The OPG/RANKL system and zinc ions are promoters of bone remodeling by osteoblast proliferation in postmenopausal osteoporosis

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Abstract
RANKL and its decoy receptor osteoprotegerin (OPG) is a mediator system involved in bone resorption and may be responsible for the homeostatic mechanism of normal bone remodeling. The serum levels of both OPG and soluble RANKL (sRANKL), the level of RANKL in primary cultures of osteoblasts, and the bone level of Zn2+ were measured in six women with postmenopausal osteoporosis and three women without osteoporosis (control group). As compared to control cases, patients with less than 15 years of estrogenic deprivation (cohort 1, n=3) presented increased levels of OPG (109.82%, p<0.002), sRANKL (229.13%, p<0.001) and RANKLOBL (272.35%, p<0.001), and decreased levels of Zn2+ (67.81%, p<0.001), whereas patients with more than 15 years of estrogenic deprivation (cohort 2, n=3) showed decreased levels of OPG (70.44%, p<0.003), and Zn2+ (61.41%, p<0.001), and increased levels of sRANKL (181.69%, p<0.002) and RANKL_OBL (201.1%, p<0.002). The significantly increased levels of sRANKL and RANKL_OBL in postmenopausal osteoporosis demonstrate osteoclastogenesis activation. According to the length of the estrogenic deprivation period, postmenopausal women with osteoporosis presented either increased (cohort 1) or decreased (cohort 2) OPG levels demonstrating osteoblast activation and osteoblast apoptosis stimulation, respectively. The bone levels of Zn2+ were significantly decreased showing limited proliferation and differentiation of the osteoblasts.

Keywords: bone Zn2+ ions, OPG/RANKL, osteoblasts, osteoporosis postmenopausal.

Introduction
Osteoporosis is the most common bone metabolic disease. Osteoporosis pathogenesis is complex and multifactor, characterized by the decrease of the bone mineral density (a focal bone decalcification process) and by bone microarchitecture deterioration (deep resorption cavities, delimited by bone lamellas more thin, with low strength, bone microfractures areas) [1, 2].

The bone is permanent liable to a process of bony remodeling, which has cyclic character in which resorbed bone quantity (through osteoclasts action) is equal to the one which it is formed (through osteoblasts action), first stage being the resorption one, and than the formation stage [2, 3].

Osteoclasts develop from precursors of the mononuclear monocyte-macrophage cell line after stimulation by macrophage colony-stimulating factor (M-CSF) and receptor for activated nuclear factor kappa ligand (RANKL). Receptor activator of nuclear factor-κB (RANK) is a member of the tumor necrosis factor family expressed by osteoclasts and their precursors [4, 5].

Osteoblasts, bone-forming cells are of mesenchymal origin and share a common precursor cell with adipocytes. During normal bone remodeling, marrow stromal cells and osteoblasts produce RANKL, which binds to the transmembrane receptor RANK on osteclast precursors and induces differentiation and activation. Osteoprotegerin (OPG) is a soluble member of the tumor necrosis factor receptor family (TNFR family) and inhibits the differentiation and fusion of the osteoclastic precursor cells, and blocks the activation of mature osteoclasts. Osteoblasts also produce osteoprotegerin (OPG), a soluble “decoy receptor” that blocks RANKL and maintains control of the bone remodeling process [4, 6].

Materials and Methods

Patients’ selection

The study was made using two cohorts of patients
with postmenopausal osteoporosis, in comparison with a control group (patients without osteoporosis), which suffered a surgical procedure for femoral cervix fracture in the Orthopedic–Traumatology I Clinic of the County Hospital, “Victor Babeș” University of Medicine and Pharmacy, Timișoara, Romania, in the years 2007–2008:

- Cohort 1 (patients in menopause, with 15 years of estrogenic deprivation), includes three patients (n=3) having their age under 65 years and T-score ≤-2.5 DS at lumbar spine and femoral level.
- Cohort 2 (patients in menopause, with over 15 years of estrogenic deprivation) was formed from three patients (n=3), having the age over 65 years and T-score ≤-2.5 DS at lumbar spine and/or femoral level.
- Control group (patients without osteoporosis) was formed from three patients (n=3), age under 60 years and T-score ≥-2.5 DS at lumbar spine and/or femoral level.

