Original article

Evaluation of Osteoblastic/ Osteoclastic Activity in Postmenopausal Osteoporosis

Evaluarea activității osteoblastice/osteoclastice în osteoporoza postmenopausală

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Abstract

Background: In postmenopausal osteoporosis, bone loss is due to an increased bone resorption with an inadequately increased bone formation. The aim of this study was to evaluate the serum levels of bone markers implicated in the bone remodelling process. The resorption markers were represented by: receptor activator of nuclear factor-κB ligand (RANKL), while bone formation markers were represented by: osteoprotegerin (OPG), estradiol (E_2) and zinc ions ($Zn^{(2+)}$). Materials and methods: The study included 134 women with postmenopausal osteoporosis and 68 postmenopausal women without osteoporosis, as a control group. The serum levels of the discussed markers were measured by the enzyme-linked immunosorbent assay (ELISA) technique. Serum zinc ions concentrations were determined using the flame atomic absorption spectrometry (FAAS) technique. Bone mineral density (BMD) was measured using the dual energy X-ray absorptiometry (DXA) technique with the assessment of T score. Results: The serum levels of sRANKL were significantly higher in the postmenopausal osteoporosis group vs. the control group ($p<0.0001$). E_2 and OPG levels in these subjects were significantly decreased as compared to controls ($p<0.0001$; respectively $p<0.0001$). The $Zn^{(2+)}$serum levels were lower in the postmenopausal osteoporosis group ($p<0.0001$) vs. the control group. Conclusions: In postmenopausal osteoporosis the OPG/sRANKL ratio was lower than 1.0, secondary to the increasing osteoclastogenesis. Bone resorption begins gradually and outruns the new bone formation rhythm, this fact being associated with low BMD.

***Keywords**: postmenopausal osteoporosis, bone mineral density, bone markers.*

Rezumat

Introducere: În osteoporoza postmenopausală, pierderea masei osoase se datorează unei resorbții osoase crescute cu formare osoasă inadecvată. Scopul acestui studiu a fost dea evalua nivelele serice ale markerilor osoşi implicați în procesul de remodelare osoasă. Markerii resorbției osoase sunt reprezentați de: ligandul receptorului activa-

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tor al factorului nuclear kappa B (RANKL), în timp ce markerii formării osoase sunt reprezentați de: osteoprotegerina (OPG), estradiol (E_2) şi ionii de zinc ($Zn^{(2+)}$). Materiale şi metode: Acest studiu cuprinde 134 de femei cu osteoporoză postmenopausală şi 68 de femei la menopauză fără osteoporoză, ca şi grup de control. Nivelele serice ale markerilor enumerați au fost măsurate prin-o tehnică imuno-enzimatică (ELISA). Concentrațiile serice ale zincului au fost determinate utilizând tehnica spectrofotometriei de absorție atomică în flacără (FAAS). Densitatea minerală osoasă (DMO) a fost măsurată utilizând tehnica DXA, cu măsurarea scorului T. Rezultate: Nivelele serice ale RANKL au fost semnificativ crescute în osteoporoza postmenopausală vs. control group ($p<0.0001$). Nivelele de E_2 şi OPG la aceste paciente au fost semnificativ scăzute comparativ cu grupul de control ($p<0.0001$, respectiv $p<0.0001$). Nivelele serice ale $Zn^{(2+)}$ au fost scăzute în osteoporoza postmenopausală ($p<0.0001$) vs. grupul de control. Concluzii: În osteoporoza postmenopausală raportul OPG/RANKL a fost scăzut sub 1.0, secundar creşterii osteoclastogenezei. Resorbția osoasă începe treptat depăşind ritmul de formare osoasă, ceea ce duce in consecinta la scăderea DMO.

