Genetic determinants of heel bone properties: Genome-wide association meta-analysis and replication in the GEFOS/GENOMOS consortium.

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ABSTRACT

Quantitative ultrasound of the heel captures heel bone properties that independently predict fracture risk and, with bone mineral density (BMD) assessed by x-ray (DXA), may be convenient alternatives for evaluating osteoporosis and fracture risk. We performed a meta-analysis of genome-wide association (GWA) studies to assess the genetic determinants of heel broadband ultrasound attenuation (BUA, n=14,260), velocity of sound (VOS, n=15,514) and BMD (n=4,566) in 13 discovery cohorts. Independent replication involved 7 cohorts with GWA data (*in silico* n=11,452) and new genotyping in 15 cohorts (*de novo* n=24,902). In combined random effects meta-analysis of the discovery and replication cohorts, 9 SNPs had genome-wide significant ($p<5\times10^{-8}$) associations with heel bone properties. Alongside SNPs within or near previously identified osteoporosis susceptibility genes including *ESR1* (6q25.1: rs4869739, rs3020331, rs2982552), *SPTBN1* (2p16.2: rs11898505), *RSPO3* (6q22.33: rs7741021), *WNT16* (7q31.31: rs2908007), *DKK1* (10q21.1: rs7902708), and *GPATCH1* (19q13.11: rs10416265), we identified a new locus on chromosome 11q14.2 (rs597319 close to *TMEM135*, a gene recently linked to osteoblastogenesis and longevity) significantly associated with both BUA and VOS ($p<8.23\times10^{-14}$). In meta-analyses involving 25 cohorts with up to 14,985 fracture cases, six of 10 SNPs associated with heel bone properties at $p<5\times10^{-6}$ also had the expected direction of association with any fracture ($p<0.05$), including 3 SNPs with $p<0.005$: 6q22.33 (rs7741021), 7q31.31 (rs2908007), and 10q21.1 (rs7902708). In conclusion, this GWA study reveals the effect of several genes common to central DXA-derived BMD and heel ultrasound/DXA measures and points to a new genetic locus with potential implications for better understanding of osteoporosis pathophysiology.

INTRODUCTION

Bone structure *in vivo* has largely been evaluated using the attenuation of a photon beam by hydroxyapatite, the principle mineral in bone. This is positively related to the mass of hydroxyapatite in the path of the beam conventionally termed bone mineral content and normalised to bone area to produce an entity termed areal bone mineral density (BMD). To allow for the reduced attenuation of the beam by overlying non-bone tissues in central areas of the body, two photon beam energies are used, resulting in a clinical technique termed dual-energy X-ray absorptiometry (DXA), which at peripheral skeletal sites is termed pDXA.

Over the past 60 years, ultrasonic material analysis has been developed as a method of determining material properties of a variety of structures. In the last 30 years this methodology has been applied to the *in vivo* assessment of bone structure and fragility termed Quantitative Ultrasound (QUS). This consists of the use of two separate ultrasound measurement techniques, velocity of sound (VOS) and bone ultrasound attenuation (BUA). While much remains to be discovered about the exact physical determinants of quantitative ultrasound (QUS) measures in the intact living calcaneum (1), cadaver studies have established a strong correlation of such indices with bone quantity and trabecular structure (2). Assessment of bone properties in the heel using QUS can predict the risk of prevalent osteoporotic fractures, such as those in the spinal vertebrae, comparably with DXA of the spine or hip, the so-called gold standard clinical techniques (3-5). Pearson correlation coefficients of heel QUS or pDXA with central DXA of the hip or spine in population-based studies are modest, typically in the range 0.4-0.6 (6). Moreover, twin- and family-based studies have found genetic correlations of the order of 0.3-0.6 and environmental correlations of the order of 0.1-0.3 (7-9); yet relative risk estimates for fracture using QUS are of similar magnitude to those derived from central DXA (5,10,11). A recent meta-analysis showed that

heel QUS predicts risk of various fractures (hip, vertebral, and any clinical fractures) independently from hip BMD (12). Overall, these results suggest that QUS of the calcaneum might capture additional genetic determinants of bone structure beyond those associated with central DXA.

A genetic contribution to osteoporosis is well established with heritability estimates reaching 84% for central BMD (13), 74% for heel QUS (7,14), 47% for bone loss (15), and 48% for hip fracture (16). Previous genome-wide association (GWA) studies have identified several chromosomal regions associated with BMD in the hip and lumbar spine regions (17,18). The most recent meta-analysis of GWA studies, performed in the context of the Genetics Factors for Osteoporosis (GEFOS) consortium, identified 56 genome-wide significant loci (32 new) associated with hip/spine BMD (19). Fourteen out of these 56 BMD-associated loci were also associated with fracture risk in a case-control meta-analysis involving about 31,000 fracture cases among 133,000 individuals (19). Using data from the GEFOS consortium, we aimed to extend the findings for central DXA-derived BMD phenotypes by searching for single nucleotide polymorphisms (SNPs) associated with heel QUS or heel DXA measures across the human genome.

RESULTS

Key features of the discovery and replication phases are summarised in **Figure 1**. In aggregate, the initial discovery phase meta-analysis in 11 cohorts (**Supplementary Table 1**) identified 42 loci of at least suggestive significance in relation to heel bone measures, of which 9 overlapped with loci previously found to be potentially associated with hip or spine BMD in the GEFOS-BMD meta-analysis (19). Regional conditional analyses results were available for QUS measures from 9 cohorts (comprising 7 of the initial discovery cohorts and a further 2 new cohorts that joined later). Based on the results of the conditional analyses (that identified two secondary signals for the QUS measures) and final combined meta-analysis of the unconditional results from all 13 discovery cohorts, a total of 25 independent SNPs were selected for replication in the next phase (i.e. *in silico* studies and *de novo* genotyping). Including the two secondary signals, the selected SNPs comprised 15 SNPs that were primarily associated with either BUA or VOS, and 12 SNPs that were associated with heel DXA BMD (**Table 2**).

Associations between the 15 SNPs that were considered for replication primarily on the basis of their association with heel BUA or VOS are shown in **Figure 2**. The SNP characteristics are summarized in **Table 2.** In the combined meta-analysis of the discovery and replication cohorts using a random-effects model, 9 SNPs showed genome-wide significant associations, of which 7 were previously reported to be associated with central DXA BMD (19). Two of the SNPs (rs7741021 and rs2908007) also showed genome-wide significant association with heel DXA BMD (**Table 2**). Three SNPs on chromosome 6q25.1 (rs4869739, rs3020331, rs2982552) mapped to intronic or regulatory regions around the *ESR1* (estrogen receptor 1) and *CCDC170* (coiled-coil domain containing 170, previously known as *C6orf97*) genes (**Figure 3**), and 5 other SNPs mapped to loci within or near previously identified osteoporosis

susceptibility genes, including 2p16.2 (*SPTBN1*, rs11898505); 6q22.33 (*RSPO3*, rs7741021); 7q31.1 (*WNT16*, rs2908007); 10q21.1 (*DKK1*, rs7902708); and 19q13.11 (*GPATCH1*, rs10416265). We identified a new locus on chromosome 11q14.2 (*TMEM135*, rs597319) significantly associated with both BUA and VOS ($p < 8.23 \times 10^{-14}$).

Subsidiary comparisons with fixed-effect meta-analysis results (**Supplementary Table 2**, **Supplementary Figure 2**, and **Supplementary Figure 3**) suggested two additional genome-wide significant loci; one at 7p14.1 upstream of *EPDR1* (rs6974574, $p < 4.92 \times 10^{-8}$ for BUA and VOS) and the other at 13q14.11 upstream of *AKAP11* (rs9533090, $p = 5.33 \times 10^{-8}$ for VOS), although there was statistically significant between-study heterogeneity in these two loci for the respective phenotypes (**Supplementary Table 3**), necessitating some caution in generalizing the fixed-effect meta-analysis results. **Figure 4** provides a comparison of the magnitudes of association of the 25 SNPs with heel bone measures and central DXA BMD, suggesting generally concordant associations in the overlapping genome-wide significant or suggestive loci.