**Bone densitometry**

Dual energy X-ray absorptiometry (DXA) scans measure the bone mineral density (BMD) at your spine and/or femur and assigns a T-score. The T-score is defined as the number of standard deviations above or below the mean BMD for normal young females. Bone mineral density of the femoral neck and lumbar spine (L1–L4) was measured using dual-energy X-ray absorptiometry (DXA-Hologic Inc. QDR–1000; Bedford, USA). The osteoporosis diagnosis was made according to the World Health Organization criteria, after DXA assessment (spine and/or neck of femur T-score, sT between -2.5 DS at lumbar spine and/or femoral level.

**Biochemical markers**

OPG, RANKL levels (soluble and osteoblast culture) were measured by a sandwich ELISA (enzyme-linked immunosorbent assay) technique using the following tests:

- The kit human RANKL (sRANKL and RANKL_sul), from BioMedica Med. GmbH & Co KG, Vienna was also measured by a set of specific antibodies and standards of the same company.
- The assay performance was characterized by a lower detection limit of (0 pmol/L + 3 SD): 0.08 pmol/L, conversion factor pg/mL to pmol/L its 1 pg/mL= 0.05 pmol/L and the mean of precision (intra-assay of variation) was 3–5%, inter-assay of variation was 6 and 9% respectively) [10].
- The kit human Osteoprotegerin ELISA, from BioVendor Laboratory Medicine, Inc., Czech Republic according to the manufacturer’s protocol (the kit is a biotin labeled antibody based sandwich enzyme immunoassay for the quantitative measurement of human osteoprotegerin in serum, specificity – approx. 1% cross-reactivity with recombinant mouse OPG, and the standard or sample is incubated with a mouse monoclonal anti-human osteoprotegerin antibody coated in microtiter wells [11].
- Osteoblasts cultures supernatant was obtained through growth and differentiation of the bone cells, using specific Growth Medium (Osteoblast Growth Medium PromoCell), from PromoCell GmbH, Heidelberg.

Zinc concentrations of bone were determined by flame atomic absorption spectrometry (Varian AA240FS Fast Sequential AAS, from Mecro System, USA), by direct aspiration methods with five standards levels (0.2, 0.5, 1.0, 1.5, and 2.0 µg/mL solution) and lower detection limit 0.1 µg/mL solution [13].

**Statistical analysis**

All the values are reported as a mean ± SD (standard deviation); the statistical analysis was made using a Student’s t-test for the paired data, and the Pearson’s correlation. Were calculated coefficients values: p<0.05 has a significantly statistical value. Were calculated correlation coefficients, the values of correlation >0.5 (positive and negative values) considered respectively.

**Results**

The bones were cut into small fragments that were dissociated to cell suspensions by enzymatic digestion with 10 ml 0.1% collagenase-I solution/100 mg bones, for 15 minutes at 37°C.

The bone tissue were washed repeatedly in 20 mL DMEM (Dulbecco’s modified Eagle medium), and supplemented the culture medium with 10% fetal bovine serum (FBS) were incubated in a humidified atmosphere with 5% CO2 at 37°C for 4–24 hours to hasten attachment. This was followed by the addition of normal culture medium, which was DMEM supplemented with 10% FBS and penicillin/streptomycin solution (1 mL 1% solution), to the dish after two days, cells were observed emerging from the cells, the confluent 70–80% after approximately seven days.

The cells were dishes by using 3 mL 0.05% trypsin-EDTA solution. The cells were placed into 24-well microplates at a density of 2×10⁴ cells/cm², and continuously incubated in a humidified atmosphere with 5% CO2 at 37°C, utilization 20 mL Osteoblast Growth Medium-Promo Cell after 14 days allows us the obtaining of some, “clusters” of mesenchymal stem cells (MCS) with characteristic fusiform cells, having a “whirl” arrangement.

