Postmenopausal Lumbar Spine Osteoporosis in Hungarian Women is Characterised By Increased Serum Levels of Nerve Growth Factor

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ABSTRACT

Purpose: In addition to its neuronal specificity, nerve growth factor (NGF) plays a role in immuno-inflammatory events. Cells involved in osteoporosis, in particular mesenchymal stem/stromal cells and monocytes/macrophages, are capable of producing NGF. The question was whether NGF might be involved in osteoporotic bone loss independent of age.

Methods: Sixty postmenopausal Hungarian women were studied for serum NGF-β and MCP-1 levels, total lumbar spine and forearm osteoporosis, and femoral neck osteoporosis. Biochemical data were measured by enzyme-linked immunosorbent assay and presented as geometric mean (95% CI). Dual-energy X-ray absorptiometry was used to measure bone mineral density (BMD) and T-score values in three bone regions. Multiple regression analysis was used to identify the independent variables involved in the elevated NGF-β levels.

Results: Serum NGF-β levels were significantly increased in the osteoporotic subgroup compared to the normal and osteopenic subgroups [15.85(8.99-27.91) vs. 12.42(8.28-18.64) pg/ml, p<0.004 and 12.49(9.36-16.65) pg/ml, p<0.002, respectively] in the total lumbar spine region. Using multiple regression analysis, serum MCP-1 levels alone and their interaction with lumbar spine BMD were significant with elevated serum NGF-β levels, age was not included in the model. Increased serum NGF-β levels were restricted to the lumbar spine, but not to forearm and femoral neck osteoporosis.

Conclusion: Increased serum NGF-β levels were associated with the severity of lumbar spine osteoporosis. Increased sympathetic and sensory neural activity may contribute to the presence of pain and neuropathy in osteoporosis.

Keywords
Osteoporosis, Lumbar spine, NGF-β levels, MCP-1 levels.

Introduction
Nerve growth factor (NGF) is a neurotrophin that plays a role in the survival of sympathetic and sensory neurons in nervous system differentiation, neuronal growth and sprouting [1]. NGF regulates axon growth and synapse formation, neurotransmitter and neuropeptide synthesis, organ innervation density and neuronal plasticity. In addition to its neuronal specificity, NGF production is involved in immuno-inflammatory events, and in skeletal bone development and health [2-4]. Local NGF secretion is strongly associated with immune cell activation and increased levels of inflammatory cytokines such as IL-1β, tumor necrosis factor-α (TNF-α) and IL-6 [5]. Prostaglandins and histamine are mediators of NGF production [6,7]. Epithelial, endothelial, connective, muscle, neuronal and glial cells are capable of synthesising NGF [8-11]. NGF acts through two receptors, the high-affinity tyrosine kinase (TrkA) receptor and the low-affinity p75 neurotrophin (p75NTR) receptor [12,13]. Their receptors are present on immune and inflammatory cells, endothelial, epithelial, connective and muscle cells, as well as adipocytes, astrocytes and keratinocytes.
Mesenchymal stem cells, osteoblastic cells, stromal cells and bone marrow cells can express both NGF receptors with the characteristic of NGF production [14,15]. NGF plays an important role in bone healing, regeneration and tissue repair through the involvement of the immune system [16,17]. Macrophages, derived from monocytes are ubiquitous cells in tissues. Cytokines, chemokines and growth factors secreted by macrophages are involved in tissue repair and regeneration processes as well as osteoclastogenesis [18,19]. Macrophages are involved in all stages of bone healing and support osteoblast differentiation and proliferation [20]. Increased NGF levels are also responsible for pain during tissue damage and repair [21,22]. The chemokine monocyte chemoattractant protein-1 (MCP-1) is not only an initiating factor in foam cell formation, but also recruits its receptor (CCR2) bearing cells into the local immuno-inflammatory events [23]. Cells expressing CCR2 receptor have a broad spectrum: antigen-presenting cells, NK and T cells, fibroblasts, monocytes, vascular smooth muscle, epithelial, endothelial, skeletal muscle, microglial and mesangial cells. MCP-1 can induce the maturation of osteoclasts from hematopoietic stem cells in the bone marrow under estrogen-deficient conditions.

