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Original Article

Effects of the CASR rs104893706 (A843E) gain-of-function mutation on bone mineral density in postmenopausal women by advanced age and smoking

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ABSTRACT

Background and Aims: The G protein-coupled calcium-sensing receptor (CaSR) plays an important role in extracellular calcium homeostasis and regulation of parathyroid hormone (PTH) secretion. There are more than 300 activating/inactivating mutations in the *CASR* gene. However, the effects of both CaSR protein and *CASR* gene on bone mineral density (BMD) have not been investigated enough. The aim of this study was therefore to determine the effect of rs104893706 (A843E, (Ala to Glu at codon843)), a gain-of-function mutation of the *CASR* gene, on BMD in postmenopausal women.
Methods: We studied the CASR A843E variation using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism methods in 180 postmenopausal women. Statistical analyses were performed using SPSS software package (version 21.0 SPSS Inc.,Chicago,IL,U.S.A.).
Results: No minor A allele, homozygous (AA) or heterozygous (CA), was observed in the study population. In other words, the frequency of the *CASR* A843E common CC genotype was 100%. BMD levels of the lumbar spine (L1-L4), femoral neck, and total hip were 1.03±0.13g/cm^2, 0.87±0.11g/cm^2, and 0.93±0.11g/cm^2, respectively. Although the femoral neck (p=0.017), upper neck (p=0.040), lower neck (p=0.011), and Ward's triangle BMD values (p=0.005) were found significantly higher in younger postmenopausal women (age<55 years) compared to older postmenopausal women (age>=55 years), there were no significant differences on BMD value of the lumbar spines, trochanter, and total hip between the age groups (p>0.05).
Conclusion: Our findings confirm the effects of advanced age in favor of decreased BMD in postmenopausal women. This study suggests that the *CASR* A843E gain-of-function mutation may not be associated with bone mineral density and osteoporosis risk since we did not detect the A843E variation in Turkish postmenopausal women.

Keywords: Osteoporosis, Calcium-sensing receptor, Gain-of-function mutation, Bone mineral density, Smoking

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INTRODUCTION

Osteoporosis is characterized by decreased bone mineral density (BMD) and a high risk of fractures. It is especially prevalent among postmenopausal women due to estrogen deficiency (North American Menopause Society, 2010). Human bones are composed of bone minerals and bone matrix, the main components of which are calcium and collagen, respectively. Collagen degradation and a decrease in levels of calcium are key contributors to osteoporosis development (Li, Zhao, Tang, & Qu, 2010; NIH Consensus Development Panel on Osteoporosis, 2001). Additionally, advanced age, genetic factors, and smoking are associated with osteoporosis development (Bjarnason & Christiansen, 2000; Rapuri, Gallagher, Balhorn, & Ryschon, 2000).

It is crucial to maintain calcium balance for the structural integrity of the bone, however, the plasma calcium level is highly affected by environmental and physiological conditions (North American Menopause Society, 2010). The total body calcium balance is regulated by the absorption of calcium from the intestine and its excretion in the urine. When calcium levels increase in blood, the calcium-sensing receptor (CaSR) inhibits the secretion of parathyroid hormone (PTH) and reabsorption of calcium in renal tubules (Brown, 1999; Brown & MacLeod, 2001). Calcium level is decreased by the inhibition of PTH secretion, increasing urinary calcium excretion, and increasing calcitonin secretion due to the effect of extracellular calcium on CaSR. (Brown & Hansen, 2005; Hannan et al., 2018). CaSR, a seven-transmembrane-spanning extracellular G-protein-coupled receptor, is primarily expressed in the parathyroids and the kidney. It can also monitor the extracellular calcium in the body and regulate calcium metabolism (Brown, Gamba, & Riccardi, 1993; Garrett et al., 1993). There are three domains in CaSR; i) a large extracellular domain that interacts with Ca^{+2}, ii) a transmembrane domain with seven helixes among the membrane, and iii) an intracellular domain with carboxyl-terminal that activates some signal pathways of the cell (Garrett et al., 1993; Aida, Koishi, Tawata, & Onaya, 1995). After binding Ca^{2+} to CaSR, phospholipase C is activated by Gq/11. Second messengers such as diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) are produced. IP3 releases Ca^{2+} from intracellular stores and DAG activates protein kinase C (PKC) and the mitogen-activated protein kinase (MAPK) pathway. Also, the CaSR stimulates the Gi/o protein and then cAMP production is inhibited. These signaling pathways give rise to a reduction in PTH secretion and a decrease in renal tubular Ca^{2+} reabsorption (Hannan et al., 2018).

