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Association study of estrogen receptor alpha gene polymorphisms with bone mass assessed by quantitative ultrasound in young adults

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Abstract Different genetic variants in estrogen receptor alpha (*ESR1*) have been shown to influence bone phenotypes including quantitative bone ultrasound in elderly. We aimed to investigate the role of *ESR1* polymorphisms in bone mass assessed by calcaneal quantitative ultrasound (QUS) in a population of young adults. The study sample consisted of 466 healthy individuals of Caucasian ancestry (315 females and 152 males) aged 18 and 25 years (median age 20.39 ± 2.70). Six *ESR1* polymorphisms (rs302033, rs2982552, rs2982575, rs2504063, rs2234693-*PvuII* and rs9340799-*XbaI*) were selected as genetic markers and genotyped. Bone mass in the right calcaneus was estimated with QUS. In the unadjusted analysis, rs2982575 polymorphism was significantly associated with quantitative ultrasound parameter in the whole sample ($p = 0.014$, β (95% CI) = −0.114 (−1.023, −0.115). However, after adjusting for multiple confounding factors, this association did not remain significant. For the rest of the selected polymorphisms in *ESR1,* no significant association was observed with calcaneal parameter. Linkage disequilibrium analysis identified a single LD block for the *ESR1* gene including *PvuII* and *XbaI* SNPs (pair-wise $r^2 = 0.66$). Our results revealed a lack of significant association between *ESR1* polymorphisms and calcaneal quantitative ultrasound in a cohort of young adults suggesting that *ESR1* gene do not play a major role in the acquisition of bone mass during early adulthood.

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Introduction

Osteoporosis is a common skeletal disorder characterized by low bone mass and structural deterioration of bone tissue predisposing to an increased risk of fractures [1]. Currently, osteoporosis is a major public health concern affecting over 200 million people worldwide [2]. Genetic factors are considered as determinant in several phenotypes relevant to the pathogenesis of osteoporosis including bone microarchitecture assessed by quantitative ultrasound (QUS) [3]. QUS has been proposed as a non-invasive and alternative method to dual-energy X-ray absorptiometry (DXA) for assessment of bone status [4]. Twin and family studies have estimated that the heritability (h^2) of QUS ranges from 53 to 74% at calcaneus [5, 6]. However, due to the fact that most previous studies investigating the genetic factors contributing to bone mass have been focused on bone mineral density (BMD) measured by DXA, there is limited evidence on the influence of genetic factors in QUS parameters.

Optimizing peak bone mass (PBM), defined as the amount of bone gained at the end of the skeletal maturation, is a crucial factor for the prevention of osteoporosis later in life [7]. PBM is usually acquired around the age of 30 [7, 8] and is known to be genetically determined with heritability estimates reaching 50–85% [9, 10]. Therefore, identifying genetic factors that influence bone accrual during growth is of relevance since it could contribute to the early identification of individuals at risk of developing osteoporosis in the elderly.

Estrogens are known to exert beneficial effects on the regulation of skeletal growth and maintenance of bone mass through the estrogen receptor α (*ERα*, *ESR1*) located at 6q25 [11]. *ESR1*, the major mediator of estrogen action in bone, has been widely studied as a candidate gene in association studies of osteoporosis-related phenotypes. In particular, rs2234693-*PvuII* and rs9340799-*XbaI* polymorphisms have shown association with several osteoporosis outcomes but with inconclusive results [12–21]. In recent years, genome-wide association studies (GWAS) and meta-analysis of GWAS have been performed leading to the identification of several genetic variants, including different *ESR1* polymorphisms, involved in bone phenotypes including BMD and QUS measurements [22–24]. Most of these studies have been conducted in mixed populations with samples of premenopausal, postmenopausal women and men considering wide age ranges. To the best of our knowledge, there have been limited prior studies carried out to investigate genetic markers that influence bone status in early adulthood, a period that corresponds to the most crucial years of PBM attainment.

To contribute to the identification of genetic markers involved in bone mass acquisition early in lifespan, in the present study we aimed to investigate the possible role of *ESR1* as a genetic marker of bone phenotypes assessed by calcaneal QUS in a population of young adults.