Postmenopausal osteoporosis is a T-cell mediated inflammatory process that results in bone loss due to decreased estrogen levels [24]. The role of bone remodeling is to maintain bone mass throughout life through the repair mechanism. Several hormonal, environmental and nutritional factors are involved in bone remodeling. Estrogen loss is associated with chronic low-grade inflammation due to increased inflammatory cytokines (TNF-α, IL-6, IL-17, IL-15) [25]. In particular, TNF-α and IL-17 are the main cytokines that promote osteoclastogenesis with the predominance of bone resorption. The interaction between receptor-activator of NF-κB (RANK) and its ligand (RANKL) is crucial for the development of multinucleated osteoclasts [26]. RANK belongs to the TNF receptor superfamily and is mainly expressed on osteoclast precursors, microglia, immune cells such as dendritic cells and macrophages [27]. Its ligand is expressed on immune cells (T and B lymphocytes), osteoblasts, osteocytes, chondrocytes, mesenchymal cells and megakaryocytes, and RANKL has also been detected in extrskeletal tissues (lymph nodes, lung, mammary gland, heart, brain, kidney, skin) [28,29]. RANKL plays an important role in initiating bone loss by binding to its receptor on multinucleated osteoclasts.

The change in serum NGF-β levels was investigated in postmenopausal Hungarian women with regard to the degree of bone loss and the relationship with serum MCP-1 levels. The question was whether there was an association between increased serum NGF-β levels and bone mineral density (BMD) or T-scores in postmenopausal osteoporosis. Bone loss was measured as the decrease in BMD and T-score values [based on World Health Organization (WHO) age-based criteria]. Three bone regions, the total lumbar spine and forearm, and the femoral neck regions were measured using dual-energy X-ray absorptiometry (DXA), and the results were analysed with the biochemical data.

**Patients and methods**

**Patients**

Sixty postmenopausal Hungarian women (mean age 64±10 years) were studied for age, bone mineral density (BMD), T-score, serum levels of NGF-β and MCP-1. Patients were randomly selected from the immunoendocrinology outpatient clinic for a DXA scan if they were not already being treated for osteoporosis. Exclusion criteria were autoimmune, allergic, tumour, endocrine and acute diseases. The extensive exclusion criteria and personal factors of the outpatient clinic partly limited the adequate number of patients who could be included in the study.

The postmenopausal period was 15±11 years. The serum estradiol level was 62.58(52.2-75.04) pmol/l. None of the 60 women were receiving estrogen replacement therapy at the time of the study. Forty-nine women had serum estradiol levels below the cut-off value of 73.4 pmol/l and 2 women had estradiol levels between 73.4-80 pmol/l. BMD with the calculated T-score was measured by DXA at the total lumbar spine (L1-L4), femoral neck and total forearm regions using the Hologic Discovery Wi device. The total group of women was divided into three subgroups (normal, osteopenic, osteoporotic) based on T-score values according to World Health Organization (WHO) criteria: values below -2.5 represented osteoporosis, values between -1 and -2.5 represented osteopenia, and values above -1 were considered normal.

**Detection of serum NGF-β and MCP-1 levels**

Serum NGF-β and MCP-1 levels were measured by enzyme-linked immunosorbent assay (ELISA) using kits from PeproTech (USA). The detailed method was described in our previous work [30]. Briefly, 96-well plates were coated with capture anti-hNGF-β or anti-hMCP-1 antibodies at concentrations of 0.05 µg/100 µl for NGF-β and 0.025 µg/100 µl for MCP-1 at room temperature overnight. After washing the plates, 300 µl of blocking buffer containing 0.05% Tween-20 and 1% BSA (bovine serum albumin) in PBS (phosphate buffered saline) was added to the wells at room temperature for 1 h. The plates were washed three times, then 100 µl of standards and 100 µl of patient sera (diluted 1:30) were added to the wells at room temperature for 2 h. The standard concentrations were as follows: 500, 61.25, 30.63, and 3.06 pg/ml for NGF-β; and 125, 61.25, 30.63 and 3.06 pg/ml for MCP-1. The plates were washed three times and 100 µl/well of biotinylated rabbit anti-hNGF-β or anti-hMCP-1 detector antibody at a concentration of 0.05 µg/100 µl was added at room temperature for 2 h. The washed plates were labelled with 100 µl/well of avidin-HRP (horseradish peroxidase) conjugate at a dilution of 1:2000 at room temperature for 30 min. Colour development was initiated with 100 µl/well of ABTS (2,2’-azinobis-[3-ethylbenzothiazoline-6-sulphonic acid]-di-ammonium salt) liquid substrate. The optical density (OD) of the wells was measured using an ELISA reader at 405 nm with a wavelength correction set at 650 nm and monitored at 5 minute intervals for approximately 20 minutes. All samples were tested in duplicate. Serum concentrations of NGF-β and MCP-1 were calculated from the corresponding standard curve.
Statistics
Age, BMD and T-score data are presented as mean±SD. Biochemical estradiol, NGF-β and MCP-1 data were skewed and therefore their logarithms, which were approximately normally distributed, were used. These data are presented as geometric mean (GM) with error bars of GM + 1SD and GM -1SD replaced in the text by the sign of the 95% confidence interval (95%CI). Student’s t-test was used to compare data between two groups. One-way ANOVA with Bonferroni post hoc test was used to compare data among three groups with equal variances. Regression analysis was used to demonstrate a strong relationship between two data using curve estimation and 95%CI of the correlation coefficient (r). Multiple regression analysis helped us to demonstrate the relevant relationship between the dependent variable, NGF-β, and the independent or calculated variables (demonstrating the interactions). P values less than 0.05 were considered significant. Statistical analyses were performed using Medcalc 17.9.7 software, but one-way ANOVA analysis and calculated variables were performed using SPSS 15.0.0 software.

