CYP11A1 expression in bone is associated with aromatase inhibitor-related bone loss

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Abstract

Aromatase inhibitors (AIs) used as adjuvant therapy in postmenopausal women with hormone receptor-positive breast cancer cause diverse musculoskeletal side effects that include bone loss and its associated fracture. About half of the 391 patients treated with AIs in the Barcelona–Aromatase induced bone loss in early breast cancer cohort suffered a significant bone loss at lumbar spine (LS) and/or femoral neck (FN) after 2 years on AI-treatment. In contrast, up to one-third (19.6% LS, 38.6% FN) showed no decline or even increased bone density. The present study aimed to determine the genetic basis for this variability. SNPs in candidate genes involved in vitamin D and estrogen hormone-response pathways (CYP11A1, CYP17A1, HSD3B2, HSD17B3, CYP19A1, CYP2C19, CYP2C9, ESR1, DHCR7, GC, CYP2R1, CYP27B1, VDR and CYP24A1) were genotyped for association analysis with AI-related bone loss (AIBL). After multiple testing correction, 3 tag-SNPs (rs4077581, s11632698 and rs900798) located in the CYP11A1 gene were significantly associated (P<0.005) with FN AIBL at 2 years of treatment. Next, CYP11A1 expression in human fresh bone tissue and primary osteoblasts was demonstrated by RT-PCR. Both common isoforms of human cholesterol side-chain cleavage enzyme (encoded by CYP11A1 gene) were detected in osteoblasts by western blot. In conclusion, the genetic association of CYP11A1 gene with AIBL and its expression in bone tissue reveals a potential local function of this enzyme in bone metabolism regulation, offering a new vision of the steroidogenic ability of this tissue and new understanding of AI-induced bone loss.

Key Words
- CYP11A1
- aromatase inhibitors
- bone loss
- breast cancer
- genetic association

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Introduction

Aromatase inhibitors (AIs) are commonly used as adjuvant therapy in postmenopausal women with hormone receptor-positive breast cancer. Although AIs are generally well tolerated with few serious adverse events, a number of musculoskeletal side effects affecting quality of life have been noted and often lead to discontinuation of therapy (Eastell et al. 2006, Henry et al. 2008).

The most common side effects reported with AIs are exacerbation of menopausal symptoms as a result of low estrogen levels; these include pain syndromes, bone loss and associated fracture (Eastell et al. 2008, Laroche et al. 2014). Several prospective studies and clinical trials have noted the need for assessment and treatment of these musculoskeletal adverse events (Reid et al. 2008, Bruksy et al. 2009, Servitja et al. 2012). In particular, bone mineral density (BMD) reduction and fracture incidence associated with AI therapy can be significantly reduced by oral bisphosphonates (BP) treatment (Bouvard et al. 2014).

We are currently conducting a prospective, non-selected, observational, clinical cohort study (Barcelona–Aromatase induced bone loss in early breast cancer (B-ABLE)) to investigate the musculoskeletal side effects of AI in postmenopausal women with early breast cancer. Previously, we reported a genetic association with AI-related arthralgia and therapy discontinuation in this cohort. SNPs in CYP17A1, CYP27B1 and VDR genes (involved in the estrogen and vitamin D signaling pathways) could predict the risk of pain syndromes (Garcia-Giralt et al. 2013).

About half of the B-ABLE cohort patients had more than 3% bone loss after 2 years on AI treatment at lumbar spine (LS) and/or at femoral neck (FN). In contrast, up to a third of patients did not lose, or even gained, bone density. Clearly, susceptibility to side effects from AI-treatment varies between individuals and this variability may be explained, in part, by individual genetic background.

