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Polymorphisms in *ALOX12*, but not *ALOX15*, Are Significantly Associated With BMD in Postmenopausal Women

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Abstract The murine arachidonate 15-lipoxygenase gene (*Alox15*) has recently been identified as a negative regulator of peak bone mineral density (BMD). The human *ALOX15* gene shares significant sequence homology with the murine *Alox15* gene; however, the human arachidonate 12-lipoxygenase gene (*ALOX12*) is functionally more similar to the mouse gene. Multiple single-nucleotide polymorphisms (SNPs) in the human *ALOX15* and *ALOX12* genes have previously been reported to be significantly associated with BMD in humans. On the basis of these data, we carried out our own investigation of the human *ALOX15* and *ALOX12* genes and their relationship with hip and spine BMD parameters. The study population consisted of 779 postmenopausal women with a mean (± standard deviation) age of 62.5 ± 5.9 years at BMD measurement and was recruited from a single large general practice in Chingford, northeast London. Three SNPs from *ALOX15* and five from *ALOX12* were analyzed. None of the SNPs that we analyzed in *ALOX15* were significantly associated with any of the BMD parameters or fracture data. However, we found that three SNPs from *ALOX12*, all previously associated with spine BMD in women, were significantly associated with spine and various hip BMD parameters in our cohort ($P = 0.029$–0.049). In conclusion, we found no association between polymorphism in *ALOX15* and BMD phenotypes but were able to replicate previous findings that genetic variation in *ALOX12* seems to play a role in determining bone structure in Caucasian women.

Keywords Bone mineral density · *ALOX15* · *ALOX12* · Osteoporosis

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Introduction

Postmenopausal osteoporosis is a systemic bone disease characterized by low bone mass and disturbed microarchitecture of bone tissue. This results in an increased fragility and is a major risk factor for fracture [1]. Peak bone mass is attained in early adult life but declines in postmenopausal women due to a reduction in estrogen production, with effects on bone and intestinal and renal calcium handling [2]. Thirty percent of postmenopausal women are afflicted with osteoporosis according to the World Health Organization definition of the disease, which is based on the use of dual-energy X-ray absorptiometry (DXA) as a noninvasive measurement of bone mass and size.

In addition to the effects of estrogen and calcium on bone structure and fracture, there is a strong genetic effect on bone structure both for peak bone mass and for bone loss and fracture rates in postmenopausal women. Twin and family studies show that 50–90% of the variance in peak bone mass is heritable [3–9] and 27–68% of the variance in osteoporotic fracture is heritable [10].

It is clear that the genetic effect for common variation in bone structure as assessed by DXA bone mineral density (BMD) is under polygenic control [11]. Many allelic variations in genes contributing to variation in BMD in postmenopausal women have already been identified using the candidate gene approach; some examples include the genes *LRP5, TGFB1, APOE*, and *COL1A1* [12–17]. In addition, the whole-genome scanning approach often using a linkage design has identified many potential positional candidates [18–29]. Thus, although the proof of principle for gene discovery for bone structure is clear, there is much work to be undertaken to identify the most important and consistent gene polymorphisms across populations.

The murine arachidonate 15-lipoxygenase gene (*Alox15*) has recently been identified as a negative regulator of peak BMD [30]. *Alox15* knockout mice had significantly higher BMD than normal mice, and pharmacological inhibitors of the *Alox15* gene product increased BMD in mice and reduced the rate of bone loss in a rat model of osteoporosis [30]. The human *ALOX15* gene shares significant sequence homology with the murine *Alox15* gene; however, the human arachidonate 12-lipoxygenase gene (*ALOX12*) is functionally more similar to the mouse gene.

The 17p13 region of the human genome has been identified as containing a quantitative trait locus (QTL) for BMD of the hip, spine [19], and wrist [18]. The *ALOX15* and *ALOX12* genes are both situated within this locus. Multiple single-nucleotide polymorphisms (SNPs) in the human *ALOX15* and *ALOX12* genes have been previously reported to be significantly associated with BMD in humans [31–33].