More than 300 *CASR* gene mutations have been identified related to the activation or inactivation of the CaSR receptor (Hend, Guarnieri, & Canaff, 2009). There are more than 95 different *CASR* mutations called activating mutations (gain-of-function). These mutations generally exist in the extracellular domain .However, they can also occur in the transmembrane domain. Some activated *CASR* mutations have been associated with autosomal dominant hypocalcemia (ADH) and Bartter Syndrome Type V (Thakker, 2004; Regala, Cavaco, Domingues, Limbert, & Lopes, 2015). A novel missense heterozygous *CASR* gene mutation, a leucine substitution for serine at codon 123 (p.Leu123Ser), was identified in a male with ADH (Regala et al., 2015). rs104893706, a constitutive A843E mutation of the *CASR* gene in exon7 is a substitution that involves a cytosine to adenine at nucleotide 2528, resulting in a substitution of Alanine (A, with C allele) to Glutamic acid (E, with A allele) in codon 843 (Zhao, Hauache, Goldsmith, Collins, & Spiegel, 1999; Sato et al., 2002). This mutation results in conformational changes in transmembrane domain 7 and gives a novel function to the receptor. A843E mutation in the *CASR* gene has been associated with high basal activity in absence of calcium and the ligand. The A843E mutation most likely stabilizes the active state of the receptor (Zhao et al., 1999).

There are numerous investigations focusing on the association of *CASR* gene variations with hyperparathyroidism (Yamauchi, 2001; Jeong et al., 2016), chronic kidney disease (Guha et al., 2015), hypertension (Jung et al., 2009), and breast cancer (Li et al., 2014). Many studies have focused on A986S loss-of-function mutation in exon 7 of the *CASR* gene (Lorentzon, Lorentzon, Lerner, & Nordstrom, 2001; Takács et al., 2002; Young et al., 2003), which is an alanine substitution to serine in codon 986. The association of the S allele with increased serum calcium levels due to decreased urinary calcium excretion indicates that this polymorphism may affect extracellular calcium levels (Young et al., 2003). There are only a few studies focusing on the association between *CASR* A986S loss-of-function mutation and osteoporosis (Lorentzon et al., 2001; Takács et al., 2002; Young et al., 2003). In a study conducted on premenopausal and postmenopausal Iraqi women, A986S was reported to be associated with osteoporosis risk and it was also reported to have an effect on mineral levels (Al-Azzawie, 2001). In contrast, no association was found between BMD values and *CASR* A986S genotypes between osteoporosis and control groups in Hungarian postmenopausal women (Takács et al., 2002). However, the effects of the *CASR* gain-of-function mutations on osteoporosis development have not been well studied in the literature. For this reason, our aim in this study was to investigate the effect of the *CASR* A843E gain-of-function mutation on osteoporosis and to determine the effects of this variation on BMD comparatively depending on the presence of the postmenopausal period.

MATERIAL AND METHODS

Selection and description of participants

One hundred and eighty Turkish postmenopausal women (57.53±7.44 mean age) were included in the study. Participants were selected from the Uskudar State Hospital in Istanbul, Turkey. Inclusion criteria for the selection of the study group were determined as being in the post-menopausal period with the absence of a menstruation period for at least a year. All participants underwent a standard inquiry including queries about the risk factors for osteoporosis (such as menopausal condition and age, smoking, alcohol consumption, family history of osteoporosis, drugs, and other medical conditions). Demographic and morphometric features were also noted. Participants having conditions or medications known to affect metabolisms of bone, such as malignities, endocrine diseases (hyper-

thyroidism, hypo/hyperparathyroidism, Cushing's syndrome), drastic liver or gastroenteric diseases, bone diseases (arthritis, Paget's disease, osteomalacia, and osteogenesis imperfecta), corticosteroids, androgenic-anabolic steroids, female steroid sex hormones, estrogen-related molecules or anticonvulsives were excluded from the study. The study protocol was approved by the Local Ethical Committee of Istanbul University, Istanbul Medical Faculty Protocol No: 2006/2145). Informed written permission was obtained from all participants before the collection of blood samples.