We further tested if the genome-wide significant or suggestive genetic loci were associated with fracture risk based on data available from 25 cohorts with up to 54,245 participants, among whom there were 14,958 cases of any fracture (excluding fractures of the skull and extremities i.e. fingers and toes), 10,663 non-vertebral fractures, and 3,220 clinical vertebral fractures (**Supplementary Table 4**). Ten out of ten SNPs associated with heel bone properties at $p < 5 \times 10^{-6}$ showed the expected directions of association with any fracture outcome based on the point estimates (**Figure 5**). Furthermore, 6 of these 10 SNPs showed nominally significant ($p < 0.05$) associations with fractures, including 3 SNPs with $p < 0.005$ (i.e. corrected for multiple comparisons using Bonferroni method) at 6q22.33 (rs7741021),

7q31.31 (rs2908007), and 10q21.1 (rs7902708). Fixed-effect meta-analysis gave similar results (**Supplementary Figure 4**).

Supplementary Figure 5 presents forest plots of the study-specific results and summary estimates by random effects meta-analysis for the 15 SNPs that were considered for replication primarily on the basis of their association with heel BUA or VOS in GWA discovery meta-analysis, suggesting generally consistent results across cohorts for a majority of the SNPs. **Supplementary Figure 6** shows the regional association plots within a one megabase window of the top SNP in each locus in the GWA discovery meta-analysis, demonstrating strong credible association signals for a number of SNPs underlying the loci selected for replication.

Subsidiary investigation of potential sex differences in the association of SNPs and heel BUA or VOS measures did not reveal convincing evidence of potentially important differences, considering the secondary nature of the hypothesis and multiple comparisons done (**Supplementary Figure 7**).

DISCUSSION

This is the first large-scale collaborative GWA study for heel bone properties assessed by quantitative ultrasound and DXA of the heel. Its conception was inspired by the observational evidence of association of heel QUS measures and fracture risk (12), independent of central DXA BMD measures (20), demonstration of a reasonably high genetic heritability of heel QUS measures (7), and suggestions of pleiotropic effects of genes in the determination of bone phenotypes (8). Indeed, consistent with the expected similarities and differences in the physical properties of bone determined by DXA and QUS and prior evidence of moderate genetic correlations between the measures (7-9), we found evidence for some genetic loci common to heel QUS measures and central DXA BMD as well as a novel locus for heel QUS at 11q14.2 (*TMEM135*, rs597319) that had not been previously identified as associated with BMD or other bone phenotype.

Seven out of 9 genome-wide significant loci found in the present study were previously reported to be associated with BMD of the hip and/or spine (**Figure 4**). This complements our previous findings (17-19) and lends support to the hypothesis of partially shared genetic determinants between QUS and BMD measures (7-9). A comparison of the standardised effect sizes (**Figure 4**) also revealed existence of some quantitative differences for some SNPs. For example, in the 7q31.31 locus (*WNT16*), the effect of rs2908007 on heel measures was about 3 times as great as its effect on hip or spine BMD, supporting Karasik *et al.*'s finding that there is significant pleiotropy in the effects of genes on bone phenotypes at different measurement sites (8). Similar quantitative differences were also observed for rs7741021 at the 6q22.33 locus (*RSPO3*). In the absence of bias and assuming minimal type II errors (i.e. adequate power), such quantitative differences in effect sizes of SNPs at different skeletal sites might indicate heterogeneity in genetically mediated responses of the

skeleton to environmental stimuli, including for example, ground reaction forces that are particularly high at the heel but are dampened at more proximal sites such as the lumbar spine (21,22).

Perhaps the most intriguing finding was that we identified a new locus for bone phenotypes on chromosome 11q14.2 (rs597319) near the transmembrane protein 135 (*TMEM135*) gene, that was genome-wide significant for both BUA and VOS. The *TMEM135* gene was first identified in a human lung adenocarcinoma cell line cDNA library (23). It has been suggested that it is critically involved in the process of osteoblastogenesis from human multi-potent adipose tissue-derived stem cells (24). Marrow fat cells and osteoblasts share a common stromal precursor and there is currently great interest in the role of increased marrow fat in osteoporotic conditions and the metabolic inter-relationships between these neighbouring cell types (25). In-depth protein sequence analysis showed that *TMEM135* is a multi-transmembrane protein with 7 transmembrane helices of high confidence. Homologies exist between *TMEM135* and the transmembrane region of frizzled-4 (24), a known component of the Wnt signaling pathway (26). ENCODE project (27) data shows that 2 SNPs in the intronic region of *TMEM135* and close to our lead signal (rs502580 and rs603140, both with high linkage disequilibrium with rs597319 [>0.92], and both highly associated with QUS outcomes in our discovery cohorts [P ~ 1.3E-07 for both]) are associated with changes in MIF-1 and Cart1 motifs in osteoblastic cell lines. Interestingly, both of these transcription factors have been previously shown to be associated with skeletal development and bone density (28,29). Furthermore, *TMEM135* was previously reported to be associated with longevity in *C. elegans* models (30) as well as with longevity and walking speed in humans (31). In summary, the associations observed in our study might be the results of direct effects of increased osteoblastogenesis on heel bone properties, or indirect effects mediated through increased mechanical loading of the calcaneum, associated with faster movements.

The other genetic loci with significant associations with heel bone measures have previously been reported to be associated with BMD or fractures. The *ESR1* gene has been shown to be related to osteoporosis susceptibility in both candidate gene (32) and GWA studies (18,33). SNPs in *SPTBN1* gene were significantly associated with central DXA BMD in a previous meta-analysis of GEFOS cohorts (18), as were SNPs in *WNT16*, *DKK1*, and *GPATCH1* genes in the recent GEFOS-BMD meta-analysis (19). The *RSPO3* gene has recently been suggested as a bone-related locus by a GWA study of extreme low and high BMD populations (34). The spectrin, beta, non-erythrocytic 1 (*SPTBN1*) gene located at chromosome 2p16.2 codes for the β-subunit of spectrin, which is a molecular scaffold protein essential in linking plasma membrane to the actin cytoskeleton. Spectrin plays an important role in determination of cell shape, positioning of trans-membrane proteins, resilience of membranes to mechanical stress, and organization of organelles and molecular traffic in cells. β-subunits coded by *SPTBN1* are responsible for most of the spectrin binding activity. Despite several GWA studies confirming the association between *SPTBN1* and osteoporosis (18,19,33,35), its role in bone pathophysiology is unclear.

The estrogen receptor 1 (*ESR1*) gene located at chromosome 6q25.1 codes for the estrogen receptor type 1 (also known as ER-α). Two isoforms of estrogen receptors in humans (α and β) are encoded by two different genes (*ESR1* and *ESR2*) and have distinct tissue and cell patterns of expression. Estrogen receptor is a DNA-binding transcription factor that regulates the activity of many different genes. Estrogen is well known to inhibit bone resorption through both direct and indirect actions on osteoclasts, and it is a major anabolic steroid in bone, particularly evident in the establishment of peak bone mass. Postmenopausal bone loss is complex, involving many genetically regulated processes. After menopause, bone is lost rapidly but variably for several years by most women as osteoclastic bone resorptive activity increases in association with osteocyte apoptosis (36). In an osteoporosis GWA study by

deCODE Genetics in 2008 (33), several markers close to *ESR1* were reported to show association with BMD, including intronic variants and upstream SNPs close to *CCDC170* (previously known as *C6orf97*). This association was replicated in both GEFOS-BMD meta-analyses (18,19), and we found three independent SNPs in this region associated with heel BUA and VOS. Most recently, this locus has been shown to be more associated with cortical volumetric BMD (as opposed to trabecular BMD), which implies a role of *ESR1* products in osteoblastogenesis and cortical porosity (37).