It is well known that estrogen and vitamin D endocrine systems play an important role in bone metabolism (Felson et al. 1993, Cranney et al. 2007). Depletion of any of these hormones leads to a bone mass reduction, which in some cases is very severe (Eriksen & Glerup 2002). In postmenopausal women, the only sources of estrogens are through the aromatase function in peripheral tissues and through steroid sulfatase activity (Reed et al. 2005), which is also active in bone (Muir et al. 2004). Therefore, the aromatase inhibition, especially at the bone tissue level, would cause a major BMD loss (Bouvard et al. 2014). However, remnant estrogen levels after aromatase inhibition may modulate the degree of bone loss. Hence, the estrogen synthesis pathway can play an important role in the final amount of available estrogen. In addition, serum 25-hydroxy-vitamin D (25(OH)D) status affects the rate of bone loss in patients with AI therapy and vitamin D supplementation to target threshold of ≥40 ng/ml protects against AI-related bone loss (AIBL) (Prieto-Alhambra et al. 2011). Therefore, both the vitamin D metabolism and the response to vitamin D treatment may be essential to the regulation of bone metabolism.

We hypothesized that genetic variants in genes involved in the estrogen and vitamin D synthesis and response can explain part of the variability in bone loss observed in AI-treated patients. Hence, we genotyped tag-SNPs in the genes of the estrogen and vitamin D signaling pathway to analyze their association with bone loss at 1 and 2 years after starting AI treatment.

Materials and methods

Ethics statement

The study protocols were approved by the corresponding ethics committee (Hospital del Mar Human Research Ethics Committee). The approved protocols for obtaining DNA from blood samples and fresh bone (and primary osteoblasts) from hip samples otherwise discarded at the time of orthopedic surgery were explained to potential study participants, who provided written informed consent before being included in the study.

Study population

Details on study design, recruitment methods and study population have been described elsewhere (Nogues et al. 2010) and are briefly summarized below. B-ABLE is a prospective, observational, clinical cohort study. Postmenopausal women diagnosed with hormone receptor-positive breast cancer and candidates for AI treatment attending the outpatient Breast Cancer Unit at Hospital del Mar (Barcelona, Spain) were consecutively invited to participate in the study. Eligible participants with history of any bone disease, rheumatoid arthritis, metabolic or endocrine diseases, prior diagnosis of Paget’s bone disease or osteomalacia, oral corticosteroids, or any other bone-active drug except tamoxifen were excluded.

Interventions  Participants were treated with AIs (letrozole, exemestane or anastrozole) according to the American Society of Clinical Oncology (ASCO)
recommendations (Winer et al. 2005), starting within 6 weeks after surgery or 1 month after the last cycle of chemotherapy, or alternatively, after 2 to 3 years of tamoxifen therapy.

All patients received supplemental calcium and vitamin D tablets (1000 mg and 800 IU daily), and those with baseline vitamin D deficiency (<30 ng/ml) received an additional dose of 16 000 IU of oral cholecalciferol every 2 weeks.

**Measurements**  
**Bone mineral density** At baseline and annually thereafter until end of treatment, BMD was measured at the LS (L1–L4), FN and total hip using a dual-energy X-ray densitometer QDR 4500 SL (Hologic, Waltham, MA, USA), following the usual protocol in our unit. In our department, the in vivo CV of this technique ranges from 1.0% at LS to 1.65% at FN. Images were subjected to rigorous scrutiny, especially when interpreting follow-up scans; those presenting scan artifacts causing falsely elevated BMD (degenerative disc disease) were excluded from analysis.

**Other assessments** Information on a large number of clinical variables was recorded at the time of enrollment, including age at recruitment, age at menarche and menopause, lactation, parity, previous chemotherapy and radiotherapy, adjuvant treatments, weight, height, plasma levels of 25(OH)D, calcium intake and smoking status.

**Selection of candidate genes**

Genes encoding key factors in the synthesis and response of the estrogens and vitamin D hormones were selected for the genetic association study (Fig. 1). Eight candidate genes were selected to test the estrogen hypothesis: CYP11A1, CYP17A1, HSD3B2, HSD17B3, CYP19A1, CYP2C19, CYP2C9 and ESR1. Six genes were selected for the vitamin D hypothesis: GC, CYP27B1, VDR and CYP24A1.