These factors make the *ALOX15* and *ALOX12* genes strong positional and functional candidates for the regulation of BMD. On the basis of the published data mentioned above, we carried out our own investigation of the human *ALOX15* and *ALOX12* genes. We chose three SNPs from *ALOX15* and five SNPs from *ALOX12* to analyze in relation to hip and spine BMD parameters in a population of Caucasian postmenopausal women from the United Kingdom.

Materials and Methods

Subjects

The population was recruited in 1988 to participate in a longitudinal epidemiological study of rheumatic diseases [34, 35]. Women between the ages of 45 and 64 were recruited from a single large general practice in Chingford, northeast London, using a population-based method in which 1,353 were approached and 1,003 agreed to participate. DNA samples were obtained from 811 individuals, with hip and spine DXA BMD data obtained from 775 and 779 of these individuals, respectively. This cohort has similar demographics and anthropometry to other UK populations regarding height, weight, smoking status, hysterectomy rates, and the use of hormone replacement therapy [34, 35]. Informed consent was obtained from each individual for a blood sample to be taken for genotyping for study of bone genotype/phenotype effects, and the study was approved by the local ethics committee.

Demographic, Anthropometric, and Lifestyle Factors

Demographic and lifestyle factor data were obtained by questionnaires [36] completed in 1988 for the population. Height and weight were measured by standard methods at this time, and weight was measured again at BMD measurement.

DXA Bone Density and Quantitative Ultrasound Measurements

Bone density measurements were undertaken in 1998, approximately 10 years after the study participants were initially recruited, using a Hologic (Waltham, MA) QDR-1000 densitometer. The measurement coefficient of variation was 0.8% at the spine and 1.4% at the total hip site.

Fracture Status

The subjects were categorized as having had a previous fracture on the basis of self-report.

Genotyping

Genomic DNA was extracted and purified from ethylenediaminetetraacetic acid (EDTA) whole blood obtained from each subject. The genotype for each SNP in each DNA sample was determined using TaqMan (Applied Biosystems, Foster City, CA) allelic discrimination 5′-nuclease assays. Polymerase chain reactions (PCRs) were performed in a reaction volume of 5 μL containing assay-specific primers and allele-specific TaqMan probes. The two labeled probes competitively bind to the template DNA during the PCR cycle, whereby the reporter dye is separated from the quencher dye resulting in an increase in fluorescence from the reporter specific for that allele. Fluorescence was measured by a Victor^2 Multilabel Plate-

Reader (Perkin-Elmer, Waltham, MA). We genotyped the SNPs rs2664593 (assay ID C__12117948_10), rs2619112 (assay ID C__3109408_10), and rs916055 (assay ID C__8685434_10) from *ALOX15* and rs312466 (assay ID C__1552908_10), rs2070590 (assay ID C__15867474_10), rs2292350 (assay ID C__1552902_1), rs1126667 (assay ID C__1552900_1), and rs312462 (assay ID C__749063_20) from *ALOX12*. All the SNPs we studied are either haplotype tagging SNPs (HT-SNPs) [37] or in strong linkage disequilibrium (LD > 0.8) with HT-SNP. This indicates that most of the common variation within these two genes is likely to be represented in our analysis.

Statistical Analysis

Statistical analysis was performed using Statistica for Windows, version 5.1 (Statsoft, Tulsa, OK). Chi-square testing was used to confirm that the SNPs were in Hardy-Weinberg equilibrium. Differences between genotype groups were examined using one-way analysis of variance (ANOVA) or the Kruskal-Wallis rank sum test. BMD data were adjusted for age, weight, and smoking habits by univariate analysis of covariance. Genotype effects on fracture rates were examined using a Chi-square test. Assessment of LD within the *ALOX15* and *ALOX12* genes was undertaken using the JLIN program [38]. Haplotype analysis was undertaken using UNPHASED software [39], which is a suite of programs for association analysis of multilocus haplotypes from unphased genotype data. QTPHASE, a program for quantitative trait analysis under UNPHASED software, was used to investigate the effect of haplotypes on quantitative traits. The program employs likelihood ratio tests in a log-linear model [39]. The comparison of phenotypic means for individual haplotypes with the means for all other haplotypes together was investigated by a step-up test. Throughout, $P \leq 0.05$ was considered significant.

