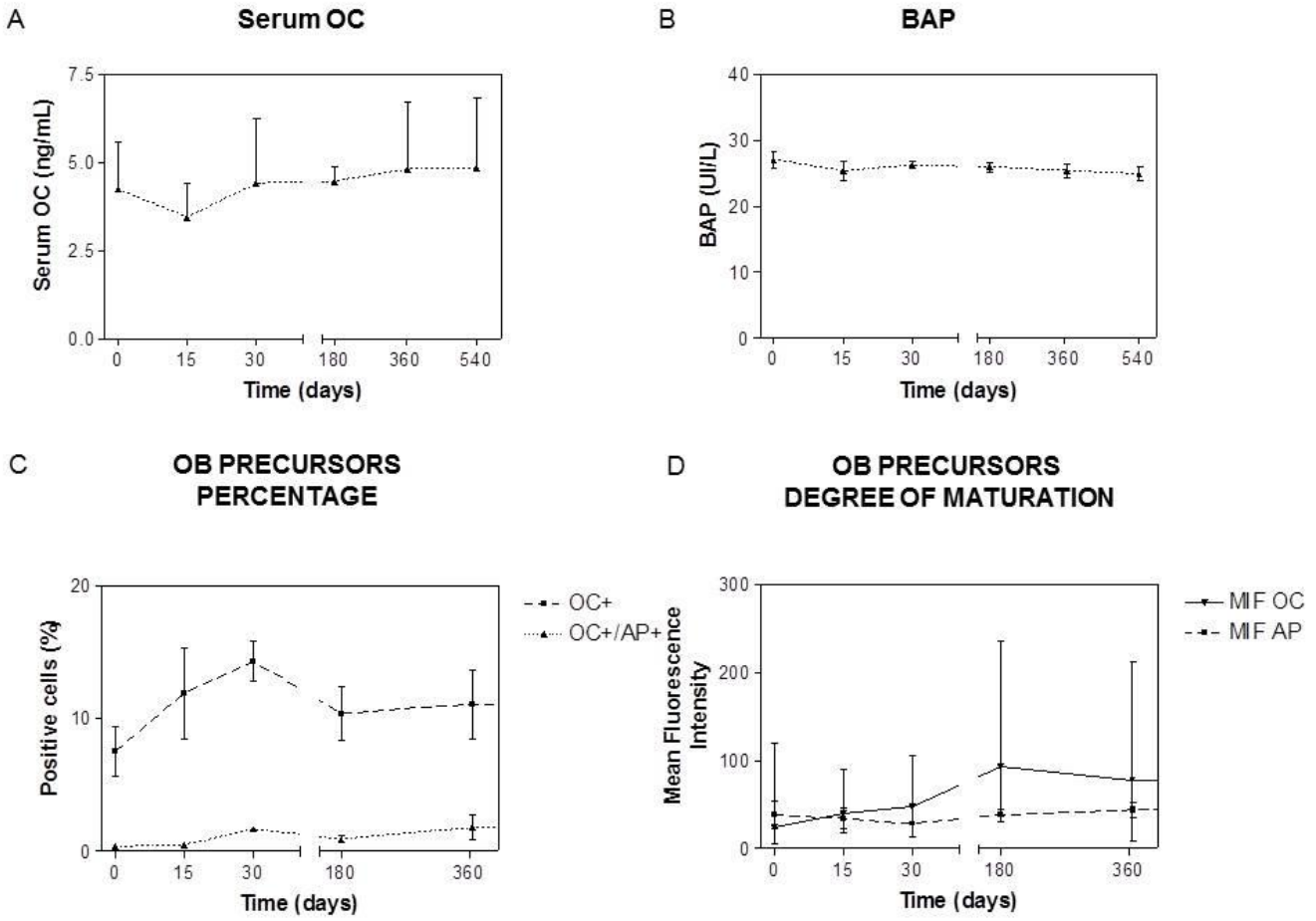
E. Graph shows the variation during treatment of serum OC in osteoporotic patients.

F. Graph shows the variation during treatment of BAP in serum of osteoporotic patients.

G. Graph shows the variation during treatment of OC+ and OC+/AP+ cells in the PBMCs of osteoporotic patients.

H. Graph shows the OC and AP MIF in the PBMCs of osteoporotic patients during treatment.

Symbols represent mean and standard deviations calculated on 5 patients, p values were calculated with Students paired T test (A,B,C) or with the Wilcoxon test (D).



