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Meta-analysis of Genome-Wide Association Studies Reveals Genetic Variants for Hip Bone Geometry

Short title: A GWAS of hip geometry in 27,000 subjects

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ABSTRACT:

Hip geometry is an important predictor of fracture. We performed a meta-analysis of GWAS studies in adults to identify genetic variants that are associated with proximal femur geometry phenotypes. We analyzed four phenotypes: 1) Femoral neck length; 2) Neck-shaft angle; 3) Femoral neck width, and 4) Femoral neck section modulus, estimated from DXA scans using algorithms of hip structure analysis. In the Discovery stage, 10 cohort studies were included in the fixed-effect meta-analysis, with up to 18,719 men and women ages 16-93 years. Association analyses were performed with ~ 2.5 million polymorphisms under an additive model adjusted for age, body mass index, and height. Replication analyses of meta-GWAS significant loci (at adjusted genome-wide significance, GWS, threshold $p \leq 2.6 \times 10^{-8}$) were performed in 7 additional cohorts *in-silico*. We looked up SNPs associated in our analysis, for association with height, bone mineral density (BMD), and fracture.

In meta-analysis (combined Discovery and Replication stages), GWS associations were found at 5p15 (*IRX1* and *ADAMTS16)*; 5q35 near *FGFR4;* at 12p11 (in *CCDC91*); 11q13 (near *LRP5* and *PPP6R3* (rs7102273)). Several hip geometry signals overlapped with bone mineral density (BMD), including *LRP5* (chr. 11). Chr. 11 SNP rs7102273 was associated with any-type fracture ($p = 7.5 \times 10^{-5}$).

We used bone transcriptome data and discovered several significant eQTLs, including rs7102273 and PPP6R3 expression (p=0.0007), and rs6556301 (intergenic, chr.5 near *FGFR4*) and PDLIM7 expression (p=0.005).

In conclusion, we found associations between hip geometry measures and several genes being part of biological pathways relevant to BMD and fractures. The results provide a defined set of genes facilitating further experimental exploration and validation to understand biological mechanisms underlying human bone geometry and etiology of bone fragility.

KEYWORDS: Hip Bone Geometry; Fracture, Genome-Wide Association Study; Meta-analysis; Candidate Genes; polymorphisms

Introduction:

Osteoporosis and associated fractures are common worldwide. In the United States alone, a total of 340,000 hip fractures occur each year, and the number of hip fractures is predicted to more than triple worldwide from 1.66 million in 1990 to 6.26 million in 2050.[1] In older individuals, hip fractures are a source of increased mortality, morbidity, and healthcare expenses. They are associated with a more than two-fold increase in the likelihood of mortality, a four-fold increase in the probability of requiring institutional care, and a two-fold increase in entering low-income status one year post-fracture as compared to patients who do not sustain hip fractures.[2] Hip fractures may be preventable in as many as 50% of cases through the use of the available pharmacotherapies [3], but improvements in the identification of high-risk patients are necessary [4, 5].

The most often studied phenotype for genetic studies of osteoporosis is bone mineral density (BMD) as assessed by dual energy x-ray absorptiometry (DXA) of the hip. BMD is a two dimensional measure and does not account for the cross-sectional distribution of bone mass in the femur. BMD is an imperfect predictor of hip fracture and increasing attention has focused on contribution of other factors to bone strength, such as hip geometry.[6, 7] Indeed, hip geometry traits have been associated with fracture risk, independent of BMD in most [8, 9, 10, 11] but not all studies [11]. Like BMD, hip geometry traits have a strong genetic component with a heritability of 28% to 70%[12]. While some candidate-gene association studies [13, 14, 15] and modest-size genome-wide association studies (GWAS) have been performed [16, 17, 18], a powerful large-scale GWAS meta-analysis is essential in order to provide comprehensive picture of the genetic architecture of hip geometry and to determine if novel genetic pathways in and above those controlling BMD influence hip geometry. Understanding the genetics of hip geometry provides the potential to identify additional genetic architecture of fracture risk above and beyond BMD. There is also a complex relationship between adult height, bone geometry, and fracture risk. The premise of this study is that geometry of the proximal femur influences its predilection to fracture and that genetic factors responsible for determining the geometric features of the proximal femur could be involved in hip fracture risk.

In this study we performed a GWAS discovery analysis of hip geometry indices measured by DXA derived hip structural analysis conducted on a large sample of women and men of predominantly European ancestry from the cohorts of the Genetic Factors for Osteoporosis (GEFOS) consortium [19]. The model adjusted for covariates, including body mass index and height, was tested. This was followed by replication of the top findings in seven

additional independent human cohorts. In order to prioritize variants which were novel for hip geometry, we then determined whether the genome-wide association findings had also been found in previous GWAS of BMD phenotypes, height or fracture. Finally, we assessed the functional relevance of the identified loci for bone biology using information from relevant sources, including gene expression data on cellular or whole animal models, as well as transcriptome and experimental studies in skeletal biopsies from human donors.

