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Research Article

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Association of Estrogen Receptor Alpha Gene Polymorphisms with Bone Mineral Density in Postmenopausal Saudi Women

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ABSTRACT

Background: Estrogen and estrogen receptor alpha (ERα) play a central role in bone metabolism. Specifically, estrogen deficiency is a major pathogenetic factor in bone loss after menopause. Polymorphisms in the ERα gene as defined by the restriction enodonucleases PvuII (T/C) and XbaI (A/G) are reported to be associated with bone mineral density (BMD), which is considered the major determinant of osteoporosis in postmenopausal women. In this study we tested the association between the polymorphisms in the first intron of the ERα gene with
Method: BMD in 80 postmenopausal Saudi women aged 46–88 years (average age, 59.82 ± 8.24 years) living in Jeddah, Saudi Arabia. We excluded women with oophorectomy or hysterectomy or using estrogen replacement therapy. All subjects were classified according to the world health organization (WHO) definitions on the basis of the BMD T-score values as measured by dual-energy X-ray absorptiometry (DXA) at two sites, the lumbar spine (LS) and femoral neck (FN). Genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.
Result: At the LS, 30 postmenopausal women had osteoporosis, 21 had osteopenia where 29 had normal bone density. At the FN, 11 had osteoporosis, 34 had osteopenia and 35 had normal bone density. The frequencies of the ERα genotypes for PvuII and XbaI polymorphisms were similar to the previously reported genotype frequencies in Caucasian and Asian populations. There were no statistically significant relationships between the BMD values (g/cm2 or T-score) and the PvuII and XbaI genotypes. In the case-control, there were no significant differences in terms of frequencies of alleles and genotypes between all groups at either site. However, in the osteopenia and osteoporosis groups, the XX genotype was associated with a higher risk of bone loss than the xx genotype at the LS (odds ratio (OR)= 1.9 and 1.3, respectively) and at the FN (OR= 2.2 and 2.6, respectively). When we combined the two polymorphisms, only six genotypes were detected: 13.75% PPXX, 1.25% PpXX, 3.75% PPXx, 43.75% PpXx, 3.75% Ppxx and 33.75% ppxx. Linkage disequilibrium (LD) analysis between the PvuII and XbaI polymorphisms showed a strong association (Δ= 0.91, P<0.01). The PPXx genotype was significantly associated with higher FN-BMD (0.99±0.09) compared to the PPXX (0.78±0.13, P=0.02) and ppxx (0.81±0.11, P=0.01) genotypes.
Conclusion: We found that ERα gene individually polymorphisms had no major effect on BMD, but ERα polymorphisms combination may be associated with BMD at the FN site in postmenopausal Saudi women.

Keywords: *Bone mineral density (BMD); Postmenopausal Saudi women; Osteoporosis; Estrogen receptor alpha (ERα) gene; PvuII and XbaI Polymorphisms; PCR-RFLP*

INTRODUCTION

Osteoporosis is a complex multifactorial polygenic disease of the skeleton characterized by decreased bone mass and deterioration of bone tissue architecture leading to increased bone fragility and facture [1]. Osteoporotic bone changes are initially silent and can progress undetected until a low trauma fraction occurs [2]. Menopause represents

a critical life step characterized by complex endocrine changes which affect the musculoskeletal system and its neurological control [3]. During growth, in a state of normal bone remodeling bone formation closely matches bone resorption (positive balance) [4]. With aging as menopause stat, the greatest change in bone remodeling occurs at menopause when there is an increase in the number of resorption cavities but bone formation does not increase proportionately and resorption cavities are not completely filled in with new bone (negative balance) this results in gradual attrition of bone [4,5]. The main pathogenetic factor age-related bone loss may be contributing factors, a hormone-dependent increase in bone resorption and accelerated loss of bone mass in the first 5 or 10 years. Postmenopausal osteoporosis is a heterogeneous disorder characterized by a progressive loss of bone tissue which begins after natural or surgical menopause and leads to fracture within 15–20 years from the cessation of the ovarian function and consider the most common primary type [3]. Multiple population-based cross-sectional and longitudinal studies over the last 25 years using BMD assessed by DXA have helped, women losing BMD more rapidly with onset of menopause [6].

Sex hormone-binding globulin (SHBG), that binds with high affinity to sex steroids and regulating their access to target cells and bioavailability, may have an important role in the pathophysiology of osteoporosis. Many studies suggested that SHBG had an independent impact on bone strength (7). In 1941, Fuller Albright was the first to note the importance of estrogen in bone metabolism. Since then, estrogen deficiency has clearly been identified as the major pathogenetic factor contributing to postmenopausal bone loss and oophorectomy with the subsequent risk to develop osteoporosis, that strongly supported by the higher prevalence of osteoporosis in women than in men which can be prevented by estrogen therapy [3,4,8]. Oophorectomy is commonly cited as an example of hypoestrogenism. Cross-sectional data of Stepan et al. (1987) suggested a mean loss of 2.8% of the metacarpal cortical area and 8% of the lumbar spine (by dual photon densitometry) in the first year following oophorectomy. Estrogen plays a central role in the control of bone strength and has a positive effect on calcium balance, and its loss at menopause leads to up-regulation of RANKL causes osteoporosis in millions of women [4, 9, 10]. The identification of estrogen receptors on human osteoblasts by Eriksenet al. (1988) led to the notion that estrogen likely had direct skeletal effects [11]. Estrogens exert both antiapoptotic effects on bone-forming cells and proapoptotic effects on mesenchymal cells and osteoclasts through classical ER regulation of target genes [12]. Estrogen act through binding to two different ERs, estrogen receptors alpha (ERα, ESR1, NR3A) and estrogen receptor beta (ERβ, ESR2, NR3b) which are members of the nuclear receptor superfamily of ligand-activated transcription factors [13,14]. Both ER isoforms (ERα and ERβ) are expressed in osteoblasts, osteocytes, osteoclasts, bone marrow stromal, monocytes [3,13]. The genomic effects of estrogen on bone are complex and mediated by ER through multiple signaling pathways [3]. ERα appears to be the major receptor mediating estrogen action in bone, it's a predominance on ERβ because it occupies most of the common sites causing a major shift of ERβ to novel EREs, these findings is associated to the different affinities for the EREs of the ERα homodimers (higher affinity), heterodimers (medium affinity) and ERβ homodimers (lower affinity), which increases the residence time of ERα homodimers on its DNA target [13, 14].

