**ALOX12** gene polymorphisms and serum selenium status in elderly osteoporotic patients

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**Conflict of interest**
None declared

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**Abstract**

**Background.** Osteoporosis is a systemic bone disease which leads to a reduction in bone mass. Many studies have shown that up to 80% of bone mineral density (BMD) variations are attributed to genetic factors. Arachidonate 12-lipoxygenase enzyme, encoded by the **ALOX12** gene, produces lipid peroxides as reactive oxygen species (ROS), leading to oxidative stress and the development of osteoporosis. Selenium (Se) is incorporated into selenoproteins, which may reduce the risk of osteoporosis.

**Objectives.** We aimed to investigate the association of **ALOX12** single nucleotide polymorphisms (SNPs) and serum Se level with lumbar spine and femoral neck BMD among elderly individuals living in Amirkola, Iran.

**Material and methods.** The study consisted of 180 individuals aged ≥60 years (90 healthy and 90 osteoporotic patients). We examined the effect of 2 **ALOX12** SNPs (rs2292350 and rs9897850), using the polymerase chain reaction – restriction fragment length polymorphism (PCR–RFLP) on both BMD regions measured by dual energy X-ray absorptiometry (DXA). Serum Se level was measured using an atomic absorption spectrophotometer PGG990 AAS (PG Instruments Ltd., Luterworth, USA).

**Results.** The rs2292350 SNP showed a significant association with femoral neck BMD (p = 0.04). Moreover, in terms of serum Se level, a significant difference was found between the patient group (57.58 ± 25.54 µg/L) and the control group (81.09 ± 25.54 µg/L) (p < 0.001). In addition, individuals with higher serum Se levels also had higher BMD of the lumbar spine (r² = 0.392; p < 0.001) and the femoral neck (r² = 0.478; p < 0.001).

**Conclusions.** The results suggested that genetic variation in **ALOX12** might influence BMD variations in our recruited participants. As for the patients with lower serum Se levels, it was observed that serum Se deficiency was accompanied by some **ALOX12** variation, contributing to the development of osteoporosis.

**Key words:​** osteoporosis, single nucleotide polymorphism, bone mineral density, **ALOX12**, selenium
Introduction

Osteoporosis is a systemic bone disease mostly occurring in elderly individuals. In this disease, disturbance in bone remodeling (bone resorption and formation) leads to a bone mass reduction, bone fragility, and eventually, to fracture. Osteoporotic fracture may cause disability, decreased quality of life, and ultimately, mortality – it affects all aspects of the patient’s life.1 It has been estimated that over 200 million people suffer from osteoporosis worldwide.2 There have also been studies reporting the rate of this disease in a local region; for example, in 2009, an Iranian multi-center study indicated that 70% of women and 50% of men aged ≥50 years suffered from osteoporosis or osteopenia.3

Peak bone mineral density (BMD) as a major determinant of bone strength achieved in early adulthood plays an important role in the prediction of osteoporotic fracture in later life.1 In addition to many confirmed factors, such as race, sex, age, nutrition, hormonal status, menopausal state, smoking, alcohol intake, and physical activity, there are many studies that support the remarkable influence of genetic factors on bone strength. Studies show that up to 80% of BMD variation is attributable to genetic factors.4-7

A link between hip, spine and wrist BMD and the arachidonate 12-lipoxygenase (ALOX12) gene has been reported, and some researchers have suggested that ALOX12 is a susceptible gene for BMD variation. ALOX12 belongs to the arachidonate lipoxygenase enzyme super-family, which catalyzes the insertion of molecular oxygen into polyunsaturated fatty acids, such as arachidonic acid.8,9

The product of ALOX12 activity, i.e., 12-hydroperoxyeicosatetraenoic acid (12-HPETE), serves as an endogenous ligand for the peroxisome proliferator-activated receptors (PPARs), which inhibit osteoblastogenesis and increase adipogenesis from a common progenitor – the mesenchymal stem cells (MSCs) of bone marrow.10-12 Therefore, ALOX12 activation could result in the upregulation of the pathway of PPARs, subsequently decreasing osteoblastogenesis and BMD.13,14 Accordingly, several single nucleotide polymorphisms (SNPs) in ALOX12 have been suggested as being associated with BMD variations in humans, but the results are controversial.14,15