Results

The demographic and morphometric characteristics of the population are detailed in Table 1. All eight of the SNPs genotyped were found to be in Hardy-Weinberg equilibrium (data not shown). The chromosomal position and allele distributions of the SNPs are detailed in Table 2. The three SNPs analyzed in *ALOX15* – rs2664593, rs2619112, and rs916055 – are located in the promoter region, intron 12, and the 3′ untranslated region (UTR), respectively. The five SNPs analyzed in *ALOX12* – rs312466, rs2070590, rs2292350, rs1126667, and rs312462 – are located in the promoter region, intron 2, intron 2, exon 6, and exon 14, respectively.

Table 1 Demographics and bone density of the population

Variables	Chingford population
Demographics (*n*)	811
Height (cm)	161.6 ± 5.9
Weight (kg)	69.1 ± 12.6
Ever smoked (%)	46.5
Prevalent fractures (%)	34.4
Hip DXA BMD (*n*)	775
Total hip (mg/cm^2)	869 ± 128
Femoral neck (mg/cm^2)	747 ± 119
Trochanter (mg/cm^2)	672 ± 107
Intertrochanter (mg/cm^2)	1,018 ± 154
Spine DXA BMD (*n*)	779
Spine L1-L4 (mg/cm^2)	955 ± 155

Results are given as mean ± standard deviation

Effects of Individual SNP Genotypes on Phenotypic Data

The effect of each SNP genotype on hip and spine BMD parameters was examined using ANOVA. Clear trends were observed involving SNPs in *ALOX12;* however, these failed to reach statistical significance (data not shown). However, when the BMD data were adjusted for the covariates age, weight, and smoking (ever smoked), several significant associations became apparent involving three SNPs in *ALOX12* and both hip and spine BMD parameters (Table 3). The *A* allele of rs312466 was significantly associated with an increased total hip, intertrochanter, and spine BMD. At the total hip, intertrochanter, and spine sites, the differences were –3.3%, –3.3%, and –2.0%, respectively, compared to individuals homozygous for the *G* allele and –2.3%, –1.9%, and –3.6%, respectively, compared to individuals with the *AG* heterozygote genotype. The *A* allele of rs2292350 was significantly associated with significantly increased spine BMD compared to the heterozygous group and decreased intertrochanter BMD. At the intertrochanter and spine sites, the differences were 2.3% and –1.0%, respectively, compared to individuals homozygous for the *G* allele and –0.3% and –3.4%, respectively, compared to individuals with the *AG* heterozygote genotype. This latter result may be spurious as it is inconsistent with the trends observed throughout the data presented in Table 3. The *A* allele of rs1126667 was associated with an increased total hip, trochanter, intertrochanter, and spine BMD. At the total hip, intertrochanter, intertrochanter, and spine sites, the differences were –3.5%, –3.6%, –3.5%, and –2.6%, respectively, compared to individuals homozygous for the *G* allele and –2.4%, –2.8%, –2.0%, and –3.7%, respectively, compared to individuals with the *AG* heterozygote genotype. Data on

Table 2 Position and allele distribution of the analyzed SNPs in relation to the *ALOX15* and *ALOX12* genes

SNP	rs2664593	rs2619112	rs916055	rs312466	rs2070590	rs2292350	rs1126667	rs312462
Gene	*ALOX15*	*ALOX15*	*ALOX15*	*ALOX12*	*ALOX12*	*ALOX12*	*ALOX12*	*ALOX12*
Chromosome position*	4491881	4482134	4481583	6839945	6841168	6842396	6843484	6854376
Function/ location*	Promoter region 172 bases from transcription start site	Intron 12	3′ UTR	Promoter region 183 bases from transcription start site	Intron 2	Intron 2	Exon 6: change of aa 261 (Arg/Gln)	Exon 14: synonymous change of aa 634 (Leu/Leu)
Allele distribution (%)	*CC* 4.6	*AA* 23.1	*CC* 11.4	*AA* 19.7	*TT* 19.2	*AA* 17.9	*AA* 19.7	*TT* 0.6
	CG 35.2	*AG* 46.8	*TC* 41.8	*AG* 46.5	*TG* 46.9	*AG* 48.2	*AG* 46.9	*TC* 18.6
	GG 60.2	*GG* 30.1	*TT* 46.8	*GG* 33.8	*GG* 33.9	*GG* 33.9	*GG* 33.5	*CC* 80.8