The human ERα gene is located on chromosome 6q25 and contains 595 amino acid residues. It also comprises eight exons separated by seven intronic regions and spans more than 140 kilobases (Figure 1) [9, 13]. Structurally similar to all nuclear receptors, ERα are composed of six functional domains (named A-F) and are generally classified as ligand-dependent transcription factors because, after the association with their specific ligands, they bind specific genomic sequences (named EREs) and interact with co-regulators to modulate the transcription of target genes [14]. Among the ERα polymorphisms most widely studied are the two single nucleotide polymorphisms (SNPs) the PvuII (397T>C) and XbaI (−351A>G), it known by the name of the detecting restriction enzyme PvuII (from Proteus vulgaris, recognition site 5'...CAG▼CTG...3') and XbaI (from Xanthomonasbadrii, recognition site 5'...T▼CTAGA...3') underline the SNPs or by their reference ID numbers, rs2234693 and rs9340799, respectively [15, 16]. Both PvuII and XbaI lie in the first intron of ERα gene, apparently nonfunctional area of the gene, have been widely associated with low BMD in many studies but not in all populations studies [13, 17]. Kobayashi et al. (1996) first reported that two genetic variations PvuII and XbaI - RFLPs were significantly associated with low BMD in Japanese postmenopausal women [18, 15]. In different studies, these polymorphisms have been associated with several pathologic conditions such as breast and prostate cancer, osteoporosis, Alzheimer's disease and cardiovascular diseases. Results are still conflicting and molecular mechanisms by which these polymorphisms influence receptor activity are as yet unclear [16, 17].

The results of many studies in Saudi Arabia found the development of osteoporosis among postmenopausal Saudi females it should be considered as a matter of public health [1,19]. Therefore, the study aims to establish these polymorphisms (PvuII and XbaI) as a genetic marker for osteoporosis in postmenopausal Saudi women by study the association of commonly studied polymorphisms of ERα with BMD and the possible effect by the frequency distribution of ERα genotypes in the women with osteoporosis and normal bone mass within the group to confirm or refute the association to predict diagnosis before it become development.

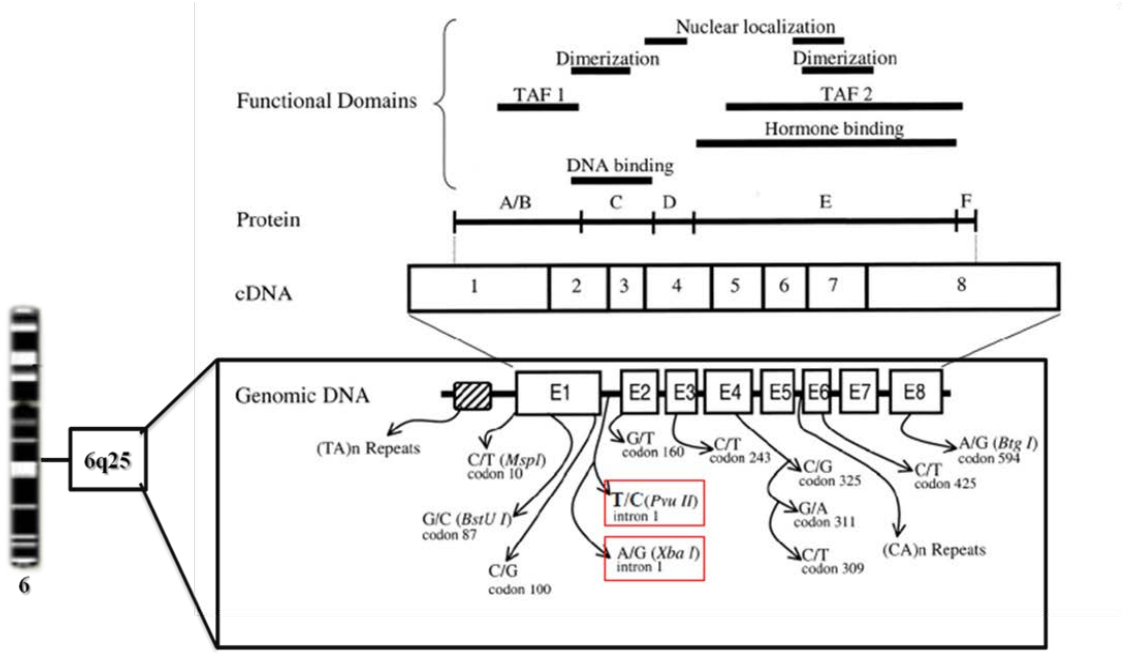

Figure 1. The human ERα gene location and genetic screening [Adapted from 9, 13].

MATERIALS AND METHODS

Subjects

All subjects gave their written informed consent of their participation in the study after it had been cleared by author which approved by King Fahad General Hospital, Ministry of Health, Jeddah, K.S.A. The study included 80 postmenopausal Saudi women, who attending King Fahad General Hospital, aged 46 to 88 years (average age 59.82 ± 8.24 years) with an average BMI 30.25 ± 4.97. We excluded women with oophorectomy or hysterectomy or had problems in parathyroid hormone or using estrogen replacement therapy (HRT). Menopause was confirmed as the absence of menses at least one year.

Bone Mineral Density (BMD) Measurement

All the subjects had undergone BMD (expressed as grams of mineral per unit projected area of the bone; g/cm^2) measurements by using Dual Energy X-ray absorptiometery (DXA) (Lunar iDXA, GE Healthcare, United Kingdom) in X-ray department in King Fahad General Hospital, Jeddah, K.S.A. BMD was measured, in grams per square centimeter, at the lumbar spine L_1-L_4 (LS-BMD; g/cm^2), right and left femoral neck then the mean was taken (FN-BMD; g/cm^2). An individual BMD value is calculated as a T-score which is the number of standard deviations above or below the mean BMD for normal young adults [20]. Subjects were categorized according to the World Health Organization (WHO) definition [21, 22].