Cuvinte cheie: *osteoporoza postmenopausală, densitatea minerală osoasă, markeri osoşi.*

Introduction

Osteoporotic fragility fractures occur due to a decreased bone mass and bone tissue disorganization (1). The alteration of bone architecture is determined by a disproportion between the rising number of active osteoclasts and the reduced number and activity of osteoblasts (2, 3).

The osteoblasts express the receptor activator of nuclear factor-κB ligand (RANKL) on their surface. The osteoclasts express the receptor activator of nuclear factor-κB (RANK) on their surface (both precursor and mature forms). The connection of RANKL to the RANK molecule triggers the development of osteoclasts and stimulates the activity of osteoclasts (3-5).

Osteoprotegerin (OPG), a member of the tumor necrosis factor receptor family, is synthesized by the bone cells. It is formed of 401 amino acids. Its monomer molecular weight is 60 kDa and the dimers form's is 120 kDa. Its main function is to inhibit the RANK-RANKL coupling and hence, it suppresses the osteoclast development and activity (3- 6).

In women, postmenopausal hypogonadism is the most important cause of osteoporosis. Estrogen controls the balance between the osteoblasts and the osteoclasts, in part by triggering osteoclasts apoptosis and by stimulating OPG synthesis by the osteoblasts (1, 5, 7).

Another factor that stimulates the osteoblasts' proliferation and thus bone formation, is represented by zinc. Zinc insufficiency induces an accelerated bone resorption and a decreased bone formation, leading to bone mass loss (1, 7, 8).

In osteoporosis, bone resorption gradually exceeds bone formation, resulting in low bone mineral density (BMD) (2, 9, 10).

The aim of this study was to determine the serum levels of bone resorption markers (RANKL) and bone formation markers (osteoprotegerin, estradiol, and zinc ions). We tried to establish a relationship between bone mineral density value and the levels of bone markers in postmenopausal osteoporosis.

Materials and methods

Patients

This study included a group with postmenopausal osteoporosis (n=134 patients, with less than 15 years of estrogenic deprivation), and a control group (n=68 postmenopausal women without osteoporosis).

The inclusion criteria were as follows: women with postmenopausal osteoporosis, aged over 55 (with physiological menopause).

The exclusion criteria were as follows: women under 40 years, with precocious menopause, and cases with secondary osteoporosis.

Each patient was informed about the study protocol and signed an informed consent. The institutional ethics committee of the University of Medicine and Pharmacy "Victor Babeş" Timişoara, Romania, approved the study design.

Bone densitometry

Dual energy X-ray absorptiometry (DXA) scan measured bone mineral density at different skeleton sites (usually spine and/or hip), assessed by the T-score. BMD of the hip (neck, trochanter, femoral, and total) and lumbar spine (L_1-L_4 and L_{total}) was measured using a DXA Hologic device (QDRC. 1000 Inc., Bedford, MA, USA). The diagnosis of osteoporosis was established using the WHO criteria (lumbar and/or femoral T score below -2.5 SD).

Biochemical markers

Venous blood samples were collected into preservative-free tubes and allowed to clot. After centrifugation at 2000g for 15 minutes, serum samples were decanted and frozen in aliquots. The serum samples were stored frozen at -20 °C for 5 days, and at -40°C for 1 month.

Bone markers serum levels were measured by the ELISA (enzyme linked immunosorbent assay) technique, as described below.

For osteoprotegerin determination, in the BioVendor OPG human ELISA kit, the standards, quality controls and samples were incubated in microtiter wells coated with a mouse anti-human OPG monoclonal antibody . The performance characteristics of this assay were as follows: the lower detection limit of 0.13 pmol/L; intra-assay precision CV% of 2.4-7.0%; inter-assay precision CV% of 3.4-7.4% (BioVendor Laboratory Medicine Inc., Brno, Czech Republic).

