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

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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 ($n = 4566$) in 13 discovery cohorts. Independent replication involved seven 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, nine single nucleotide polymorphisms (SNPs) had genome-wide significant ($P < 5 \times 10^{-8}$) 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 ($P < 8.23 \times 10^{-14}$). In meta-analyses involving 25 cohorts with up to 14 985 fracture cases, six of 10 SNPs associated with heel bone properties at $P < 5 \times 10^{-6}$ also had the expected direction of association with any fracture ($P < 0.05$), including three 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 principal mineral in bone. This is positively related to the mass of hydroxyapatite in the path of the beam conventionally termed bone mineral content and normalized 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 broadband ultrasound attenuation (BUA). While much remains to be discovered about the exact

physical determinants of 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 of 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 ~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

Participant characteristics are summarized in Table 1 and key features of the discovery and replication phases are summarized in Figure 1. In aggregate, the initial discovery phase meta-analysis in 11 cohorts (Supplementary Material, Table S1) 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 (Table 2) 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 comprise 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 and 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 (Fig. 3), and five 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 Material, Table S2 and Figs S2 and S3) 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 Material, Table S3), 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 3220 clinical vertebral fractures (Supplementary Material, Table S4). Ten of 10 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 (Fig. 5). Furthermore, 6 of these 10 SNPs showed nominally significant ($P < 0.05$) associations with fractures, including three 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 Material, Fig. S4).

Supplementary Material, Figure S5 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 Material, Figure S6 shows the regional association plots within a one megabase window of the top SNP in each locus in the GWA discovery meta-analysis, demonstrating

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					
							BUA (dB/MHz)		VOS (m/s)		Heel BMD (g/cm^2)	
		N	Females (%)	Age (years) Mean (SD)	Weight (kg) Mean (SD)	Height (cm) 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		20 569	66	60.3 (11)	73.3 (14.1)	165 (9)	14 260	86 (18)	15 514	1593 (42)	4556	0.478 (0.138)
In silico replication												
AOGC[a]	Australia/UK[b]	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	46.0 (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		13 407	52	69.2 (6.9)	75.4 (14.2)	167 (9)	10 466	73 (17)	10 465	1544 (30)	2236	0.550 (0.138)
De novo 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[b]	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		24 902	64	60.2 (10.0)	71.6 (12.7)	165 (8)	24 901	89 (16)	23 913	1568 (40)	–	–
Total		58 878	62	62.3 (9.7)	73.0 (13.6)	165 (8)	49 627	85 (17)	49 892	1570 (39)	6792	0.502 (0.138)

[a]The AOGC cohort contributed to *in silico* lookups of SNP-fracture associations only.
[b]The EMAS study comprises cohorts in Belgium, Estonia, Hungary, Italy, Poland, Spain, Sweden and UK.