The wingless-type MMTV integration site family, member 16 (*WNT16*) gene located at chromosome 7q31.31 is part of the Wnt/LRP pathway, which is a known major anabolic pathway in bone (38). The effects of activation of this pathway include differentiation of mesenchymal precursors into osteoblasts, osteoblast proliferation, bone mineralization, and avoidance of osteoblast apoptosis, and inhibition of osteoclastogenesis through effects on expression of *OPG* and *RANKL*. Other members of this pathway such as *LRP5, LRP4, SOST, WLS, DKK1* and *CTNNB1* have previously been associated with BMD at genome-wide significance level (18,19,33,35).

The variant rs7902708 on chromosome 10q21.1 locates between the *MBL2* and *DKK1* genes, and is in close linkage disequilibrium with another SNP in this locus (rs1373004, $R^2 = 0.87$ in HapMap CEU population) that was previously found to have a significant association with BMD and fracture risk in GWA meta-analyses (19). Since the *MBL2* (mannose-binding lectin 2) gene product is active in the innate immune system, it is more likely that these variants have a *cis* regulatory effects on Dickkopf-1 (*DKK1*), which is a known Wnt signalling pathway inhibitor (39). Several functional studies have showed the role of *DKK1* in osteolytic bone lesions in patients with advanced multiple myeloma (40) and its inverse relationship with bone mass has been shown in knockout mouse models (41). A similar relationship to the

Wnt signalling pathway has also been proposed for the *RSPO3* gene (21). Although *GPATCH1* was also found to be associated with hip and spine BMD in a previous GEFOS meta-analysis (19), there is no functional information about it in genomic databases.

Caution must be exercised in interpreting the results of the heel DXA BMD analyses because there were fewer than 7,000 participants contributing to the combined meta-analysis. The obtained results, however, were consistent with the work of Portero *et al.* suggesting that heel DXA BMD and BUA measure comparable properties of the calcaneum, which reflect the amount of bone mineral in the field of view of the detector (2).

While the current study had limited statistical power in the meta-analysis of SNP associations with fracture outcomes, it was nevertheless encouraging to observe nominally statistically significant and expected directions of associations with fractures for 6 SNPs associated with heel bone measures, including 3 SNPs at 6q22.33 (rs7741021), 7q31.31 (rs2908007), and 10q21.1 (rs7902708) whose p-values for association surpassed the multiple testing chance-corrected threshold of $p < 0.005$. The concordant findings may, albeit indirectly, suggest that some of the genetic susceptibility to fracture could partly be mediated through bone properties (e.g. structural or material) captured by QUS or DXA measures; but larger well-powered studies are needed to appropriately assess such relevance.

In conclusion, the present GWA study reveals the effect of several genes common to central DXA-derived BMD and heel ultrasound/pDXA measures and points to a new genetic locus with potential implications for better understanding of osteoporosis pathophysiology. Quantitative differences seen in the standardised effect sizes of some SNPs at different skeletal sites are potentially indicative of heterogeneity in genetically mediated responses of the skeleton to environmental stimuli, including ground reaction forces that are particularly high at the heel than at central sites.

MATERIALS AND METHODS

Study subjects and measurements

The GEFOS consortium is an international collaboration of investigators dedicated to identify the genetic determinants of osteoporosis (http://www.gefos.org/). In particular, the GEFOS consortium extended the breadth of its predecessor, the Genetic Markers for Osteoporosis (GENOMOS) consortium, into meta-analysis of GWA discovery studies. In the current GEFOS/GENOMOS project we performed GWA discovery and replication of genetic loci associated with heel bone properties, including QUS (measures: Broadband Ultrasound Attenuation [BUA] and Velocity of Sound [VOS]) and DXA (measure: heel BMD).

The discovery phase comprised 13 cohort studies with GWA data and relevant heel bone phenotypes (including BUA in 14,260 participants from 9 cohorts; VOS in 15,514 participants from 9 cohorts; and heel DXA BMD in 4,556 participants from 3 cohorts) arising from populations across North America, Europe, and East Asia. Independent replication was performed using summary results from 7 cohorts with GWA data (*in silico* n=11,452) and analysis of individual-level data from 15 other cohorts in the GENOMOS consortium that were centrally genotyped for candidate polymorphisms by the Kbioscience laboratory in the UK (*de novo* n=24,902). Characteristics of the study cohorts are summarized in **Table 1**. All studies were approved by institutional ethics review committees at the relevant organizations and all participants provided written informed consent. Further descriptive information about the participating cohorts is available from the GEFOS/GENOMOS websites (http://www.gefos.org/?q=studies and http://www.genomos.eu/index.php?page=cohorts).

Genotyping and imputation methods

All the discovery cohorts were genotyped using commercially available Affymetrix (Affymetrix Inc., Santa Clara, CA, USA) or Illumina (Illumina Inc., San Diego, CA, USA) genotyping arrays. Quality control was performed independently for each study according to standard manufacturer protocols and within study procedures. To facilitate meta-analysis, each group performed genotype imputation with IMPUTE or MACH software using genotypes from the HapMap Phase II release 22, NCBI build 36 (CEU or CHB/JPT as appropriate) as reference panels. Each imputation software estimates an overall imputation quality score for each SNP. These quality scores and minor allele frequencies for up to ~2.5 million SNPs available from each cohort were considered in the 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) genetic model. Phenotypes for the association analyses were defined as the sex-specific standardized residuals from linear regression of each outcome variable (BUA, VOS, or heel BMD) on age, age-squared, weight, height, and machine type (if more than one machine was used). 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. The final results submitted to the coordinating centre for meta-analysis were the per-allele regression coefficients with corresponding standard errors and p-values for the associations of up to 2.5 million SNPs and standardized residuals of each outcome variable. Analysis of imputed genotypes used either the dosage information from MACH or the genotype probabilities from

IMPUTE. The replication analyses used the same analytical procedures as above where applicable (e.g. using study-specific standardized residuals as outcomes).

Meta-analysis

Meta-analysis of the GWA discovery summary results was conducted in two independent collaborating centres (Cambridge, UK, and Boston, USA). Because of potentially limited power to detect sex-specific associations, we pre-specified the primary analyses to involve meta-analysis of the pooled data (i.e., males and females combined). Quality control filters applied for exclusions of SNPs from the meta-analysis were: imputation quality score < 0.3 for MACH and < 0.4 for IMPUTE, average minor allele frequency of <1% across studies, and SNPs missing from >50% of the cohorts contributing to each outcome. Inverse-variance fixed-effects meta-analysis (using METAL software) was conducted in the discovery set with double genomic correction (42) to control for potential inflation of the test statistics in individual studies and in the meta-analysis. The genome-wide level of statistical significance was set at $p < 5\times10^{-8}$ and suggestive level of significance at $5\times10^{-8} \leq p < 5\times10^{-6}$. There were no extreme genomic inflation factors noted in the discovery phase studies or in the GWA meta-analysis (**Supplementary Table 1**). QQ-plots for the combined GWAS meta-analysis results are provided in **Supplementary Figure 1**.

To help refine the choice of SNPs to be taken forward for replication, conditional analyses were conducted within a one megabase window of the best-associated SNP in each locus in the discovery cohorts, if there was more than one SNP with a suggestive level of significance. These secondary analyses took the SNP in the locus with the lowest p-value and conditioned the analysis of all of the other SNPs in the locus by including it in the regression models. In addition, for loci containing SNPs previously associated with hip or spine BMD in GEFOS (19), we performed additional conditioning on the nearby "BMD SNP".

