

# Assessment of Gene-by-Sex Interaction Effect on Bone Mineral Density

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## ABSTRACT

Sexual dimorphism in various bone phenotypes, including bone mineral density (BMD), is widely observed; however, the extent to which genes explain these sex differences is unclear. To identify variants with different effects by sex, we examined gene-by-sex autosomal interactions genome-wide, and performed expression quantitative trait loci (eQTL) analysis and bioinformatics network analysis. We conducted an autosomal genome-wide meta-analysis of gene-by-sex interaction on lumbar spine (LS) and femoral neck (FN) BMD in 25,353 individuals from 8 cohorts. In a second stage, we followed up the 12 top single-nucleotide polymorphisms (SNPs;  $p < 1 \times 10^{-5}$ ) in an additional set of 24,763 individuals. Gene-by-sex interaction and sex-specific effects were examined in these 12 SNPs. We detected one novel genome-wide significant interaction associated with LS-BMD at the Chr3p26.1-p25.1 locus, near the *GRM7* gene (male effect = 0.02 and  $p = 3.0 \times 10^{-5}$ ; female effect = -0.007 and  $p = 3.3 \times 10^{-2}$ ), and 11 suggestive loci associated with either FN- or LS-BMD in discovery cohorts. However, there was no evidence for genome-wide significant ( $p < 5 \times 10^{-8}$ ) gene-by-sex interaction in the joint analysis of discovery and replication cohorts. Despite the large collaborative effort, no genome-wide significant evidence for gene-by-sex

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Additional Supporting Information may be found in the online version of this article.

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interaction was found to influence BMD variation in this screen of autosomal markers. If they exist, gene-by-sex interactions for BMD probably have weak effects, accounting for less than 0.08% of the variation in these traits per implicated SNP. © 2012 American Society for Bone and Mineral Research.

**KEY WORDS:** GENE-BY-SEX INTERACTION; BMD; ASSOCIATION; AGING

## Introduction

Osteoporosis is a common condition that affects at least 30% of women and 12% of men at some point in life.<sup>(1)</sup> Women show a greater incidence of both stress fractures early in life<sup>(2)</sup> and fragility fractures later in life.<sup>(3)</sup> Areal bone mineral density (BMD), evaluated by dual-energy X-ray absorptiometry (DXA), is to date the most widely used assessment of bone strength and a reliable clinical predictor of osteoporotic fracture<sup>(4)</sup> in both men and women. Twin studies have demonstrated that BMD is a highly heritable trait ( $h^2 \sim 75\%$ ) in both women and men up to the age of 70 years.<sup>(5)</sup>

Strong sexual dimorphism in bone phenotypes, including BMD, has been reported, possibly explaining the observed differences in fracture risk between the sexes.<sup>(6)</sup> One explanation for this sex-specific predisposition to osteoporosis and fracture risk is the possibility that the observed differences between men and women are driven by genetic effects determining bone fragility. There are known sex differences in bone traits in mice.<sup>(7–10)</sup> Similarly, some genome-wide linkage analyses in humans have reported sex-specific results. In a whole-genome linkage analysis stratified by sex, sex-specific quantitative trait loci (QTLs) were found in the Framingham sample,<sup>(11)</sup> as well as by other groups.<sup>(6)</sup> Furthermore, in a meta-analysis by Ioannidis and colleagues<sup>(12)</sup> that included data from the nine whole-genome linkage scans for BMD, several sex-specific QTLs were observed. Notably, some sex-specific QTLs did not appear in any of the individual studies of BMD linkage, possibly because the individual studies had limited power.

Previously published genome-wide association studies (GWAS) of bone phenotypes, such as BMD,<sup>(13–18)</sup> did not test for single-nucleotide polymorphism (SNP)-by-sex interactions, nor did they mainly investigate sex-specific results. As the sample sizes of GWAS expanded with the growth of consortia, it has become more feasible to study sex-specificity of BMD.<sup>(19)</sup> However, direct comprehensive assessment of SNP-by-sex interactions for BMD has not been previously reported. The availability of large-scale GWAS collaborations allows for massive testing of SNP-by-sex interaction effects<sup>(20)</sup> and for rigorous replication of proposed discovered interactions that would allay the risk of false positives.<sup>(21)</sup> To study potential genetic contributions to the sexual dimorphism in BMD, we first performed a comprehensive study of genome-wide gene-by-sex interactions in cohorts with both men and women who are part of the Genetic Factors for Osteoporosis (GEFOS) consortium. In addition, we tested SNPs previously reported to be associated with BMD in a recent meta-analysis of GWAS studies for SNP-by-sex interactions.

## Materials and Methods

### Subjects and bone mineral density measurements

The discovery samples included 25,353 subjects of European ancestry (9056 men and 16,297 women) from 8 cohorts (AMISH, CHS, DECODE, ERF, FHS, HABC, RSI, RSI); see Supplemental Table S1) who are members of the GEFOS consortium. For replication, we recruited an additional 24,763 individuals (6814 men and 17,949 women) from 19 independent studies. Two (HKO: Asian and SAFOS: Mexican-American) of the replication cohorts consist of samples of non-European ancestry. All cohorts measured BMD ( $g/cm^2$ ) at the femoral neck (FN) and lumbar spine (LS) using DXA. Sample size varied (340 ~ 7605) across different cohorts, and average age for each cohort ranged from 19 to 80 years. Supplemental Table S1 displays the sample characteristics for each cohort.