In the BioVendor sRANKL human ELISA kit, calibrators, quality controls and samples were incubated in microtitration wells together with the excess of recombinant OPG. The sRANKL/OPG complex formed during the two-hour incubation step was captured by the immobilized anti-sRANKL monoclonal antibody. The performance characteristics of this assay were: the lower detection limit of 1.5 pg/mL; intra-assay precision CV% of 4.5-5.5%; inter-assay precision CV% of 7-9%; Unit conversion: 1pg/ml=0.016pmol/L (60 kDa) (BioVendor Laboratory Medicine, Inc., Brno, Czech Republic).

The E_2 (Estradiol) was measured by the ELISA human kit. The microtiter wells were coated with an antibody directed towards a unique antigenic site on the estradiol molecule. Assay characteristics of the E_2 ELISA kit were as follows: standards of 0; 25; 100; 250; 500; 1000; 2000 pg/mL, control 4.5 pmol/L (4.2±1.3 pmol/L). The basal values in postmenopausal women are: 11-65 pg/mL, the maximum inter- and intra-assay CV% were 6.8 and 7.25% respectively, analytic sensitivity of 3-6 pg/ml and the conversion factor was 1 pg/mL= 3.67pmol/L (Human Gesellschaft fűr Biochemica und Diagnostica Hmb, Wiesbaden, Germany).

Ions Concentration

The serum levels of $Zn^{(2+)}$ were determined using flame atomic absorption spectrometry (FAAS), a direct aspiration method, using an air/acetylene flame (Varian AA240FS Fast Sequential AAS, MecroSystem USA). The protocol includes deprotenization by diluting the serum (1:9) with trichloro-acetic acid 5%. The assay performance was characterized by a stock solution of 1.0 mg/L (dilution of serum with water soluble n-butanol 6%) from which we established 5 standards (0.0; 50; 100; 200; 250 µg/dL).

Statistical analysis

The data were analyzed using SPSS version 14 for Windows (SPSS Inc, Chicago, Ill., USA). Kolmogorov-Smirnov test was used to establish the normal distribution of continuous variables. All values were reported as mean ± SD. The level of significance was established at 0.05. Student´s t-test was applied to compare normal distributed values, while Mann-Whitney *U*-test was used for not normal distributed values. Pearson or Spearman correlation tests were used appropriately.

Results

Osteoporotic women were significantly older than control ones (for the osteoporotic group the mean age was 65.5±1.5 yrs, ranging from 61 to 70 years, respectively, for the control group the mean age was 56.5 ± 1.5 yrs, ranging from 55 to 58 years, $p<0.002$).

Table 1. Demographic and osteodensitometric data in study groups

Parameter (mean ± S.D.)	Study group (n=134)	Control group (n=68)	P (t-test)
Age (yrs)	65.5 ± 4.5	56.5 ± 1.5	<0.002
Menopause age (yrs)	46.5 ± 1.5	47.5 ± 0.5	* <0.215
Duration of E_2 deprivation (yrs)	17.5 ±1.25	9.5 ± 0.5	<0.001
L_1 spine T-score	–3.15 ± 0.08	–1.55 ± 0.12	<0.001
L_2 spine T-score	–3.54 ± 0.12	–1.6 ± 0.1	<0.001
L_3 spine T-score	–3.85 ± 0.2	–1.72 ± 0.15	<0.001
L_4 spine T-score	–3.4 ± 0.17	–1.37 ± 0.08	<0.001
Total spine T-score	–3.65 ± 0.6	–1.7 ± 0.2	<0.001
Total spine BMD (g/cm^2)	0.616 ± 0.02	0.775 ± 0.145	<0.0001
Femoral neck T-score	–3.55 ± 0.25	–1.55 ± 0.13	<0.001
Trochaner T-score	–3.37 ± 0.18	–1.62 ± 0.1	<0.001
Inter femoral T-score	–2.85 ± 0.1	–1.75 ± 0.15	<0.001
Total femoral T-score	–3.45 ± 0.5	–1.6 ± 0.3	<0.001
Total femoral BMD(g/cm^2)	0.624 ± 0.05	0.785 ± 0.128	<0.0001

($p<0.05$ - significant difference from the control group and $p^*>0.05$ no significant difference from the control group; these were determined using the Student's t test).