*From GenBank reference sequence NM_001140, Genome Build 36.1. aa, amino acid

age, height, weight, and smoking (ever smoked) for subjects grouped by rs1126667 genotype are detailed in Table 4; no significant associations were observed. Data for the other SNPs studied also showed no association (data not shown). No significant associations were observed involving any of the SNPs from *ALOX15* (data not shown).

Fifty-one women in the cohort (6.3% of the total population) were on osteoporosis therapy at the time of BMD measurement. The effect of removing these individuals from the analysis was to further strengthen the majority of the associations involving rs312466 ($P = 0.012$–0.095) and rs1126667 ($P = 0.013$–0.055), although it removed the significant associations observed for rs2292350 (data not shown).

Fracture Rates

Two hundred and seventy-nine subjects in the population had suffered a fracture in the past, giving a prevalent fracture rate of 34%. No significant associations between any of the SNPs and prevalent fracture was observed (data not shown).

LD and Haplotype Analysis

Pairwise LD D′ and r^2 values for the three SNPs in *ALOX15* and the five SNPs in *ALOX12* are given in Figures 1 and 2, respectively. These data are consistent with values observed for white American subjects in the International HapMap Project [37]. The three SNPs in *ALOX12* that showed significant associations with BMD parameters were found to be in strong LD with each other. The D′ values were 0.996 (rs312466 vs. rs2292350), 0.997 (rs312466 vs. rs1126667), and 1.0 (rs2292350 vs. rs1126667). The r^2 values were 0.53 (rs312466 vs. rs2292350), 0.98 (rs312466 vs. rs1126667), and 0.55 (rs2292350 vs. rs1126667). There were four haplotypes found in each gene with a frequency of 4% or greater. The four haplotypes found in *ALOX15* are G-G-T (34%), G-A-C (29.7%), C-G-T (18.8%), and G-A-T (13.4%). The four haplotypes found in *ALOX12* are G-G-A-G-C (40.4%), A-T-G-A-C (33.9%), G-G-G-G-C (15.1%), and A-T-G-A-T (9.9%). These haplotypes represented 95.9% and 99.3% of the population for *ALOX15* and *ALOX12*, respectively. None of these haplotypes were significantly associated with any BMD parameters (data not shown).

Discussion

Associations have been reported between multiple SNPs in the human *ALOX15* and *ALOX12* genes and BMD in more than one population [31–33]. Ichikawa et al. [31] reported significant associations between several SNPs in the 5′ region of *ALOX12* (rs3840880, rs9897850, rs312466, rs2292350, rs1126667, rs434473, rs1042357) and lumbar spine BMD in Caucasian men and women. They also reported associations between one SNP in *ALOX15* (rs9894225) and one in *ALOX12* (rs312462) and femoral neck BMD in women, although these were of marginal significance. Urano et al. [32] reported an association between SNP in the 5′ flanking region of *ALOX15* (rs748694) and lumbar spine and total-body BMD in postmenopausal Japanese women. Xiong et al. [33] reported a significant association between SNP in the 5′ region of *ALOX12* (rs2073438) and BMD at the ultradistal radius as well as a haplotype block covering the entire gene and BMD at the hip in their population of Caucasian men and women. They also highlighted *ALOX12* as showing female-specific association with BMD at the spine and hip.