Also, the cultured incubated which under osteoinductive factors (ascorbic acid 2×10⁻³ M, β-glycerol phosphate 7×10⁻¹ M, dexamethazone 1×10⁻⁶ M; 10 µL solution/1 mL medium of cultures) suffers an osteoblast (OBL) differentiation for up to 28 days (Figure 1).

The obtained osteoblasts (OBL) have a characteristic morphology: big cells, “nest” disposal in “star arrangement”, which forms bone trabecule, and after prolongations retreat delimitates bone canaliculus (Figure 2).
The obtained results of this study allow us to observe an increase of the serum levels of RANKL (sRANKL), determinate through ELISA method: in cohort 1, an increase of 229.13% of the average ($p<0.001$) and in cohort 2, an increase of 181.69% of the average ($p<0.002$), comparing the control group. In cohort 2, the sRANKL levels are more reduced in comparison with cohort 1 (Table 1, Figure 3).

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<tr>
<td>RANKL-OBL [pg/mL]</td>
<td>79.31±5.92</td>
<td>29.12±1.0</td>
<td>58.56±3.08</td>
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<td>$p&lt;0.001$</td>
<td>r=0.949</td>
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<td>r=0.898</td>
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<td>sRANKL [pg/mL]</td>
<td>68.35±4.74</td>
<td>29.7±10.93</td>
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</tr>
<tr>
<td>$p&lt;0.001$</td>
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<td>RANKL-OBL/sRANKL ratio</td>
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<tr>
<td>OPG [pg/mL]</td>
<td>41.69±0.48</td>
<td>35.96±0.48</td>
<td>26.7±0.37</td>
</tr>
<tr>
<td>$p&lt;0.002$</td>
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<td>Zn$^{2+}$ [µg/g bone]</td>
<td>7.31±0.43</td>
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The values reported as a mean ± SD; coefficient values $p<0.05$ were considered statistically significant in comparing to control group, and coefficient of correlation $r>0.5$, values negative or positive, were considerate respectively.

The obtained results of this study allow us to observe an increase of the RANKL levels in osteoblast primary cultures supernatant (RANKL-OBL), determinate through ELISA method: in cohort 1, an increase of 272.35% of the average ($p<0.001$), and in cohort 2, an increase of 201.1% of the average ($p<0.002$), comparing the control groups. In cohort 2, the RANKL-OBL levels are more reduced in comparison with cohort 1, phenomena specific to osteoporosis (Table 2, Figure 3).

Also, we can observe an increase of the RANKL-OBL/sRANKL ratio in cohort 1, an increase of 117.9% of the average (RANKL-OBL/sRANKL ratio >1.0; $p<0.001$) and in cohort 2 an increase of 109.86% of the average (RANKL-OBL/sRANKL ratio >1.0; $p<0.001$) characteristic to osteoporosis with bone fracture. In comparing, the control group in case of

![Figure 1](image1.png)

Figure 1 – The “cluster” of mesenchymal stem cells (MCS). Phase-contrast micrographs, magnification at ×100: the “cluster” of mesenchymal stem cells with characteristic fusiform cells, having a “whirl” arrangement, after 7 days of culture.

![Figure 2](image2.png)

Figure 2 – The culture of osteoblasts (OBL). Phase-contrast micrographs, magnification at ×200: the characteristic morphology in osteoblasts culture, after 28 days of culture.

Analyzing the obtained results in the cells cultures realized in our study we can observe: a reduction of mesenchymal stem cells (MCS) to 68.46% of the cells density in cohort 1 ($p<0.003$), and a reduction to 48.42% of the cells density in cohort 2 ($p<0.001$), comparing the control group (Table 1).

Table 1 – Mesenchymal stem cells and osteoblast number in case of postmenopausal women with osteoporosis as compared to control groups

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<td>MSC ($&lt;10^5$ cells/cm$^2$)</td>
<td>0.65±0.15</td>
<td>1.41±0.1</td>
<td>0.16±0.07</td>
</tr>
<tr>
<td>$p&lt;0.003$</td>
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<td>OBL ($&lt;10^5$ cells/cm$^2$)</td>
<td>0.78±0.06</td>
<td>1.63±0.07</td>
<td>0.55±0.05</td>
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MSC – mesenchymal stem cells; OBL – osteoblasts; SD – standard deviation. The values reported as a mean ± SD; coefficients value $p<0.05$ were considered statistically significant, compared with control group.