Selenium (Se) is an essential trace element that incorporates into selenoproteins as selenocysteine – the 21st amino acid. Various members of the glutathione peroxidase (GPx) family (including phospholipid hydroperoxide glutathione peroxidase – PHGPx) are well-known selenoproteins with antioxidant capacity that play an important role in the scavenging of lipid peroxide products.16,17 Therefore, 12-HPETE serves as a reactive oxygen species (ROS), quickly converting to 12-hydroxyeicosatetraenoic acid (12-HETE) by peroxidase 2, 4. Therefore, a decrease of Se can interfere with the turnover of lipid peroxidation, resulting in ROS accumulation that leads to cellular and extracellular damage in bone turnover, such as inhibition of osteoblastic differentiation, which is a major contributor to the development of osteoporosis.18,19

Although there is no clear mechanism indicating a relationship between the Se status and osteoporosis development, reports show that antioxidant supplementation reduces the risk of osteoporosis via an improvement in antioxidant capacity.16,18,20,21 Furthermore, Se intake would reduce the risk of osteoporotic hip fracture in a population-based case-control study and Se deficiency also resulted in a reduction in femur and tibia BMD in rats. Therefore, Se deficiency can be considered a putative risk factor of osteoporosis.22,23

To the best of our knowledge, there have been no reports on the status of ALOX12 polymorphisms and Se in osteoporotic individuals in our area. Therefore, we aimed to investigate the association of 2 ALOX12 polymorphisms (rs2292350 and rs9897850) and serum Se with lumbar spine and femoral neck BMD in this population.

Material and methods

Participants

From among 1,616 elderly participants in the Amirkola Health and Aging Project (AHAP), we randomly selected 90 out of 558 osteoporotic individuals (45 males and 45 females) as the study group and 90 out of 326 age- and gender-matched individuals (45 males and 45 females) as control subjects.24 The remaining participants had osteopenia. Informed consent was obtained from each participant and the study was approved by the Zanjan University of Medical Sciences (Iran) Ethics Committee. The osteoporosis status was determined by BMD measurement. All selected participants were ≥60 years old and they had never taken any medication related to BMD or bone turnover, or Se supplementary drugs, and they were all non-smokers. None of the participants had renal or metabolic bone disease.

Bone mineral density measurement

Bone mineral density [g/cm²] of the lumbar spine (L1–L4) and proximal femur were measured by a dual energy X-ray absorptiometry (DXA) densitometer, using a Lexxos densitometer (DMS, Montpellier, France). According to the World Health Organization (WHO) criteria, the participants were categorized into 2 separate groups, osteoporotic and normal. Subjects with a BMD of 2.5 standard deviations (SD) or below the average value for young healthy adults (i.e., a T-score of <–2.5 SD) were considered osteoporotic patients, and subjects with a T-score of >–1.0 SD were considered normal subjects.25

Serum selenium measurement

Serum Se levels were measured by an atomic absorption spectrophotometer PG990 AAS (PG Instruments Ltd., Lutterworth, USA) equipped with a graphite furnace. Each serum sample was first diluted with deionized water (1:1); then, 10 µL of each diluted sample was injected into the graphite
furnace. The working standard solution was prepared from stock standards of Se, according to Standard Reference Material (SRM) from National Institutes of Standards and Technology (NIST); 1000 mg/L for AAS (selenium dioxide in nitric acid 0.5 mol/L (Merck KGaA, Darmstadt, Germany)). The operating parameters for measuring serum Se levels were set as recommended by the manufacturer (wavelength: 196 nm; bandwidth: 0.4 nm; and lamp current: 5 mA).

Genotyping

Polymorphism selection
Using extensive literature searches, among all known polymorphisms of ALOX12, 2 SNPs (rs9897850 and rs2292350) were selected on the basis of other researchers' findings.1,4,5,7 Both SNPs were listed in the National Center for Biotechnology Information (NCBI) SNP database and happened to be very common (minor allele frequencies >0.35).

Polymerase chain reaction
Genomic DNA was extracted from whole blood using a QIAamp DNA Blood Mini Kit (QIAGEN Korea Ltd., Seoul, Korea) and stored at −20°C. The polymerase chain reaction (PCR) was performed using 2 pairs of forward and reverse primers (CinnaGen, Tehran, Iran) as follows: forward – 5’AGTGTTCTCACTATGTTCGC3’, reverse – 5’CCCCAGACTAGCCAAACC3’ for rs9897850 targeting the promoter region, and forward – 5’AGTAGGTGTAGGTGTATAGGTGAC3’, reverse – 5’TGTTTAGCCTATTCC3’ for rs2292350 targeting the intron 2 region of the ALOX12 gene.