### Genotyping and genotype imputation

Discovery GWAS cohorts were genotyped with various platforms, including Illumina (San Diego, CA USA) 370CNV (CHS and DECODE); Illumina Infinium HumanHap550 BeadChip (RSI and RSI); the Affymetrix (Santa Clara, CA USA) Nsp 250K chip, the Sty 250K chip, and the 50K gene-centered MIP chip (FHS); Illumina Human 1M-Duo BeadChip (HABC); Affymetrix 500k or 6.0 (AMISH); and both Illumina and Affymetrix (ERF). Imputation for nongenotyped SNPs was used to perform meta-analysis of results from the same set of SNPs across all individual cohorts. Imputation quality was assessed using the ratio of empirical observed variance of the allele dosage to the expected binomial variance. The imputations were conducted using Hidden-Markov Model implemented in MACH,<sup>(22,23)</sup> IMPUTE,<sup>(24,25)</sup> or BIMBAM.<sup>(26)</sup> The imputation reference panel was the HapMap CEU Phase II (release 22, build 36). Details of the imputation procedures in each discovery cohort and the filtering criteria for each SNP can be found in Supplemental Table S2.

Five of the 19 cohorts for follow-up used various genotyping platforms and applied their cohort-specific filtering criteria. Samples from the other 14 replication studies, all members of the GENOMOS consortium, were de novo genotyped by K-Biosciences (Hoddesdon, UK) using a competitive allele-specific PCR (KASPar) assay. Most of the de novo genotyped studies opted for amplified DNA. We evaluated the genotype concordance between genomic DNA genotypes and amplified DNA genotypes in an independent panel of 82 SNPs within 96 samples.<sup>(19)</sup> The genotype accuracy between before and after amplification was 99.97%. A Y-chromosome-specific assay was evaluated in all samples to confirm the sex of the individual

sample. Sample mismatches between the sex-specific assay and the reported sex in the questionnaire were removed from analysis. Aspects of reproducibility (ie, assessment of duplicate sample, positive control, and negative control genotyping) were considered during the stage of assay design for all markers. For the quality control of cohort-specific genotyping, sex concordance checks were assessed using a Y-chromosome assay to identify potential plating errors for which if unresolved samples were excluded. Genotyping concordance using a different platform was also assessed in the EDOS study using Illumina OmniExpress technology for a distinct project on a subset of the EDOS cohort, which was originally genotyped at K-Biosciences. The concordance of the shared markers with the microarray and the SNPs genotyped by K-Biosciences was 99.7%. Genotyping comparisons between discovery and follow-up samples were not compared. The following inclusion thresholds were applied for the cohorts typed by K-Biosciences: sample call rate >80%, SNP call rate >90%, Hardy-Weinberg equilibrium  $p > 1 \times 10^{-6}$ . Detailed information on all replication cohorts can be found in Supplemental Table S2.

### Statistical methods for phenotype-genotype association analysis and meta-analysis

A two-stage approach was applied to test gene-by-sex interaction. In stage 1, using a fixed effects inverse variance approach, we performed a meta-analysis of summary statistics for the gene-by-sex interaction effects in genome-wide analyses of FN- and LS-BMD obtained from 8 discovery cohorts. We used a  $p$  value threshold of  $p \leq 5 \times 10^{-8}$  for genome-wide significance and  $p \leq 1 \times 10^{-5}$  for suggestive signals. In the second stage, we then evaluated SNPs with interaction  $p$  values  $< 1.0 \times 10^{-5}$  obtained from stage 1 in replication cohorts. In addition, we evaluated the sex-specific effects for these top SNPs from our GWAS analysis. We also conducted candidate gene analysis to investigate the gene-by-sex interaction for previously reported BMD-associated loci from the largest GWAS meta-analysis to date.<sup>(19)</sup>

### Stage 1: GWAS discovery analysis

Only cohorts with both sexes were included in our discovery stage. Each discovery cohort conducted cohort-specific genome-wide association analyses using linear regression with a main effect for each SNP and SNP-by-sex interaction terms using an additive model for each SNP. We adjusted for age, height, weight, study site (for multisite cohort studies), sex, and principal components to control for population stratification. The “dosage” information for imputed genotype was used to account for uncertainty of imputation. In addition, we conducted sex-specific association analyses for the top SNPs ( $p \leq 1 \times 10^{-5}$ ) to examine their effect on BMD within each sex. We excluded SNPs with poor imputation quality (observed variance/expected variance  $< 0.3$  for MACH and BIMBAM imputed data sets and  $< 0.4$  for IMPUTE imputed data sets) and low minor allele frequency (MAF  $< 0.03$  for sex-specific analysis and MAF  $< 0.05$  for gene-by-sex interaction analysis).