The DerSimonian and Laird random-effects model was used for meta-analysis of studies in the replication set and also in the final combined analysis of the discovery and replication studies (43). For each SNP included in the replication phase, we meta-analyzed its association with all three phenotypes, simply for completeness, but interpreted the findings while taking into account the primary outcome that the SNP was associated with in the discovery phase. Fixed-effect meta-analysis results were used for subsidiary comparison. We also conducted meta-analysis of the associations of SNPs with fracture outcomes, using only SNPs that were associated with BUA, VOS, or heel DXA BMD at $p < 5 \times 10^{-6}$ in the combined analyses, to assess their potential relevance to this clinical outcome.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online and the GWAS meta-analysis results are made available at the GEFOS website (http://www.gefos.org/?q=content/data-release).

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Conflict of Interest statement. None declared.

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Tables and Figures

Main Tables and Figures

Table 1. Characteristics of studies that contributed to GWAS discovery and replication of SNP-associations with heel QUS/DXA BMD measures.

Stage\ Cohort	Country	Demographics					Heel QUS/DXA BMD outcomes					
		N	Females	Age (yrs)	Weight (kg)	Height (cm)	BUA (dB/MHz)		VOS (m/s)		Heel BMD (g/cm^2)	
			(%)	Mean (SD)	Mean (SD)	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)
GWAS Discovery												
EPIC	UK	2630	56%	62.1 (8.6)	80.5 (15.4)	167 (9)	2630	83 (19)	2630	1632 (40)	-	-
FHS	USA	3229	58%	64.6 (11.9)	76.8 (17.2)	166 (10)	3229	73 (21)	3225	1548 (38)	-	-
HKOS	China	730	100%	48.7 (15.4)	54.8 (10.4)	155 (7)	730	74 (22)	730	1551 (41)	-	-
NSPHS06	Sweden	495	55%	51.4 (19.1)	71.9 (12.8)	164 (10)	495	96 (21)	-	-	-	-
RSI	Netherlands	1615	54%	66.5 (8.2)	74.3 (11.8)	169 (9)	1615	112 (13)	1615	1525 (37)	-	-
SHIP	Germany	1198	54%	58.0 (13.5)	80.2 (15.8)	168 (9)	1198	115 (15)	1198	1565 (35)	-	-
SHIP-TREND	Germany	687	56%	50.8 (13.6)	78.7 (15.1)	170 (9)	687	116 (14)	687	1571 (33)	-	-
TWINSUK1	UK	1701	100%	46.2 (12.1)	65.8 (12.5)	163 (6)	1701	76 (18)	1701	1658 (49)	-	-
TWINSUK23	UK	1975	100%	46.9 (12.5)	66.1 (12.2)	163 (6)	1975	76 (18)	1975	1653 (50)	-	-
H2SS	Korea	1753	53%	60.8 (6.6)	61.9 (10.0)	158 (8)	-	-	1753	1591 (45)	-	-
AGES	Iceland	3179	58%	76.4 (5.4)	75.8 (14.3)	167 (9)	-	-	-	-	3179	0.491 (0.152)
CroatiaKorcula	Croatia	878	64%	56.3 (14.2)	79.0 (14.2)	168 (9)	-	-	-	-	878	0.443 (0.098)
CroatiaSplit	Croatia	499	57%	49.3 (14.7)	80.6 (16.3)	172 (9)	-	-	-	-	499	0.459 (0.101)
Subtotal		**20569**	**66%**	**60.3 (11)**	**73.3 (14.1)**	**165 (9)**	**14260**	**86 (18)**	**15514**	**1593 (42)**	**4556**	**0.478 (0.138)**
Insilico replication												
AOGC‡	Australia/UK*	1955	100%	69.6 (8.6)	69.6 (17.3)	158 (16)	-	-	-	-	-	-
B-PROOF	Netherlands	1092	59%	74.0 (6.7)	76.0 (12.4)	168 (9)	1092	69 (17)	1091	1535 (32)	-	-
HABC	USA	1493	48%	74.8 (2.9)	73.8 (14.3)	167 (9)	1493	73 (18)	1493	1541 (30)	-	-
MICROS	Italy	588	45%	46 (16.6)	70.2 (14.9)	167 (9)	588	73 (16)	588	1544 (29)	-	-
MrOS-USA	USA	3925	0%	73.9 (5.9)	83.1 (12.7)	175 (7)	3925	79 (17)	3925	1551 (30)	-	-
SOF	USA	2103	100%	80.1 (4.2)	66.3 (12.5)	158 (6)	2103	59 (17)	2103	1527 (30)	-	-
YFS	Finland	1265	58%	37.9 (5.0)	75.8 (15.5)	172 (9)	1265	80 (16)	1265	1559 (29)	1250	0.560 (0.110)
HCS-AUS	Australia	986	49%	66.2 (7.6)	79.4 (15.5)	166 (9)	-	-	-	-	986	0.538 (0.166)
Subtotal		**13407**	**52%**	**69.2 (6.9)**	**75.4 (14.2)**	**167 (9)**	**10466**	**73 (17)**	**10465**	**1544 (30)**	**2236**	**0.550 (0.138)**
Denovo replication												
AUSTRIOS-B	Austria	448	85%	83.6 (5.9)	62.0 (12.3)	156 (8)	448	90 (17)	448	1496 (36)	-	-
CABRIO-C	Spain	1274	62%	62.4 (9.2)	73.7 (13.1)	161 (8)	1274	70 (23)	1273	1545 (41)	-	-
CAIFOS	Australia	1113	100%	80.0 (2.6)	67.5 (12.1)	157 (6)	1113	101 (9)	1113	1516 (28)	-	-
CALEX-FAM	Finland	983	79%	37.0 (22.4)	64.3 (16.9)	164 (11)	983	83 (16)	-	-	-	-
EMAS	Europe*	2870	0%	59.9 (11.0)	83.1 (13.6)	173 (7)	2870	80 (19)	2870	1550 (34)	-	-
EPICNOR	UK	5723	54%	63.6 (9.2)	73.2 (12.4)	167 (9)	5723	79 (20)	5718	1638 (43)	-	-
EPOLOS	Poland	684	56%	53.4 (16.0)	73.2 (13.7)	166 (10)	684	112 (13)	684	1548 (35)	-	-
FLOS	Italy	1000	84%	59.8 (12.7)	64.8 (12.3)	163 (9)	1000	58 (7)	1000	1503 (83)	-	-
GEOS	Canada	5495	100%	55.8 (10.3)	65.4 (11.9)	158 (6)	5495	111 (10)	5495	1546 (32)	-	-
LASA	Netherlands	894	51%	75.6 (6.5)	74.2 (12.6)	166 (9)	894	71 (20)	894	1611 (44)	-	-
MrOS-SWE	Sweden	1718	0%	75.4 (3.2)	80.6 (12.0)	175 (7)	1718	81 (21)	1718	1555 (38)	-	-
OPRA	Sweden	821	100%	75.2 (0.1)	67.6 (11.3)	160 (6)	821	102 (10)	821	1523 (27)	-	-
OSTEOSII	Greece	307	87%	50.5 (12.6)	74.1 (15.7)	163 (7)	307	112 (16)	307	1556 (36)	-	-
PEAK25	Sweden	857	100%	25.5 (0.2)	64.5 (11.2)	168 (6)	857	118 (11)	857	1575 (32)	-	-
SWS	UK	715	100%	29.7 (3.7)	72.4 (14.8)	163 (7)	714	72 (13)	715	1548 (27)	-	-
Subtotal		**24902**	**64%**	**60.2 (10)**	**71.6 (12.7)**	**165 (8)**	**24901**	**89 (16)**	**23913**	**1568 (40)**	**-**	**-**
TOTAL		**58878**	**62%**	**62.3 (9.7)**	**73 (13.6)**	**165 (8)**	**49627**	**85 (17)**	**49892**	**1570 (39)**	**6792**	**0.502 (0.138)**

‡ The AOGC cohort contributed to insilico lookups of SNP-fracture associations only. * The EMAS study comprised cohorts in Belgium, Estonia, Hungary, Italy, Poland, Spain, Sweden, and UK.