Ethical approval
All enrolled patients gave informed consent, and all procedures were performed in accordance with the 1964 Declaration of Helsinki and the Research Committee of the Medical University of Debrecen.

Results
Serum NGF-β and MCP-1 levels in relation to total lumbar spine BMD and T-score values
The total group of women was divided into three subgroups according to T-score values: above -1 represented the normal subgroup, between -1 and -2.5 the osteopenic subgroup, and below -2.5 the osteoporotic subgroup. Serum NGF-β levels were significantly elevated in the osteoporotic subgroup compared to the normal and osteopenic subgroups [15.84(8.99-27.91) pg/ml vs. 12.42(8.28-18.64) pg/ml, p<0.004 and 12.49 (9.36-16.65) pg/ml, p<0.002, respectively] (Figure 1). The differences among the subgroups were not relevant for serum MCP-1 levels: [16.37(13.36-20.06) pg/ml for the normal; 16.17(13.73-19.05) pg/ml for the osteopenic and 17.19(12.75-23.18) pg/ml for the osteoporotic subgroup]. The distribution of age in the subgroups was similar to the distribution of serum NGF-β levels. Older women were included in the osteoporotic subgroup than in the normal and osteopenic subgroups [71±8.77 years vs. 62.94±9.17 years, p<0.025 and 60.04±7.99 years, p<0.001, respectively].

Our results showed that increased serum NGF-β levels can be detected in osteoporosis. The role of age in relation to increased serum NGF-β levels needs further investigation.

Linear regression analysis was used to demonstrate the relationship between serum NGF-β levels and total lumbar spine BMD or T-score values or serum MCP-1 levels
The relationship between serum NGF-β levels as the dependent variable and the independent variables: total lumbar spine T-score or BMD values, age and serum MCP-1 levels was investigated. The results showed inverse and moderate relationships with total lumbar spine T-score and BMD values with the following regression equations: log(NGF-β)=1.0704-(0.0284*Lumbar spine-T-score), r = - 0.3585, p<0.0049 and log(NGF-β)=1.3365-(0.2507*Lumbar spine-BMD), r = - 0.3415, p=0.0087 (Figure 2). The positive relationship between serum NGF-β and MCP-1 levels was stronger than the relationship between serum NGF-β levels and the Years of Age variable [log(NGF-β)=(1.2344*log(MCP-1))-0.3794, r = 0.5958, p<0.0001 and log(NGF-β)=0.8514 + (0.0045*Years of Age), r = 0.4030, p=0.0014]. Surprisingly, the relationship between total lumbar spine T-score or BMD values and the Years of Age variable was also moderate and inverse [Lumbar spine-Tscore=2.0181-(0.0609*Years of Age), r = - 0.4557, p<0.0003 and Lumbar spine-BMD= 1.2628-(0.0066*Years of Age), r = - 0.4523, p<0.0004].

The regression analysis between two variables did not seem to be sufficient to decide the contribution of age to the involvement of increased serum NGF-β levels in osteoporosis.