Determination of PvuII and XbaI Genotypes in ERα gene

Genomic DNA was extracted from whole blood that stored in EDTA coated tubes using spin protocol from QIAamp DNA Blood Mini Kit (QIAGEN Inc., USA) according to manufacturer's instructions. Two polymorphic loci of ERαGene (T\C: PvuII, A\G: XbaI) were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. They were amplified using forward primer : 5'CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC TCC'3 and reverse primer 5'TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA'3 (Biolegio, Holand) by MultiGene™ Gradient Thermal Cycler (LabnetIntrnational Inc., Edison, NJ, USA) [23]. All the PCR reaction was performed using PromegaGoTaq® Hot Start Green Master Mix Kit (Promega Co.,

Madison, WI, USA) in a final volume of 50 µl containing 1 µl of each primer (with final concentration 0.2 µM), 25 µlGoTaq® Hot Start Green Master Mix (1X), 23 µl nuclease free water and 1 µl genomic DNA. The PCR running program conditions were as follows: initial denaturation at 95°C for 5 min followed by 35 cycle of denaturation at 95°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min and 30 sec followed by final extension at 72°C for 5 min. The PCR products were then electrophoresed in 2% (w/v) of agarose gel stained with ethidium bromide (10 mg/ml) using 1X of TAE as gel running buffer to ensure the PCR product's size (1400 bp). The PCR product was purified by using the Isolate PCR kit (Bioline Inc., Taunton, USA).Then, the PCR products were digested separately with fast digest restriction endonucleases PvuII–HF and XbaI (New England BioLabs® Inc, Pickering, Ontario, Canada) at 37°C for 3h then XbaI was inhibited at 64°C for 20 min where PvuII-HF was inhibited at 80°C for 20 min according to manufacturer's instructions. For polymorphism: 5 µl of 10X NEB buffer (final conc. 1X), 34 µl of nuclease free water, 0.5 µl of 100X BSA (final conc. 1X), 10 µl of pure PCR product and 0.5 µl (10 units) of restriction were added. The digested products were analyzed, after mixed 10µl of digested product with 2µl of loading dye, by electrophoresis in 2% (w/v) of a garose gel [23]. The sizes of the bands were estimated using HyperLadder II (50-2000 bp ladder (200 Lanes), Bioline, UK). The DNA fragment (digestion products) carried out by the gel was visualized under UV – light and photographed using gel documentation (Moecular Imager® Gel DocTM XR + Systems with Image LabTM 2.0 Software, Bio-Rad, USA). The genotyping was done on the basis of the presence or absence of the endonuclease restriction site. Lowercase letters indicated the presence for endonuclease restriction site (p/T and x/A, for PvuII and XbaI, respectively) while the absence of the restriction site is indicated with uppercase letters (P/C and X/G, for PvuII and XbaI, respectively).

Statistical Analysis

Statistical analyses were performed by using the Statistical Package for Social Sciences (SPSS) version 18.0 (SPSS Inc., Chicago, IL, USA). The numerical data are expressed as mean±standard deviations (SD) and the categorical variables as number and percentage. A case-control study was designed to compare patients (who had osteoporosis or osteopenia) with control (who had normal bone mass). Deviation from Hardy–Weinberg equilibrium for ERα genotypes was calculated by the chi-square test to test the frequency's constant of alleles and genotypes in a population from generation to generation by comparing observed allele and genotype frequencies with expected one (in equilibrium). The associations between BMD values and other parameters with genotypes or haplotypes were assessed using analysis of independent samples T-test. Contingency analysis was applied to compared the frequency distribution of genotypes between women with osteoporosis, osteopenia and normal bone, the odds ratio (OR) and risk ratio (RR) at 95% confidence interval (CI) also were assessed. The probability of P-value was two-sided and 0.05 or less was considered to be statistically significant. Linkage disequilibrium (LD) resulting from the association between alleles of PvuII and XbaI polymorphisms was assessed by the χ^2 test. The linkage disequilibrium coefficient was calculated according to [23, 24, 25] $\Delta = \frac{\mathbf{fPXfpx - fPxfpX}}{\sqrt{(\mathbf{fPX + fPx})(\mathbf{fpX + fpx})(\mathbf{fPX + fpX})(\mathbf{fPx + fpx})}}$

fs are the frequencies of the corresponding haplotypes. Δ is the correlation coefficient between the uniting gametes at the two loci. When Δ = 0 the association is random and when Δ = ±1 the association is complete, with a positive or negative sign implying an excess or deficiency of + + and - - haplotypes relative to + - and - + haplotypes, respectively [25].

RESULTS

Subject Characteristics

The subjects for the study were 80 postmenopausal Saudi women aged 46 to 88 years (average age 59.82 ± 8.24 years) with an average BMI 30.25 ± 4.97. On the basis of BMD measurements (T-score value) of DXA at two sites (lumbar spine and femoral neck) all the subjects were classified according to Word health Organization (WHO) definition. 38.75% (n= 31) of postmenopausal women had osteoporosis, 31.25% (n= 25) were osteopenic and 30% (n= 24) had normal bone (control). Descriptive analysis and bone mineral density (BMD) measurement were presented in Table 1.