### Stage 2: replication analysis

Twelve SNPs from the first stage were followed for replication. For each cohort, we performed sex-specific association analysis, and for the cohorts with both sexes, SNP-by-sex interaction was examined directly as with the discovery cohorts. For cohorts with both sexes, we meta-analyzed their association results. For cohorts with only one sex, we meta-analyzed the sex-specific association results for the main effects of a SNP and then estimated the gene-by-sex interaction effects by  $\hat{\beta}_{Male} - \hat{\beta}_{Female}$  with its corresponding standard error  $\sqrt{SE^2(\hat{\beta}_{Male}) + SE^2(\hat{\beta}_{Female})}$ , where  $\hat{\beta}_{Male}$  and  $\hat{\beta}_{Female}$  are sex-specific SNP effects from sex-stratified analysis. Utilizing all samples, the final result was obtained through meta-analyzing, with inverse variance as weight, the direct gene-by-sex interactions test result from two-sex sample cohorts, and the interaction results estimated from contrasting the one-sex sample cohorts.

### Power analysis for interaction

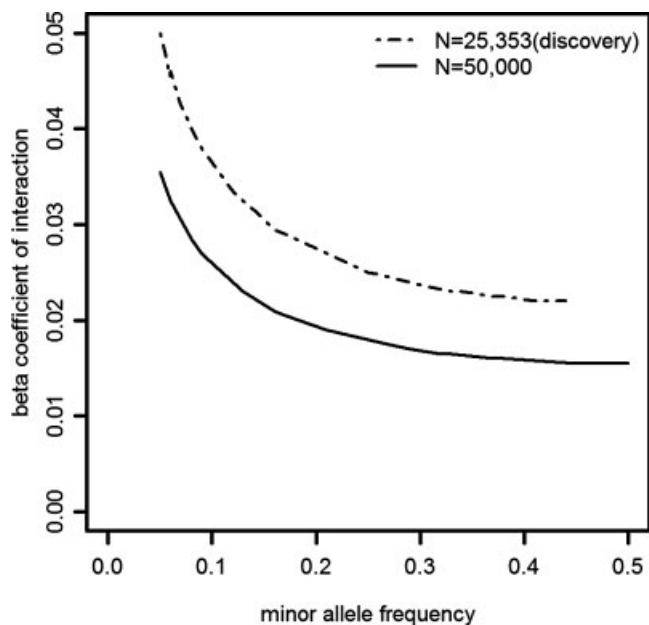
Before beginning second-stage analyses, we estimated that we would have samples of size 50,000 in total. So we conducted a power analysis on the detection of gene-by-sex interaction based on the sample size of our discovery cohorts (25,353) and the estimated sample size of discovery plus follow-up cohorts (50,000). Using the effect sizes of top SNPs we observed in our discovery cohort, we explored the relationship between detectable (with 80% power at genome-wide significant level of  $5 \times 10^{-8}$ ) interaction effect sizes and allele frequency. We explored all different scenarios corresponding to the range of observed effect sizes of our top SNPs, but their power analysis results are similar, so we only present one scenario in Fig. 1, corresponding to the parameter estimates of rs1405534 in LSBMD (effect size of  $-0.0048$  for SNP and  $-0.0219$  for SEX).

### Gene expression quantitative trait loci (eQTL) analysis

In parallel to our second-stage analysis, we also investigated our top findings from the first stage for the possibility of a meaningful biologic process underlying the genes that these SNPs belonged to. We conducted cis-eQTL analysis within a 500-kb flanking region of each of the top SNP to evaluate whether the SNP-by-sex interactions also influence transcript levels of genes in human primary osteoblasts and lymphocytes. In each locus, we selected either the gene in which the interacting SNPs were located or its closest nearby gene. Expression experiments in primary osteoblasts and lymphocytes were conducted in different study samples. For ungenotyped SNPs, surrogate SNPs with LD  $r^2 \geq 0.5$  and within 100 kb of the targeted SNPs were used in primary osteoblast samples.

### Lymphocytes

A gene expression profile with 24,385 RefSeq annotated genes (Illumina Sentrix Human-6 BeadChips) and genome-wide genotyping of  $\sim 2.5$  million SNPs were available from HapMap samples (lymphoblastoid cell lines from 128 women and 142 men). Blood sample collection, RNA and DNA isolation,



**Fig. 1.** Power analysis. This plot presents the relationship between detectable gene-by-sex interaction effect size with 80% power and minor allele frequency. Specifically, we used the parameter estimates of rs1405534 in LSBMD ( $SD = 0.187$  at genome-wide significant level of  $5 \times 10^{-8}$ ) along its relevant parameter estimates (effect size of  $-0.0048$  for SNP and of  $-0.0219$  for SEX), as the setting for our power analysis. The sample size used is 25,353 for discovery cohort and 50,000 (estimated) for discovery + replication cohort. With the sample of size 50,000, we would have 80% power to detect gene-by-sex interaction effect of 0.018 or larger in LSBMD for the SNP with minor allele frequency of 0.25. This would explain 0.08% variation of LSBMD. With 25,353 individuals in the discovery stage, we did not have adequate power to identify interaction reaching genome-wide significant level ( $p < 5 \times 10^{-8}$ ) at any allele frequency.

expression profiling, and DNA genotyping have been described in detail.<sup>(27)</sup> A mixed-effect regression model implemented in R was used to test SNP-sex interactions, adjusting for sex, ethnicity, and age.