Table 2. Serum levels of bone markers in postmenopausal women with osteoporosis, compared to the control group

Parameter (mean ± S.D.)	Study group (n=134)	Control group (n=68)	P (t-test)
E_2 (pg/mL)	28.2 ± 1.7	42.5 ± 4.4	<0.0001†
OPG (pg/mL)	42.1 ± 0.5	47.1 ± 1.5	<0.0001†
sRANKL (pg/mL)	67.6 ± 3.6	43.6 ± 2.4	<0.0001‡
OPG/sRANKL ratio	0.63 ± 0.04	1.18 ± 0.03	<0.0001‡
$Zn^{(2+)}$ (µg/dL)	57.6 ± 4.6	80.4 ± 3.4	<0.0001†

(† t-test; ‡Mann-Whitney non-parametric test)

Although menopause occurred around the same age in both groups, E_2 deprivation period was longer in the osteoporotic group, as compared to the control group (17.5 ± 1.2 years, *vs.* 9.5±0.5 yrs, $p<0.001$) (*Table 1*).

Spine and femoral BMD values were significantly lower in the study group as compared to the control group. Demographic and osteodensitometry parameters are presented in *Table 1*.

Serum levels of E_2 were significantly lower in the study group (ranging from 18.5 to 30.5 pg/mL) as compared to the control group (ranging from 36.5 to 49.7 pg/mL, $p<0.0001$) (*Table 2*).

Osteoporotic women showed significantly lower values of serum OPG (ranging from 41.1 to 43.2 pg/mL) as compared to the control subjects (ranging from 45.8 to 48.5 pg/mL, $p<0.0001$, *t*-test).

The serum sRANKL levels were significantly higher in the study group (ranging from 61.4

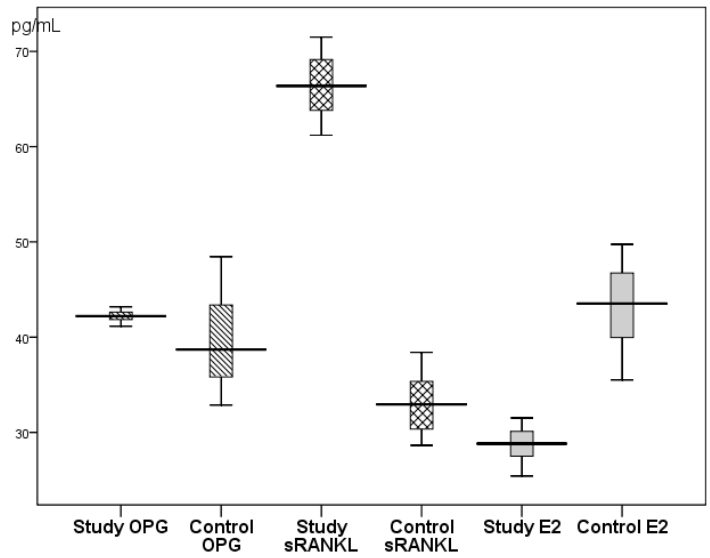

Figure 1. Serum levels (pg/mL) of bone markers (OPG, sRANKL) and E_2 in osteoporotic women *vs.* control group.

to 73.3 pg/mL) as compared to the control group (range: 30.5-47.5 pg/mL), ($p<0.0001$, non-parametric test). Hence, the OPG/sRANKL ratio was significantly lower in the osteoporotic group (-85.8%, $p<0.0001$, non-parametric test) (*Figure 1*).

Serum $Zn^{(2+)}$ levels were significantly lower in the study group (ranging from 52.6 to 70.1 μg/dL), as compared to the control subjects (ranging from 89.6 to 100.5 μg/dL, $p<0.0001$, t-test) (*Table 2*).