Table 2. Summary of P-values for association of SNPs in 25 loci with heel BUA, VOS, or heel DXA BMD in GWAS discovery/replication meta-analysis. P-values smaller than the genome-wide significance threshold (**P < 5x10^{-8}**) or suggestive significance threshold (**P < 5x10^{-6}**) are indicated in bold typeface[‡].

				Discovery P-values[†]			Replication P-values[†]			Combined P-values[†]		
Locus	**SNP**	**Closest gene**	**Genetic function**	**BUA**	**VOS**	**DXA**	**BUA**	**VOS**	**DXA**	**BUA**	**VOS**	**DXA**
Combined p < 5 x10^{-8}				**9 cohorts, 14258 participants**			**21 cohorts, 35082 participants**			**30 cohorts, 49335 participants**		
2p16.2	rs11898505	*SPTBN1*	Intronic, Regulatory region	**7.78E-08**	**2.92E-08**	7.68E-01	**6.66E-12**	1.10E-04	9.63E-02	**4.24E-13**	6.25E-06	2.65E-01
6q22.33	rs7741021	*RSPO3*	Intronic, Regulatory region	**8.52E-07**	**1.72E-07**	7.69E-06	**1.19E-18**	**2.54E-21**	1.49E-03	**9.26E-21**	**9.58E-20**	**4.11E-08**
6q25.1	rs4869739	*CCDC170*	Intronic	**5.25E-10**	**4.75E-11**	**7.73E-10**	1.02E-03	**3.92E-08**	3.82E-01	**1.93E-09**	**2.64E-18**	1.21E-02
6q25.1	rs3020331*	*ESR1*	Intronic	1.27E-02	7.94E-06	2.01E-04	**3.04E-10**	**3.79E-17**	1.95E-01	**2.91E-09**	**6.64E-15**	1.26E-03
6q25.1	rs2982552	*ESR1*	Intronic, Regulatory region	2.87E-02	**3.31E-06**	3.83E-04	**6.16E-17**	**1.14E-18**	1.00E-01	**1.70E-10**	**7.32E-16**	1.21E-04
7q31.31	rs2908007	*WNT16*	Upstream	**8.59E-21**	**5.02E-23**	**4.31E-11**	**1.31E-22**	**2.06E-39**	3.47E-02	**4.32E-35**	**1.62E-59**	**1.34E-09**
10q21.1	rs7902708	*MBL2/DKK1*	Intronic	8.23E-03	**1.46E-07**	9.51E-01	**1.02E-08**	**6.99E-09**	2.60E-03	**1.30E-08**	**5.29E-15**	2.47E-01
11q14.2	*rs597319*	*TMEM135*	*Intronic*	*2.62E-04*	***1.18E-08***	*5.05E-03*	***2.01E-12***	***2.70E-17***	*2.20E-02*	***8.23E-14***	***4.86E-26***	*3.05E-04*
19q13.11	rs10416265	*GPATCH1*	Non-synonymous coding	**8.30E-07**	**2.99E-08**	1.15E-01	**5.84E-08**	2.92E-05	3.45E-01	**2.37E-13**	**4.08E-12**	6.72E-02
Combined p ≥ 5 x10^{-8}				**9 cohorts, 14258 participants**			**21 cohorts, 35082 participants**			**30 cohorts, 49335 participants**		
5p13.3	rs9292469	NPR3	Upstream	**3.09E-06**	6.01E-03	9.27E-01	5.95E-01	1.69E-01	9.96E-01	1.43E-01	6.12E-01	9.42E-01
7p15.2	rs11520772	TAX1BP1	Intronic	**9.71E-07**	4.84E-04	6.24E-01	8.43E-02	1.32E-01	5.48E-01	2.86E-04	7.07E-03	8.79E-01
7p14.1	rs6974574*	EPDR1	Upstream	5.81E-03	1.34E-05	2.56E-04	2.51E-04	4.84E-03	7.31E-01	8.25E-05	3.89E-05	9.25E-03
7q11.23	rs38664	UPK3B	Intronic	9.10E-04	**1.52E-06**	6.60E-01	4.39E-02	1.58E-02	5.35E-01	3.25E-04	**1.02E-07**	8.79E-01
13q12.3	rs3000634	USPL1	Upstream	2.10E-05	**1.27E-07**	2.18E-01	6.80E-03	1.91E-01	5.38E-01	8.12E-01	8.00E-02	1.70E-01
13q14.11	rs9533090	AKAP11	Upstream	3.78E-02	5.04E-03	**5.05E-10**	7.60E-03	2.44E-04	6.44E-01	1.02E-03	1.40E-05	6.97E-03
16q24.1	rs7188801	FOXL1	Upstream	3.32E-04	**3.09E-06**	2.16E-02	3.91E-01	1.66E-02	5.48E-01	9.70E-03	7.62E-06	2.90E-02
				9 cohorts, 14258 participants			**6 cohorts, 10466 participants**			**15 cohorts, 24723 participants**		
2p21	rs17032452	CAMKMT	Intronic	8.73E-01	5.30E-01	**1.74E-06**	5.49E-01	4.24E-01	3.59E-01	6.26E-01	9.67E-01	1.56E-03
3p14.2	rs6414591	C3orf67	Upstream	3.49E-01	2.39E-01	**1.72E-06**	1.31E-01	9.13E-02	6.83E-01	7.86E-01	8.17E-01	9.22E-02
5q31.2	rs11959305	TGFBI	Intronic	1.89E-02	1.82E-02	**6.84E-08**	6.52E-01	2.80E-01	8.61E-01	8.47E-02	7.74E-03	1.15E-01
7p15.3	rs7787266	STEAP1B	Intronic	4.08E-01	4.93E-01	**2.53E-06**	2.93E-01	3.14E-01	6.21E-01	1.97E-01	2.70E-01	9.71E-03
9q21.33	rs10868487	GAS1	Downstream	6.10E-01	3.81E-01	**2.37E-06**	2.61E-01	2.50E-01	7.03E-01	8.57E-01	7.46E-01	8.92E-02
13q31.1	rs9574655	SPRY2	Downstream	2.58E-01	1.38E-01	**9.09E-08**	8.59E-01	6.43E-01	8.67E-02	5.81E-01	4.72E-01	3.50E-01
16q12.2	rs923220	IRX5	Upstream	1.24E-03	7.98E-03	**6.05E-07**	7.85E-01	7.28E-01	9.34E-01	2.58E-02	3.95E-02	1.56E-02
20q11.22	rs3746429	EDEM2	Missense variant	4.42E-01	8.27E-01	**3.80E-07**	2.07E-01	9.14E-02	3.35E-01	7.23E-01	3.93E-01	4.35E-04
21q22.2	rs2836789	FLJ45139	Upstream	1.56E-01	1.36E-02	**1.51E-06**	2.09E-03	4.09E-02	5.07E-01	3.77E-03	2.27E-03	1.57E-03

[‡] The P-values in the GWAS discovery are based on a fixed effect meta-analysis model, while those in the replication and combined analyses are based on a random effects meta-analysis model.