Three of the five SNPs that we analyzed in *ALOX12* had been reported by Ichikawa et al. [31] to be associated with lumbar spine BMD in women (rs312466, rs2292350, rs1126667). We found all three of these SNPs to be significantly associated with spine BMD in our cohort, as well as several hip BMD parameters. We also analyzed the

Table 3 BMD parameters in relation to the allele distribution of *ALOX12* SNPs associated with BMD

	rs312466				rs2292350				rs1126667			
	AA	*AG*	*GG*	*P*	*AA*	*AG*	*GG*	*P*	*AA*	*AG*	*GG*	*P*
Hip DXA BMD	*n* = 148	*n* = 347	*n* = 255		*n* = 136	*n* = 367	*n* = 258		*n* = 150	*n* = 355	*n* = 256	
Total hip (mg/cm^2)	888 (886) ± 130	868 (867) ± 132	860 (863) ± 121	0.042 (0.188)	868 (871) ± 114	864 (865) ± 131	882 (877) ± 131	0.119 (0.505)	889 (887) ± 131	868 (867) ± 132	859 (861) ± 119	0.029 (0.131)
Trochanter (mg/cm^2)	688 (686) ± 112	669 (668) ± 110	667 (669) ± 100	0.088 (0.203)	675 (678) ± 104	668 (668) ± 109	679 (676) ± 108	0.331 (0.544)	689 (688) ± 113	670 (669) ± 110	665 (667) ± 100	0.048 (0.128)
Intertrochanter (mg/cm^2)	1,038 (1,037) ± 155	1,019 (1,018) ± 160	1,005 (1,008) ± 147	0.047 (0.199)	1,013 (1,017) ± 131	1,010 (1,012) ± 159	1,036 (1,031) ± 159	0.047 (0.313)	1,039 (1,037) ± 157	1,019 (1,018) ± 161	1,004 (1,006) ± 143	0.039 (0.148)
Femoral neck (mg/cm^2)	764 (763) ± 118	742 (741) ± 121	744 (746) ± 117	0.072 (0.161)	744 (746) ± 109	745 (746) ± 124	757 (753) ± 118	0.319 (0.770)	765 (764) ± 120	742 (742) ± 121	742 (744) ± 115	0.056 (0.138)
Spine DXA BMD	*n* = 150	*n* = 347	*n* = 256		*n* = 137	*n* = 368	*n* = 259		*n* = 152	*n* = 355	*n* = 257	
L1-L4 (mg/cm^2)	978 (978) ± 166	944 (943) ± 152	959 (960) ± 150	0.049 (0.052)	974 (975) ± 146	942 (943) ± 152	964 (962) ± 164	0.042 (0.091)	979 (979) ± 168	944 (943) ± 153	954 (954) ± 149	0.039 (0.051)

Results are given as adjusted mean (unadjusted mean) ± standard deviation. *P* values are given as values for adjusted means (unadjusted means). Adjusted means are adjusted for the covariates age, weight, and smoking habits

Table 4 Demographics of study subjects grouped by rs1126667 genotype

	rs1126667			
	AA (*n* = 150)	*AG* (*n* = 355)	*GG* (*n* = 256)	*P*
Age (years)	62.4 ± 6	62.4 ± 5.9	62.8 ± 5.8	0.777
Height (cm)	162.2 ± 5.6	161.4 ± 6.0	161.9 ± 6.0	0.200
Weight (kg)	68.7 ± 11.8	68.8 ± 12.6	69.6 ± 13	0.672
Ever smoked (%)	53.9	44.0	42.4	0.080