Methods

Study subjects

The population study comprised four hundred and sixty-six healthy individuals of Caucasian ancestry (315 females and 151 males, median age 20.38 ± 2.70) from different academic centers of Granada (Spain). Inclusion criteria were subjects between 18 and 25 years of age. Exclusion criteria were history of bone disease, metabolic or endocrine diseases, hormone contraception therapy or treatments that could affect bone mass such as anticonvulsants or systemic corticosteroids for the previous 6 months. This information was collected by asking the subjects about their medical history. None of the subjects were taking calcium or vitamin D supplements. The study was approved by local Ethical Committee and conducted in accordance with the Declaration of Helsinki. A signed and informed consent was obtained for each participant.

Body composition and lifestyle variables

Body weight measurement was performed using Body Composition Analyzer (TANITA BC-418MA®). Height was measured using a Harpenden stadiometer to the nearest 0.1 cm (Holtain 602VR®). Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Physical activity (PA) were determined using the self-administered International Physical Activity Questionnaire (IPAQ) that calculates the respective total minutes for vigorous PA, moderate PA, and walking [25]. A wide range of PA activity level was found in study population from 0 MET (none PA) to 23184.00 MET-min. Most of individuals showed a MET-min value corresponding with moderate PA activity (median 2785.34 MET-min). Dietary calcium intake (DCI) was assessed using the 72-h recall method that covers intake on a Thursday, Friday and Saturday [26]. To improve the accuracy of the food descriptions, standard household measures and pictorial food models were employed during the interviews to define amounts when requested. Food records were converted to nutrient intake with Nutriber® software (Nutriber 1.1.5).

Calcaneal ultrasound

QUS of the right calcaneus was determined by measuring Broadband ultrasound attenuation (BUA) (dB/MHz) using the CUBA clinical ultrasound bone densitometer (McCue Ultrasonics Limited, Compton, Winchester, UK). QUS method has been postulated as a non-invasive, portable, inexpensive and useful tool for assessment bone mass alternative to DXA [4]. Heritability estimates for QUS of the heel appears to have comparable with BMD measured by DXA [5]. Daily calibrations were made with physical phantom to control the long-term stability of the apparatus.

ESR1 genetic markers selection and genotyping

Saliva samples for DNA extraction were collected from study participants using the OG-500 Collection Kit (DNA Genotek Inc, Ontario, Canada). DNA was isolated from saliva samples according to manufacturer´s protocol. Six single-nucleotide polymorphisms (SNPs) of *ESR1* (rs302033, rs2982552, rs2982575, rs2504063, rs2234693 and rs9340799) previously associated with osteoporosis-related phenotypes (BMD and QUS parameters) in candidate gene and/or GWAS were selected as genetic markers in this study [15, 16, 22–24].

Genotyping was performed at the Genomic and Genotyping unit of GENYO center (Pfizer-University of Granada-Junta de Andalucía Centre for Genomics and Oncological Research) using the Open Array technology (Life Technologies, Carlsbad, CA, USA). A TaqMan OpenArray genotyping plate was custom designed including six predesigned TaqMan genotyping assays for each of the selected SNPs. Standard cycling conditions were used as recommended by the manufacturer. Thermal cycling and fluorescence detection were performed using QuantStudio

12 K Flex Real-Time PCR System (Applied Biosystems). Genotyping call rate for the seven Taqman assays included in the array was higher than 95%. To guarantee accuracy of genotyping duplicate samples and negative controls were included in all genotyping arrays, showing 100% identical genotypes.

Statistical analysis

The Chi-squared test was used to determine whether the observed genotype frequencies were compatible with the Hardy–Weinberg equilibrium (HWE). Linear regression analysis was used to analyze the relationships between each SNP in *ESR1* and calcaneus QUS unadjusted and adjusted for confounding factors (age, sex, weight, height, physical activity and calcium intake). Results are reported as a percentage change (β) in a standard deviation (SD) with 95% confidence intervals (95% CI) for each copy of the minor allele. Haploview program (Broad Institute of MIT and Harvard) was used to calculate the linkage disequilibrium (LD) coefficient and determine SNPs haplotypes [27]. *P* values less than 0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA).