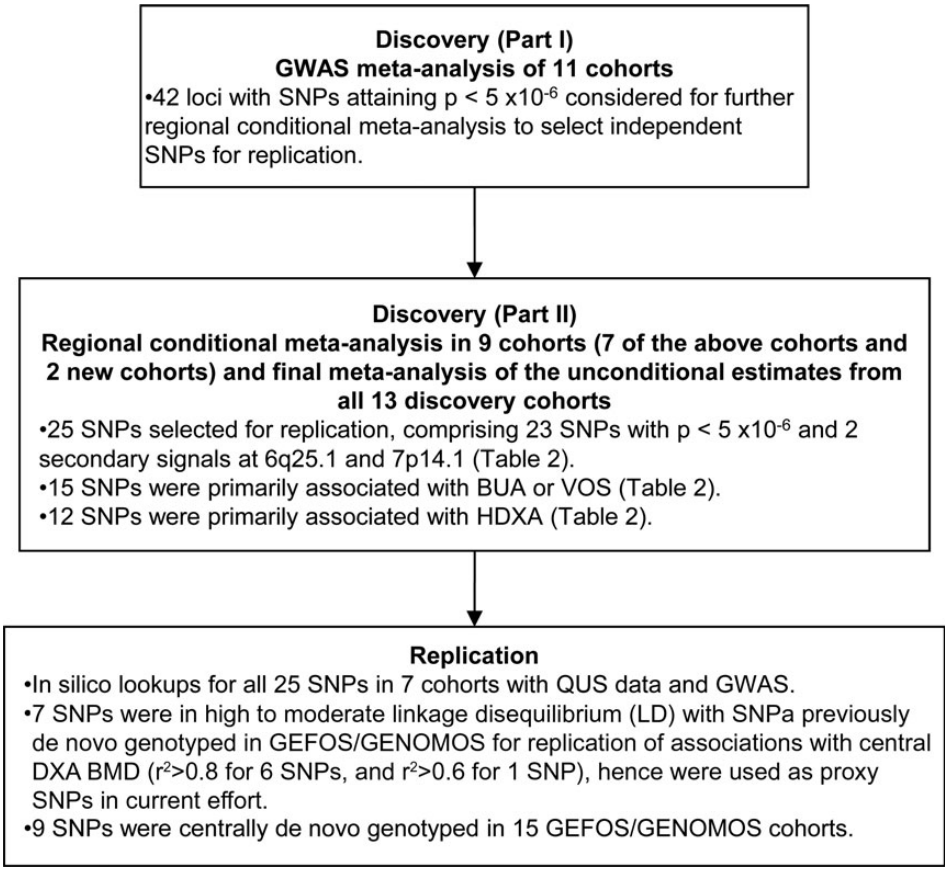

Figure 1. Flow chart summarizing key features of the discovery and replication phases.

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 Material, Fig. S7).

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 of nine genome-wide significant loci found in the present study were previously reported to be associated with BMD of the hip and/or spine (Fig. 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 standardized effect sizes (Fig. 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 three 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