Methods

Study subjects and Hip Geometry phenotypes

The Discovery stage of this study utilized data from 10 cohort studies and totaled data from 18,719 adult men and women for whom genome-wide SNP data and relevant hip geometry phenotypes were available. These individuals were from populations across North America, Europe, and East Asia, members of the Genetic Factors for Osteoporosis (GEFOS) consortium (Supplemental **Table S1a).** The GEFOS consortium is an international collaboration of investigators dedicated to the identification of genetic determinants of osteoporosis and fragility fracture[20, 21, 22]. Additional descriptive information about the participating cohorts is available in the Supplementary Materials (Supplemental **Table S1a**).

We sought *in silico* independent replication, using summary results from 7 cohorts (n=8,334 men and women) with genome-wide SNP data that became available after the initial discovery meta-analysis was completed. Characteristics of the replication cohorts are summarized in Suppl. **Table S1b**. All studies were approved by institutional ethics review committees at the relevant organizations and all participants provided written informed consent.

Hip geometry measures were derived from dual-energy x-ray absorptiometry (DXA) scans using the Hip Structural Analysis (HSA)[23] algorithm or a software supplied by a DXA machine manufacturer (see Suppl. **Tables S1a,b**). In each cohort study the following hip geometry indices were measured: femoral neck length (FNL), neck-shaft angle (NSA), the narrowest width of the femoral neck (NNW) and its section modulus (NNZ).[12] Coefficients of variation for hip geometry traits were reported to range from 3.3% (NN outer diameter) to 9.1% (FNL)[24].

In each cohort, at the time of the DXA exam or on a visit preceding it, standing height was measured and body mass index (BMI) calculated (Suppl. **Tables S1a, b)**.[25]

GWAS of bone phenotypes

Genotyping and imputation methods

All the cohorts were genotyped using commercially available Affymetrix (Affymetrix Inc., Santa Clara, CA, USA) or Illumina (Illumina Inc., San Diego, CA, USA) genotyping arrays (Suppl. **Table S2)**. Quality control was performed independently in each study according to standard manufacturer-provided protocols and within study procedures. To facilitate meta-analysis, each group performed genotype imputation with IMPUTE[26] or MACH[27] software (see Suppl. Table **S2** for details) using genotypes from the HapMap Phase II release 22, NCBI build 36 (CEU or CHB/JPT as appropriate (for Hong-Kong cohort)) as reference panels. More recent reference panels such as HRC42 were not available at the time of the analyses. Each imputation software provides an overall imputation quality score for each single-nucleotide polymorphism (SNP). Analysis of imputed genotypes used either the dosage information from MACH or the genotype probabilities from IMPUTE (Supplemental Table **S2**). Before performing an analysis, poorly imputed and low frequency or rare polymorphisms were excluded. Specifically the quality control filters applied for exclusions of SNPs were: imputation quality score <0.3 for MACH and <0.4 for IMPUTE, average minor allele frequency (MAF) of <1% across studies, and SNPs missing from ≥50% of the cohorts contributing to each outcome (at the meta-analysis stage). After quality control, up to ~2.5 million SNPs were available from each cohort for the Discovery meta-analysis.

Association analyses

In the Discovery phase, each cohort conducted analyses according to a standard pre-specified analysis plan under an additive (i.e. per allele count) genetic model. Phenotypes were defined as the sex-specific standardized residuals derived from linear regression of each outcome variable on age, age^2, body mass index and height. The assumption of normality of residuals in the linear regression model was checked within each cohort for each phenotype and no deviations were reported. The SNP-phenotype associations in each study were adjusted for potential confounding by population substructure using principal components as appropriate; pedigree and twin-based studies additionally corrected for family structure (using the R Kinship2 package: https://cran.r-project.org/web/packages/available_packages_by_name.html). Sex-specific analyses were performed, except for the family-based cohorts, where combined-sex models were generated with additional adjustment for sex. Cohort-specific summary statistics

(beta-coefficients, standard errors, and p-values) were used for the meta-analysis of each outcome variable (standardized residuals of FNL, NSA, NNW, and NNZ) with the genome-wide SNPs. The replication analyses used the same analytical procedures as above (e.g. using study-specific standardized residuals from the covariate-adjusted model, as outcomes).