Table 1 . Descriptive analysis and bone mineral density measurement for all subjects 80 postmenopausal Saudi women

Variables	Osteoporosis (n= 31)	Osteopenia (n= 25)	Control (Normal) (n= 24)
Frequency %	38.75%	31.25%	30%
Age (years)	62.69±10.13 (48-88)	60.16±7.26 (47.1-77.1)	55.75±8.53 (46-81.6)
Weight (kg)	74.73±12.93 (51.5-98)	76.27±11.82 (56.1-96)	77.54±12.42 (57.5-107)
Height (cm)	162.13±6.3 (144-173)	156.46±6.78 (142-168)	157.04±6.65 (145-170)
BMI (kg/m^2)	28.43±4.62 (20.31-37.81)	31.01±3.28 (25.78-37.67)	31.63±6.1 (23.53-49.75)
LS-BMD* (g/m^2)	0.71±0.08 (0.51-0.95)	0.87±0.15 (0.19-1.08)	1.09±0.11 (0.93-1.33)
T-score*	-3.17±0.75 (-4.8-(-0.9))	-1.57±0.55 (-2.4-(-0.4))	0.04±0.83 (-1-1.8)
RFN-BMD* (g/m^2)	0.68±0.12 (0.43-1.07)	0.8±0.07 (0.66-0.95)	1.01±0.11 (0.83-1.35)
T-score*	-2.11±1.01 (-4.2-1)	-1.14±0.6 (-2.3-0.1)	0.44±0.81 (-0.9-2.4)
LFN-BMD* (g/m^2)	0.69±0.1 (0.44-1)	0.81±0.08 (0.69-1.03)	1.01±0.14 (0.84-1.41)
T-score*	-1.99±0.85 (-4.2-0.5)	-1.1±0.64 (-2.1-0.8)	0.53±1.18 (-0.9-3.9)
MFN-BMD* (g/m^2)	0.69±0.1 (0.43-1.04)	0.81±0.06 (0.71-0.95)	1.01±0.11 (0.83-1.25)
T-score*	-2.02±0.89 (-4.2-0.75)	-1.12±0.55 (-1.9-(-0.5))	0.47±0.93 (-0.9-2.6)

Results are expressed as mean±standard deviation (SD)

Between the parentheses (minimum – maximum values)

BMI: Body Mass Index , LS-BMD: Bone mineral density at lumbar spine L_1-L_4

RFN-BMD: Bone mineral density at right femoral neck, LFN-BMD: Bone mineral density at left femoral neck MFN-BMD: Bone mineral density at mean femoral neck, *highly significance ($P<0.05$) between all groups

Analysis of the PvuII and XbaI Polymorphisms in the ERα Gene

At PvuII site the two allele polymorphism was: PP (absence of the restriction site PvuII on both alleles) produced one fragment with size= 1400 bp, Pp (heterozygous for the restriction site) produced three fragments with size= 1400 bp, 950 bp, 450 bp and pp (presence the restriction site on both alleles) produced two fragments with size= 950 bp, 450 bp lengths. For XbaI site: XX (absence of the restriction site XbaI on both alleles) produced one fragment with size= 1400 bp, Xx (heterozygous for the restriction site) produced three fragments with size= 1400 bp, 1000 bp, 400 bp and xx (presence the restriction site on both alleles) produced two fragments with size= 1000 bp, 400 bp lengths (Figure 2).

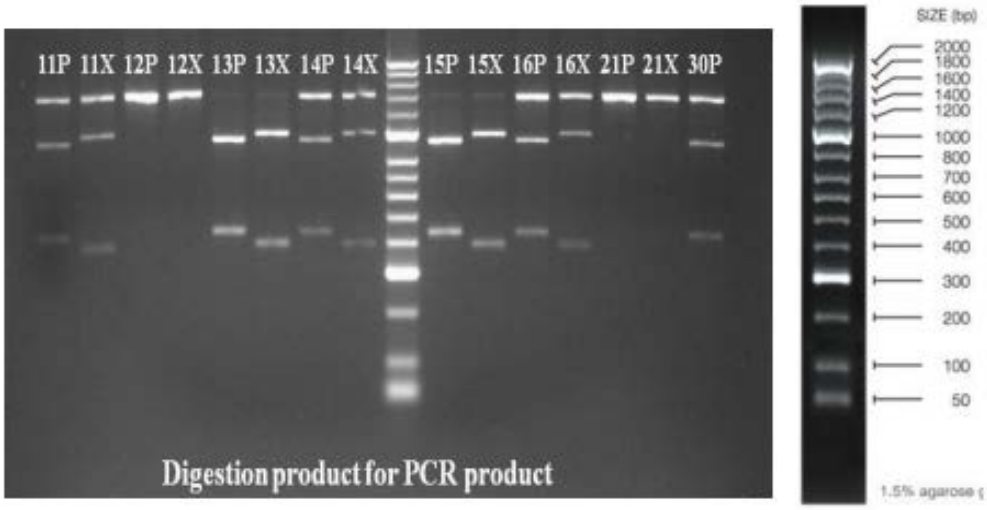

Figure 2. Electrophoretic analysis of the digested PCR products for two genetic polymorphisms in *ERα* on 2% (w/v) agarose gel.
Lane M= molecular size marker (50 bp – 2000 bp).

For *PvuII* polymorphism: lane 11P, 14P, 16P and 30P: Heterozygous Pp
lane 12P and 21P: Homozygous PP
lane 13P and 15P: Homozygous pp

For *XbaI* polymorphism: lane 11X, 14X and 16X : Heterozygous Xx
lane 12X and 21X: Homozygous XX
lane 13X and 15X: Homozygous xx

General Characteristic of the Study Subjects According to ERα Genotypes

The clinical characteristics of the study groups according to PvuII & XbaI genotypes are presented in Table 2. Genotypes distributions for whole group were in Hardy-Weinberg equilibrium (Goodness of fit X^2= 0.00016, df = 1, P= 0.98 for PvuII, Goodness of fit X^2= 3.47, df= 1, P= 0.99 for XbaI). There were no statistically significant differences in all variables between the PvuII or XbaI genotypes. There were trend for women with pp genotype to have the highest lumbar BMD (g/m^2) values and those with xx genotypes showed higher BMD values (normal bone) in all sites comparing with XX genotype.

Genotype and Allele Frequencies in the Intron 1 of ERα Gene Polymorphism at Lumbar Spine and femoral neck

The genotype and allele frequencies of PvuII and XbaI polymorphisms according to disease status at lumbar spine (LS) and femoral neck (FN) were given in Table 3. In comparing the results between osteoporosis vs. control groups and between osteopenia vs. control groups at both sites, no statistically significant differences were observed in the frequency of PvuII genotypes and alleles. The frequency PP and pp genotype were more in controls whereas in osteoporosis and osteopenia groups the frequency of Pp genotype was high. The frequency of the P allele was more pronounced among osteoporosis and osteopenia groups whereas p allele more pronounced among control group.