BMD measurement

During the scanning procedure, participants were stationed motionlessly in the scanner per standard procedures. BMD values of the lumbar spine (L1–L4) and hip (femoral neck and total hip) were evaluated by GE-Lunar DPX Pro (GE Healthcare, Madison, WI, USA) Pencil Beam DXA densitometer. All DEXA scans were analyzed using software (encore version 2005, 9.30.044) provided by the manufacturer.

PCR-Based Detection of the *CASR* A843E mutation

Genomic DNA was extracted from the blood by salting-out procedure (Miller et al., 1998). A843E (rs104893706) C>A in exon 7 of *CASR*, an activating mutation, was determined by carrying out a polymerase chain reaction (PCR) at a thermal cycler (Biorad T100 Thermal Cycler). The specific primers for rs104893706 C>A mutation (the forward primer sequence: 5'-GCCTTCAAGTCCCGGAAGCTGC-3', and reverse primer sequence: 5'-CGGTGCTGCAACGCACCTCCTC-3') were designed by in silico method and used in PCR. PCR reactions were carried out in a total volume of 25 µl containing 150-200 ng genomic DNA, sterile deionized water, 10xTaq polymerase buffer with $(NH4)_2SO_4$ (MBI Fermentas), 25mM $MgCl_2$ (MBI Fermentas), 5 mM deoxynucleotide triphosphates (MBI Fermentas), 50 pmol/µl of each primer (IDT DNA Technologies) and 1.5 U Taq DNA polymerase (MBI Fermentas). PCR cycling conditions were as follows: an initial denaturing step for 5 minutes at 94°C, an amplification step including 35 cycles of denaturation (45 seconds at 94°C), annealing (45 seconds at 61°C), and extension (45 seconds at 72°C), followed by a final extension step for 5 minutes at 72°C. The A843E PCR products (259 bp) were directly digested with BglI restriction enzyme (10units/µl) (Promega), and the digested DNA fragments were characterized on 3% agarose gel in 1X Tris-borate-EDTA buffer by gel electrophoresis. Results were analyzed considering that a single fragment of 259 bp was the A allele, and two fragments of 154 bp and 105 bp were the C allele.

Statistics

Statistical analyses were performed by the SPSS software package (version 21.0 SPSS Inc., Chicago, IL, U.S.A.). Clinical parameters were given as mean ± SD. Mean values were compared between the groups using the unpaired Student's t-test. Values of $p<0.05$ were accepted as statistically significant.

RESULTS

The characteristics and bone mineral density (BMD) values of the subjects are shown in Table 1. In our study group consisting of postmenopausal women, the frequency of the *CASR* A843E common CC genotype was 100%, that is, rare A allele (AA and CA genotypes) was not observed.

Table 1. Characteristics and BMD values of the study population.

	Study population (n=180)	Min.	Max.
Age (year)	57.53±7.44	44	80
Age of menopause (year)	47.10±4.56	30	56
BMI (kg/m^2)	30.75±4.92	20.17	46.11
Lumbarspine L1 BMD (g/cm^2)	0,95±0.14	0.71	1.56
Lumbarspine L2 BMD (g/cm^2)	1.02±0.13	0.76	1.75
Lumbarspine L3 BMD (g/cm^2)	1.07±0.15	0.71	1.76
Lumbarspine L4 BMD (g/cm^2)	1.06±0.16	0.68	1.66
Lumbarspine L1-L2 BMD (g/cm^2)	0.98±0.13	0.74	1.67
Lumbarspine L1-L3 BMD (g/cm^2)	1.03±0.13	0.75	1.70
Lumbarspine L1-L4 BMD (g/cm^2)	1.03±0.13	0.76	1.69
Lumbarspine L2-L3 BMD (g/cm^2)	1.04±0.14	0.76	1.76
Lumbarspine L2-L4 BMD (g/cm^2)	1.05±0.14	0.74	1.72
Lumbarspine L3-L4 BMD (g/cm^2)	1.06±0.15	0.70	1.71
Femoral neck BMD (g/cm^2)	0.87±0.11	0.61	1.26
Upper neck BMD (g/cm^2)	0.72±0.11	0.51	1.09
Lower neck BMD (g/cm^2)	1.01±0.11	0.79	1.42
Ward's triangle BMD (g/cm^2)	0.70±0.12	0.44	1.08
Trochanter BMD (g/cm^2)	0.76±0.11	0.50	1.12
Shaft BMD (g/cm^2)	1.01±0.17	0.27	1.57
Total hip BMD (g/cm^2)	0.93±0.11	0.69	1.27

BMI: Body mass index, BMD: Bone mineral density. The values were given as means ± SD.