Meta-analysis

Meta-analysis of the GWAS discovery results was conducted and tested independently in two collaborating centers (Broad Institute and Hebrew SeniorLife, both in Boston, MA, USA). Because of potential power limitations to detect sex-specific associations (N males = 5,510 and N females = 11,701, see Suppl. **Table S1**), we performed sex-combined meta-analysis. A fixed effects, sample size weighted meta-analysis (using METAL software) was conducted in the Discovery set. Double genomic correction to control for potential inflation of the test statistics was performed, in individual studies and in the meta-analysis. The genome-wide level of statistical significance (GWS) was adjusted for multiple correlated traits (effective n=1.92); thus the genome-wide significance was proclaimed at $p \leq 2.6 \times 10^{-8}$ and the suggestive level of significance at $p < 5 \times 10^{-6}$. The QQ plots were generated for each phenotype, by pruning association results at a linkage disequilibrium (LD) threshold of 0.50 (calculated with SNAP [28] using 1000 Genomes [29] data). Regional plots were generated with LocusZoom with modifications[30], using chromosome position coordinates as provided in GrCh37/hg19.

In-silico Replication and Combined meta-analysis

Since the *in-silico* replication was performed in cohorts with existing genome-wide data (and not by *de-novo* genotyping), all the region-wide SNPs in LD (r^2 threshold of 0.7) interval with a genome-wide significantly associated SNP(s) for hip geometry were taken forward for replication. Meta-analysis of the replication results was conducted by 3 collaborating centers (Broad Institute, Hebrew SeniorLife, and Ioannina, Greece). A fixed-effects model was used for meta-analysis of studies in the replication set and also in the final combined analyses of the discovery and replication sets, for each phenotype. Replication was proclaimed for any SNP when in the combined (joint) meta-analysis, (a) GWS threshold was achieved and (b) combined analysis p-value was lower than that in the Discovery.

In-silico search for independent signals (conditional analyses, Discovery stage)

To identify secondary (independent) association signals in the regions containing SNPs that were genome-wide significant, we performed region-wide association analyses conditioning on the most significant hip-geometry SNP within a one megabase window of the SNP with the lowest p-value in a given locus, by including the other SNPs as a covariate in the regression models.

In-silico search for Height-associated SNPs, BMD-associated SNPs, and any type of fracture GWAS (Discovery stage)

In order to test for independence between the hip geometry and adult height signals, we looked up SNPs at least suggestively associated ($p < 5 \times 10^{-6}$) in our Discovery analysis, in the dataset of SNPs associated with height in the Genetic Investigation of ANthropometric Traits (GIANT) Consortium meta-analysis of 183,727 subjects [25]. Furthermore, in order to determine if our GWS SNPs for hip geometry phenotypes were co-localized with previously identified SNPs for BMD, we looked up nearby "BMD SNPs"[31]. To assess the potential relevance of discovered loci to fracture risk, we looked up hip-geometry SNP associations (SNPs that were at least suggestively associated in our discovery analysis with one of the four hip geometry phenotypes, adjusted for covariates) with fracture using a large study from the GEFOS consortium (37,857 cases and 227,116 controls) [32, 33].

LD score regression: SNP heritability and Genetic correlations

LD score regression was used to estimate the SNP heritability (h^2) of the studied traits and to estimate the genetic correlation (r_g) i) between hip geometry phenotypes and ii) between hip geometry phenotypes and 235 traits and diseases with publicly available summary GWAS data using LD Hub [34]. The toll is a centralized database of summary-level GWAS results for hundreds of diseases/traits, as well as a web interface that automates the LD score regression analysis pipeline [35]. To correct for multiple testing the r_g was deemed significant at α=0.0002 (0.05/235, Bonferroni corrected for 235 tests).

Bioinformatic annotations and Functional validation

Annotation of SNPs used NCBI's dbSNP build hg19.

Gene Expression Analysis in Human Bone

Global gene expression profiling was performed in trans-iliac bone biopsies obtained from postmenopausal white women from Oslo, as described in [36]. This permitted us to calculate the correlation values between hip geometry SNPs (and their proxies) and the mRNA levels of genes in the vicinity of the identified loci. In brief, the women undergoing bone biopsies (50–86 years old) were free from diseases other than osteoporosis or receiving medication (past or present) possibly affecting bone remodeling or representing secondary causes of osteoporosis[36, 37]. RNA was purified and analyzed using Affymetrix HG U133 2.0 plus arrays as described elsewhere [36]. Bone total RNA was subjected to global transcript profiling using HG-U133 plus 2.0 microarrays (Affymetrix). These data are available at the European Bioinformatics Institute (EMBL-EBI) ArrayExpress repository, ID: E-MEXP-1618 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-1618/).[17, 38] DXA scans from the bone donors were subjected to HSA. Filtered transcript levels from 80 bone biopsies were correlated with hip geometry data; results were adjusted for multiple testing using false-discovery rate (FDR).