RANKL concentrations correlated positively and significantly with age in both study groups (r=0.920, 95% confidence interval 0.810-0.965, $p<0.001$). OPG values increased gradually with age for all patients (Pearson correlation coefficient r=0.326, 95% confidence interval 0.126-0.500, p=0.001).

Neither in the study group, nor in the control group did BMD values correlate with OPG levels. Although BMD values did not correlate with sRANKL concentrations in the control group, they showed a significantly negative but rather weak correlation with sRANKL values in the study group (Spearman coefficient r = -0.304, 95% confidence interval -0.455 -0.137, p=0.0003).

In the study group, E_2 levels did not correlate with BMD values, but in the control group, there was a positive weak correlation (Pearson correlation coefficient r = 0.285, r^2=0.08, p=0.01).

OPG levels correlated negatively with sRANKL concentrations in the study group (r= -0.564, 95% confidence interval -0.672 -0.432, $p<0.0001$). Nonetheless, in the control group, the correlation was strong and positive (r=0.699, 95% confidence interval 0.548-0.806, $p<0.0001$). Serum OPG correlated positively with E_2 levels in both groups (study group: r = 0.542, r^2 =0.293, $p<0.0001$, control group: r = 0.694, r^2=0.481, $p<0.0001$).

Discussions

The bone tissue suffers a constant remodelling process. The resorption phase (mediated by osteoclasts) is followed by the formation phase (induced by osteoblasts) (2, 12, 13).

The estrogen contributes to the bone turnover, mainly by enhancing osteoblastmediated bone formation. E_2 exerts its effect on osteoblasts by binding to two estrogen receptor isoforms (ERα and ERβ) (6, 12, 14, 15).

In our study, estradiol serum levels were below the ”critical” level of 40 pg/mL. Osteoporotic women presented a difference of 50.7%, as compared to the control group. E_2 concentrations were significantly lower ($p<0.0001$) in postmenopausal women with osteoporosis, associated with low BMD.

In the study group, BMD presented reduced values, especially at spine level. Total lumbar spine BMD presented a difference of 84% compared to the controls, while total femoral BMD showed a difference of 80.5% (*Table 1*).

The diminished values of BMD could be explained by a decreased function of estrogen re-

ceptor ERα (associated with postmenopausal estrogenic deficiency). Subsequently, dissociation occurs between the bone resorption and bone formation processes. The resorption phase will prevail, leading in the end to osteoporosis and bone microfractures.

Furthermore, Rogers A. *et al*. demonstrated the important role of the postmenopausal endogenous circulating estradiol concentrations in the bone turnover process (6, 14, 16).

OPG, a new described cytokine, hampers the coupling of RANKL with the RANK molecule, and therefore prevents osteoclast differentiation and mature osteoclast activation (3, 5, 15, 17).

The OPG/RANKL ratio is involved both in the regulation of osteoclast formation mechanism and in the activation of mature cells. Estrogen deficiency lowers the OPG/RANKL ratio, promoting thus osteoclastogenesis, and accelerating bone resorption (5, 6, 12, 16).

There are many heterogeneous data in the literature about the complex relationships between circulating bone-turnover marker levels (OPG, RANKL), bone mineral density and vertebral fractures in postmenopausal women (4, 14, 18, 19).

Our study group showed lower OPG serum levels than in the control subjects (a decrease up to 12%, $p<0.0001$). This could be explained by the age-related reduction of osteoblastic multiplication capacity and also by osteoblastic apoptosis, correlated with aging (during severe, advanced osteoporosis) (*Table 2*). Being an antiresorptive parameter, the age-related OPG increase could illustrate the inability of bone cell mechanisms to overcome the bone loss.