**Selection of SNPs**

SNPs were selected on the basis of the following criteria: i) minor allele frequency (MAF) >0.05; ii) haplotype tagging (tag-SNPs) according to HapMap project in CEU population; iii) putative functional polymorphisms; and iv) previous association with other musculoskeletal phenotypes: plasma 25(OH)D concentrations (Wang et al. 2010), BMD (Kobayashi et al. 1996, Langdahl et al. 2000, Zarrabeitia et al. 2004, Enjuanes et al. 2006) and AI-related-arthralgia (Garcia-Giralt et al. 2013).

**DNA extraction and polymorphism genotyping**

DNA extraction from peripheral blood was performed at the LGC genomics facilities. Polymorphism genotyping was carried out using KASPar v4.0 genotyping systems at the LGC genomics facilities (LGC, Hoddesdon, Hertfordshire, UK). To ensure genotyping quality, a random sample (5% of the total number of samples) was also genotyped in a separate control plate. There was 100% concordance between these results.

**Statistical analyses**

Hardy-Weinberg equilibrium (HWE) was calculated by $\chi^2$. HWE $P$ values for all the SNPs were calculated using the Tufts University website template (http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20%20HW%20calculator.xls).

The outcome was AIBL, calculated as the cumulative LS and FN BMD percent changes at each assessment (1 and 2 years follow-up). BMD changes from baseline were evaluated using Student $t$-test for paired samples.

Multivariate linear regressions (log-additive, dominant and recessive models) were used to assess the association between the studied SNPs and AIBL at 1 and 2 years follow-up. Models were adjusted for age, BMI and previous tamoxifen and chemotherapy. Potential confounding for baseline 25(OH)D concentrations and type of AI was assessed. To minimize false discovery due to multiple testing, we performed the false discovery rate correction (FDR) (Benjamini et al. 2001), accepting all predictions with $q<0.05$ as significant.

Haplotype frequencies for the significant SNPs on CYP11A1 were estimated using expectation maximization algorithm. Association between haplotypes and AIBL was tested by haplo.glm, based on a glm regression analysis, controlling for age, BMI and previous tamoxifen and chemotherapy. We assumed an additive model, where haplotype-specific parameters represent the coefficient of AIBL. The most common haplotype was used as the reference.

All analyses were two-tailed. Statistical analyses were performed using R for Windows version 2.13.2 (packages: SNPassoc, foreign, multtest and haplo.stats).

**Human osteoblast culture**

Human primary osteoblasts (hOB) were obtained from trabecular bone of three post-menopausal women who...
underwent hip replacement due to osteoarthritis. Bony tissue was cut up into small pieces, washed in phosphate buffered solution (PBS, Gibco by Life Technologies) to remove non-adherent cells and placed on a 140 mm culture plate. Samples were incubated in DMEM (Gibco, Invitrogen), supplemented with 10% FBS (Sigma–Aldrich), 100 U/ml penicillin/streptomycin (Sigma–Aldrich), 0.4% fungizone (Gibco by Life Technologies) and 100 μg/ml ascorbic acid (Sigma). This allowed osteoblastic cells to migrate from the fragments and proliferate. All experiments were performed at passage 0.

Total RNA extraction

RNA from hOB cultures \((n=2)\) was extracted using High Pure RNA Isolation kit (Roche Diagnostics) according to manufacturer instructions. Total bone RNA was obtained from FN trabecular bone of two post-menopausal women who underwent hip replacement due to osteoarthritis. Bone samples were cut up into small fragments, washed in PBS and incubated 10 min in Tri-Reagent solution (Sigma–Aldrich). After a vigorous vortex, chloroform (Merk, Darmstadt, Germany) was added to the sample, followed by centrifugation for 15 min at 12,000 g. The upper water phase was collected and extraction continued according to manufacturer instructions (TRI Reagent Protocol, Sigma–Aldrich). Concentration of the purified RNA was analyzed on a spectrophotometer (Nanodrop, Thermo Fisher Scientific, Inc., Wilmington, DE, USA). RNA preparations were considered acceptable for RT-PCR when their A260 (nm)/A280 (nm) ratios were > 1.8. The integrity of the RNA preparations was verified by 1% (w/v) agarose (Agarose D-1 Low EEO, Conda, Pronadisa, Madrid, Spain) bleach gel (1% (v/v) commercial chlorine bleach) electrophoresis (Aranda et al. 2012).