Results are given as mean ± standard deviation

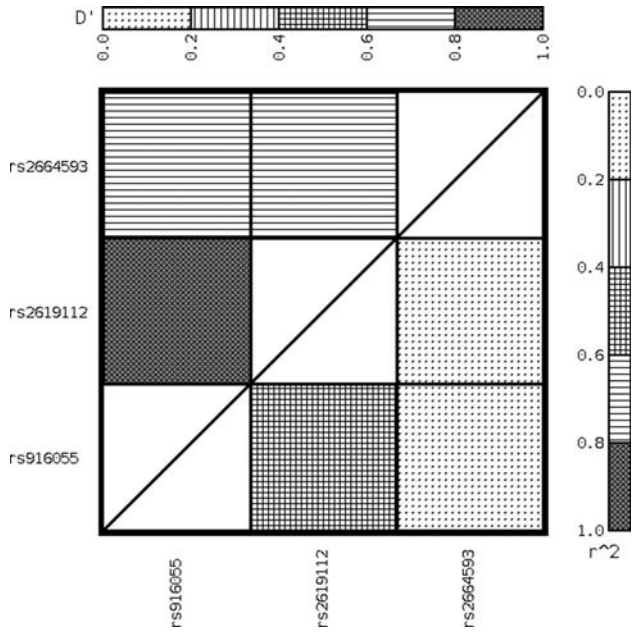

Fig. 1 LD for three SNP pairs in the *ALOX15* gene. Gray-shaded coding represents the strength of LD according to the scale shown on the top (D′) and the right (r^2)

ALOX12 SNP reported by Ichikawa et al. [31] to be associated with femoral neck BMD in women (rs312462). We did not find any associations between this SNP and spine or hip BMD parameters in our cohort.

The strongest association reported by Ichikawa et al. [31] in women related to rs312466 and lumbar spine BMD. They reported an association between the *A* allele at this SNP site and an increased spine BMD in a recessive allele expression mechanism. We observed an association between the *A* allele of rs312466 and increased total hip, intertrochanter, and spine BMD, although the expression mechanism is not clear. The SNPs rs2292350 and rs1126667 are in strong LD with rs312466, and these generally show consistent relationships with BMD, the rare allele being associated with increased mass.

In relation to the possible functional role of the SNPs, rs312466 is located within the promoter region of *ALOX12*

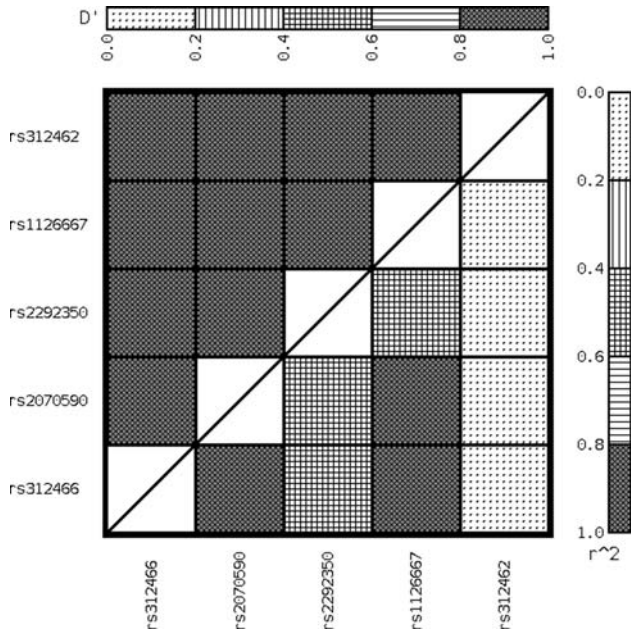

Fig. 2 LD for five SNP pairs in the *ALOX12* gene. Gray-shaded coding represents the strength of LD according to the scale shown on the top (D′) and the right (r^2)

and is predicted to lie within a putative vitamin D receptor binding site [1,25-$(OH)_2$D response element] [31]. It is possible that polymorphism at this SNP site affects the binding abilities of transcriptional machinery, which in turn affects expression of the gene. Polymorphism at rs1126667, the SNP where we observed our strongest associations with BMD parameters, causes a nonsynonymous coding change of amino acid 261 (Arg/Gln). This amino acid substitution could well affect the function of the ALOX12 protein. The fact that the glutamine residue at this position is highly conserved among species would lend support to the theory that a change to arginine would affect the function of the gene product. The SNP rs2292350 is in an intron and is less likely to play a functional role, which is supported by generally weaker evidence for association compared to rs312466 and rs1126667.