There is a decrease of the differentiated osteoblasts (OBL): to 63.41% of the cells density in cohort 1 ($p<0.002$), and a decrease to 44.71% of the cells density in cohort 2 ($p<0.004$), comparing the control group (Table 1).

The obtained results of this study allow us to observe an increase of the serum levels of RANKL (sRANKL), determinate through ELISA method: in cohort 1, an increase of 229.13% of the average ($p<0.001$) and in cohort 2, an increase of 181.69% of the average ($p<0.002$), comparing the control group. In cohort 2, the sRANKL levels are more reduced in comparison with cohort 1 (Table 1, Figure 3).

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Also, we can observe an increase of the RANKL-OBL/sRANKL ratio in cohort 1, an increase of 117.9% of the average (RANKL-OBL/sRANKL ratio >1.0; $p<0.001$) and in cohort 2 an increase of 109.86% of the average (RANKL-OBL/sRANKL ratio >1.0; $p<0.001$) characteristic to osteoporosis with bone fracture. In comparing, the control group in case of
which RANKL-OBL/sRANKL ratio had the average (RANKL-OBL/sRANKL ratio <1.0, characteristic to menopause without osteoporosis) (Table 2, Figure 3).

In cohort 1, an increase of OPG serum levels (increase of 109.82% of the average, \( p<0.002 \)), comparing the control groups. In cohort 2, exists a decrease of the OPG serum levels (decrease to 73.15% of the average, \( p<0.003 \)), comparing the control groups (Table 2, Figure 3).

In this study, we could demonstrate that OPG/sRANKL ratio in cohort 1, a reduction to 47.77% of the average (\( p<0.001 \)), comparing the control group. In cohort 2, a reduction to 38.54% of the average (\( p<0.001 \)), comparing the control group. To both groups (with postmenopausal osteoporosis) OPG/sRANKL ratio is lower than 1.0 (OPG/sRANKL ratio <1.0), common cause osteoporosis disease. In case of the control group OPG/sRANKL ratio has the average, higher than 1.0 (OPG/sRANKL ratio >1.0), menopause characteristic, but without osteoporosis (Table 2, Figure 3).

Levels of ionic zinc (Zn\(^{2+}\)) from the bone tissue were determinate through atomic absorption spectrometry analyzed (FAAS, in acetylene flame). Analyzing the obtained results it is observed: in cohort 1 a decrease of Zn\(^{2+}\) bone levels to 67.81% of the average (\( p<0.001 \)), and in cohort 2 a decrease of Zn\(^{2+}\) bone levels to 61.41% of the average (\( p<0.001 \)), comparing the control group (Table 2).

In our study, we could demonstrate the existence of good positive correlation in cohort 1 between the low levels of the Zn\(^{2+}\) bone ions and high serum levels of OPG (\( r=0.704 \)), some powerful negative correlations between the low levels of the Zn\(^{2+}\) bone ions or increased serum levels of sRANKL (\( r=-0.970 \)), or increased of the RANKL-OBL (\( r=-0.949 \)) (Table 2, Figure 4).

In control group exists a good positive correlation between OPG serum levels and bone Zn\(^{2+}\) levels (\( r=0.977 \)), a powerful negative correlation between sRANKL levels (\( r=-0.958 \)), and a powerful negative correlation between RANKL-OBL levels and bone Zn\(^{2+}\) levels (\( r=-0.971 \)), characteristics of postmenopausal women without osteoporosis (Figure 6).