When comparing PP and Pp at LS between osteoporosis and control groups the odd ratio was 2.13 (95%CI: 0.49-9.2). This estimate indicates that the odds of osteoporosis is 2.13 times higher in Pp genotype than control group and the Pp is more likely to occur in the osteoporotic group than in the control group (RR= 1.21, 95%CI: 0.82-1.79). The odd ratio: PP vs. pp was 1.23 (95%CI: 0.26-5.73), Pp vs. pp was 0.58 (95%CI: 0.18-1.82).Where the odd ratio between osteopenia and control groups for PP vs. Pp genotype was 1.25 (95%CI: 0.27-5.71), PP vs. pp genotype was 0.95 (95%CI: 0.19-4.63), Pp vs. pp the odd ratio was 0.76 (95%CI: 0.23-2.71). This observed showed the protective effect of pp from disease by 28% relative risk reduction from osteoporosis and 14% from osteopenia.

At FN between osteoporosis and control groups, the odd ratio between PP vs. pp genotype was 0.5 (95%CI: 0.06-4.33), Pp vs. pp genotype was 0.31 (95%CI: 0.05-1.73), this indicated the protective effect of pp genotype. For both PP+Pp the odd of osteoporosis 2.86 times higher than in control group (the odd of osteoporosis 3.2 times higher in Pp than control group (RR)= 2.17), the odd of osteoporosis 2 times higher in PP than control group(RR= 1.3). No

significant differences in allele frequencies of XbaI polymorphisms at both sites were between osteoporosis and control groups (P= 0.53 at LS, P=0.3at FN) and between osteopenia and control groups (P= 0.39 at LS, P=0.27 at FN) and in the frequencies of XbaI genotypes.

Between osteoporosis vs. control groups and osteopenia vs. control at LS the odd ratio between XX and xx genotypes were 0.77 (95%CI: 0.15-3.85) and 0.54 (95%CI: 0.1-2.84), between Xx and xx genotype were 0.58 (95%CI: 0.18-1.76) and 0.65 (95%CI: 0.19-2.24), respectively. At FN between osteoporosis vs. control groups and osteopenia vs. control the odd ratio between XX and xx genotypes were 0.38 (95%CI: 0.05-3.1) and 0.46 (95%CI: 0.1-2.01), between Xx and xx genotype were 0.5 (95%CI: 0.11-2.35) and 0.69 (95%CI: 0.24-1.93), respectively. These data indicated the protective effect of xx genotype from osteoporosis and osteopenia at both site.

Table 2. General characteristic in relations to PvuII and XbaI genotypes in all 80 postmenopausal Saudi women

Variables	PP (n= 14) 17.5%	Pp (n= 39) 48.75%	pp (n= 27) 33.75%	XX (n= 12) 15%	Xx (n= 38) 47.5%	xx (n= 30) 37.5%
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Age (years)	59.24±9.38	58.84±10.03	61.54±7.96	59.64±9.09	58.47±9.62	61.59±8.80
Weight (kg)	73.40±14.07	76.68±11.19	76.78±13.09	74.80±14.24	75.23±9.62	77.76±13.85
Height (cm)	156.68±7.48	159.21±5.72	159.28±8.23	156.46±7.79	159.56±5.57	158.76±8.17
BMI (kg/m2)	30.07±6.77	30.26±4.26	30.32±4.93	30.80±7.21	29.5±3.39	30.94±5.52
LS-BMD (g/m2)	0.89±0.18	0.84±0.23	0.9±0.14	0.88±0.19	0.84±0.23	0.90±0.14
T-score	-1.68±1.49	-1.92±1.63	-1.47±1.31	-1.81±1.54	-1.92±1.65	-1.46±1.29
RFN-BMD (g/m2)	0.83±0.16	0.81±0.20	0.81±0.12	0.79±0.14	0.82±0.19	0.83±0.16
T-score	-0.89±1.34	-1.105±1.54	-1.01±1.05	-1.28±1.16	-1.02±1.57	-0.97±1.11
LFN-BMD (g/m2)	0.82±0.15	0.84±0.20	0.81±0.11	0.77±0.12	0.84±0.18	0.83±0.16
T-score	-0.99±1.32	-0.82±1.67	-1.09±0.92	-1.37±1.09	-0.84±1.50	-0.91±1.33
MFN-BMD (g/m2)	0.83±0.15	0.83±0.19	0.81±0.11	0.78±0.13	0.84±0.18	0.83±0.15
T-score	-0.97±1.29	-0.92±1.53	-1.06±0.97	-1.35±1.06	-0.89±1.48	-0.94±1.16

Results are expressed as mean±standard deviation (SD)

BMI: Body Mass Index, LS-BMD: Bone mineral density at lumbar spine L1-L4

RFN-BMD: Bone mineral density at right femoral neck, LFN-BMD: Bone mineral density at left femoral neck

MFN-BMD: Bone mineral density at mean femoral neck

Table 3. PvuII and XbaI genotypes and alleles frequencies distribution according to disease status