#### Primary osteoblasts

A gene expression profile with 18,144 known genes (Illumina Human Ref8v2 BeadChips) and genome-wide genotyping of 561,303 SNPs (Illumina 550k Duo chips) were available (GSE15678) in 95 human Caucasian primary osteoblast samples (42 women and 53 men). Human trabecular bone came from the shaft of proximal femora obtained from donors undergoing total hip replacement. Primary osteoblasts were derived from bone tissue. Tissue collection, RNA and DNA isolation, expression profiling, and DNA genotyping have been described in detail.<sup>(28)</sup> A linear regression model implemented in PLINK (<http://pnu.gmh.harvard.edu/~purcell/plink/>) was used to test SNP-sex interactions, adjusting for sex and year of birth.

#### Gene-set enrichment tests of functional similarity

To explore functional similarity of the genes from our top SNP-sex interactions, we performed a gene-set enrichment test to

examine the probability of selected candidate genes clustering in particular biological/functional pathways as defined by the Gene Ontology (GO) project.<sup>(29)</sup> The GO Consortium provides controlled vocabularies, which model “Biological Process,” “Molecular Function,” and “Cellular Component” that are structured into directed acyclic graphs based on published literature and databases. Gene products may be annotated to one or more GO nodes. To determine whether any GO terms annotate a specified list of genes at a frequency greater than that would be expected by chance, a  $p$  value was calculated using the hypergeometric distribution.<sup>(30)</sup> To correct for multiple testing, the false discovery rate (FDR) was estimated.<sup>(31)</sup>

## Results

### GWAS SNP-by-sex interaction for bone mineral density

We observed one significant locus associated with SNP-by-sex interaction for LS-BMD at 3p26 with the most significant marker being rs10510373 (closest gene *GRM7*,  $p \leq 3.41 \times 10^{-8}$ ) and 11 SNPs with suggestive signals for interactions for either LS-BMD or FN-BMD. We carried forward these 12 SNPs from stage 1 GWAS for replication in 24,763 replication samples. The genome-wide association plots are shown in Supplemental Fig. S1 and the quantile-quantile plots are displayed in Supplemental Fig. S2, which shows that there was no systematic inflation of test statistics (with genomic inflation factor 1.010 for FN-BMD and 1.003 for LSBMD). The results for stage 1, stage 2, and stage 1 + stage 2 analysis for the 12 SNPs are displayed in Table 1. Of the 12 SNPs carried forward for replication, no SNP reached genome-wide significance for the combined stage 1 + stage 2 analyses.

We also conducted sex-specific analysis for the 12 SNPs with at least suggestive interaction signals in stage 1 to examine their sex-specific signal with BMD (Table 2). None of these 12 SNPs showed a genome-wide significant sex-specific signal.

### Differential expression and eQTL

As listed in Table 3, except for *RELL1*, gene expression levels of the top 11 associated genes were obtained in human lymphocytes. Gene expression levels in lymphocytes of these 11 genes were higher in men than the expression levels in women. However, none of them were considered statistically significant after correcting for multiple comparisons using Bonferroni correction ( $p$  value cutoff =  $0.05/11 = 0.0046$ ). A significant eQTL was found for SNP rs3748371, as a polymorphic allele T in rs3748371 was associated with higher *SERPINA1* expression (data not shown). However, no significant SNP-sex interactions on gene expression were shown in human lymphocyte samples.

Except for *RELL1*, gene expression levels of the top 11 associated genes were obtained in human primary osteoblasts. Gene expression levels in these 11 genes were not significantly different between men and women. For targeted SNPs not genotyped in human primary osteoblast samples, surrogate SNPs with  $r^2 \geq 0.5$  and within 100 kb of the targeted SNPs were selected and their eQTL estimated (ie, SNP rs6696978 as a surrogate SNP of targeted SNP, rs2295294 in *UBE4B* loci). No