The PCR was carried out using a PCR Master Mix 2X (CinnaGen) according to the manufacturer’s protocol, adjusted to a total reaction mixture of 25 μL, containing 50 ng of total DNA template. Amplification was performed using a DNA thermal cycler (Analytik Jena AG, Jena, Germany) for 35 cycles (93°C, 60 s; 57°C, 60 s; 72°C, 60 s) with an initial heating at 95°C for 5 min and a final extension for 5 min at 72°C. The PCR products were analyzed by 1% agarose gel electrophoresis and visualized with a gel documentation and analysis system (Gel Doc™EZ System; Bio-Rad Laboratories, Hercules, USA) after staining by DNA safe stain (CinnaGen). The PCR product size was 215 bp for rs9897850 and 300 bp for rs2292350.

Restriction Fragment Length Polymorphism (RFLP)
Each PCR product in a dose of 10 μL was digested using 5 U of HinfI (BIORON GmbH, Ludwigshafen, Germany) and PscI (PciI) (Thermo Scientific, Waltham, USA) restriction enzyme per 20 μL of reaction mixture at 37°C for 3 h. The digestion products were analyzed by 1.5% agarose gel electrophoresis after staining by DNA safe stain, and were then visualized using UVdoc.

Statistical analysis
Statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, USA). The genotype frequencies of both SNPs were estimated by allele counting for all participants, and the Hardy-Weinberg equilibrium (HWE) was assessed using the χ² test.26 The linkage disequilibrium (LD) and haplotyping were analyzed using CubeX online software (www.oegge.org/software/cubex) (Gaunt et al.; licensee BioMed Central Ltd., London, UK).27 Values of p ≤ 0.05 were considered statistically significant.

Differences in the frequency of genotypes between the osteoporotic patients and the gender-matched normal controls were tested using χ² tests. The correlation of the genotypes and lumbar spine or femoral neck BMD adjusted for age and gender was analyzed using a one-way analysis of variance (ANOVA) followed by Tukey’s test.

In the next step, differences in serum Se level between the patients and the controls were tested using an independent sample t-test. In addition, the correlation between Se level and lumbar spine or femoral neck BMD was evaluated using Pearson’s bivariate correlation.

Results

Clinical characteristics
A total of 90 individuals with osteoporosis (45 males and 45 females) were recruited as the study group and 90 aged-

<table>
<thead>
<tr>
<th>Participants</th>
<th>Normal controls</th>
<th>Osteoporotic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>male</td>
<td>female</td>
</tr>
<tr>
<td>Number of patients</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>Age [years]</td>
<td>68.11 ±4.56*</td>
<td>64.93 ±4.69*</td>
</tr>
<tr>
<td>Height [cm]</td>
<td>164.93 ±7.8*</td>
<td>154.04 ±5.82*</td>
</tr>
<tr>
<td>Weight [kg]</td>
<td>77.58 ±10.88*</td>
<td>76.30 ±9.89*</td>
</tr>
<tr>
<td>BMI [kg/m²]</td>
<td>28.50 ±6.2*</td>
<td>32.18 ±3.96*</td>
</tr>
<tr>
<td>Lumbar spine BMD [g/cm²]</td>
<td>1.12 ±0.12**</td>
<td>1.04 ±0.09*</td>
</tr>
<tr>
<td>Femoral neck BMD [g/cm²]</td>
<td>1.05 ±0.11**</td>
<td>0.98 ±0.07*</td>
</tr>
<tr>
<td>Serum Se [µg/L]</td>
<td>87.15 ±26.08**</td>
<td>73.68 ±23.19*</td>
</tr>
</tbody>
</table>

Data is presented as mean ± standard deviation (SD); BMI – body mass index; BMD – bone mineral density; * statistical significance between normal controls and osteoporotic patients; ** statistical significance between males and females of each group.
matched normal participants (45 males and 45 females) were selected to be the control group. Table 1 represents the demographic and morphometric characteristics and the BMD values of all participants.