The statistical power of the study was estimated using Quanto version 1.2 software (Department of Preventive Medicine, University of Southern California, CA) considering a BUA mean of 81.96 standard deviation (SD) 27.65, 5% type I error, MAFs of 0.34–0.49, 466 individuals and an additive genetic model.

Results

Table 1 summarizes the descriptive characteristics of the 466 study subjects by gender and as a whole. The mean calcaneal ultrasound measurement for the total population was 81.96, SD 27.65 (dB/Mhz), similar to that previously observed for young adults [28, 29].

Position, function and minor allele frequency (MAF) of the six *ESR1* SNPs selected as genetic markers are showed in Table 2. None of the SNPs failed the missingness test (genotyping >0.05) or the frequency test (MAF <0.01) and all SNPs were observed to be in HWE.

Association analyses of SNPs in the *ESR1* gene and calcaneal ultrasound parameter without adjustment for confounding factors in the combined population as well as stratifying individuals according to gender are shown in Table 3. Linear regression analysis revealed that the rs2982575 polymorphism was significantly associated with calcaneal QUS in the whole sample ($p = -0.014$, β (95% CI) $= -0.569$ (-1.023, -0.115). However, this association did not remain statistically significant after adjusting for multiple covariates such as age, sex, weight, height, PA and calcium intake (Table 4). In the unadjusted and adjusted analyses, none of the rest of SNPs in *ESR1* (rs3020331, rs2982552, rs2982575, rs2504063, *XbaI* and *PvuII*) were statistically significantly associated with quantitative ultrasound (Tables 3 and 4).

Table 2 General information for the selected single-nucleotide polymorphisms of *ESR1* (6q25)

Marker ID	Chr position	Function	Alleles[a]	MAF	HWE[b]
rs3020331	151687645	Intron	C/T	0.43	0.10
rs2982552	151738428	Intron	C/T	0.49	0.19
rs2982575	151748656	Intron	C/T	0.49	0.17
rs2504063	151769572	Intron	G/A	0.47	0.51
rs2234693	151842200	Intron	T/C	0.44	0.19
rs9340799	151842246	Intron	A/G	0.34	0.75

HWE Hardy–Weinberg; *MAF* minor allele frequency

[a] The second allele is the minor allele

[b] *p* values of HWE equilibrium test

Table 1 Descriptive characteristic of study participants

Characteristic	Females ($n = 315$)		Males ($n = 152$)		Overall ($n = 466$)	
	Mean	SD	Mean	SD	Mean	SD
Age (years)	20.31	2.67	20.54	2.76	20.38	2.70
Height (m)	1.63	0.06	1.75	0.06	1.67	0.08
Weight (kg)	60.01	10.71	73.55	13.24	64.40	13.20
BMI (kg/m^2)	22.31	3.81	23.70	3.82	22.79	3.87
Calcium intake (mg/day)	787.98	335.17	827.583	365.11	800.81	345.26
Physical activity (MET/min)*	2256.17	(0–15624.00)	3889.25	(0–23184.00)	2785.34	(0–23184.00)
Calcaneal ultrasound (dB/Mhz)	77.62	28.80	91.01	27.31	81.96	27.65

BMI bone mineral index; *BUA* broadband ultrasound attenuation

* MET-min are expressed as mean and range

Table 3 Unadjusted analysis of association between *ESR1* gene and quantitative bone ultrasound

SNP	Calcaneal ultrasound (dB/MHz)								
	Overall		*P* value	Females		*P* value	Males		*P* value
	β	(95% CI)		*β*	(95% CI)		*β*	(95% CI)	
rs3020331	0.015	(−0.481, 0.678)	0.739	0.019	(−0.610, 0.855)	0.742	0.021	(−0.785, 1.019)	0.798
rs2982552	−0.024	(−0.664, 0.383)	0.599	0.030	(−0.451, 0.781)	0.599	−0.112	(−1.555, 0.280)	0.172
rs2982575	−0.114	(−1.023, −0.115)	0.014	−0.067	(−0.966, 0.241)	0.238	−0.083	(−1.054, 0.338)	0.311
rs2504063	−0.027	(−0.616, 0.334)	0.561	−0.076	(−0.923, 0.170)	0.176	0.108	(−0.290, 1.460)	0.188
rs2234693	0.039	(−0.289, 0.721)	0.401	0.025	(−0.465, 0.740)	0.654	0.004	(−0.852, 0.901)	0.956
rs9340799	−0.010	(−0.717, 0.579)	0.835	0.025	(−0.644, 1.022)	0.655	−0.037	(−1.208, 0.762)	0.655