cis-Expression Quantitative Trait Loci (cis-eQTLs) in Human Bone Tissues

Genome-wide genotyping in the sample of women from Oslo was performed by Affymetrix Genome-Wide Human SNP Array 6.0/Affymetrix Axiom Biobank array (1,000,000 and 700,000 SNPs assessed, respectively)[37]. We conducted cis-eQTL analysis within a 2Mb flanking region (1 Mb upstream and 1 Mb downstream) of each of the replicated SNPs to evaluate whether they influence transcript levels of genes in human whole bone (using the same resource of iliac bone biopsies from 84 postmenopausal women).[17, 38]

Gene expression in Primary Murine Osteoblasts and Osteoclasts

Gene expression profiles (“Trajectories”, i.e. increase or decrease within the days post differentiation) of candidate genes in close proximity to genome-wide associated SNPs were examined in primary mouse osteoblasts undergoing differentiation. These data have been described in detail previously[39] and are publicly available from the Gene Expression Omnibus (GSE54461). For details, see Supplementary Methods.

We also mined publically-available osteoclast expression data (GEO Accession GSM1873361; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72846) for expression (presence/absence) of candidate genes during osteoclastogenesis.

Co-expression network from mouse cortical bone

We used a co-expression network constructed from mouse cortical bone expression profiles [40]. By mapping mouse homologs of human genes located in GWAS regions associated with BMD, the network previously identified an Osteoblast Functional Module containing 33 genes implicated by GWAS [40]. For the association with hip geometry phenotypes, the following algorithm was applied:

Step 1: Using *plink2*, we generated LD intervals based on LD data from 1000 Genomes Phase I. The interval was defined by the up- and downstream-most SNP in LD with the index SNP at an r^2 threshold of 0.7, as described in Calabrese et al. [40]. This resulted in the file "*plink.ld*" that contains all the proxies for the index SNPs based on build hg19.

Step 2: To define the set of genes mapped to the intervals, we converted the hg19 LD intervals defined in step one to hg38 intervals using the UCSC lift-over tool. A total of 12 unique genes were identified for the 5 (GWAS Discovery) regions.

Step 3: We then identified mouse homologs for all 12 unique human genes and mapped these onto the bone co-expression network [40].

ATAC-seq Epigenetic Intersection Analyses

We used two functional epigenomic datasets derived using the Assay for Transposase-Accessible Chromatin followed by sequencing (ATAC-seq) on embryonic day (E) 15.5 mouse proximal femora: (1) proximal femur (head+neck+proximal femur growth plate up to but not including the osseous diaphysis) ATAC-seq dataset, which consists of 24,804 called peaks (N=2 biological replicates)[41] and (2) proximal femoral head ATAC-seq dataset, which consists of 22,727 called peaks (3 biological replicates, unpublished). ATAC-seq assays and computational pipelines were performed as described in Guo et al. [41]. For both types of samples, we used stringent ENCODE recommended IDR (irreproducible discovery rate) cutoff of 0.05. Raw sequencing *fastq* files and processed peak *bed* files were previously deposited on NCBI GEO (GSE100585).

To perform computational intersections of these ATAC-seq datasets with height adjusted hip-geometry-associated loci, *Mus musculus* (mm10) peak calls were first lifted over to the UCSC Genome Browser human genome (hg19). Second, we focused on the top 5 significant loci (**Table 1**), and for each lead (index) SNP we used the Broad Institute HaploReg v.4.1 tool [42] to identify all hg19 variants with an $r^2 \geq 0.4$ in the European 1000 Genomes Population dataset. This yielded 513 height adjusted hip geometry variants that could serve as linked, putatively causal variants. Third, we used BEDTools v2.18 [43] and the UCSC Genome Table Browser tool [44] to identify any overlap (≥1 bp) of a height adjusted hip geometry variant and an ATAC-seq peak. Fourth, for loci showing intersections, we used Mouse Genome Informatics to identify expression patterns for nearby genes, when appropriate.

RESULTS

Each participating study analyzed hip geometry phenotypes by GWAS using standard best practices (See Methods). A meta-analysis of the individual genome-wide association studies was performed first on the first set of studies with available GWAS results. The meta-analysis Q-Q plots did not provide evidence of genomic inflation of association test statistics (ƛ's ranging from 1.01 to 1.07, (Supplemental **Table S3**). Results of SNP-phenotype associations (hip geometry phenotype, top associated SNPs, their MAF and functional impact) are presented in Table 1. Thus, the discovery analysis identified five loci with genome wide significant associations, in *IRX1/ADAMTS16* and near *FGFR4, NSD1*, and *RAB24* (chr.5), a gene-dense region (*LRP5/PPP6R3/GAL*) on chr. 11, *CCDC91* (chr.12), and *RUNX1* (chr. 21). The identified signals were either intronic or intergenic. Most of the loci were phenotype-specific. The chromosome region-wide association analyses with conditioning on the most significant SNP did not reveal secondary (independent) association signals.