The percentage of smokers in the study group was 8.88% . The mean age of the smokers was 52.69±4.54, while the mean age of the nonsmokers was 58.00±7.51 (p=0.001). The percentage of smokers was 43.8% in the subgroup of postmenopausal women aged 55 years or over, while that of smokers in the subgroup of postmenopausal women younger than 55 years old was 56.3% (p=0.136) (Data not shown).

The subjects were placed into two groups according to their age (classified as age≥55 vs. ages<55) and smoking status (smoking vs. non-smoking). It was observed that the BMD values of the femoral neck (p=0.017), upper neck (p=0.040), lower neck (p=0.011), and Ward's triangle (p=0.005) were significantly higher in postmenopausal women younger than 55 years old compared to postmenopausal women aged 55 and older. However, there was no significant difference between age groups in terms of BMD values of lumbar vertebra, trochanter, and total hip (p>0.05). Also, there were no differences in BMD values between the smoking and non-smoking groups (Table 2).

Table 2. Effects of smoking and age on critical BMD values in postmenopausal women

	AGE			SMOKING		
	Age<55	**Age≥55**	**p-value**	**(-)**	**(+)**	**p-value**
L1 BMD (g/cm²)	0.96±0.12	0.94±0.14	0.271	0.95±0.14	0.93±0.12	0.685
L2 BMD (g/cm²)	1.03±0.13	1.00±0.14	0.347	1.01±0.13	1.04±0.14	0.489
L3 BMD (g/cm²)	1.08±0.14	1.06±0.15	0.624	1.07±0.15	1.10±0.12	0.401
L4 BMD (g/cm²)	1.06±0.15	1.06±0.16	0.978	1.06±0.16	1.04±0.14	0.668
L1-L2 BMD (g/cm²)	0.99±0.12	0.98±0.13	0.265	0.98±0.13	0.99±0.13	0.864
L1-L3 BMD (g/cm²)	1.03±0.13	1.00±014	0.373	1.01±0.13	1.03±0.12	0.671
L1-L4 BMD (g/cm²)	1.04±0.13	1.02±0.14	0.452	1.03±0.14	1.03±0.12	0.862
L2-L3 BMD (g/cm²)	1.05±0.13	1.04±0.14	0.482	1.04±0.14	1.07±0.13	0.452
L2-L4 BMD (g/cm²)	1.05±0.13	1.04±0.15	0.674	1.05±0.14	1.06±0.12	0.760
L3-L4 BMD (g/cm²)	1.07±0.14	1.06±0.15	0.809	1.06±0.15	1.07±0.12	0.848
Femoral neck BMD (g/cm²)	0.89±0.12	0.85±0.10	**0.017***	0.87±0.11	0.85±0.10	0.534
Upper neck BMD (g/cm²)	0.74±0.13	0.70±0.10	**0.040***	0.72±0.11	0.70±0.10	0.489
Lower neck BMD (g/cm²)	1.04±0.12	1.00±0.10	**0.011***	1.01±0.11	1.01±0.10	0.948
Ward's triangle BMD (g/cm²)	0.73±0.12	0.68±0.12	**0.005***	0.70±0.13	0.68±0.09	0.478
Trochanter BMD (g/cm²)	0.76±0.12	0.76±0.09	0.897	0.76±0.10	0.73±0.13	0.236
Shaft BMD (g/cm²)	1.14±0.18	1.10±0.17	0.128	1.12±0.07	1.06±0.18	0.288
Total hip BMD (g/cm²)	0.95±0.13	0.92±0.09	0.193	0.93±0.11	0.91±0.12	0.410

BMD: Bone Mineral Density. BMD values were given as Mean ± SD.