Figure 1
Schematic diagram of metabolites and enzymes of steroid and vitamin D signaling pathways. Selected genes for the association analyses are marked in grey boxes and bold font.
CYP11A1 and CYP17A1 mRNA expression analysis

RT-PCR was performed using the High Capacity cDNA RT Kit (Applied Biosystems) according to manufacturer protocol. The reaction volume was set to 20 µl containing 1.1 µg of total RNA.

CYP11A1 primer pairs for PCR were: F 5'-CCGTGACCCTGCAGAGATAT-3' and R 5'-TGGTCATCTCTAGCAGCG-3', encompassing exons 7 and 8 of isoform 1 (NM_000781.2) and resulting in an amplification product of 249 base pairs.

CYP17A1 primer pairs for PCR were: F 5'-AAGGGCAAGGACTTCTCTGGGCGG-3' and R 5'-AGGGTTTTGTTGGGGAAAAT-3', encompassing exons 2 and 3 of isoform 1 (NM_000102.3) and resulting in an amplification product of 422 base pairs.

Western blotting

Cultured hOBs (*n* = 5) were washed with PBS and then incubated with loading buffer (62.5 mM Tris–HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 0.004% (w/v) SDS) and resulting in an amplification product of 422 base pairs.

Table 1  Baseline patient characteristics

<table>
<thead>
<tr>
<th>Patient characteristic (N=391)</th>
<th>Mean ± s.d.</th>
<th>Median (IQ)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years) (s.d.)</td>
<td>61.3 ± 8.5</td>
<td>49.3 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>Mean BMI (s.d.)</td>
<td>29.5 ± 5.4</td>
<td>12 (3)</td>
<td></td>
</tr>
<tr>
<td>Mean age of menopause onset (years) (s.d.)</td>
<td>23.3 ± 4.5</td>
<td>3 (11)</td>
<td></td>
</tr>
<tr>
<td>Median age of menarche (IQ)</td>
<td>12 (3)</td>
<td>2 (2)</td>
<td></td>
</tr>
<tr>
<td>Median breastfeeding (in months) (IQ)</td>
<td>3 (11)</td>
<td>2 (2)</td>
<td></td>
</tr>
<tr>
<td>Prior tamoxifen therapy n (%)</td>
<td>159 (40.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior chemotherapy n (%)</td>
<td>235 (60.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aromatase inhibitor n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Letrozole</td>
<td>262 (67.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exemestane</td>
<td>124 (31.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anastrozole</td>
<td>5 (1.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone mineral density</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumbar spine</td>
<td>0.961 ± 0.109</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral neck</td>
<td>0.747 ± 0.085</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IQ, interquartile range; s.d., standard deviation.
bromophenol blue and 30% (v/v) 2-mercaptoethanol) containing complete protease inhibitors solution (Protease Inhibitor Cocktail Tablets, Roche). Cell lysates were boiled 5 min at 95°C, fractionated by SDS–PAGE and transferred to a nitrocellulose membrane using a Mini-Protean cell (Bio-Rad Laboratories S.A.) according to manufacturer instructions.