The *in-silico* replication was performed in the cohorts with existing GWAS, followed by the combined (joint) analysis of the Discovery and Replication stages, with two models of adjustment as above. Thus, in the combined Discovery and Replication analysis, SNPs near *IRX1/ADAMTS16* (chr.5), a gene-dense region (*LRP5/PPP6R3/GAL*) on chr. 11, and *CCDC91* (chr.12) became GWS. SNP rs6556301 near *FGFR4, NSD1,* and *RAB24* (chr.5), although still meeting the GWS threshold, became slightly less significant than that in the Discovery ($p = 2.3 \times 10^{-8}$ and 1.4×10^{-8}, respectively). SNPs in *RUNX1* (chr. 21) did not replicate.

Some of the hip geometry associated regions had previously been associated with DXA BMD (GEFOS Consortium, **Table** 2). Indeed, there were several signals overlapping with BMD at the suggestive level of significance ($p < 5\times10^{-6}$), mostly for narrow-neck section modulus phenotype: in/near *LRP5* (chr. 11). We further compared the hip-geometry-associated regions discovered by us with GWAS meta-analysis for height performed by the GIANT Consortium [25](**Table** 3). Only rs11049605 in *CCDC91* (chr.12) was GWS-associated with height.

We also looked up the SNPs that were suggestively ($p < 5 \times 10^{-6}$) associated with hip geometry in our Discovery analysis in the recent large GWAS of any type of fracture [32]. No SNP was nominally-significantly associated with fracture at p<0.05 (**Table** 4).

Genetic correlations with Hip Geometry by LD score regression

LD score regression (Supplemental **Table S4**) estimated that SNPs nominally associated with hip geometry phenotypes explained from 12.1±2.7% heritability (NSA) and 12.9±3.3% (FNL) to 17.5±2.9% (NNZ) to 22.0±3.2% of NN Width. NNZ and NN Width significantly correlated (0.369±0.095, p<0.001), while FNL and NSA negatively correlated (-0.526±0.179, p< 0.01). We next determined the genetic correlations between hip geometry phenotypes and several traits and diseases (Supplemental **Table S5**). Only other DXA-derived traits, including femoral, lumbar spine, and forearm BMD, demonstrated strong genetic correlation with NNZ (positive) or NNW (negative), at Bonferroni-corrected α threshold.

Gene Expression Analysis in Human Bone

We correlated hip geometry phenotypes with gene expression from human trans iliac bone biopsies (**Table** 5). 102 different transcripts were correlated with the various structural parameters at 10% FDR. Among Physiological System Development and Function categories the most significant were: “Connective Tissue Development and Function” ($4.62 \times 10^{-2} – 3.12 \times 10^{-4}$); “Embryonic Development” ($4.63 \times 10^{-2} – 3.12 \times 10^{-4}$), “Organ Development” ($4.63 \times 10^{-2} – 3.12 \times 10^{-4}$); “Organismal Development” ($4.93 \times 10^{-2} – 3.12 \times 10^{-4}$); “Skeletal and Muscular System Development and Function” ($4.96 \times 10^{-2} – 3.12 \times 10^{-4}$). Notably, several bone structure relevant subcategories (containing at least two genes) were identified within these categories, including: “Development of vertebral body” (CLEC3B), $p=3.12 \times 10^{-4}$; “Differentiation of connective tissue cells” (IL1A,LRP8,MRPS18B,NIPBL,PIAS1,SOST,TOB1), $p=8.67 \times 10^{-3}$;

"Quantity of osteoclasts" (IL1A,SOST,TOB1), p=1.43 x 10^{-2}; "Ossification of bone" (NIPBL,SOST,TOB1), p= 2.42 x 10^{-2}.

cis-eQTLs in Human Bone Tissues

The results of the *cis*-eQTL obtained from whole bone biopsies are shown in **Table** 6. For genome-wide significant SNPs we found two significant eQTLs after multiple testing correction (adjusted for number of SNP-gene pairs by Bonferroni correction), namely rs7102273 (intergenic, chr. 11) and *PPP6R3* (protein phosphatase 6 regulatory subunit 3) expression (p=0.0007), and rs6556301 (intergenic, chr.5) and *PDLIM7* (PDZ and LIM domain 7) expression (p=0.005).

Primary Murine Cells

We tested whether mouse homologs of human genes were expressed in mouse calvarial osteoblasts and found that for some, the expression changed during cell differentiation. **Table** 7 presents a list of genes whose expression profiles (trajectories) underwent changes. Thus, Lrp5 expression increased with osteoblast differentiation; Rab24 expression slightly decreased; Irx1 expression increased and then reached a plateau at the late differentiation stage. Expression profiles for other genes were less obvious.