***** Secondary signals at the discovery phase following conditional analyses within the region (see Supplementary Figure 6 for the regional association plots).

[†] The number of cohorts and participants contributing to the analysis of each SNP at each stage slightly varied depending on quality control filters as well as successful imputation or denovo genotyping of the particular SNP. Figure 1 and Supplementary Figure 3 show the exact numbers that were available for each SNP at each stage for the confirmed loci.

The novel locus is italicized.

Figure 1. Flow chart summarising key features of the discovery and replication phases.

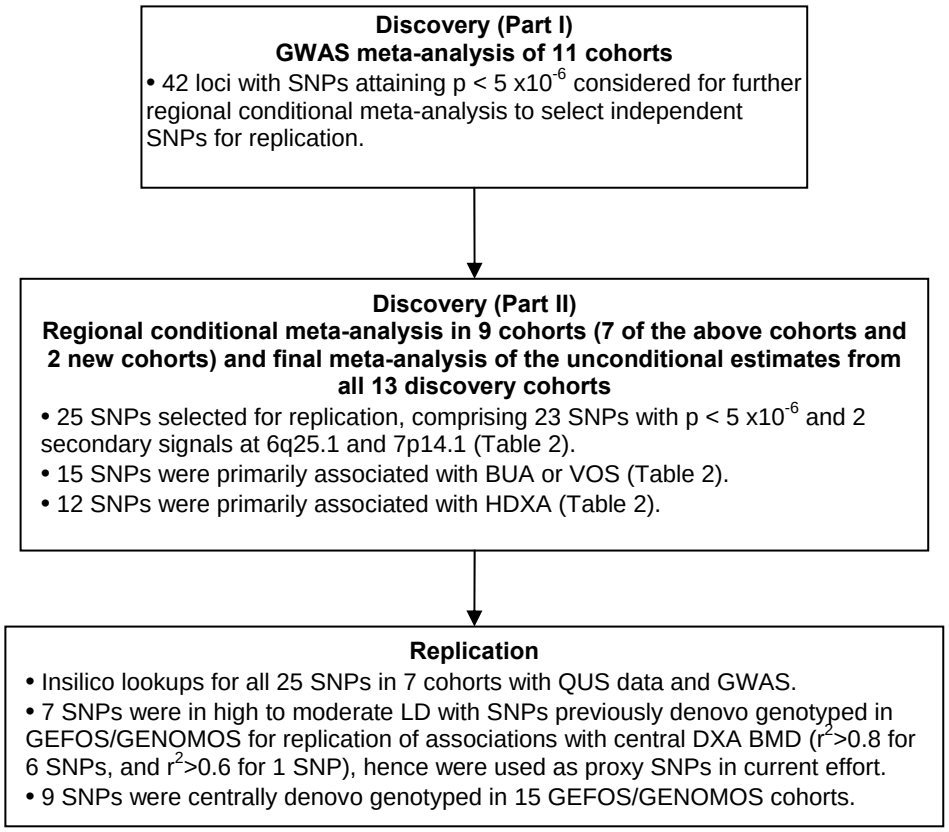
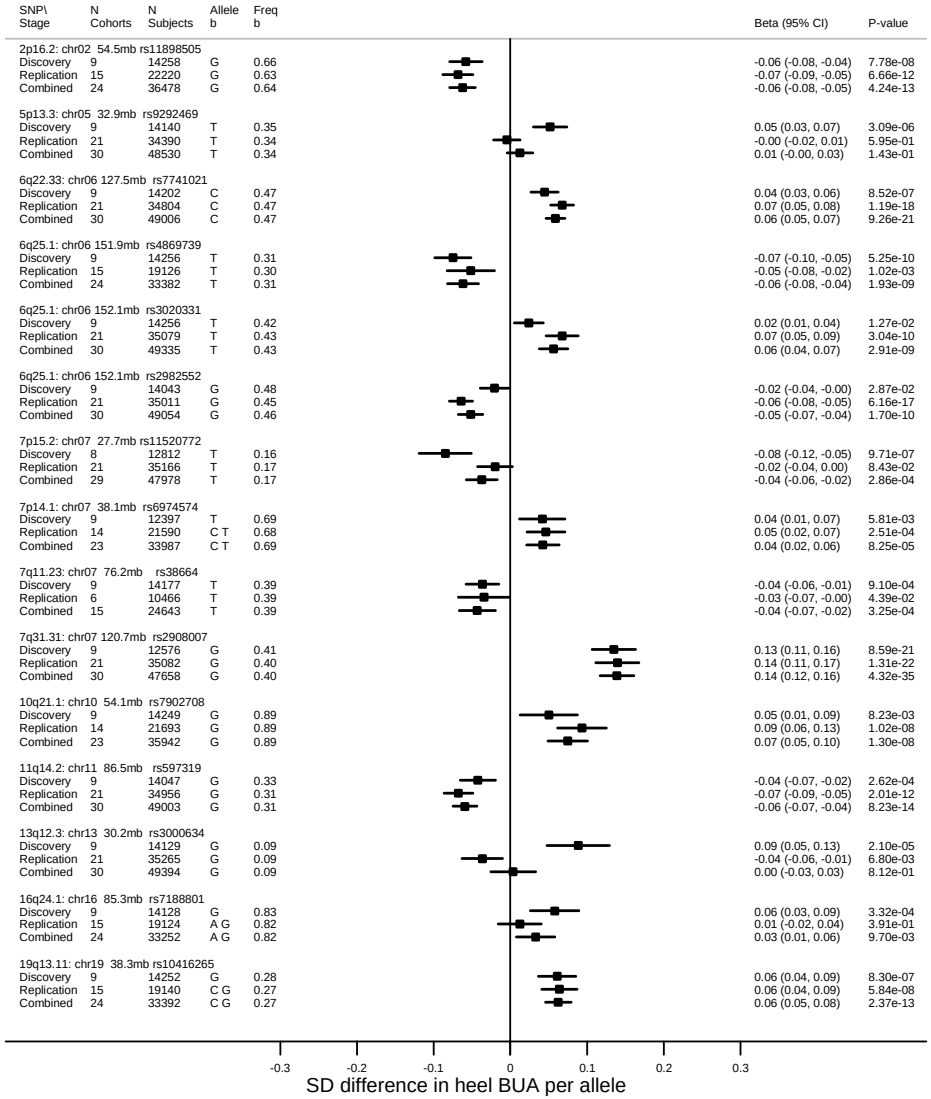
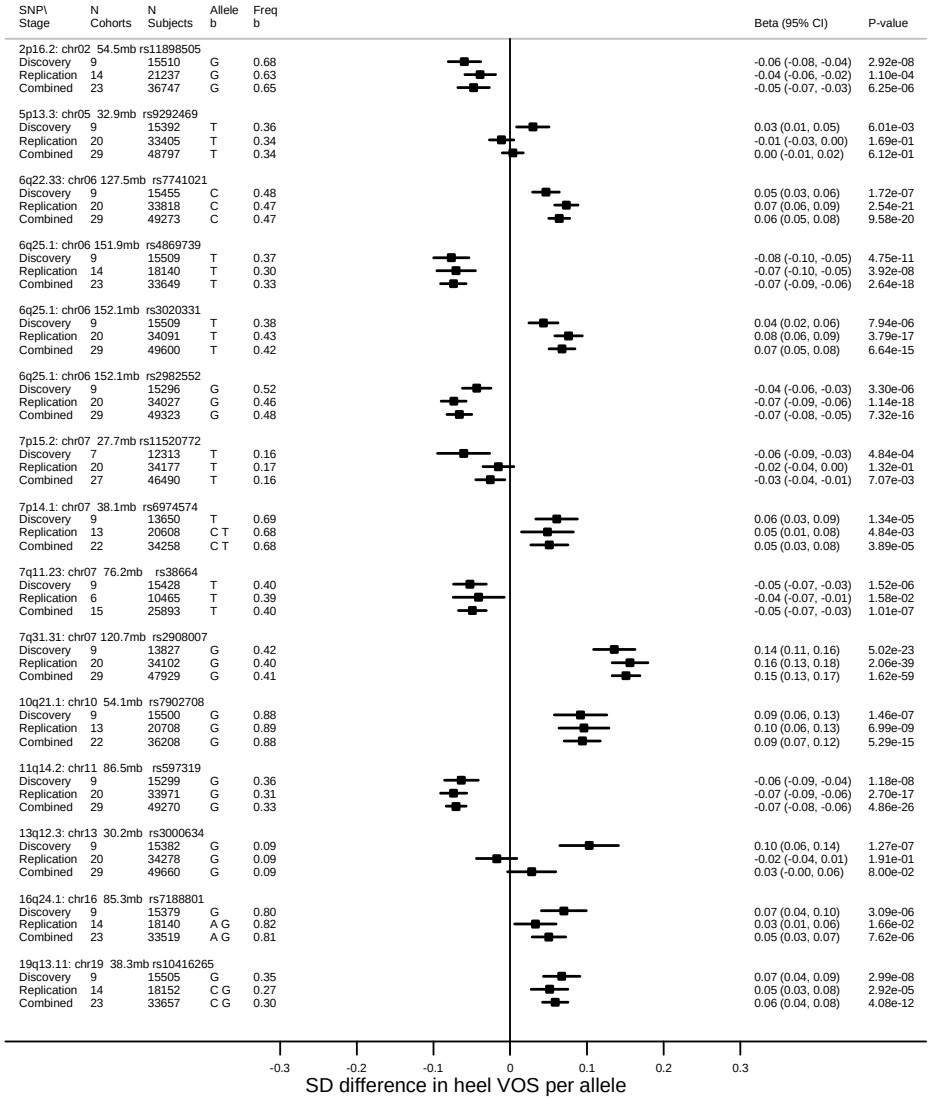