**Table 1.** Stage 1 and Replication Results for Meta-analysis of Sex-by-Gene Interaction<sup>a</sup>

SNP ID	Chr	BP (b36)	Genes in or nearby	Alleles <sup>b</sup>	Allele frequency	Stage 1 (n = 25,353)			Stage 2 (n = 24,763)			Stage 1 + Stage 2		
						Beta	SE	p value	Beta	SE	p value	Beta	SE	p value
<b>FNBM</b>														
rs17284960	5	163556080	MAT2B	t/c	0.378	-0.011	0.002	7.50 × 10 <sup>-6</sup>	0.0012	0.003	6.84 × 10 <sup>-1</sup>	-0.0061	0.0019	1.05 × 10 <sup>-3</sup>
rs10756362	9	12334883	TYRP1	t/g	0.427	0.011	0.002	5.76 × 10 <sup>-6</sup>	0.0031	0.0031	3.05 × 10 <sup>-1</sup>	0.0079	0.0019	2.16 × 10 <sup>-5</sup>
rs11788458	9	113657842	UGCG	a/g	0.855	0.017	0.003	5.42 × 10 <sup>-7</sup>	-0.0045	0.0037	2.20 × 10 <sup>-1</sup>	0.0072	0.0025	3.50 × 10 <sup>-3</sup>
rs3748317	14	93923432	SERPINA1	a/c	0.150	-0.016	0.004	7.39 × 10 <sup>-6</sup>	0.0024	0.0035	4.97 × 10 <sup>-1</sup>	-0.0067	0.0025	7.22 × 10 <sup>-3</sup>
<b>LSBMD</b>														
rs2295294	1	10113471	UBE4B	a/t	0.623	0.019	0.004	8.60 × 10 <sup>-6</sup>	-0.0057	0.0035	9.84 × 10 <sup>-2</sup>	0.0043	0.0026	1.07 × 10 <sup>-1</sup>
rs7417366	1	166730211	XCL2	a/g	0.648	0.021	0.004	7.42 × 10 <sup>-8</sup>	0.0027	0.0039	4.92 × 10 <sup>-1</sup>	0.0118	0.0028	2.18 × 10 <sup>-5</sup>
rs10510373	3	7886027	GRM7	c/g	0.112	0.034	0.006	3.41 × 10 <sup>-8</sup>	-0.0138	0.0052	7.59 × 10 <sup>-3</sup>	0.006	0.0039	1.29 × 10 <sup>-1</sup>
rs4832734	4	37379393	RELL1	t/c	0.834	0.023	0.005	1.89 × 10 <sup>-6</sup>	-0.0046	0.0051	3.71 × 10 <sup>-1</sup>	0.01	0.0035	4.35 × 10 <sup>-3</sup>
rs6830890	4	112738575	C4orf32	a/g	0.127	-0.028	0.006	7.98 × 10 <sup>-7</sup>	0.0074	0.0048	1.19 × 10 <sup>-1</sup>	-0.0073	0.0036	4.22 × 10 <sup>-2</sup>
rs1405534	8	25066043	DOCK5	c/g	0.491	0.015	0.003	8.99 × 10 <sup>-6</sup>	0.0004	0.0033	8.92 × 10 <sup>-1</sup>	0.0076	0.0024	1.29 × 10 <sup>-3</sup>
rs12900333	15	76465331	CRABP1	t/c	0.119	-0.028	0.006	6.20 × 10 <sup>-6</sup>	0.0096	0.0051	5.81 × 10 <sup>-2</sup>	-0.0056	0.0039	1.48 × 10 <sup>-1</sup>
rs2717096	18	73058030	GALR1	a/g	0.442	-0.019	0.004	1.50 × 10 <sup>-6</sup>	0.0022	0.0033	5.08 × 10 <sup>-1</sup>	-0.0065	0.0025	8.97 × 10 <sup>-3</sup>

<sup>a</sup>Positive Beta implies larger effect of coded allele in men.

<sup>b</sup>Alleles = coded allele/noncoded allele.

**Table 2.** Gender-Specific Results for the Top SNPs From the Discovery Cohorts

SNP ID	FNBM	Chr	BP (b36)	Genes in or nearby	Alleles <sup>a</sup>	Male sample						Female sample											
						Stage 1			Stage 2			Stage 1 + Stage 2			Stage 1			Stage 2			Stage 1 + Stage 2		
						Beta	SE	p value	Beta	SE	p value	Beta	SE	p value	Beta	SE	p value	Beta	SE	p value	Beta	SE	p value
rs17284960	MAT2B	t/c	-0.008	0.002	1.69 × 10 <sup>-5</sup>	-0.003	0.005	5.76 × 10 <sup>-1</sup>	-0.005	0.002	2.21 × 10 <sup>-3</sup>	0.001	0.001	3.00 × 10 <sup>-1</sup>	-0.001	0.002	4.89 × 10 <sup>-1</sup>	0.001	0.001	3.95 × 10 <sup>-1</sup>			
rs10756362	TYRP1	t/g	0.006	0.002	3.08 × 10 <sup>-3</sup>	0.000	0.005	9.38 × 10 <sup>-1</sup>	0.005	0.002	3.67 × 10 <sup>-3</sup>	-0.003	0.001	1.22 × 10 <sup>-2</sup>	-0.002	0.002	3.52 × 10 <sup>-1</sup>	-0.002	0.001	9.90 × 10 <sup>-3</sup>			
rs11788458	UGCG	a/g	0.007	0.003	1.18 × 10 <sup>-2</sup>	-0.004	0.005	3.93 × 10 <sup>-1</sup>	0.003	0.002	1.98 × 10 <sup>-1</sup>	-0.007	0.002	5.40 × 10 <sup>-5</sup>	0.001	0.002	8.11 × 10 <sup>-1</sup>	-0.004	0.001	3.67 × 10 <sup>-3</sup>			
rs3748317	SERPINA1	a/c	-0.009	0.003	1.09 × 10 <sup>-3</sup>	0.001	0.005	7.71 × 10 <sup>-1</sup>	-0.004	0.002	9.24 × 10 <sup>-2</sup>	0.005	0.002	4.60 × 10 <sup>-3</sup>	0.001	0.002	7.37 × 10 <sup>-1</sup>	0.002	0.001	6.66 × 10 <sup>-2</sup>			
<b>LSBMD</b>																							
rs2295294	UBE4B	a/t	0.011	0.003	3.77 × 10 <sup>-4</sup>	-0.003	0.004	4.30 × 10 <sup>-1</sup>	0.003	0.002	2.11 × 10 <sup>-1</sup>	-0.006	0.002	9.84 × 10 <sup>-3</sup>	0.000	0.002	8.46 × 10 <sup>-1</sup>	-0.001	0.001	5.54 × 10 <sup>-1</sup>			
rs7417366	XCL2	a/g	0.011	0.003	5.75 × 10 <sup>-4</sup>	-0.006	0.006	2.88 × 10 <sup>-1</sup>	0.007	0.002	1.91 × 10 <sup>-3</sup>	-0.008	0.002	1.22 × 10 <sup>-4</sup>	-0.004	0.003	9.41 × 10 <sup>-2</sup>	-0.004	0.001	9.02 × 10 <sup>-4</sup>			
rs10510373	GRM7	c/g	0.020	0.005	3.03 × 10 <sup>-5</sup>	0.002	0.007	7.05 × 10 <sup>-1</sup>	0.009	0.003	6.03 × 10 <sup>-3</sup>	-0.007	0.003	3.32 × 10 <sup>-2</sup>	-0.001	0.004	8.98 × 10 <sup>-1</sup>	-0.001	0.002	6.66 × 10 <sup>-1</sup>			
rs4832734	RELL1	t/c	0.018	0.004	1.99 × 10 <sup>-6</sup>	-0.009	0.007	2.19 × 10 <sup>-1</sup>	0.010	0.003	7.10 × 10 <sup>-4</sup>	-0.003	0.003	2.14 × 10 <sup>-1</sup>	0.008	0.003	6.35 × 10 <sup>-3</sup>	0.001	0.002	7.55 × 10 <sup>-1</sup>			
rs6830890	C4orf32	a/g	-0.021	0.005	5.64 × 10 <sup>-6</sup>	0.007	0.006	2.18 × 10 <sup>-1</sup>	-0.007	0.003	1.56 × 10 <sup>-2</sup>	0.004	0.003	1.98 × 10 <sup>-1</sup>	-0.004	0.003	2.36 × 10 <sup>-1</sup>	-0.001	0.002	7.29 × 10 <sup>-1</sup>			
rs1405534	DOCK5	c/g	0.009	0.003	1.35 × 10 <sup>-3</sup>	-0.005	0.004	2.37 × 10 <sup>-1</sup>	0.001	0.002	5.57 × 10 <sup>-1</sup>	-0.004	0.002	2.40 × 10 <sup>-2</sup>	-0.007	0.002	1.71 × 10 <sup>-3</sup>	-0.005	0.001	1.17 × 10 <sup>-5</sup>			
rs12900333	CRABP1	t/c	-0.019	0.005	7.56 × 10 <sup>-5</sup>	0.001	0.007	8.56 × 10 <sup>-1</sup>	-0.008	0.003	1.36 × 10 <sup>-2</sup>	0.000	0.003	9.20 × 10 <sup>-1</sup>	-0.002	0.003	4.60 × 10 <sup>-1</sup>	-0.002	0.002	1.99 × 10 <sup>-1</sup>			
rs2717096	GALR1	a/g	-0.012	0.003	2.74 × 10 <sup>-4</sup>	-0.001	0.004	8.67 × 10 <sup>-1</sup>	-0.005	0.002	2.85 × 10 <sup>-2</sup>	0.002	0.002	4.00 × 10 <sup>-1</sup>	-0.002	0.002	2.89 × 10 <sup>-1</sup>	0.000	0.001	7.49 × 10 <sup>-1</sup>			