Table 4 Adjusted analysis of association between *ESR1* gene and quantitative bone ultrasound

SNP	Calcaneal ultrasound (dB/MHz)								
	Overall		*P* value	Females		*P* value	Males		*P* value
	β	(95% CI)		*β*	(95% CI)		*β*	(95% CI)	
rs3020331	0.005	(−0.526, 0.585)	0.917	−0.005	(−0.752, 0.692)	0.934	0.004	(−0.876, 0.918)	0.963
rs2982552	−0.028	(−0.662, 0.340)	0.529	0.016	(−0.516, 0.693)	0.773	−0.107	(−1.516, 0.301)	0.188
rs2982575	−0.070	(−0.797, 0.092)	0.120	−0.070	(−0.972, 0.212)	0.207	−0.078	(−1.025, 0.349)	0.333
rs2504063	−0.023	(−0.577, 0.335)	0.602	−0.085	(−0.952, 0.116)	0.124	0.101	(−0.347, 1.446)	0.227
rs2234693	0.005	(−0.461, 0.516)	0.911	0.015	(−0.512, 0.679)	0.782	−0.009	(−0.921, 0.828)	0.917
rs9340799	0.011	(−0.543, 0.704)	0.800	0.037	(−0.538, 1.092)	0.504	−0.029	(−1.165, 0.807)	0.720

Adjusted for sex, age, weight, height, physical activity and calcium intake

Linkage disequilibrium analysis pattern between the tested SNPs in our population is shown in Fig. 1. A single LD block for the *ESR1* gene including *XbaI* and *PvuII* SNPs (pair-wise $r^2 = 0.66$) was identified. The observed *XbaI-PvuII* haplotypes frequencies were: TA 55.0%, CG 34.3% and CA 9.8%. No significant association was observed between none of the possible haplotypic combinations and calcaneal ultrasound after un-adjusted and adjusted regression analyses (data no shown).

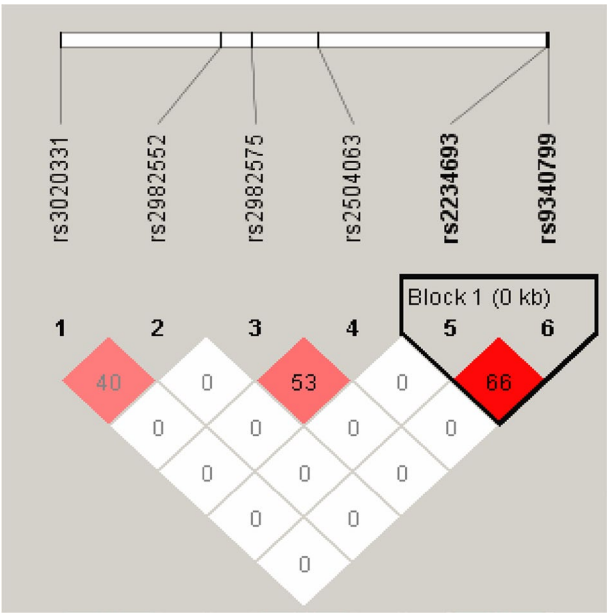

Fig. 1 Location and pair-wise linkage disequilibrium values of *ESR1* polymorphisms in Caucasian young adults. *Darker color* indicates higher LD and *lighter color* indicates less LD (r^2)

Discussion

Due to the growing occurrence of osteoporosis worldwide, community-based genetic screening programs may become particularly relevant to identify individuals at risk of developing the disease. The use of genetic tests, as a novel preventive approach, might be of great significance in the implementation of early strategies to reduce osteoporosis risk [30]. Otherwise, given the affordability of the technology and the potential to provide information on bone properties, quantitative ultrasound has been postulated as a valuable technique for assessing bone mass status in primary

care services. Thus, the identification of genetic markers associated with calcaneal ultrasound parameter has become an issue of particular interest.