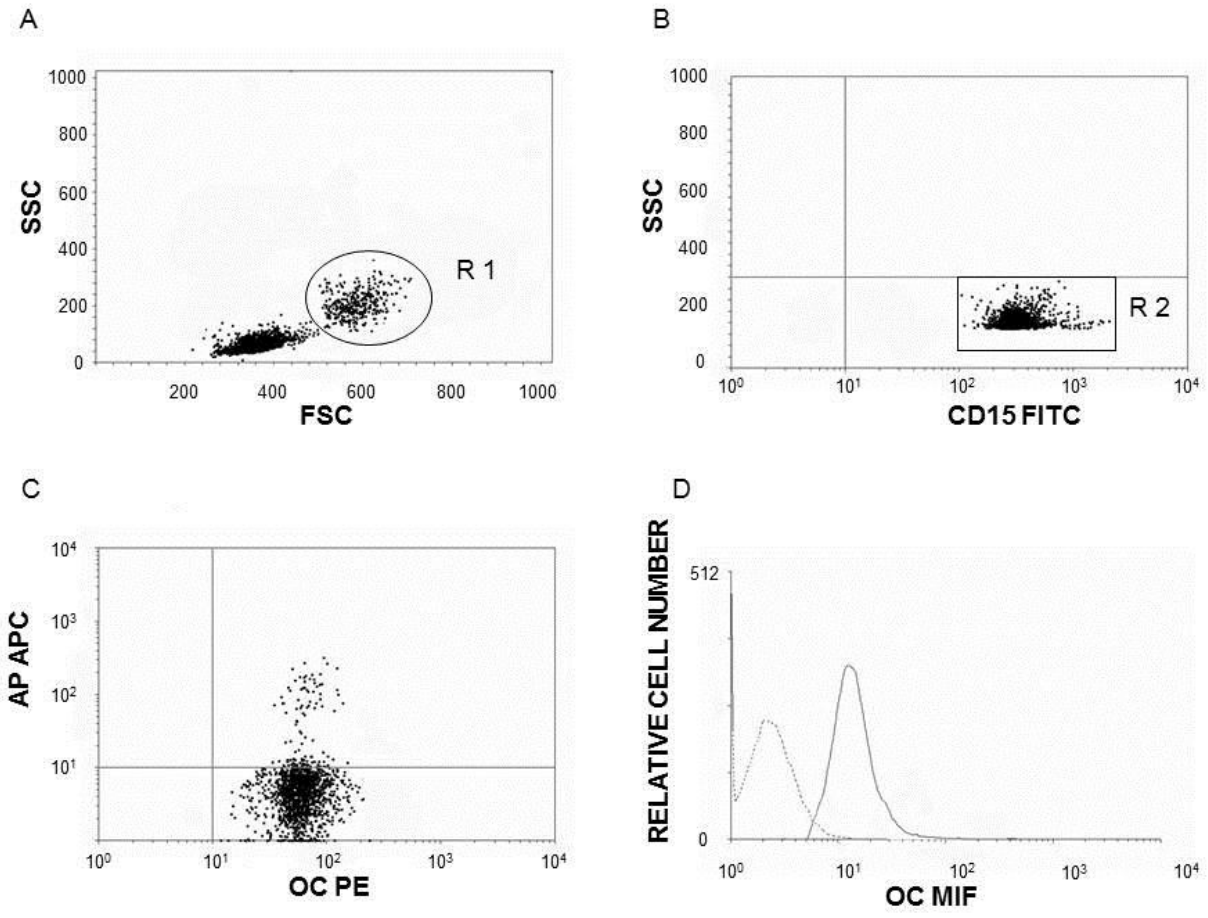
Supplemental figure. Flow cytometry analyses of OB precursors.

A. Dot plot represents PBMCs, monocytes are indicated (R1).

B. Dot plot represents CD 15 positive cells (R2) analysed on the whole PBMCs population.

C. Dot plot represents monocytes positive for AP and OC, CD 15 positive cells (R2) were excluded by the analyses.

D. Histogram represents the mean fluorescence intensity (MFI) of OC + cells (gated on monocytes after exclusion of CD 15+ cells). Broken line is the isotype control unbroken line is the stained sample.



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