<sup>a</sup>Alleles = coded allele/noncoded allele.

**Table 3.** Expression Analysis (eQTL and Differential Expression by Sex) for Top SNPs Identified From the Discovery Stage

Genes	Targeted SNPs	Human lymphocytes eQTL		Human primary osteoblast eQTL						
		Differential <sup>a</sup> expression by sex <i>p</i> values	SNP x sex interaction <i>p</i> values	Differential <sup>a</sup> expression by sex <i>p</i> values	Surrogate SNPs	LD (r2 or D')	Distance (kb)	MAF	Alleles	SNP x sex interaction <i>p</i> values
FN BMD										
<i>MAT2B</i>	rs17284960	4.06E-04	0.682	0.043	rs17284960	1.00	0.0	0.42	C/T	0.47
<i>TYRP1</i>	rs10756362	0.117	0.398	0.711	rs7860071	0.67	98.7	0.30	T/C	0.68
<i>UGCG</i>	rs11788458	0.048	0.867	0.723	rs11788458	1.00	0.0	0.14	C/T	0.62
<i>SERPINA1</i>	rs3748317	0.677	0.418	0.558	rs709932	1.00	4.5	0.14	T/C	0.47
LS BMD										
<i>UBE4B</i>	rs2295294	0.251	0.166	0.960	rs6696978	0.95	18.7	0.30	G/A	0.36
<i>XCL2</i>	rs7417366	0.490	0.615	0.509	rs7417366	1.00	0.0	0.35	C/T	0.66
<i>GRM7</i>	rs10510373	0.097	0.014	0.948	rs10510378	0.95	55.9	0.13	C/T	0.08
<i>RELL1</i>	rs4832734	NA	NA	NA	NA	NA	NA	NA	NA	NA
<i>C4orf32</i>	rs6830890	0.250	0.974	0.620	NA	NA	NA	NA	NA	NA
<i>DOCK5</i>	rs1405534	0.038	0.910	0.857	rs1358268	1.00	4.0	0.46	T/C	0.40
<i>CRABP1</i>	rs12900333	0.879	0.737	0.309	rs2037348	0.84	24.7	0.22	C/T	0.74
<i>GALR1</i>	rs2717096	0.463	0.565	0.988	rs2717096	1.00	0.0	0.45	A/G	0.76

MAF = minor allele frequency. NA = not available.