The presented data showed significantly higher sRANKL serum concentrations in the study group (an increase of 55%; *vs*. control group, $p<0.0001$). This illustrates osteoclast activation with further augmented bone resorption (*Table 2*). In postmenopausal women with significantly high bone turnover, the decrease in bone formation and the increase in bone resorption will promote osteoporosis. In postmenopausal osteoporosis, the decreased OPG serum levels may be an indicator for a higher fracture risk.

The OPG/sRANKL ratio plays a substantial role in providing information regarding bone structure. The Fracture Risk Assessment Tool (FRAX) calculates the 10-year absolute fracture risk for hip fractures and other major osteoporotic fractures (1, 11, 13, 17, 19).

In our study, the osteoporosis group had an OPG/sRANKL ratio lower than 1.0 (a decrease of 85.8%, *vs.* controls, $p<0.0001$), secondary to the enhanced osteoclastogenesis. The control group presented an OPG/sRANKL ratio above 1.0, typical for menopausal cases without osteoporosis (*Table 2; Figure 1*).

The serum biomarkers of bone turnover have an established role in clinical monitoring of patients treated with skeletally active drugs. RANKL inhibitors might be active in suppressing bone resorption to a greater extent than any other class of drugs, without toxic effects. Denosumab was developed as a human monoclonal anti RANKL antibody, which suppresses RANKL activity (2, 12, 14, 20).

Postmenopausal osteoporosis is characterized by a high rate of bone remodelling, inducing an increased bone loss and a significant risk for fractures. In this study, the serum levels of the assessed bone markers were associated with low BMD (a decrease in spine BMD of 84%, $p<0.0001$ and of 80.5%, $p<0.0001$ in femoral BMD) (*Table 1*).

The differentiation of osteoblasts from their mezenchimal precursors is a process dependent upon the major transcription factor Cbfa-1/Runx-2, which regulates OPG transcription. Zinc ions stimulate osteoblast proliferation and differentiation, serving as promoters of Cbfa-1/Runx-2. In osteoporosis, this mechanism diminishes. As a result, the osteoblastic activity is suppressed and bone microfractures can occur. Low-zinc diet decreases the number of osteoblasts (1, 5, 7, 10, 18).

In our study, the $Zn^{(2+)}$ concentrations were lower (by 39.5%, *vs.* controls, $p<0.0001$), as a result of increased bone turnover. This also illustrates the negative bone balance, in favor of bone resorption (*Table 2*).

Conclusions

Decreased OPG serum levels represent the consequence of osteoblasts' apoptosis, secondary to estrogen deprivation. High serum sRANKL concentrations (a resorption bone marker) in osteoporotic patients confirmed the activation of osteoclasts. The lower OPG serum levels in the osteoporotic group, in comparison to the controls, illustrate the age-related reduction of osteoblastic multiplication capacity and also osteoblastic apoptosis. In our study group of postmenopausal osteoporosis the OPG/sRANKL ratio was lower than 1.0.

In postmenopausal osteoporosis $Zn^{(2+)}$ serum levels decreased, because of diminished mineralizing capacity of the bone tissue.

The bone turnover markers may be useful for osteoporosis screening in postmenopausal women and also for the calculation of the Fracture Risk Assessment Tool.

Abbreviations

ARORC = age-related osteoblasts replicate capacity;
AMG = Amgen's;
BMD = bone mineral density ;
BMT = bone turnover marker;
Cbfa-1/Runx-2 = corebinding factor-1/runt-related transcription factor-2;
DXA = dual energy X-ray absorptiometry;
ELISA = enzyme-linked immunosorbent assay;
ER = estrogen receptor;
FAAS = flame atomic absorption spectrometry;
FRAX = Fracture Risk Assessment Tool;
MP = menopause;
OPG = osteoprotegerin;
OCIF = osteoclastogenesis inhibitory factor;
RANK = receptor for activated nuclear-factor kappa;
SD = standard deviations;
sRANKL = soluble receptor for activated nuclear-factor kappa ligand;
TNFR = tumor necrosis factor receptor;
WHO = World Health Organization;
Zn = zinc

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