**Allelic frequencies and haplotype structure**

Two SNPs (rs9897850 and rs2292350) in the ALOX12 gene were genotyped using the polymerase chain reaction – restriction fragment length polymorphism (PCR–RFLP). Neither SNP was found to be in HWE due to high p-values of the χ² test. The genotype characteristics of the participants are outlined in Table 2. The 2 SNPs analyzed in ALOX12 – rs9897850 and rs2292350 – are located in the promoter region and in the intron 2 region of chromosome 17p13, respectively. The LD was calculated for the ALOX12 gene polymorphisms (rs9897850 and rs2292350) for the whole population (D’ = 0.305; r² = 0.0628). There was no significant correlation between any of the 4 possible haplotypes and the risk of disease.

**Association between single nucleotide polymorphisms and bone mineral density variations**

The effect of each SNP genotype on the lumbar spine and femoral neck BMD parameters was examined using ANOVA. For rs9897850, there was no significant difference in either lumbar spine or femoral neck BMD, but a marginal trend was observed involving rs2292350.

The rs2292350 SNP showed the most significant association for femoral neck BMD (p = 0.04), as individuals homozygous for the G allele at rs2292350 had 0.17 ±0.7 g/cm² higher mean femoral neck BMD than those homozygous for the A allele (p = 0.037) (Table 3).

**Serum selenium concentration**

A significant difference was found between the osteoporotic patients (57.58 ±25.54 µg/L) and the controls (81.09 ±25.58 µg/L) in terms of serum Se levels (p < 0.001). However, the mean Se concentration in male subjects happened to be significantly higher compared to females (p = 0.002). Individuals with higher Se levels also had higher BMD of the lumbar spine (r² = 0.392; p < 0.001) and the femoral neck (r² = 0.478; p < 0.001), but no association was found for genotype frequency of either SNP.

**Discussion**

Previous studies have shown a linkage between hip, spine and wrist BMD and the 17p13 chromosomal region where the ALOX12 gene has been mapped to.8,9 12-hydroperoxyeicosatetraenoic acid, i.e., the product of ALOX12 activity, serves as an endogenous ligand for PPARs, which inhibits osteoblastogenesis.11,12 Therefore, ALOX12 has been considered a candidate gene for the development of osteoporosis and several SNPs in the human ALOX12 gene have been suggested as being associated with BMD in humans.1,4,5,7,15

In the present study, 2 polymorphisms (rs9897850 and rs2292350) of ALOX12 were investigated for a probable association with BMD in the elderly population of Amirkola, Iran. None of the mentioned polymorphisms were found to be in HWE. Deviations from HWE may have happened as a result of a new mutation, inbreeding, selective mating, or a genotyping error.28,29

The rs2292350 polymorphism was significantly associated with reduced femoral neck BMD in both genders.

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**Table 2. The genotype properties of ALOX12 gene SNPs**

| Variable               | Common Hz n (% within the group) | Het n (% within the group) | Rare Hz n (% within the group) | Overall p-value for χ² test | OR rare Hz vs common Hz (CI), p-value |
|------------------------|----------------------------------|---------------------------|--------------------------------|----------------------------|_____________________________________|
| rs9897850              |                                  |                           |                                |                           |                                       |
| normal controls        | C/C                              | C/T                       | T/T                            | 0.784                     | 0.85 (0.33–2.15), 0.728               |
| osteoporotic patients  | C/C                              | C/T                       | T/T                            | 0.784                     | 0.85 (0.33–2.15), 0.728               |
|.rs2292350              |                                  |                           |                                |                           |                                       |
| normal controls        | G/G                              | G/A                       | A/A                            | 0.054                     | 9.15 (1.06–79.11), 0.044              |
| osteoporotic patients  | G/G                              | G/A                       | A/A                            | 0.054                     | 9.15 (1.06–79.11), 0.044              |

SNPs – single nucleotide polymorphisms; Hz – homozygote; Het – heterozygote; N – number of patients; OR – odds ratio; CI – confidence interval.