<sup>a</sup>Gene expression is considered significantly higher in men than the expression level in women for  $p < 4.55 \times 10^{-3}$  (0.05/11).

surrogate SNP (genotyped) can be found for targeted SNP rs6830890 in *C4orf32* loci; therefore, the eQTL in this region was not estimated. No significant SNP-sex interactions on gene expression (eQTL) were found in human primary osteoblast samples.

### Gene-set enrichment tests

To test the probability of our candidate genes clustering into a particular biological pathway, we performed a gene set enrichment test on 12 genes listed in Table 3. Because of lack of biological or functional annotation, *RELL1* and *C4orf32* were excluded from analyses. The most significant clustering (Fisher exact test  $p = 6.03 \times 10^{-4}$ ; Benjamini-Hochberg multiple testing correction  $p = 0.045$ ) of genes involved nervous system development and function, including *UGCG*, *GRM7*, *TYRP1*, and *UBE4B* genes. However, by looking at more specific biological function ontology terms under nervous system development and function, no significantly enriched functions were found.

Previously, we have identified 55 genome-wide significant loci associated with BMD with the most significant  $p$  values  $< 5 \times 10^{-8}$ ,<sup>(19)</sup> and we found an additional 10 loci that were considered as suggestive signals with the most significant  $p$  values  $< 5 \times 10^{-6}$ . We performed a gene-set enrichment analysis on these 65 loci and found functional enrichments on ossification ( $p = 1.8 \times 10^{-7}$ ), bone formation ( $p = 2.8 \times 10^{-6}$ ), mineralization of trabecular bone ( $p = 6.1 \times 10^{-6}$ ), cell-cell connection among osteoclast and osteoblast and chondrocyte ( $p = 1.2 \times 10^{-6}$ ), degenerative mitral valve disease ( $p = 1.5 \times 10^{-6}$ ), and Wnt/ $\beta$ -catenin signaling ( $p = 3.4 \times 10^{-5}$ ). To estimate whether additional enriched functional pathways could be identified by adding gene-sex interaction loci to the 65 BMD associated loci, we performed a gene-set enrichment analysis on

75 loci (65 BMD associated loci with 10 gene-sex interaction loci with functional information). These 10 gene-sex interaction loci were not significantly clustered together with any of the 65 BMD-associated loci in any known functional and biological pathways or gene sets. No additional enriched functional and biological pathways were found.

### Discussion

To our knowledge, this is the first genome-wide association analysis focusing on SNP-by-sex interaction of BMD phenotypes. We identified 12 SNP-sex interaction loci with suggestive genome-wide significance ( $p < 10^{-5}$ ) in 25,353 adult men and women that did not replicate in an additional 24,763 adult men and women. Also we observed no replicated SNP-by-sex interaction for any of the top SNPs found to be significantly associated with BMD in the largest GWAS meta-analysis performed to date (detail described in Supplemental Text and Supplemental Table S3). To account for the varying linkage disequilibrium structure across different populations, we also analyzed the data from the sample of European ancestry (data not shown). Although we observed improvement of the  $p$  values (smaller  $p$  values in European ancestry samples only), no SNP-by-sex interaction was replicated for any of the top SNPs in this sample. Hence, the conclusion remained the same.

Recent studies<sup>(32)</sup> suggest that sex-specific genetic architecture influences many human phenotypes, including reproductive, physiological, and complex disease traits. Some of the underlying mechanisms might be attributed to differential gene regulation in males and females, particularly in sex steroid responsive genes (different hormonal milieu). In the field of skeletal genetics, in particular, sex-specific findings were

reported in linkage studies (both in humans and animal models) and candidate gene association studies (reviewed in Karasik and Ferrari<sup>(6)</sup>).

Similar to the studies in mice, whole-genome linkage studies in humans for BMD also provided evidence of sex-specific QTLs with max LOD of 3.29.<sup>(12)</sup> More recently, this was confirmed by Peacock and colleagues,<sup>(33)</sup> who found male-specific QTLs on chromosomes 7q34, 14q32, and 21q21 to be linked with aBMD. Several groups studying candidate genes also found evidence of sex-specific associations. For example, an SNP in the glucocorticoid receptor (*GR*) gene was associated with extreme BMD in Chinese men but not women;<sup>(34)</sup> SNPs in the *VKORC1* gene were associated with decreased BMD in Mexican-American and black men but not women from NHANES III.<sup>(35)</sup> Finally, sex-specific associations of the Pirin (*PIR*) gene with lumbar spine BMD was shown in a large Chinese cohort.<sup>(36)</sup> None of these findings overlap with our top hits.

Analyzing sex-specific associations between SNPs and BMD tests a different hypothesis than showing that there are interactions between genetic variants and sex on BMD. The former hypothesis tests whether the association between SNPs and BMD differs from zero either within males or females, whereas interaction tests whether the magnitude of association between SNPs and BMD *differs significantly between sexes*. Given that most genetic variants have very subtle effects, it is expected that the lists of discovered genes and variants associated with BMD in analyses limited to men and in analyses limited to women may differ simply because of power considerations, even if the effects of these variants are not genuinely different in the two sexes. In addition, the observation that men have higher BMD than women does not necessarily imply that genetic variation contributes to the differential distribution of BMD between men and women, despite the fact that sex is by itself genetically determined.