We also mined osteoclast expression data (GEO Accession GSM1873361; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72846) and found evidence of expression of some of our genes of interest in control mice in mature osteoclasts. On chr. 5 (proximal locus), Adamts16 expression, but not Irx1, was present during osteoclastogenesis; in distal locus on chr. 5, among the 3 genes of interest, expression of Rab24 and Nsd1 was detected but reads mapping to Fgfr4, were not observed in mature osteoclasts suggesting a lack of expression of this gene in these cells. On chr. 11, notably Lrp5 and Ppp6r3 were expressed in osteoclast whereas Gal was not.

Co-expression network from mouse cortical bone expression profiles

We previously used a network-based approach to nominate potentially causal genes at BMD GWAS associations [40]. In this work, Calabrese et al. identified two modules from a bone co-expression network that were enriched for genes implicated by BMD GWAS. For the hip geometry GWAS results we determined if implicated genes were members of these two modules (referred to as modules 6 and 9). We hypothesized that genes implicated by hip geometry GWAS and members of modules 6/9 might play a role in the regulation of hip geometry and be strong candidates to underlie associations with hip geometry.

A total of 12 genes were identified for the 5 GWAS regions and all 12 had clear mouse homologs. Two (*LRP5* and *PPP6R3*) of the 12 mouse genes belonged to either module 6 and or 9. Both of these modules have previously been shown to be enriched for genes with well-known roles in osteoblast activity.

ATAC-seq *epigenomic intersections in mice*

We performed computational intersections between two ATAC-seq datasets derived from mouse embryonic proximal femora (see Methods) and 513 height adjusted hip-geometry variants from 5 associated loci to identify putative regulatory variants influencing proximal femur geometry. Our intersections with a first dataset derived from all proximal femur tissues revealed 5 hip geometry variants from 2 loci that overlap with an ATAC-seq peak (Supplemental **Table S6**). At the *IRX1/ADAMTS16* locus on Chr. 5, four variants fall within the same ATAC-seq peak, with rs6871994 in the strongest LD to lead variant (r^2 = 0.88) and associated with FNL. At the *RUNX1* locus on Chr. 21, rs8129061 is at r^2 = 0.51 to the lead variant, resides within an ATAC-seq peak within a gene desert, and is associated with FNL. Intersections with a second dataset, focused specifically on the proximal femoral head cartilage, did not yield any SNPs. All remaining loci (incl. *FGFR4*, *NSD1*, *RAB24*, *LRP5*, *PPP6R3*, *GAL*, and *CCDC91*) did not yield variant intersections with either dataset, although ATAC-seq peaks were identified in the vicinity of each gene (Supplemental **Figure 2**).

DISCUSSION

This is the largest study to comprehensively assess genetic variants associated with proximal femur geometry using a GWAS approach with functional validation in human bone transcript profiles, cell and animal data. Despite the contribution of bone geometry of the femur to fracture risk [11], and the known heritability of hip geometry traits measured using HSA (28% to 70%[12]), there have been no large-scale GWAS for hip geometry. We assessed the evidence of association of the novel markers identified here, in large GWAS meta-analyses of height (183,727 participants [25]), DXA-derived lumbar spine (N~32,000) and femoral neck BMD (N~32,000)[31], as well as fracture (37,857 cases and 227,116 controls from the GEFOS consortium).

Our GWAS findings identified several noteworthy genes. In the combined discovery and replication meta-analysis, significant associations were found for FNL at 5p15 where *IRX1* and *ADAMTS16* are mapped ($P = 2.4 \times 10^{-8}$). In height-adjusted NNW, significant association was

observed at 12p11 ($P = 4.9 \times 10^{-12}$) in the intron of *CCDC91*. Height-adjusted NNZ was associated with variants at 11q13 near *LRP5* and *PPP6R3* ($P = 4.3 \times 10^{-11}$), which are known genes for BMD. SNPs in *FGFR4/NSD1*/*RAB24* (chr.5) and in *RUNX1* (chr. 21) did not replicate. It is of interest that FGFR4 interacts with FGF23, an inhibitor of mineralization; and RUNX1 is involved in hematopoiesis and osteogenesis.

Since stature is an important contributor to hip fracture, and since measures of hip geometry are dependent on an individual's size, we performed a systematic study of the genetic associations of hip geometry with adjustment for height, in a sample of >27,000 adults. Importantly, we performed GWAS of hip geometry with adjustment for height at a whole-genome scale, not only for the subset of SNPs previously shown to be associated with height (to prevent "collider bias"[45]). We did not adjust our hip geometry measures for areal BMD, since these measures are both DXA-derived (therefore "collider bias"[45] might be expected). We also performed non-height-adjusted analysis (not shown here). The following chromosomal loci/genes were identified at suggestive-GWS levels: *HHIP* (chr.4), *ENPP2* (on chr. 8), *ASTN2* / *TMEM38B* (chr. 9), near *FAM10A4/DLEU2* (chr.13), *GDF5* and *DDX27* (both on chr. 20). Not surprising, SNPs in most of these genes were also strongly associated with adult height. Of note, in our height-adjusted analysis, only rs11049605 in *CCDC91* (chr.12) was still GWS-associated with height, therefore indicating that adjustment for height dramatically reduced hip geometry associated signals, probably due to body-size contribution to the HG phenotypes.