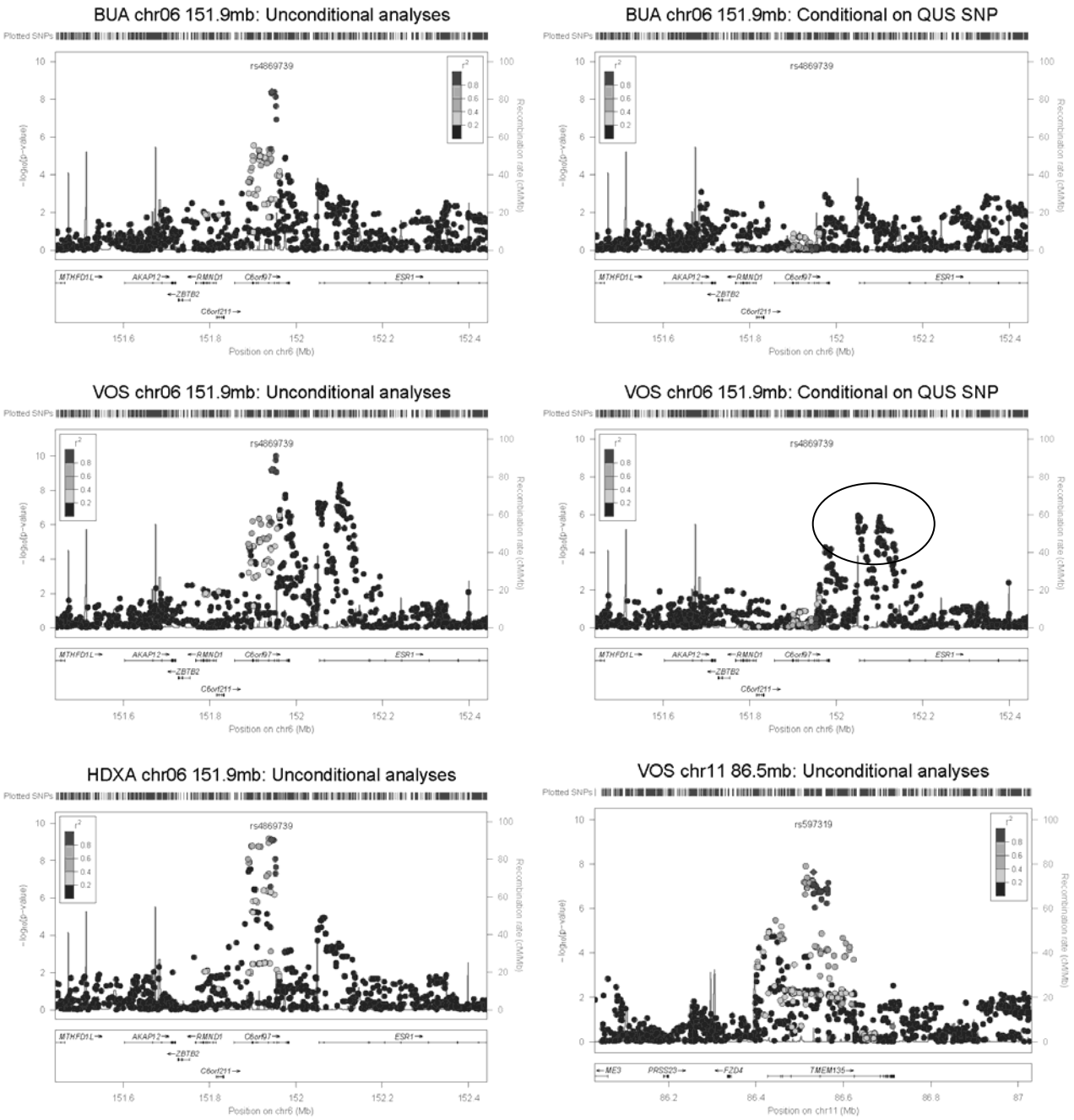
Figure 2. Summary of SNP associations with heel BUA or VOS in GWAS discovery meta-analysis and replication in independent samples of participants.