The human cholesterol side-chain cleavage enzyme (encoded by \textit{CYP11A1} gene) and the steroid 17-alpha-monooxygenase (encoded by \textit{CYP17A1} gene) were detected using the anti-CYP11A1 rabbit polyclonal antibody (ab175408, Abcam, Cambridge, UK) in a 1:500 dilution in blocking solution and the anti-CYP17A1 rabbit polyclonal antibody (ab80206, Abcam) in a 1:50 dilution, respectively. The anti-actin mouse monoclonal antibody (ab3280, Abcam) was used as loading control in a 1:500 dilution. Horseradish peroxidase-conjugated anti-rabbit antibody (Thermo scientific, Rockford, IL, USA), at 1:2500 dilution, was used for detection of the cholesterol side-chain cleavage enzyme and steroid 17-alpha-monooxygenase. Horseradish peroxidase-conjugated polyclonal Goat Anti-mouse (Dako Denmark, Glostrup, Denmark) was used for actin detection at 1:2500 dilution.

Results

Baseline patient characteristics and AIBL assessment

A total of 531 women were recruited from February 2006 to February 2013 in the B-ABLE cohort. Of these, 391 were not treated with BP and were selected for the genotyping study. Patients with spine scan artifacts or bilateral prostheses (n=12) were excluded from the study (Fig. 2). Three patients developed osteoporosis during the second year of AI treatment. They were immediately offered oral BP treatment and, from then on, their recorded data were also omitted from analysis.

Baseline patients eligible for the AIBL study totaled 296 for LS and 379 for FN. Of these, 263 patients with LS measurement (88.8%) and 346 (91.3%) with FN measurement completed 1 year of AI-treatment. At 2 years, 233 patients (78.7%) with LS and 307 (81%) with FN measurements had available data. Baseline clinical characteristics of the study participants are shown in Table 1.

The intra-individual cumulative percent change in BMD at LS was $-2.79\%$ (95% CI: $-3.11$ to $-2.46$; $P<0.001$) at 1 year and $-3.82\%$ (95% CI: $-4.24$ to $-3.40$; $P<0.001$) at 2 years and at FN was $-1.22\%$ (95% CI: $-1.64$ to $-0.80$; $P<0.001$) at 1 year and $-2.14$ (95% CI: $-2.62$ to $-1.67$; $P<0.001$) at 2 years (Fig. 3).

Analysis of patient distribution by categories of BMD change revealed that 80.3 and 61.6% of patients experienced BMD loss both at LS and FN, respectively, after 2 years on AI therapy, with more than half of them showing losses $>3\%$. In contrast, 19.8 and 38.4% of patients assigned to BP-non treated group at baseline reached T-scores $>2.0$ and were immediately started on active treatment.

BMD, bone mineral density; LS, lumbar spine; FN, femoral neck.
showed no BMD changes or increases, at LS and FN, respectively (Fig. 4).

Genetic association with AIBL

MAF and HWE P value for each genotyped SNP in the B-ABLE cohort are reported in Table 2. Three SNPs (rs4077581, rs11632698 and rs900798) in the CYP11A1 gene were associated with BMD variation in FN at 2 years of AI treatment. P values and β coefficients (95% CI) are shown in Table 3.

The haplotype frequencies predicted by the expectation-maximization algorithm for these SNPs are shown in Table 4. The haplotype TGG (reference haplotype) was the most frequent haplotype in our cohort. Inheritance of one copy of the haplotype CAT was associated