Of note, one previous GWAS in 1000 European-descent Americans[16] found a common genetic variant, rs7430431 in the receptor transporting protein 3 (*RTP3*) gene, to be in strong association with the buckling ratio ($p = 1.6 \times 10^{-7}$), an index of bone structural instability, and with femoral cortical thickness ($p = 1.9 \times 10^{-6}$). The *RTP3* gene is located at 3p21.31. We were unable to confirm this signal in our much larger study.

We further looked for shared associations between our hip geometry phenotypes and bone fractures. SNP rs7102273 (intergenic, 11q13.2) was associated with fracture ($p = 7.5 \times 10^{-5}$); the allele that was associated with higher NNZ, also corresponded to the lower risk of fracture. Of interest, this SNP is in LD (R^2=0.96; D'=1) with another intronic variant, rs12272917, that was associated with skull BMD in Kemp et al. [46] study (the distance between the SNPs is ~122.2K). Both SNPs are in *PPP6R3* (a.k.a. *SAPS3*) gene, whose Gene Ontology (GO) annotations include "*protein phosphatase binding"*, and related pathways are "Transport to the Golgi and subsequent modification" and "Vesicle-mediated transport". It is still unclear how the gene may be influencing bone geometry. Some of our signals fall into known loci for other bone

phenotypes. Apart from *LRP5* and *PPP6R3* (chr. 11) there were no signals overlapping with BMD at the suggestive level of significance ($p < 5\times10^{-6}$).

By correlating hip geometry phenotypes with gene expression from trans iliacal bone biopsies, we found gene transcripts falling into several functional categories. Among "Physiological System Development and Function" categories the most significant were: *"Connective Tissue Development and Function"*, "Embryonic Development", "Organ Development", "Organismal Development", and "Skeletal and Muscular System Development and Function". Notably, several bone structure relevant subcategories (containing at least two genes) were identified within these categories, including: "Development of vertebral body", "Differentiation of connective tissue cells", "Quantity of osteoclasts", and "Ossification of bone", supporting the bone-structure role of the genes associated with hip geometry.

To further understand functional implications of our hip geometry signals via molecular and cellular mechanisms, we used the bone co-expression network from prior work of Farber and colleagues[40]. They found that mouse analogues of BMD GWAS genes were enriched for genes important to bone (mostly osteoblast) biology. By mapping mouse homologs of human genes located in GWAS regions onto murine dataset, they identified an Osteoblast Functional Module containing 33 genes implicated by GWAS. These genes are candidates for 30 of the 64 BMD GWAS regions discovered by GEFOS in European-descent persons.[47] Most of the GO ontologies shared between these modules corresponded to cellular components, biological processes, and molecular functions pertinent to osteoblasts and ossification.[40, 48] The top genes for hip geometry included *PPP6R3* and *LRP5*, whose expression correlated with osteoblastic modules. We thus suggest that the identification of our GWAS top SNPs for hip geometry confirms they are excellent candidates for being potentially responsible for the signal in associated loci.

Given that greater than 95% of variants (i.e., those with r^2 >0.4 to lead hip-geometry-associated variants) fall within non-coding regions, they likely function to alter hip geometry through their effects on gene regulation. To refine the putative functional roles of hip geometry variants in this context and in the absence of inaccessible human developmental tissues, we performed computational intersections of all such variants with ATAC-seq datasets derived from embryonic mouse chondrocytes, at a stage when proximal femoral morphology is initially determined. These analyses yielded 2 associated novel loci with 5 variants in strong to modest LD with the lead variant within an ATAC-seq peak. We detected variants in distant-acting enhancers at two loci associated with FNL (*IRX1*/*ADAMTS16* and *RUNX1*). While mouse

and/or human chondrocyte or bone phenotypes have only been reported for *Runx1*/*RUNX1* [49], IRX1 has been shown to influence chondrocyte differentiation [50] and it (MGI [51]), along with *Adamts16* (MGI [52]) and *Runx1* (MGI [53]) is expressed during mouse femoral development. While follow-up experiments on variants in *IRX1* could further demonstrate its role in hip geometry, its re-identification here bolsters our computational strategy to whittle-down loci to fewer putatively causal variants.

We also mined two publicly available datasets, of primary mouse osteoblasts undergoing differentiation (GSE54461) and osteoclast expression data (GSM1873361). In mouse primary cells, among chr. 11 genes, Lrp5 (but not Ppp6r3) expression increased with osteoblast differentiation. For both Lrp5 and Ppp6r3, expression was present during osteoclastogenesis, while Gal was not expressed in either osteoblasts or osteoclasts. This evidence supports the role that *LRP5*, a well-known bone-active gene, plays in "pleiotropic" actions on most bone-related properties.