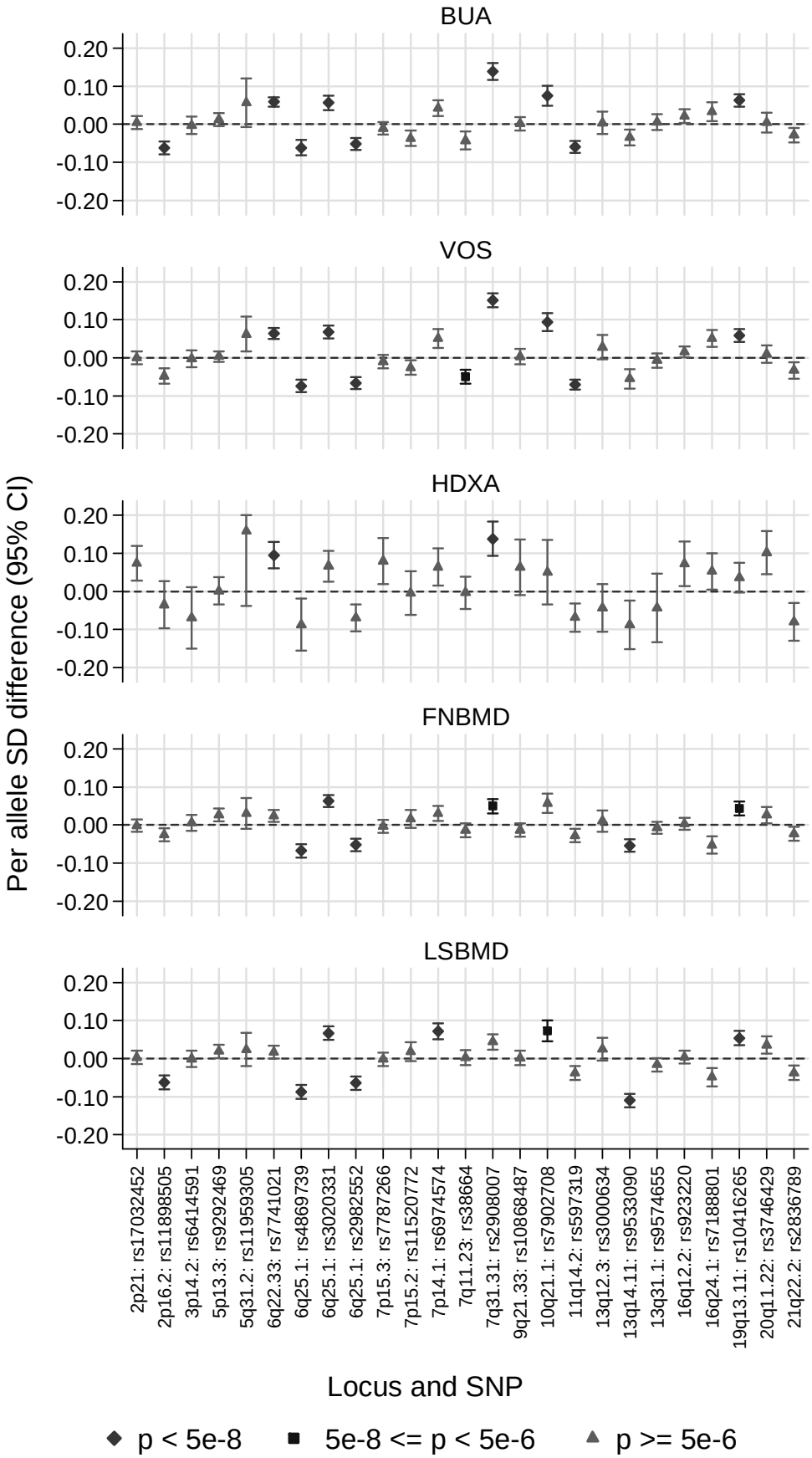
The pooled estimates in the GWAS discovery are based on a fixed effect meta-analysis model, while those in the replication and combined analyses are based on a random effects meta-analysis model. Allele b indicates the effect allele, and the presence of two alleles in this column indicates that a proxy SNP with $r^2 > 0.8$ (except for 16q24.1 locus for which $r^2 = 0.6$) was used for the replication analyses.

Figure 3. Regional association plots for chromosome 6q25.1 region with heel BUA, VOS, and heel DXA BMD in discovery cohorts before and after conditioning on the most significant SNP in the region* as well as a novel locus for heel bone properties at 11q14.2.



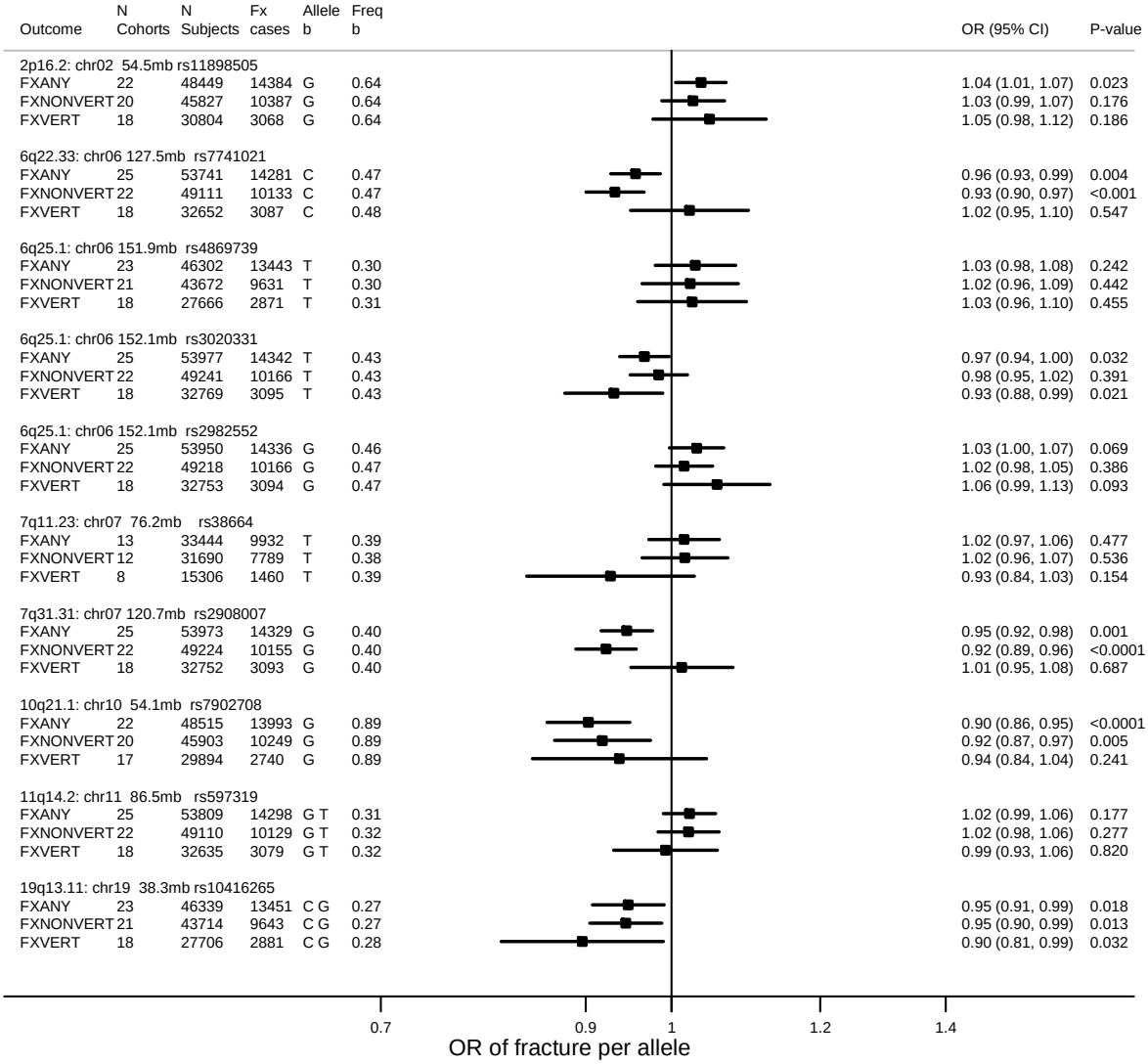
* The conditional analyses led to the identification of the highlighted secondary signal for association of 6q25.1 with VOS. Conditional analyses results for heel DXA BMD were not available from the 3 relevant discovery cohorts. Colour versions of the above figures have been made available in the online supplementary materials (Supplementary Figure 6).

Figure 4. Comparison of magnitudes of associations of 25 SNPs with heel bone properties and central DXA BMD.



The SNP associations with central DXA BMD are based on lookup of previously published results from GEFOS.

Figure 5. Per allele odds ratios for association with fracture risk for 10 SNPs that were associated with heel BUA, VOS, or heel DXA BMD at $p < 5 \times 10^{-6}$ in combined meta-analyses using a random effects model.



The pooled estimates are based on a random effects meta-analysis model. FXANY = Any fracture; FXNONVERT = Non-vertebral fracture; FXVERT = Vertebral fracture.
Allele b indicates the effect allele, and the presence of two alleles in this column indicates that a proxy SNP with $r^2 > 0.8$ was used for the replication analyses.