Table 2  Characteristics of the SNPs selected for genotyping

<table>
<thead>
<tr>
<th>Hypotheses</th>
<th>Locus</th>
<th>SNP ID</th>
<th>Alleles (R/A)</th>
<th>MAF (*)</th>
<th>HWE P value*</th>
<th>Genotyping efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroid pathway</td>
<td>CYP19A1</td>
<td>rs1062033</td>
<td>C/G</td>
<td>0.45 (G)</td>
<td>0.538</td>
<td>98.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs4775935</td>
<td>T/C</td>
<td>0.47 (T)</td>
<td>0.837</td>
<td>96.9</td>
</tr>
<tr>
<td></td>
<td>CYP17A1</td>
<td>rs4919687</td>
<td>G/A</td>
<td>0.30 (A)</td>
<td>0.113</td>
<td>98.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs6163</td>
<td>C/A</td>
<td>0.43 (A)</td>
<td>0.063</td>
<td>98.9</td>
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<tr>
<td></td>
<td>CYP11A1</td>
<td>rs4077581</td>
<td>T/C</td>
<td>0.36 (C)</td>
<td>0.741</td>
<td>97.9</td>
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<td></td>
<td></td>
<td>rs900798</td>
<td>G/T</td>
<td>0.39 (T)</td>
<td>0.392</td>
<td>97.4</td>
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<tr>
<td></td>
<td></td>
<td>rs11632698</td>
<td>G/A</td>
<td>0.47 (A)</td>
<td>0.259</td>
<td>97.4</td>
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<td>HSD3B2</td>
<td>rs2854964</td>
<td>A/T</td>
<td>0.34 (T)</td>
<td>0.910</td>
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<td>HSD17B3</td>
<td>rs3765948</td>
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<td>0.15 (C)</td>
<td>0.845</td>
<td>99.5</td>
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<td>CYP2C19</td>
<td>rs12248560</td>
<td>C/T</td>
<td>0.19 (T)</td>
<td>0.631</td>
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<td>rs3758581</td>
<td>G/A</td>
<td>0.07 (A)</td>
<td>0.000</td>
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<td></td>
<td>rs4244285</td>
<td>G/A</td>
<td>0.14 (A)</td>
<td>1.000</td>
<td>97.5</td>
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<td></td>
<td>CYP2C9</td>
<td>rs28371674</td>
<td>C/T</td>
<td>0.16 (T)</td>
<td>0.460</td>
<td>98.0</td>
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<td>ESR1</td>
<td>rs2504063</td>
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<td>0.45 (A)</td>
<td>0.182</td>
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<td>Vitamin D pathway</td>
<td>CYP27B1</td>
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<td>0.24 (T)</td>
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<td></td>
<td>rs4646536</td>
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<td>0.25 (G)</td>
<td>1.000</td>
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<td></td>
<td>GC</td>
<td>rs3755967</td>
<td>C/T</td>
<td>0.31 (T)</td>
<td>0.722</td>
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<td>CYP24A1</td>
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<td>0.22 (A)</td>
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<td></td>
<td></td>
<td>rs11907350</td>
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<td>0.04 (A)</td>
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<tr>
<td></td>
<td>VDR</td>
<td>rs4809957</td>
<td>A/G</td>
<td>0.24 (G)</td>
<td>0.160</td>
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<tr>
<td></td>
<td></td>
<td>rs2544037</td>
<td>A/G</td>
<td>0.39 (G)</td>
<td>0.286</td>
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<td></td>
<td></td>
<td>rs11568820</td>
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<td>0.23 (T)</td>
<td>0.670</td>
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<td>0.35 (A)</td>
<td>0.434</td>
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<td></td>
<td></td>
<td>rs17879735</td>
<td>C/A</td>
<td>0.47 (A)</td>
<td>0.919</td>
<td>98.2</td>
</tr>
</tbody>
</table>

* R, reference allele; A, alternative allele; ma, minor allele frequency; HWE, Hardy-Weinberg equilibrium. *(In B-ABLE cohort). In bold, HWE P values deviated from HWE.