We then interrogated a unique dataset of global gene expression from trans-iliac bone biopsies obtained from 84 postmenopausal women [36]. In human whole bone biopsies, transcripts for LRP5 correlated with FNL of the biopsied persons ($p<0.05$), while transcripts for RUNX1 correlated with their NNZ ($p<0.05$). Finding the associations with LRP5 confirm earlier results of Wnt signaling system's candidates associated with BMD and fracture risk.[36] Validation of our top genes detected in human bone samples by RT-PCR was carried out previously [36, 54]).

Since most of these postmenopausal women with trans-iliac bone biopsies were genotyped, eQTLs were also available for our analysis. Thus, for subset of SNPs we found two eQTLs, still significant after applying multiple testing correction (by FDR), namely rs7102273 (intergenic, chr. 11) to associate with PPP6R3 expression ($p=0.0007$), and rs6556301 (intergenic, chr.5) with PDLIM7 expression ($p=0.005$). *PDLIM7* (PDZ And LIM Domain 7/ Enigma) is known to code for the LIM mineralization proteins (LMP) which have an important osteogenic role [55]. It seems that alteration of gene expression by the variants located in a regulatory region in the vicinity of *PPP6R3*, similar to *PDLIM7*, may affect bone geometry. We did not observe a link between the associated SNPs in the chr. 11 region and *LRP5* gene expression in human bones. The low expression level in whole bone and small sample size in human eQTL studies may limit the statistical power to detect cis-eQTLs.

We also estimated that SNPs associated with hip geometry phenotypes explained from 12.1±2.7 heritability in NSA up to 22.0±3.2 (NN Width). NN Section Modulus and NN Width

significantly correlated (0.369±0.095, p<0.001), while FNL and NSA negatively correlated (-0.526±0.179, p<0.01). It is important to note that due to our relatively modest sample (n=18,719), many more SNPs are expected to be uncovered through larger efforts, as follows from our experience with GWAS of other phenotypes, namely BMD, height and BMI, which will explain larger portion of heritability. Moreover, only singular signals (loci) generally reached GWS for each hip geometry trait: two for each FNL and NNW, one for NNZ, and none for NSA (Discovery). The impact of a single variant on a complex polygenic trait is usually small, and multiple variants with small effects, operating within a complex network, likely underlie hip geometry variance. Therefore, identification of a larger number of gene variants that play a role in bone architecture is necessary before real gains in predicting fracture risk can be achieved by using hip-geometry-associated variants. Statistical evidence (replication in independent cohorts) and biological evidence (from mammalian cells and human whole bone), although not always agree, are both indispensable for finding the true genetic loci.

The primary advantage of HSA-measured hip geometry is that bone geometry and areal BMD, both of which contribute to bone strength, are considered. This, however, makes our task of distinguishing between genetics of hip geometry per se, independent of BMD, challenging. It is important to emphasize that hip geometry is actually measured from the DXA image data, not estimated via the BMD. The algorithm of HSA was described in detail in [56]. Of note, we found genetic correlations between some hip geometry phenotypes and DXA-derived BMD at femur, lumbar spine, and forearm. These genetic correlations were positive with NN-section modulus (bone strength measure) but negative with NN-width. The latter fully complies with the understanding that wider bones have larger cross-sectional area but not necessary higher density; this underlines the potential role of expansion of outer femoral neck diameter and cortical thinning that occurs with aging [57]. The oldest women had wider femoral necks containing less bone tissue, thinner cortices, less bending resistance, and significantly greater buckling ratios [58].

This study has several limitations that need to be noted. GWAS studies were imputed to older imputation panels because the initial imputation began at the early stages of this project several years ago; at the present, newer and denser panels exist (1000Genomes, HRC and UK10K). More detailed exploration would need fine-mapping and sequencing of the loci prioritized here. Also, animal modeling experiments were considered beyond the scope of the present study. Given the biologic plausibility of our findings, further exploration is left to future studies.

In conclusion, our GWAS-based study provides indications that hip geometry measured using HSA may reveal novel molecular pathways influencing skeletal shape and impacting mechanical properties. Our findings also suggest that HSA-measured hip geometry might capture additional genetic determinants beyond those associated with hip BMD. These variants should be prioritized for future functional validation regarding their involvement in the regulation of bone strength and risk of hip fracture. At the current stage of genetics research, there are few instances where genetic association findings are directly used clinically. However, since hip geometry contributes to hip fracture risk[2], discovery of genetic determinants of hip geometry may have utility in predicting fracture as methods to derive polygenic risk scores mature further [3, 4]. Since hip geometry still evolves in early life and even beyond, the identification of genetic determinants of hip architecture could be used as potential diagnostic tools and drug targets to improve bone strength.

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