DISCUSSION

Bone mineral density (BMD), which is regulated by fine-tuned metabolic control, is also under genetic control (Li et al., 2010; Zhang et al., 2016; Kuo, Chang, Chi, & Chu,2008). It is known that there is a positive correlation between decreased BMD levels and increased fracture risk (Hend et al., 2009). Acquired bone mass in adolescence and young adulthood partially determines the risk of developing osteoporosis and suffering from resulting fractures (Lorentzon et al., 2001). Although 60-80% of the age-specific changes in BMD are linked with genetic factors, the specific genes affecting BMD have not yet been clearly described (Lorentzon et al., 2001). Many studies have investigated the genes responsible for vitamin D receptor (VDR), the estrogen receptor (ER), and the collagen receptor (COL1A1) as candidates for osteoporosis (Ioannidis et al., 2002; Willing et al., 1998; Xie et al., 2015). Among these, the variations of the VDR gene affecting calcium levels were found to be associated with BMD levels and osteoporosis (Kurt et al., 2012). Another receptor that affects calcium metabolism is the calcium-sensing receptor (CaSR) (Wang et al., 2006). It was hypothesized that mutations in the *CASR* gene were associated with BMD levels and osteoporosis risk, however, more evidence is needed for confirmation as the results of existing studies examining certain variations were contradictory.

More than three hundred mutations/polymorphisms associated with loss/ gain-of-function in the *CASR* gene have been identified (Hend et al., 2009). These variations have been investigated in different patient groups having different diseases including hypoparathyroidism/hyperparathyroidism, chronic kidney disease, and hypertension (Yamauchi et al., 2001; D'Souza-Li, 2006; Jung et al., 2009; Guha et al., 2015; Jeong et al., 2016). Heterozygous activating mutations of the *CASR* gene have been described in autosomal dominant hypocalcemia (ADH), which is associated with hypoparathyroidism or Bartter syndrome subtype V (Egbuna & Brown, 2008). On the other hand, heterozygous inactivating mutations of the *CASR* gene have been associated with familial hypocalciuric hypercalcemia type 1. Additionally, homozygous inactivating *CASR* mutations have been associated with hypercalcemia in neonatal severe primary hyperparathyroidism (Egbuna & Brown, 2008).

The investigation of the relationship between the *CASR* gene and BMD values has mostly been limited to the inactivating A986S variation. Even though there are controversial findings related to the relationship between *CASR* A986S genotypes and osteoporosis risk in humans (Cetani et al., 2003; O'Seaghdha et al., 2010; Eller-Vainicher et al., 2014), many studies have implied an association between *CASR* A986S genotypes and BMD (Lorentzon et al., 2001; Takács et al., 2002; Young et al., 2003; Donáth et al., 2004). In a study on healthy adolescent girls, it was reported that the A986S polymorphism of the *CASR* gene was associated with circulating calcium levels and BMD (Lorentzon et al., 2001). Eller-Vainicher et al. (2014) suggested that the probability of vertebral fracture increased more than four times in Caucasian primary hyperparathyroidism patients with *CASR* 986S genotype, after adjusting for factors including age, lumbar spine, and serum calcium levels (Eller-Vainicher et al., 2014). In a genome-wide association study (GWAS) conducted on this subject, it was found that the *CASR* A986S polymorphism was associated with lower lumbar spine BMD level, but not with the femoral neck (O'Seaghdha et al., 2010). On the contrary, the *CASR* gene A986S variation was not associated with BMD and osteoporotic fractures in Italian and Hungarian postmenopausal women (Takács et al., 2002; Cetani et al., 2003). This finding may be explained by the fact that the CaSR does not play an important role in the regulation of osteoblast function (Takács et al., 2002). Additionally, an osteoblastic extracellular cation-sensing mechanism, different than CaSR, has also been identified (Pi, Garner, Flannery, Spurney, & Quarles, 2000). It is important to note that the A986S effect on bone could be modified by the osteoblastic CaSR-like receptor (Takács et al., 2002).

While the association of BMD with the loss of function mutations has been examined in many studies, the BMD relationship with gain-of-function mutations has not been studied extensively. The most common gain-of-function mutations in the *CASR* gene are R990, A843E, S122C, P569H, and I839T. The gain-of-function *CASR* mutations such as L125P, C131W, and A843E have been identified in patients with Bartter Syndrome (Pi et al., 2000) and the functional studies show that these mutations resulted in more pronounced receptor activation than other known gain-of-function mutations (Brown & MacLeod, 2001). To the author's knowledge, the present study is the first article investigating whether there is an association between gain-of-function mutation A843E of the *CASR* gene and osteoporosis.