Multiple regression analysis was used to demonstrate the variables that may be involved in the increased serum NGF-β levels
Stepwise multiple regression analysis confirmed the relationship between serum NGF-β levels and the total lumbar spine BMD variable (p<0.0072) or serum MCP-1 levels (p<0.0001) [regression equation: log(NGF-β) = (log(MCP-1)*1.1327) – (Lumbar spine-BMD *0.2119) - 0.0758, r = 0.6268, p<0.0001] (Figure 3). These independent variables showed a significance

Figure 1: Distribution of serum NGF-β, MCP-1 levels and age in normal, osteopenic and osteoporotic subgroups based on total lumbar spine T-scores.
Figure 2: Linear regression analysis was used to show the relationship between serum NGF-β levels and serum MCP-1 levels, total lumbar spine BMD or T-score values and age, and between age and total lumbar spine BMD or T-score values.

<table>
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<tr>
<th>Independent variables</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>r partial</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
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<td></td>
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<td>0.0101</td>
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<td>log(MCP-1)</td>
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<td>0.5817</td>
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<tr>
<td>Lumbar spine-BMD</td>
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<td>0.0758</td>
<td>-0.3525</td>
<td>-2.794</td>
<td>0.0072</td>
</tr>
</tbody>
</table>

Variables not included in the model:
Years of Age

Figure 3: Multiple regression analysis was used to show the independent variables that may be involved in increased serum NGF-β levels without (a) and with interactions (b) where two variables were calculated for one variable.

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>r partial</th>
<th>t</th>
<th>p</th>
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<tbody>
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<tr>
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<td>0.6244</td>
<td>5.928</td>
<td>&lt;0.0001</td>
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<tr>
<td>log(MCP-1) * Lumbar spine-BMD</td>
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<td>0.06620</td>
<td>-0.3522</td>
<td>-2.791</td>
<td>0.0072</td>
</tr>
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Variables not included in the model:
Years of Age
Lumbar spine-BMD
Years of Age * log(MCP-1)
Years of Age * Lumbar spine-BMD
with increased serum NGF-β levels. The Years of Age variable was not included in the model. Similar results were found between serum NGF-β levels and total lumbar spine T-score \((p<0.0042)\) or serum MCP-1 levels \((p<0.0001)\) [regression equation: \(\log(\text{NGF-β})=(\log(\text{MCP-1})*1.141)-(\text{Lumbar spine-T-score}*0.0237) - 0.3098, r = 0.6378, p<0.0001\)]. These independent variables were significant with increased serum NGF-β levels. The Years of Age variable was also not included in this model.

Using interactions in the model, serum MCP-1 levels alone \((p<0.0001)\) and the interaction between serum MCP-1 levels and Lumbar spine-BMD variable \((p<0.0072)\) explained 39.27% of the elevated serum NGF-β levels when the Years of Age variable was excluded [regression equation: \(\log(\text{NGF-β})=(\log(\text{MCP-1})*1.2758)-(\log(\text{MCP-1})*\text{Lumbar spine-BMD})* 0.1747)-0.249, r = 0.6267, p<0.0001\)]. Surprisingly, serum MCP-1 levels alone \((p<0.0001)\) and the interaction between the Years of Age and the Lumbar spine-T-score variables \((p<0.0013)\) also showed a significance with the elevated serum NGF-β levels [regression equation: \(\log(\text{NGF-β})=(\log(\text{MCP-1})*1.1242)-(\text{Years of Age})*\text{Lumbar spine-T-score})*0.0004)-0.2889, r = 0.6555, p<0.0001\)].

The results using multiple regression analysis excluded the role of the Years of Age variable in the relationship between serum NGF-β levels and total lumbar spine BMD. The role of the Years of Age variable was included in the relationship between serum NGF-β levels and total lumbar spine T-scores.

**Serum NGF-β levels in osteoporotic and non-osteoporotic women based on DXA scan in the total lumbar spine, forearm and femoral neck regions**

The total number of women was divided into two subgroups, osteoporotic and non-osteoporotic according to T-value scores measured in the total lumbar spine, forearm and femoral neck regions. A relevant increase in serum NGF-β levels was found only in the osteoporotic subgroup compared to the non-osteoporotic subgroup, measured in the total lumbar spine region [15.85(8.99-27.91) vs. 12.46(8.9-17.44) pg/ml, \(p<0.0002\)], but failed in the total forearm [13.52(8.44-21.66) vs. 13.34(8.33-21.36) pg/ml] and femoral neck regions [13.85(8.51-22.55) vs. 13.23(8.33-21.03) pg/ml] (Figure 4).