There are obvious gender-specific hormonal milieus contributing to the sexual dimorphism on bone health. Notably, differences in response to estrogen and testosterone have been shown for male and female chondrocytes, osteoblasts, myoblasts, and other cells.<sup>(37,38)</sup> In an earlier study, sex differences in response to progesterone have also been reported in cells derived from rat lumbar vertebrae.<sup>(39)</sup> Most recently, using human peripheral blood mononuclear precursor cells from adult males and females that were differentiated into osteoclasts, Wang and Stern<sup>(40)</sup> demonstrated sex-specific actions of estrogen and androgen. Thus, 17 $\beta$ -estradiol and testosterone largely affected expression of different genes from a custom-designed array containing 94 genes related to bone and hormone metabolism. If such sex-specific expression of genes related to bone metabolism does in fact occur, this was not reflected by our analyses.

Attempts to evaluate whether there are functionally significant sex-specific differences arising from the interaction results, differences in gene expression between males and females and eQTL on SNP-by-sex interactions were analyzed in human samples from two tissue types: primary osteoblast and lymphocytes. We failed to find significant differential gene expression between men and women among the 12 transcripts

prioritized by the SNP-by-sex interaction analyses despite the presence of significant eQTL (eSNP). However, it is important to note that a lack of evidence from expression experiments does not necessarily exclude gene interaction with sex influencing BMD variation, given that (i) experimental models such as osteoclastogenesis, osteoblastogenesis, or early skeletal development do not represent all relevant processes related to the skeleton at the organism level; (ii) variation in a gene leading to disease may affect protein function but not expression; and (iii) absence of detectable SNP-by-sex interactions may be the result of modest effect sizes or owing to different environmental conditions.

There are several potential limitations of this study. First, this analysis was restricted to autosomal SNPs, although we do not expect that many BMD-associated SNPs will be located on the sex chromosomes, as there is not much of X-linked heritability of BMD. Second, areal BMD, despite being a sexually dimorphic phenotype, is not necessarily the most optimal skeletal phenotype because it does not adequately represent bone size. To avoid identifying genes responsible for bone size, we adjusted for height in the model to account for this concern. We also repeated our analysis without height adjustment, and the results were very similar. However, given the limitation of 2D aBMD, volumetric density measures would also be worth assessing in future genetic studies of sex differences in bone. Third, our analyses were corrected for body weight and height, which are important determinants of the skeletal differences observed between men and women. From this perspective, gene-sex interaction effects influencing BMD variation through weight and height (size) parameters would have been missed. We evaluated here (mostly) direct skeletal effects. Finally, despite our large sample size, we did not identify genome-wide significant SNP-by-sex interactions from using 25,353 individuals for the discovery stage followed by 24,763 individuals for replication. As shown in Fig. 1, with the sample of size 50,000, we would have 80% power to detect gene-by-sex interaction effect of 0.018 or larger in LSBMD (SD = 0.187) for the SNP (which explains around 0.08% variation of a quantitative trait) with minor allele frequency of 0.25. With 25,353 individuals in the discovery stage, we did not have adequate power to identify interaction reaching a genome-wide significant level ( $p < 5 \times 10^{-8}$ ) at any allele frequency. The power analyses were conducted under the assumption that the interaction effects are similar to what we observed for the main effects in our first-stage analysis. We are aware that the real effect size may be relatively smaller (a "winner's curse" effect) and acknowledge that despite the large size of the populations studied, we were still underpowered to detect these magnitudes of interaction effect sizes. This result also implies that the gene-by-sex effect, if it exists, is smaller in magnitude than the previously observed SNP effects. Although we did not replicate the top SNP-by-sex interaction loci in additional samples, possibly because of insufficient statistical power and/or potential heterogeneity across discovery studies and replication studies, we cannot completely rule out the possibility that these top loci from the discovery stage are in fact involved in sex-specific regulation of BMD.

Using bioinformatics approaches, we performed biological functional interaction network analysis with a couple of

prespecified pathways, namely sex steroids and Wnt signaling (detailed Methods and Results are described in the Supplemental Text). Using this approach, we were able to indirectly link some of our top SNP-by-sex loci to  $\beta$ -estradiol (Supplemental Fig. S3). For example, UBE4B has been found to negatively regulate the level of p53 and to inhibit p53-dependent transactivation and apoptosis.<sup>(41)</sup> Based on chromatin immunoprecipitation assays, studies found p53 as being recruited to the ER promoter along with other transcription factors and that this complex was formed in a p53-dependent manner, which suggests that p53 regulates ER expression through transcriptional control of the ER promoter.<sup>(42)</sup> This type of evidence from biological experiments may suggest that if there are true SNP-by-sex interactions on BMD, they may be indirect, acting through more complex networks of genes. Thus, UGCG and SERPINA1 (two of the most significant findings from the discovery stage) were found to have a direct functional interaction with Wnt signaling pathways (Supplemental Fig. S4). SERPINA1 not only interacts with CTNBN1 protein but also decreases activation of the NF- $\kappa$ B complex,<sup>(43)</sup> which also supports its involvement in bone metabolism.

In conclusion, our results suggest that an SNP-by-sex interaction effect if present may be too small and heterogeneous to be detected with the current sample size and/or with the limitations of the areal BMD phenotypes. Therefore, future investigations of true differences in genetic associations between the sexes will require even larger samples or more sexually dimorphic skeletal phenotypes such as volumetric skeletal measurements.

## Disclosures

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