Watanabe et al. (2002) reported that the A843E mutation caused inhibition of renal external medullary potassium channel activity in two patients with hypocalcemia, insufficient parathyroid hormone secretion, and Bartter syndrome. Kinoshita et al. (2014) showed that the PTH and serum Mg^{+2} levels were lower, and fractional excretion of Mg^{+2} was increased in 12 autosomal dominant hypocalcemia patients with A843E mutations. Since we did not observe the *CASR* A843E mutation in our study group, we could not analyze the combined effects of the mutation on both osteoporosis risk and risk factors in postmenopausal women.

BMD values of the femoral upper, lower neck, and Ward's triangle were higher in younger postmenopausal women compared to older women. It was reported that calcium absorption may be defective in smokers (Zhang et al., 2016). However, the mechanisms related to the negative effect of smoking on bone mass have not yet been identified (Zhang et al., 2016). Both the status and duration of smoking have been reported to have deleterious effects on the bone mineral density of the lumbar spine (Brown & MacLeod, 2001). It was reported that women who smoke have lower bone mass and tend to lose bone faster than non-smokers. Also, postmenopausal women who smoked were reported to have significantly higher fracture rates than non-smokers (Zhang et al., 2016). Our findings did not confirm the negative effects of smoking on bone density. This different finding could be related to the low rate of smoking in the study group.

An age-dependent decrease in BMD in women occurs mostly after menopause (Brown & MacLeod, 2001). Menopause related to estrogen deficiency stimulates the expedition of bone loss due to age (Nuti et al., 2019). Age is a notable risk factor for fracture, specifically hip fracture. Hip fracture risk increases fourfold between ages 55 and 85 depending on BMD (North American Menopause Society, 2010). We therefore divided our study group accordingly into two subgroups: younger (aged under 55 years old) and older (aged 55 years old or over) women. In the present study, BMD values of the femoral neck, upper neck, lower neck, and Ward's triangle were lower in older than in younger postmenopausal women. There was no significant difference found between the age groups and the BMD values of the total hip, lumbar spines, and trochanter ($p>0.05$). Our findings showed that age was a risk factor for osteoporosis in postmenopausal women. It is important to note that hormonal changes may also affect osteoporosis along with age, especially in postmenopausal terms.

A843E mutation (rs104893706, C>A) of the *CASR* gene is responsible for alanine to glutamate substitution which affects the inactive conformation of the CaSR. A843E was also associated with high basal activity, and the activation of the receptor in the absence of calcium (Zhao et al.,1999). Mechanistically, receptor activation due to gain-of-function mutation resulted in a decrease in calcium reabsorption and an increase in calcium excretion. In the NCBI Alfa Allele Frequencies database (https://www.ncbi.nlm.nih.gov/snp/rs104893706?horizontal_tab=true#frequency_tab), the minor allele frequency (MAF) of the rs104893706 is very low in different populations around the world (A=0.00001 (1/78682, PAGE_STUDY) and A=0.000 (0/660, ALFA)). In the present study, our findings related to *CASR* A843E gain-of-function mutation were consistent with the reported findings in the NCBI Alfa Allele Frequencies database (https://www.ncbi.nlm.nih.gov/snp/rs104893706?horizontal_tab=true#frequency_tab). Our findings detected a similar allele distribution of the rs104893706 in the Turkish population as in other populations. The limitation was the small number of samples (n=180). Because allele frequencies were so low, the findings of this study offer new information for the Turkish population.

CONCLUSION

In conclusion, since the *CASR* gene A843E mutation was not observed in the selected population, our findings showed that BMD

levels were affected by age. However, the present study suggests that the variations in the *CASR* gene should be further investigated by DNA sequencing which will lead to a comprehensive understanding of the *CASR* gene as it relates to the risk of osteoporosis and BMD levels in Turkish postmenopausal women.

Ethics Committee Approval: The study protocol was approved by the Local Ethical Committee of Istanbul University, Istanbul Medical Faculty Protocol No: 2006/2145). Informed written permission was obtained from all participants before the collection of blood samples.

Peer-review: Externally peer-reviewed.

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