When serum NGF-β levels were examined, elevated levels were only associated with lumbar spine osteoporosis and not with forearm and femoral neck osteoporosis.

**Discussion**

The ovariectomised rat model has been widely used as a postmenopausal osteoporosis model to study the clinical consequences together with the serum biomarkers involved in bone loss [31]. The role of NGF-β and MCP-1 biomarkers showed strong correlations with BMDs in ovariectomised rats using an early postmenopausal osteoporosis model [32]. In our previous work, serum levels of NGF-β and MCP-1 were significantly lower in postmenopausal Hungarian women compared to obese women [30]. Immunohistochemical localisation of NGF-β showed its presence in osteoprogenitor cells, bone marrow stromal cells, osteoblasts, chondrocytes, endothelial cells, skeletal muscle and periosteal matrix of fracture callus [33]. The multipotential human mesenchymal stem cells and their ability to differentiate into osteoblasts, chondrocytes, adipocytes and myoblasts make them a critical factor in tissue and bone repair and regeneration processes [34]. All of these cells, as well as inflammatory and immune cells are capable of producing NGF-β [11,35]. Macrophages can control bone homeostasis among osteoclasts, mesenchymal stem/stromal cells, osteoblasts, osteocytes and angiogenesis [36]. Therefore, macrophages also play an important role in immunity, tissue repair and bone homeostasis. MCP-1 is a chemokine that controls monocyte/macrophage migration and infiltration during inflammation. Monocyte-derived IL-1 and TNF-α are modulators of bone remodeling. In mouse osteoblasts, expressed MCP-1 was associated with both bone resorption and bone formation, but the amount of MCP-1 was directly related to bone resorption [20].

Our results showed significantly increased serum NGF-β levels in Hungarian osteoporotic women compared to normal and osteopenic women. A moderate association was found between serum NGF-β levels and lumbar spine BMD or lumbar spine T-score values. The relationship between NGF-β and MCP-1 levels or the Years of Age variable was stronger, highlighting their role in the increased NGF-β levels in osteoporosis. Using multiple regression analysis, serum MCP-1 levels alone and their interaction with total lumbar spine BMD values without the Years of Age variable were significant with the elevated serum NGF-β levels. T-score values with the interaction of the Years of Age variable or serum MCP-1 levels alone also showed significance with the elevated serum NGF-β levels, supporting that the T-score value is an age-related index. No relevant increase in serum MCP-1 levels was seen in osteoporosis. The association between serum MCP-1 and NGF-β
levels was significant. The ability of immune and inflammatory cells to produce NGF may lead to increased serum NGF-β levels. Sympathetic and sensory nerve activity in bone may be related to bone loss by increasing bone resorption and decreasing bone formation [37,38]. The exclusive association of elevated serum NGF-β levels with the lumbar spine region highlights the role of the bone marrow in osteoporosis due to its high vascular and cellular density [39,40]. Data support that NGF is essential for the chemosensitivity of sensory neurons involved in thermal and mechanical hyperalgesia in certain neuropathic conditions [41]. NGF is present in the bone marrow environment and can influence hemopoietic stem cell development through several different pathways [42,43]. Anti-NGF therapy is available [44]. Its use attenuated cutaneous hypersensitivity and musculoskeletal discomfort, with an insignificant small increase in lumbar spine BMD in mice with osteoporosis [45].

The limitation of the study may be the small number of patients with osteoporosis. This may be due to the extensive exclusion criteria and the limited number of patients attending the outpatient clinic. For the subgrouping of women, the age-related T-scores have to be used, but the BMD showed a relevant association with the increased serum NGF-β levels in lumbar spine osteoporosis.

**Conclusion**

A significant increase in serum NGF-β levels was demonstrated in osteoporotic Hungarian women at the total lumbar spine, supporting the importance of NGF-β in bone loss and associated pain and neuropathy. Its relationship with osteoclastogenesis via serum MCP-1 and the decreased lumbar spine BMD values makes elevated serum NGF-β levels a marker for the severity of lumbar spine osteoporosis. The lumbar predominance may also suggest that accelerated NGF production may be involved in repairing trabecular microfractures.

**References**


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