		Femoral neck			Lumbar spine		
Genotypes		**Osteoporosis (n= 11)**	**Control (n= 36)**	**Osteopenia (n= 33)**	**O steoporosis (n= 30)**	**Control (n= 29)**	**Osteopenia (n= 21)**
PvuII	**PP**	18.2% (n= 2)	19.4% (n= 7)	15.2% (n= 5)	13.3% (n= 4)	20.7% (n= 6)	19% (n= 4)
		P^1= 0.68		P^1= 0.49	P^1= 0.46		P^1= 1
	Pp	63.6% (n= 7)	41.7% (n= 15)	51.5% (n= 17)	56.7% (n= 17)	41.4% (n= 12)	47.6% (n= 10)
		P^2= 0.25		P^2= 0.49	P^2= 0.34		P^2= 0.68
	pp	18.2% (n= 2)	38.9% (n= 14)	33.3% (n= 11)	30 % (n= 9)	37.9% (n= 11)	33.3% (n=7)
		P^3= 0.6		P^3= 0.88	P^3= 1		P^3= 1
XbaI	**XX**	18.2% (n= 2)	11.1% (n= 4)	18.2% (n= 6)	13.3% (n= 4)	13.8% (n= 4)	19% (n= 4)
		P^1= 1		P^1= 0.72	P^1= 1		P^1= 1
	Xx	54.5% (n=6)	44.4% (n=16)	48.5% (n= 16)	53.3% (n= 16)	41.4% (n= 12)	47.6% (n= 10)
		P^2= 0.64		P^2= 0.47	P^2= 0.33		P^2= 0.48
	xx	27.3% (n= 3)	44.4% (n=16)	33.3% (n= 11)	33.3% (n= 10)	44.8% (n= 13)	33.3% (n= 7)
		P^3= 0.56		P^3= 0.45	P^3= 1		P^3= 0.67
Alleles	**P**	50%	40%	41%	42%	41%	43%
	p	50%	60%	59%	58%	59%	57%
	P-value P vs. p	P= 0.42		P= 92	P= 1		P= 0.88
	X	45%	33%	42%	40%	34%	43%
	x	55%	67%	58%	60%	66%	57%
	P-value X vs. x	P= 0.3		P= 0.27	P= 0.53		P= 0.39

Data are presented as frequency % with number of cases between the practice.

P^1:P-value for PP vs. Pp, P^2: P-value for Pp vs. pp, P^3: P-value for PP vs. pp

Genotype Combination beteween PvuII and XbaI Polymorphisms of the ERα Gene

To evaluate a possible additive effect of ERα polymorphisms on BMD in postmenopausal Saudi women, the combination of PvuII and XbaI genotypes were used and analyzed in relation to BMD. We defined nine haplotypes (PPXX, PpXX, ppXX, PPXx, PpXx, ppXx, PPxx, Ppxx, ppxx). When we combined the two polymorphisms together in this study, six genotypes were detected (PPXX, PpXX, PPXx, PpXx,Ppxx, ppxx). The three genotypes (ppXX, ppXx, PPxx) were not detected. The frequencies of the genotype combination in all subjects were presented in Table 4. There was highly significant and strong association according to linkage disequilibrium between PvuII and XbaI polymorphisms (Δ= 0.91, X^2= 117.98, P= 0.00).

Table 4. Frequency of genotypes for both polymorphisms in all 80 subjects

Genotype	Derived Alleles	N	Frequency %	Alleles	N	Frequency %
PPXX	PX/PX 2/2	11	13.75%	px=1	92	57.5%
PpXX	PX/px 2/4	1	1.25%			
PPXx	PX/Px 2/3	3	3.75%	PX=2	61	38.13%
PpXx	PX/px 1/2	35	43.75%	Px=3	6	3.75%
Ppxx	Px/px 1/3	3	3.75%	pX=4	1	0.63%
ppxx	px/px 1/1	27	33.75%			
Total		80	100%		160	100%

Data are presented as number of cases (N) with frequency

Analysis of association between BMD and other characteristics with the genotypes in combination were showed in Table 5. Analysis of BMD data at FN showed PPXx genotype (0.99±0.09) was significant associated with higher FN-BMD with respect to those with PPXX (0.78±0.13, P= 0.02) and ppxx (0.81±0.11,P= 0.01) genotype. There were no significant difference between all groups in terms of age, height, weight, BMI, LS-BMD and FN-BMD. However, the women with Ppxx genotype had significantly higher BMI than PpXx and ppxx (P< 0.05). A higher prevalence of the ppxx genotype was observed in the control group. At FN, We only found 3 normal individuals and no osteoporotic patients with the PPXx genotype (Figure 3).

Table 5. General characteristic in relations to PvuII and XbaI genotypes combination in all 80 postmenopausal Saudi women

Variables	Genotypes					
	PPXX (n= 11)	**PpXX (n= 1)**	**PPXx (n= 3)**	**PpXx (n= 35)**	**Ppxx (n= 3)**	**ppxx (n= 27)**
Frequency %	13.75	1.25	3.75	43.75	3.75	33.75
Age (years)	59.83±9.51	57	57.07±10.54	58.59±9.69	62.1±17.27	61.54±7.96
Weight (kg)	74.05±14.69	83	71±14	75.62±10.25	90.5±23.33	76.78±13.09
Height (cm)	156.59±8.16	155	157±5.56	159.79±5.6	152±2.82	159.28±8.24
BMI (kg/m^2)	30.46±7.46	34.55	28.65±3.9	29.58±3.39	39.01±8.64**	30.32±4.93
LS-BMD (g/m^2)	0.88±0.2	0.85	0.94±0.01	0.84±0.23	0.92±0.16	0.89±0.14
T-score	-1.7±1.61	-2.2	-1.15±0.21	-1.96±1.6	-1.3±1.3	-1.47±1.31
RFN-BMD (g/m	0.79±0.14	0.78	0.99±0.08*	0.8±0.19	0.98±036	0.81±0.12
T-score	-1.27±1.22	-1.3	0.5±0.69*	-1.15±1.5	-0.47±1.77	-1.03±1.05
LFN-BMD (g/m	0.77±0.13	0.8	0.99±0.1*	0.83±0.18	1.02±0.39	0.81±0.11
T-score	-1.39±1.14	-1.1	0.47±0.9*	-0.95±1.5	0.73±3.2	-1.09±0.92
MFN-BMD (g/m^2	0.78±0.13	0.79	0.99±0.09*	0.82±0.17	1±0.32	0.81±0.11
T-score	-1.37±1.11	-1.2	0.48±0.77*	-1.01±1.4	0.13±2.3	-1.06±0.97

Results are expressed as mean±standard deviation (SD)
* Significance independent T-test; PPXx vs. PPXX and PPXx vs. ppxx (P< 0.05)
** Significance independent T-test; Ppxx vs. PpXx and Ppxx vs. ppxx (P< 0.05)