Table 3  Associated SNPs with FN BMD variation in linear-regression analysis

<table>
<thead>
<tr>
<th>Locus</th>
<th>SNP</th>
<th>Genotype groups</th>
<th>n</th>
<th>β coefficient (95% CI)</th>
<th>P value</th>
<th>FDR adj. P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP11A1</td>
<td>rs4077581</td>
<td>T/T</td>
<td>122</td>
<td>0.98 (0.30–1.66)</td>
<td>0.005b</td>
<td>0.045</td>
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<tr>
<td></td>
<td></td>
<td>T/C</td>
<td>138</td>
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<tr>
<td></td>
<td></td>
<td>C/C</td>
<td>43</td>
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<tr>
<td></td>
<td>rs900798</td>
<td>G/G</td>
<td>113</td>
<td>1.06 (0.39–1.72)</td>
<td>0.002b</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
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<td>G/T</td>
<td>140</td>
<td></td>
<td></td>
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<tr>
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<td>50</td>
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<tr>
<td></td>
<td>rs11632698</td>
<td>G/G</td>
<td>85</td>
<td>0.94 (0.30–1.58)</td>
<td>0.004b</td>
<td>0.045</td>
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<tr>
<td></td>
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<td>G/A</td>
<td>142</td>
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<td></td>
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<tr>
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<td></td>
<td>A/A</td>
<td>75</td>
<td></td>
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</table>

* Adjusted by: age, BMI, previous chemotherapy treatment and previous tamoxifen treatment.

*Additive model.
with 0.99% less BMD loss at 2 years of AI treatment, compared to the reference haplotype \( (P = 0.006) \). Therefore, at 2 years of follow-up, patients carrying 2 copies of the haplotype CAT experienced half of the BMD loss \( (-2.05\%) \) observed in patients carrying two copies of the referent haplotype \( (-4.03\%) \).

**CYP11A1 and CYP17A1 expression in human FN bone and primary osteoblasts.**

*CYP11A1* (Fig. 5A) and *CYP17A1* (Fig. 6A) mRNA expression was detected both in fresh bone tissue and in cultured osteoblasts obtained from the bone samples. Although CYP17A1 was not associated with AIBL, we considered to assess its expression in bone since it is the only enzyme in the estrogen synthesis pathway not previously described in this tissue.

At the protein level, both common isoforms of cholesterol side-chain cleavage enzyme according to the UniProt database (P05108-CP11A_HUMAN) were detected in cultured hOB: one 60 kDa band corresponding to isoform 1: NP_000772.2 and one 42 kDa band corresponding to truncated isoform 2: NP_001093243.1 (Fig. 5B). The steroid 17-alpha-monooxygenase protein was also detected in hOB cells (Fig. 6B).

**Discussion**

SNPs in candidate genes that participate in estrogen and vitamin D hormone-response pathways were genotyped to analyze their association with AIBL. Three tag-SNPs located in the *CYP11A1* gene region were significantly associated with AIBL at 2 years of treatment. The nominal association of these SNPs at 1 year of follow-up supports this association. Next, we demonstrated the *CYP11A1* RNA expression in fresh FN bone tissue and primary osteoblasts obtained from this tissue. At the protein level, both common isoforms of cholesterol side-chain cleavage enzyme were detected in hOB, suggesting a potential role of this cytochrome P450 enzyme in bone metabolism.

The *CYP11A1* gene encodes the cholesterol side-chain cleavage enzyme (alternatively, P450scc), which catalyzes...
the first, and rate-limiting step in steroidogenesis, converting cholesterol to pregnenolone by side-change cleavage (Fig. 1). In addition to converting cholesterol, P450scc can also hydroxylate vitamin D2, vitamin D3 and their precursors (Slominski et al. 2006, Tuckey et al. 2008, Nguyen et al. 2009), suggesting a broad spectrum of roles in cell metabolism. This enzyme is a mitochondrial membrane-bound protein mainly expressed in the adrenal cortex, ovary, testis and placenta (Payne & Hales 2004). Moreover, Teplyuk et al. (2009) detected the expression of one N-terminally truncated CYP11A1 isoform in mouse and human osteoblastic cell lines, regulated by RUNX2. This 32-kDa isoform was localized in cytoplasm and nucleus but not in the mitochondria; its function remains unknown.