**Table 3. The association of ALOX12 genotypes with BMD**

<table>
<thead>
<tr>
<th>BMD [g/cm²]</th>
<th>Genotype</th>
<th>rs9897850</th>
<th>p-value</th>
<th>rs2292350</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
<td>GG</td>
<td>GA</td>
</tr>
<tr>
<td>Lumbar spine</td>
<td>0.88 ±0.23</td>
<td>0.88 ±0.23</td>
<td>0.91 ±0.23</td>
<td>0.84</td>
<td>0.88 ±0.23</td>
</tr>
<tr>
<td>Femoral neck</td>
<td>0.87 ±0.19</td>
<td>0.85 ±0.18</td>
<td>0.9 ±0.22</td>
<td>0.44</td>
<td>0.86 ±0.19</td>
</tr>
</tbody>
</table>

Data is presented as mean ± standard deviation (SD); BMD – bone mineral density.
Our results showed that individuals homozygous for the A allele of this polymorphism had the lowest BMD values. Similarly to our findings, it was found by Mullin et al. that rs2292350 was significantly associated with spine and various hip BMD parameters in postmenopausal women; however, they report that homozygotes for the A allele of rs2292350 had significantly higher spine BMD compared with the heterozygous group, as opposed to our results.7

Harsløf et al. reported that heterozygous individuals for both polymorphisms (rs9897850 and rs2292350) had lower lumbar spine BMD and an increased risk of vertebral fractures compared with homozygous individuals for either allele.4 In addition, Xiao reported the rs2292350 polymorphism to be significantly associated with BMD in the lumbar spine, the femoral neck and the total hip in Chinese families.5

In another study, Ichikawa et al. investigated the relationship between 12 SNPs in ALOX12 and BMD variations in the hip and the spine in a healthy American population. They observed that up to 3% of the spine BMD variation in men and 0.8% of that in women is due to genetic variations in ALOX12. Both rs9897850 and rs2292350 were associated with lumbar spine BMD in both genders, but the most significant association was found with rs9897850 in men.1

Neither SNP in our study was in a haplotype block due to a weak LD with each other. Therefore, we did not observe any association of the abovementioned SNPs with lumbar spine or femoral neck BMD in the haplotype analysis, but Ichikawa et al. reported that a common haplotype containing both rs9897850 and rs2292350 was associated with high lumbar spine BMD in women and low lumbar spine BMD in men.1

The inhibition of osteoblastic differentiation of bone MSCs due to oxidative stress is a major contributor to the development of osteoporosis.19,30–33 Basu et al. found that the levels of 8-iso-PGF2a (a major F2-isoprostane) as a biomarker of oxidative stress negatively correlated with BMD. They concluded that an increase in oxidative stress was related to a reduction in BMD values.34

Experimental evidence suggests that Se might decrease the risk of osteoporosis via incorporating into selenoproteins, such as GPx, and can act as an antioxidant against oxidative damage.23,35 Chen et al. in their study on human epidermoid carcinoma cells observed that GPx and PHGPx activity decreased with a lowering of the glutathione (GSH) content.36 In another study, this team observed that an overexpression of the Se-dependent PHGPx enzyme could reduce ALOX12 activity, and in this manner it was possible to decrease the risk of developing osteoporosis.37,38 Besides, Liu et al. showed that selenite (Na2SeO3), as a selenium supplement, could increase the activity and gene expression of GPx. This Se supplementation is able to reverse the reduced antioxidant capacity and GSH, in addition to its ability to suppress the ROS production in H2O2-treated MSCs.18

Odabasi et al. measured the Se concentration in plasma and red blood cells in postmenopausal women with osteoporosis in comparison with BMI-matched healthy postmenopausal women. They did not observe any significant difference between the 2 groups.39 In another study, Arikan et al. investigated serum Se levels in postmenopausal women with osteoporosis or osteopenia and healthy controls, and did not find any correlation between Se and lumbar spine BMD.40 In contrast, in this study we observed higher serum Se levels in the controls than in the osteoporotic patients (p < 0.001), and individuals that had higher Se levels had higher BMD in the femoral neck and the lumbar spine.

Conclusions

The effect of 2 SNPs in ALOX12 on the BMD of both the lumbar spine and the femoral neck was investigated in the present study. Our findings suggest the significance of ALOX12 in both BMD variations and in the development of osteoporosis. In addition, the antioxidant effect of PHGPx, which is due to Se as an essential trace element acting as a cofactor, may be able to reduce ALOX12 activity. The results of this study can open the door to a better understanding of the mechanism of Se action in osteoporosis. Surely, further investigation into this area would be needed in order to improve our knowledge of osteoporosis development.

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