Our results revealed no significant associations of six tested SNPs in *ESR1* gene with calcaneal QUS in a population of young adults. Thus, it could be suggested that QUS as a complex phenotype, may be modulated by other genetic markers beyond *ESR1* in early life stage. In accordance with our findings, previous work has evidenced a lack of relationship between other phenotype related to osteoporosis such as BMD and genetic variants of *ESR1*. Interestingly, in a large meta-analysis conducted by the GENOMOS consortium in 18,917 individuals from eight European populations, XbaI and PvuII polymorphisms were not associated with BMD [14]. In this line, in a longitudinal study carried out in a population of Caucasian women, Sowers et al. reported a minimal impact of *XbaI* and *PvuII* genotypes on BMD measurements with respect to other covariates such as BMI [21]. Furthermore, no significant associations between BMD at different sites and *XbaI* and *PvuII* SNPs were reported in previous studies conducted in Caucasian women [19, 20].

In relation to previous studies analyzing the role of *ESR1* in bone mass by assessed QUS, Albagha et al. found a significant association between *XbaI* and *PvuII* haplotypes and calcaneal ultrasound parameter [16]. Similarly, Binh et al. identified that *XbaI-PvuII* polymorphisms were associated with speed of sound (SOS) parameter [12]. First, it is important to consider that although BUA and SOS are both parameters determined by QUS, BUA is influenced by connectivity and trabecular separation and SOS is directly related to the elasticity and density of the bone [4]. Thus, on the basis of our findings together with those from Binh et al., it could be postulated that these genetic variants in *ESR1* might influence SOS but not BUA. In addition, it is important to note that these two previous studies have been conducted in populations of postmenopausal women. Therefore, the possibility that *XbaI* and *PvuII ESR1* genetic variants could influence bone phenotypes only later in life when the level of oestrogens decreases should be considered. To confirm these hypotheses, further replication studies in independent populations of young adults would be of interest.

Regarding rs3020331, rs2982552, rs2982575, rs2504063 SNPs in *ESR1* gene, our findings revealed no significant associations with calcaneal QUS suggesting that these polymorphisms might not play a relevant role in bone gain during early adulthood. In contrast, rs3020331 and rs2982552 polymorphisms were identified as genetic determinants of heel bone properties in European subjects in a meta-analysis of GWAS conducted by GEFOS/GENOMOS (Genetic Markers of Osteoporosis) consortium [24]. Moreover, BMD was reported to be associated with rs2982575 and rs2504063 genetic variants in previous meta-analysis of GWAS conducted in Caucasian women [22–24, 31]. It would be relevant to consider that most meta-analysis, to maximize sample size and statistical power, have been conduced in combined samples of different ages and do not perform stratified analysis by age ranges. As we analyzed a cohort including only young adults (18–25 years), again a possible reason for discrepancies may be caused by differences in population age range between the studies of Koller et al. (20–45 years), Rivadeneira et al. (18–96 years) and Moayyeri et al. (25–80 years) and our study cohort. Additionally, contradictory findings may also be due to differences in sample size and ethnicity background. Therefore, similar to that observed for *XbaI* and *PvuII*, our findings raise the possibility that these other *ESR1* polymorphisms could be genetic markers for osteoporosis-related phenotypes later in life but not for bone mass accrual in early stages. However, given the current limited evidence concerning the potential role of *ESR1* gene polymorphisms in bone gain during early adulthood, it is difficult to completely exclude the possibility that these genetic variants are likely to be causal variants involved in PBM acquisition. Thus, further studies in young adults and functional studies are needed to confirm the preliminary findings of the present study.

There were potential limitations to this study. Due to its cross-sectional design, no causal conclusions can be drawn. In addition, we cannot discard that the limited statistical power could contribute to lack of reported significant associations since the sample size analyzed represents a power of 50% to detect four fold increments of QUS traits in our cohort assuming MAFs of 0.34–0.49 at the 5% significance level.

In summary, we investigated the possible influence of *ESR1* gene polymorphisms on bone mass status assessed by calcaneus QUS in a cohort of young adults. Our results suggest that *ESR1* polymorphisms do not contribute to heel ultrasound measurement in early adulthood.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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