In our study, SNPs in CYP11A1 gene: rs4077581 (located in the promoter region), rs11632698 (in the intron 2) and rs900798 (in the 3’ region) were associated with BMD loss at FN. A statistical trend toward significance was also found between BMD loss at LS and SNPs rs4077581 (P = 0.042) and rs900798 (P = 0.029), after 2 years of AI treatment. All the scans in the study were evaluated for artifacts and/or structure changes that might have spuriously elevated BMD. This procedure had implications mainly for LS results, since spinal degenerative changes can notably increase BMD measurements. If all vertebrae were affected, the spine was reported as ‘invalid,’ with BMD results immediately omitted from the study. This substantially reduced the number of patients available to test this hypothesis, which could explain the difference in the statistical significance obtained for LS and FN in these SNPs.

Genetic variants may alter CYP11A1 expression or activity, determining sex steroid hormone levels responsible for quantitative phenotypes that include hormone-related disorders. Supporting these hypotheses, other polymorphic variants in this gene have been previously associated with breast and endometrial (Terry et al. 2010) cancer susceptibility (Zheng et al. 2004, Sun et al. 2012) and polycystic ovary syndrome (Gao et al. 2010).

The capacity of bone to synthesize estrogens has been known for a long time. However, these findings were always confined to enzymes acting downstream of DHEA. Thus, for example, the expression of HSD3B, aromatase, 17b-HSD and steroid sulfatase enzymes in bone has been detected (Saito & Yanaihara 1998, Janssen et al. 1999). Interestingly, we found that CYP11A1 and CYP17A1 (the gene encoding for the enzyme steroid 17-alpha-monooxygenase, which acts upon pregnenolone and then upon 17-hydroxy pregnenolone to form DHEA) are also expressed in human bone tissue and osteoblasts. Taken together, all enzymes in the steroid synthesis pathway have been identified in osteoblastic cells, suggesting a potential capacity of bone to synthesize its own sex hormones from cholesterol, independently of serum steroids secreted by adrenal gland, gonad and placenta. This process takes on special importance after menopause, when plasma estrogen levels are strongly deprived and estrogen synthesis is relegated to extragonadal sites. Accordingly, localized estrogen synthesis in osteoblasts is more active in postmenopausal women (Janssen et al. 1999). Therefore, the activity of CYP11A1, CYP17A1 and other enzymes involved in estrogen synthesis within bone tissue could have a central role in the local steroid sex hormone levels responsible for AIBL.

Genetic variants in other genes in the sex-steroid synthesis pathways (HSD17B3, CYP2C9, CYP19A1 and CYP2C19) have shown a trend of association with AIBL in our cohort study, but statistical significance after multiple testing correction was not achieved (data not shown). Napoli et al. (2013) found the SNP rs700518 (G/A at
Val(80)) in the CYP19A1 gene associated with AIBL at the LS and the total hip at 12 months. Hence, we cannot rule out that genetic variants in these genes, which act downstream of CYP11A1, could also intervene in the bone metabolism regulation. The same occurred with crucial genes in the vitamin D-synthesis pathway (CYP24A1 and VDR), suggesting once more the importance of this hormone in the attenuation of AI side effects such as arthralgia and bone loss (Prieto-Alhambra et al. 2012, Garcia-Giralt et al. 2013).

One limitation of the present study is the number of patients finally included in this specific analysis of our cohort, which does not permit detection of subtle allelic effects. Hence, our results need further replication in larger studies. However, to our knowledge this is the largest cohort available to date for AIBL genetic studies.

In conclusion, the CYP11A1 gene was associated with bone loss secondary to AI therapy. Moreover, CYP11A1 and CYP17A1 expression have been detected in total human bone and in osteoblasts. This is the first time that both common isoforms of CYP11A1 protein have been detected in primary osteoblasts, revealing a potential function of this enzyme in bone metabolism regulation and especially in the action of AI in the bone tissue. The presence in bone of all enzymes in the steroid-synthesis pathway opens up the possibility that this tissue can synthesize androgens and estrogens independently of serum steroid precursors.

Declaration of interest
The Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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