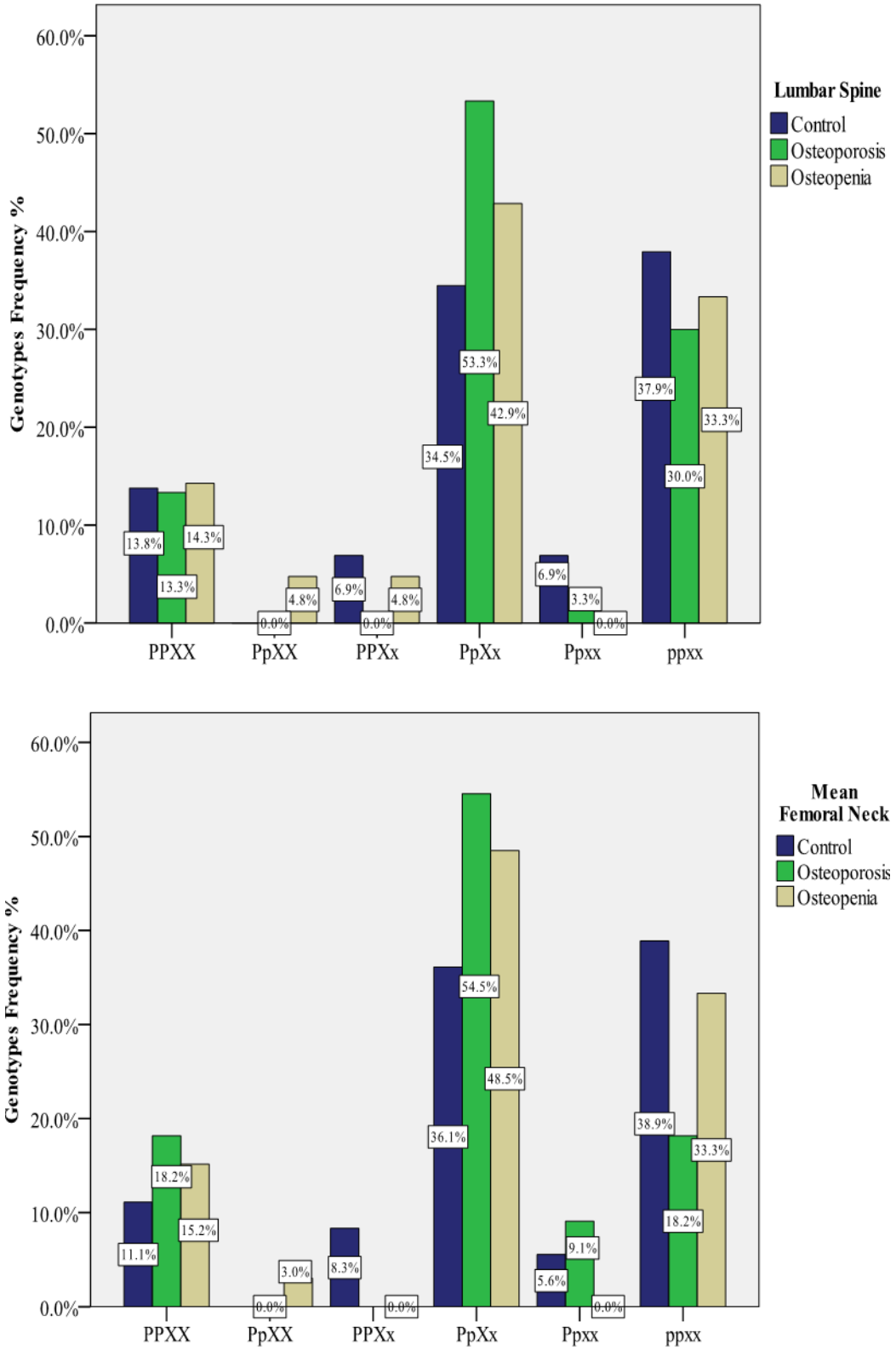

Figure 3. The frequency distribution for the combination ofPvuIIandXbaI polymorphism according to disease status at both sites.

DISCUSSION

Evidence from epidemiological and twin studies clearly demonstrates that hat osteoporosis is a complex multifactorial disease in which a genetic factor contributed to the pathogenesis of osteoporosis [13, 26]. Estrogen receptor alpha gene (ERα) is a strong candidate for regulation of bone density, bone remodeling and postmenopausal bone loss but studies to date have yielded conflicting results [27, 28].

This study reported the frequency and association of ERα genotypes with BMD in postmenopausal Saudi women. We evaluated the association of 2 SNPs (PvuII and XbaI) of ERα gene with osteoporosis in 80 postmenopausal Saudi women.

The frequencies of the ERα genotypes in 80 Saudi postmenopausal women were: Pp 48.75%, pp 33.75%, PP 17.5% and Xx 48.75%, xx 36.25% and XX 15% which were similar to previously published ERαgenotype frequencies in the Caucasian [27, 29, 30, 31, 32], Asian populations [23, 33, 34] and as distribution ofPvuII genotypes where different in XbaI genotypes in some populations studies [16, 22, 24, 35, 36, 37,38, 39].

In this study we analyzed the relation between ERαPvuII and XbaI polymorphisms genotypes individually with BMD values at lumbar spine (LS) and femoral neck (FN) site in postmenopausal Saudi women, there were no significant different as studies carried out in Danish postmenopausal women [29, 39], in postmenopausal Finnish women [32], Chinese women [24], Turkish women [31] and in 313 elderly Belgian women [40]. Also, this findings consist with reported by Albaghaet al. (2001) in Scotlanish women "individual PvuII and XbaI genotypes did not predict BMD at both LS and FN in a multiple linear regression model including age, height, weight" [27]. In contrast, in south Indian PvuII genotypes were significantly different in LS-BMD [22].

The data showed women with the pp genotype had the highest LS-BMD (g/cm^2)values, these differences were not statistically significant. This finding is consistent with the results of Korean women with pp genotype BMD was significantly higher than in women with PP genotype (P= 0.05) [33] and Japanese study [35] are different from the previous observation for postmenopausal Indian women with the PP genotype showed the highest BMD at spine than those with pp genotype (P= 0.028) [23] (P= 0.001) [26]. At FN, pp genotype related to low BMD which were statistically significant in Indian women comparing with PP genotype (P= 0.021) and with Pp genotype (P< 0.05) [23]. For RFN, women with PP genotype showed the highest BMD value than Pp and pp genotypes whereas in Korean study Pp genotype was showed the highest BMD than pp genotype (P= 0.018) and PP genotype [38] whereas in Japanese women the PP genotype was significantly lower in BMD than the pp genotype (P= 0.017) [35], also in Turkish postmenopausal women PP genotype had lower FN-BMD values compared to those with Pp (P= 0.019) and pp genotypes (P= 0.011) [41].