Lipoxygenases are a class of iron-containing nonheme enzymes that catalyze the incorporation of molecular oxygen into polyunsaturated fatty acids such as linoleic and arachidonic acids. Arachidonic acid is converted to hydroperoxyeicosatetraenoic acid (HPETE) in the lipoxygenase reaction, which is then converted to hydroxyeicosatetraenoic acid (HETE) by peroxidase. Murine Alox15 preferentially incorporates oxygen at carbon-12 of arachidonic acid (yeilds 15 and 12-HETE at a ratio of 1:3 [40]), whereas human ALOX15 preferentially incorporates oxygen at carbon-15 (yeilds 15 and 12-HETE at a ratio of 12:1 [41]). The human *ALOX15* gene shares 75% sequence homology with the murine *Alox15* gene, whereas the human *ALOX12* gene shares 57% sequence homology with murine Alox15. However, since human ALOX12 produces only 12-HETE, murine Alox15 is functionally more similar to human ALOX12 than human ALOX15 in terms of reaction products.

The products of the lipoxygenase reactions serve as endogenous ligands for the peroxisome proliferator-activated receptor-γ (PPARγ); activation of this pathway in marrow-derived mesenchymal progenitors inhibits osteoclastogenesis and increases adipogenesis [42, 43]. PPARγ-haploinsufficient mice display increased bone mass with increased osteoblastogenesis and decreased adipogenesis [44]; also, it has been shown that oxidized lipids inhibit osteoblastic differentiation from preosteoblasts *in vitro* [45, 46] and bone formation *in vivo* [47]. In theory, a decrease in the quantity or function of human ALOX12 could result in a corresponding decrease in PPARγ activation and a subsequent increase in osteoclastogenesis with a positive effect on BMD. Since the decrease in bone volume observed with osteoporosis is accompanied by an increase in marrow adipose tissues [48, 49], it seems plausible that the PPARγ pathway may play a role in the etiology of the disease, possibly through upregulation.

In this study, we investigated the role of selected *ALOX15* and *ALOX12* polymorphisms in a large, well-characterized population of ethnically homogeneous Caucasian women. For a polymorphism such as rs312466 with a minor allele frequency of 0.43, our calculations suggest this study has the power to detect a QTL explaining a 3% difference in BMD (80% power at 0.05 level) [50]; however, for a polymorphism with a lower allele frequency, such as rs2664593, the power is slightly less (i.e., 4% difference in BMD with 80% power at 0.05 level). Thus, one limitation of this study is that small effects attributable to SNPs in *ALOX15* (e.g., <2%) may not be detectable in our cohort, but such effects are likely to be of little clinical relevance.

Although there are many similarities between our results and those published by Ichikawa et al. [31], it should be pointed out that there are still discrepancies between the findings of the two studies. The expression mechanism of each allele at each SNP site is not clearly defined in our study, whereas Ichikawa et al. [31] report a recessive allele expression mechanism occurring with the *A* allele of rs312466 in women (although data relating to the alleles responsible for the effects seen at rs2292350, rs1126667, and rs312462 were not presented). They reported an association between rs312462 and femoral neck BMD in women; our data largely support this given the observed trend but did not achieve statistical significance. It should be pointed out that the study by Ichikawa et al. [31] used a cohort of women with a mean age of 33.2, whereas our population of women had a mean age of 62.5 years. Therefore, the possible effects of age-related bone loss and

menopausal status impacting the relationship between *ALOX12* polymorphisms and BMD in women must also be considered.

In conclusion, our data support the findings of Ichikawa et al. [31] that rs312466, rs2292350, and rs1126667, all of which are in the 5′ region of *ALOX12*, are significantly associated with spine BMD in women; and we extend those findings to include certain hip BMD parameters. We were not able to support the findings that rs312462, SNP in the 3′ region of *ALOX12*, is associated with femoral neck BMD in women. Both studies appear to be in good agreement that polymorphism in *ALOX15* does not appear to have a substantial influence on BMD parameters in women. More studies need to be carried out on *ALOX12* to investigate the role that this gene has to play in the regulation of bone structure.

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