![Figure 5 – Relationships between the bone levels of Zn\(^{2+}\) and the serum levels of bone markers in postmenopausal women with osteoporosis, in cohort 2 (n=3). The negative or positive values of the correlation coefficient (\( r>0.5 \)) were considerate respectively.](image5)

![Figure 6 – Relationships between the bone levels of Zn\(^{2+}\) and the serum levels of bone markers in postmenopausal women with osteoporosis, in control group (n=3). The negative or positive values of the correlation coefficient (\( r>0.5 \)) were considerate respectively.](image6)

**Discussion**

The sRANKL molecule from the TNF family and its receptor, RANK, are the key regulators of bone remodeling and have a major role in the development and activation of osteoclasts. The sRANKL from the osteoblasts mediates osteoclastogenesis and, in contrast, OPG is a “decoy” receptor that acts by binding to neutralizing both the soluble RANKL. OPG is a soluble member of the tumor necrosis factor receptor family (TNFR family) and inhibits the differentiation and fusion of the osteoclastic precursor cells and blocks the activation of mature osteoclasts [8, 14, 15].

Osteoblasts–osteoclasts couple has a continuous interaction in the presence of molecular RANK/RANKL/OPG system, with an important role in bone remodeling regulation. In postmenopausal osteoporosis, OPG/sRANKL ratio is lower than 1 (OPG/sRANKL ratio <1.0), comparing the one of the women which is in premenopause. Through a feedback-regulated mechanism, they will determine OPG secretion increase.
at osteoblasts level, but manifesting a decrease of the “decoy” receptor for soluble OPG activity. OPG will not be connected with sRANKL, determining sRANKL connection with osteoclasts RANK having as a result RANK/ RANKL complex formation, which has a role in osteoclastsogenesis stimulation and bone resorption stimulation being favorable to osteoporosis installation [3, 16, 17].

OPG is assuring “bone protection” through osteoblasts activation, turnover increase with bones formation stimulation. OPG is considered an important marker of bone synthesis. Postmenopausal, the OPG serum levels increase demonstrates osteoblasts activation and the decrease of OPG serum levels represents the consequence of age-related osteoblasts apoptosis (ARORC, age-related osteoblasts replicate capacity) [5, 18].

In osteoporosis, a high osteoblastic apoptosis leads to a decrease of multiplication capacity of the age-related osteoblasts (ARORC, age-related osteoblast respective capacity) specific to advanced age. This reduction leads to microfractures at bones level. Increased osteoblast irreparable apoptosis rate accelerates age-related osteoblast replication capacity decrease (Table 1).

In case of our study, the increased serum levels of OPG in cohort 1 (in postmenopausal osteoporosis in the initial phase) demonstrates osteoblasts activation, and the decrease in cohort 2 (in postmenopausal osteoporosis in the belated phase) demonstrates stimulation of osteoblast apoptosis, associated with will increase significantly bone turnover; producing a decrease in bone formation and increasing bone resorption. The unbalance promotes appearance the osteoporosis. In postmenopausal osteoporosis, OPG serum level decrease can be an indicator of a higher risk of bones fracture (Table 2, Figure 1).

To both groups (with postmenopausal osteoporosis) OPG/sRANKL ratio is lower than 1.0 (OPG/sRANKL ratio <1.0), common cause of the osteoblast genesis through “decoy receptor” activity decrease of the OPG due to estrogens deficit postmenopausal installed. In case of the control group OPG/sRANKL ratio has higher than 1.0 (OPG/sRANKL ratio >1.0), menopause characteristic, but without osteoporosis. This unbalance represents an indicator of a higher risk of osteoporosis bones fracture (increased the risk factor). Thus, OPG/sRANKL ratio is an important determinant of bone mass and skeletal integrity (Table 2, Figure 3).

At bone’s level the main RANKL actions are: mature osteoclasts activation for bone resorption which’s stimulating “pool” increase of the active metabolic osteoclasts, assures osteoclasts survival through their apoptosis inhibition. RANKL (sRANKL and RANKL_OBL) it is considered an important marker of the bone resorption [4, 17, 19].

In case of our study, the serum levels of sRANKL and RANKL_OBL are significantly higher (in cohort 1 and cohort 2), demonstrating osteoclast activation, associated witch will increase significantly the bone turnover; producing a decrease of bone formation and increasing bone resorption, the unbalance favors the osteoporosis appearance (Table 2, Figure 3).