According to a meta-analysis, the XbaI polymorphism was significantly associated with femoral neck BMD and lumbar spine BMD and showed XX genotype had higher BMD values than either Xx or xx [42] and the same result was found in Indian population [22, 23]. We did not find a significant effect of this genotype on BMD at any site but we observed women with XX genotype showed the lowest BMD in lumbar spine and femoral neck as in postmenopausal Korean women [34], Bulgrian women [38] and as in a large population-based Japanese study which had reported findings that contradict with the meta-analysis, BMD for the right femoral neck was significantly lower in those with the XX genotype than in those with the xx genotype (P= 0.033) and Xx and also in lumbar spine [35]. The same result found in Turkish study, women with XX genotype had statistically significance lower FN-BMD values than those with xx genotype (P= 0.056) [41]. Also, Xx genotype showed significantly lower FN-BMD than in women with the xx genotype (P= 0.041) [33], where xx genotype showed the highest lumbar BMD value as in Korean and Japanese studies [35, 34], this conflicted with Indian studies where xx genotype had the lowest BMD significantly and XX genotype had the highest BMD significantly at both lumbar spine and femoral neck (P< 0.001) [23] and for lumbar spine (P= 0.05) [22]. Xx genotype predominated in our study showed the highest femoral neck BMD value, Xx had significantly higher femoral neck BMD than xx as in Indian women (P= 0.013) [23].

Further in case-control study, we examined the association of PvuII and XbaI polymorphism individual with osteoporosis, osteopenia and control groups at LS and FN. No significant difference between the groups in prevalence of these genotypes both site. On other hand, comparison of allele frequencies for XbaI and PvuII polymorphisms between the groups yielded no significant differences. Our results were in accordance with studies in postmenopausal Turkish women, Iranian postmenopausal women and Spanish women that showing no association [31, 37, 43 respectively]. Also, Langdahlet al. (2000) found that postmenopausal Danish women have no association with bone mass and the risk of vertebral fractures [36]. The results of study carried by Ivanovaetal. (2007) found when comparing the ERα genotype frequencies in women with low BMD and normal BMD were statistically significant (P< 0.05), the PP was more common in women with low BMD (26%) than normal BMD (12%) whereas XX genotype was more frequent in women with low BMD (33%) than normal BMD (14%) [38]. Conversely, a significant association of these two SNPs with osteoporosis was found [22, 23]. The frequencies of PP and XX genotypes were significantly high in controls in comparison with osteoporotics whereas pp and xx genotype frequencies were significantly high in osteoporotics when compared with controls which indicated that the PP and

XX genotypes were associated with higher bone density in their population, the risk was significantly higher with genotype xx.

The haplotypes PPXX, PpXx, and ppxx are the most frequent in our subjects as similar in other population [23, 30, 38] and PPXx, PpXX and Ppxx are rare whereas other genotypes (ppXX and PPxx) cannot founded. It is therefore, most likely that X and P are linked and X and p are not linked [39]. We only found 3 normal individuals and no osteoporotic patients at FN with the PPXx genotype and significant associated with higher BMD than PPXX and ppxx. This genotype is represent (3.75%) in our subjects. Higher prevalence of the ppxx genotype was observed in the control group and PPXX and PpXx in osteoporotic and osteopenic at both sites. PPXX genotype was significantly associated with reduced bone mass compared with all other genotypes (P= 0.037) in Japanes study [35] whereas in Danish study no significant relation between ERα haplotypes and BMD [39]. In Iranian women PX were the major haplotypes frequency [37] where in our subject px. There was no statisticaly difference in prevalence of these combined genotypes or haplotypes between osteoporotic patients and normal controls as in Denmark study [39]. Many study were reported Px haplotypes associated significantly with low bone [27]. Rotterdam study was described an association of the px haplotype with reduced BMD and increased fracture risk, with evidence for an allele dose effect (for allele copy, odds ratio= 2.2) [13].

It has been suggested that some of these contrasting results or negative results may have been due to insufficient sample size or because of possible genetic effects were masked by different gene-gene and gene-environment interaction [23, 34].

The mechanisms by which these polymorphisms are associated with BMD remain unclear. The first intron of the estrogen receptor may not known to play a role in gene regulation [27] and how the intronic polymorphism of the ERα gene influences the receptor function [36] but there are some regulatory signal increase the possibility to bind with transcription factors. Analysis of potential regulatory elements surrounding the PvuII and XbaI polymorphisms showed that the "P" allele of PvuII polymorphism (CAGCCG, polymorphic site underlined) disrupts a potential recognition site for the transcription factor AP4 (recognition sequence: CAGCTG), raising the possibility that this polymorphism could influence gene regulation [27] and its positions in an intron near the gene promoter suggests a possible role in either transcription regulation or mRNA processing and stability [36]. Theoretically, polymorphisms in introns could affect mRNA production because it have been shown to contain regulatory sequences such as enhancers of mRNA production and that polymorphisms within the first intron can have significant effect on the level of protein synthesis [39].

Another explanation is a possible linkage disequilibrium between the PvuII and XbaI polymorphisms with other polymorphisms in the ERα gene such as TA tandem repeats polymorphism in the promoter region of ERα gene [36]. Results showed a strong linkage disequilibrium in this subjects, this was obviously due to the short distance (50 pb) between these polymorphisms, which minimized the probability of genetic rearrangements during the crossing-over phase of meiosis [36].

CONCLUSION

We conclude that PvuII and XbaI polymorphisms at the ERα gene individually were not found to be the main genetic determinants of bone mass density. ERα haplotypes (PPXx genotype) may be associated with high BMD values at the FN site in postmenopausal Saudi women.

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The authors have declared no conflict of interest regarding this work.

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