Also, we can observe an increase of the RANKL_OBL/ sRANKL ratio in cohort 1 and in cohort 2 (RANKL_OBL/sRANKL ratio >1.0, which is characteristic to osteoporosis with bone fractures) comparing the control group in case of which RANKL_OBL/sRANKL (RANKL_OBL/sRANKL ratio <1.0), characteristic to menopause without osteoporosis (Table 2, Figure 3).

This results in increased osteoclast activation through a “switch-like” diversion of osteoprogenitor cell differentiation away from monocyte-macrophage cell development and toward osteoclastogenesis. Osteoblasts differentiation from their mesenchymal forerunners is a process dependent by the major transcription factor presence Cbfa-1/Runx-2 (core binding factor-1/runt-related transcription factor-2), which regulates transcription at genomic level. Zn^{2+} ions are stimulating osteoblasts proliferation and differentiation, being promoters of the major transcription factor presence Cbfa-1/Runx-2. Previous in vitro studies have suggested a direct effect of zinc on both the proliferation and differentiation of osteoblast-like cells [4, 7, 20]. Zinc inhibits the differentiation of osteoclasts and promotes osteoblast activity affecting the bone formation. The participation of trace elements in normal development and maintenance of the skeleton is related to their catalytic functions in organic bone matrix synthesis (Figure 7) [1, 3, 20, 21].

**Figure 7 – The RANKL/OPG system in bone remodeling at post-menopausal osteoporosis.** OPG – Osteoprotegerin; sRANKL – Soluble receptor activator of nuclear factor-κB ligand; RANK – Receptor activator of nuclear factor-κB; RANKL_OBL – Osteoblast receptor activator of nuclear factor-κB ligand; Zn^{2+} – Zinc ions; ↑ – Increased; ↓ – Decreased.
In this study, analyzing the obtained results it is observed: in cohort 1 and cohort 2, a decrease of Zn²⁺ bone levels comparing the control group, as a result of bone turnover increase, but with a rate of the bone resorption superior to synthesis, determining in these conditions a negative bone balance and being favorable for microfractures and osteoporosis fractures appatition (Table 2).

Zinc is an essential trace element that increases osteoblast numbers and bone formation. Zn²⁺ is the most abundant trace element in bone, being present at a concentration of up to 300 μg/g bone, and it has been considered an important factor in bone metabolism. In osteoporosis, this mechanism is diminished, and as a result it is suppressed osteoblasts’ activity. Zinc deficiency is associated with unbalance of the bone remodeling. Low zinc intake has been reported to be associated with low bone mass in women. It is defined as a disease characterized by low bone mass and associated with low bone mass in women. It is defined as a disease characterized by low bone mass and associated with low bone mass in women.

**Conclusions**

In conclusion, the sRANKL and RANKL/OPG is significantly increased in postmenopausal osteoporosis and is a marker of increased of the bone resorption. The OPG is moderately increased in osteoporosis and is a marker of bone formation, stimulating bone turnover. The decreased of OPG serum levels represents the consequence of osteoblasts apoptosis. OPG/sRANKL ratio <1.0 and generates osteoclastogenesis through “decoy receptor” activity decrease of the OPG due to estrogens deficit postmenopausal installed.

In postmenopausal osteoporosis exists a decrease of the bone levels of Zn²⁺ because of activation and osteoblast differentiation reduction as bones mineralizing capacity decreases. In postmenopausal osteoporosis exists a powerful negative correlation between decreased levels of the bone Zn²⁺ and increased of the RANKL/OPG or sRANKL because of osteoclastogenesis and represents a major risk factor in the appearance of microfractures/fractures bone.

In postmenopausal osteoporosis in the initial phase exists a powerful positive correlation between decreased Zn²⁺ ions levels and increased OPG serum levels because of bone turnover stimulation, and in the belated phase exists a powerful positive correlation between decreased Zn²⁺ ions levels and decreased OPG serum levels because of osteoblast apoptosis increase.

The bone remodeling unbalance in postmenopausal women with osteoporosis is produced by decreased bone formation or increased bone resorption, and represents an indicator of a higher risk of osteoporosis bones fracture (increasing the risk factor).

**References**


The OPG/RANKL system and zinc ions are promoters of bone remodeling by osteoblast proliferation...


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