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

Locus	SNP	Closest gene	Genetic function	Discovery *P*-values[b]			Replication *P*-values[b]			Combined *P*-values[b]		
				BUA	VOS	DXA	BUA	VOS	DXA	BUA	VOS	DXA
Combined $P < 5 \times 10^{-8}$				9 cohorts, 14 258 participants			21 cohorts, 35 082 participants			30 cohorts, 49 335 participants		
2p16.2	rs11898505	*SPTBN1*	Intronic, regulatory region	$\mathbf{7.78\times10^{-8}}$	$\mathbf{2.92\times10^{-8}}$	7.68×10^{-1}	$\mathbf{6.66\times10^{-12}}$	1.10×10^{-4}	9.63×10^{-2}	$\mathbf{4.24\times10^{-13}}$	6.25×10^{-6}	2.65×10^{-1}
6q22.33	rs7741021	*RSPO3*	Intronic, regulatory region	$\mathbf{8.52\times10^{-7}}$	$\mathbf{1.72\times10^{-7}}$	7.69×10^{-6}	$\mathbf{1.19\times10^{-18}}$	$\mathbf{2.54\times10^{-21}}$	1.49×10^{-3}	$\mathbf{9.26\times10^{-21}}$	$\mathbf{9.58\times10^{-20}}$	$\mathbf{4.11\times10^{-8}}$
6q25.1	rs4869739	*CCDC170*	Intronic	$\mathbf{5.25\times10^{-10}}$	$\mathbf{4.75\times10^{-11}}$	$\mathbf{7.73\times10^{-10}}$	1.02×10^{-3}	$\mathbf{3.92\times10^{-8}}$	3.82×10^{-1}	$\mathbf{1.93\times10^{-9}}$	$\mathbf{2.64\times10^{-18}}$	1.21×10^{-2}
6q25.1	rs3020331[c]	*ESR1*	Intronic	1.27×10^{-2}	7.94×10^{-6}	2.01×10^{-4}	$\mathbf{3.04\times10^{-10}}$	$\mathbf{3.79\times10^{-17}}$	1.95×10^{-1}	$\mathbf{2.91\times10^{-9}}$	$\mathbf{6.64\times10^{-15}}$	1.26×10^{-3}
6q25.1	rs2982552	*ESR1*	Intronic, regulatory region	2.87×10^{-2}	$\mathbf{3.31\times10^{-6}}$	3.83×10^{-4}	$\mathbf{6.16\times10^{-17}}$	$\mathbf{1.14\times10^{-18}}$	1.00×10^{-1}	$\mathbf{1.70\times10^{-10}}$	$\mathbf{7.32\times10^{-16}}$	1.21×10^{-4}
7q31.31	rs2908007	*WNT16*	Upstream	$\mathbf{8.59\times10^{-21}}$	$\mathbf{5.02\times10^{-23}}$	$\mathbf{4.31\times10^{-11}}$	$\mathbf{1.31\times10^{-22}}$	$\mathbf{2.06\times10^{-39}}$	3.47×10^{-2}	$\mathbf{4.32\times10^{-35}}$	$\mathbf{1.62\times10^{-59}}$	$\mathbf{1.34\times10^{-9}}$
10q21.1	rs7902708	*MBL2/DKK1*	Intronic	8.23×10^{-3}	$\mathbf{1.46\times10^{-7}}$	9.51×10^{-1}	$\mathbf{1.02\times10^{-8}}$	$\mathbf{6.99\times10^{-9}}$	2.60×10^{-3}	$\mathbf{1.30\times10^{-8}}$	$\mathbf{5.29\times10^{-15}}$	2.47×10^{-1}
11q14.2	*rs597319*	*TMEM135*	*Intronic*	2.62×10^{-4}	$\mathbf{1.18\times10^{-8}}$	5.05×10^{-3}	$\mathbf{2.01\times10^{-12}}$	$\mathbf{2.70\times10^{-17}}$	2.20×10^{-2}	$\mathbf{8.23\times10^{-14}}$	$\mathbf{4.86\times10^{-26}}$	3.05×10^{-4}
19q13.11	rs10416265	*GPATCH1*	Non-synonymous coding	$\mathbf{8.30\times10^{-7}}$	$\mathbf{2.99\times10^{-8}}$	1.15×10^{-1}	$\mathbf{5.84\times10^{-8}}$	2.92×10^{-5}	3.45×10^{-1}	$\mathbf{2.37\times10^{-13}}$	$\mathbf{4.08\times10^{-12}}$	6.72×10^{-2}
Combined $P \geq 5 \times 10^{-8}$				9 cohorts, 14 258 participants			21 cohorts, 35 082 participants			30 cohorts, 49 335 participants		
5p13.3	rs9292469	*NPR3*	Upstream	$\mathbf{3.09\times10^{-6}}$	6.01×10^{-3}	9.27×10^{-1}	5.95×10^{-1}	1.69×10^{-1}	9.96×10^{-1}	1.43×10^{-1}	6.12×10^{-1}	9.42×10^{-1}
7p15.2	rs11520772	*TAX1BP1*	Intronic	$\mathbf{9.71\times10^{-7}}$	4.84×10^{-4}	6.24×10^{-1}	8.43×10^{-2}	1.32×10^{-1}	5.48×10^{-1}	2.86×10^{-4}	7.07×10^{-3}	8.79×10^{-1}
7p14.1	rs6974574[c]	*EPDR1*	Upstream	5.81×10^{-3}	1.34×10^{-5}	2.56×10^{-4}	2.51×10^{-4}	4.84×10^{-3}	7.31×10^{-1}	8.25×10^{-5}	3.89×10^{-5}	9.25×10^{-3}
7q11.23	rs38664	*UPK3B*	Intronic	9.10×10^{-4}	$\mathbf{1.52\times10^{-6}}$	6.60×10^{-1}	4.39×10^{-2}	1.58×10^{-2}	5.35×10^{-1}	3.25×10^{-4}	$\mathbf{1.02\times10^{-7}}$	8.79×10^{-1}
13q12.3	rs3000634	*USPL1*	Upstream	2.10×10^{-5}	$\mathbf{1.27\times10^{-7}}$	2.18×10^{-1}	6.80×10^{-3}	1.91×10^{-1}	5.38×10^{-1}	8.12×10^{-1}	8.00×10^{-2}	1.70×10^{-1}
13q14.11	rs9533090	*AKAP11*	Upstream	3.78×10^{-2}	5.04×10^{-3}	$\mathbf{5.05\times10^{-10}}$	7.60×10^{-3}	2.44×10^{-4}	6.44×10^{-1}	1.02×10^{-3}	1.40×10^{-5}	6.97×10^{-3}
16q24.1	rs7188801	*FOXL1*	Upstream	3.32×10^{-4}	$\mathbf{3.09\times10^{-6}}$	2.16×10^{-2}	3.91×10^{-1}	1.66×10^{-2}	5.48×10^{-1}	9.70×10^{-3}	7.62×10^{-6}	2.90×10^{-2}
				9 cohorts, 14 258 participants			6 cohorts, 10 466 participants			15 cohorts, 24 723 participants		
2p21	rs17032452	*CAMKMT*	Intronic	8.73×10^{-1}	5.30×10^{-1}	$\mathbf{1.74\times10^{-6}}$	5.49×10^{-1}	4.24×10^{-1}	3.59×10^{-1}	6.26×10^{-1}	9.67×10^{-1}	1.56×10^{-3}
3p14.2	rs6414591	*C3orf67*	Upstream	3.49×10^{-1}	2.39×10^{-1}	$\mathbf{1.72\times10^{-6}}$	1.31×10^{-1}	9.13×10^{-2}	6.83×10^{-1}	7.86×10^{-1}	8.17×10^{-1}	9.22×10^{-2}
5q31.2	rs11959305	*TGFBI*	Intronic	1.89×10^{-2}	1.82×10^{-2}	$\mathbf{6.84\times10^{-8}}$	6.52×10^{-1}	2.80×10^{-1}	8.61×10^{-1}	8.47×10^{-2}	7.74×10^{-3}	1.15×10^{-1}
7p15.3	rs7787266	*STEAP1B*	Intronic	4.08×10^{-1}	4.93×10^{-1}	$\mathbf{2.53\times10^{-6}}$	2.93×10^{-1}	3.14×10^{-1}	6.21×10^{-1}	1.97×10^{-1}	2.70×10^{-1}	9.71×10^{-3}
9q21.33	rs10868487	*GAS1*	Downstream	6.10×10^{-1}	3.81×10^{-1}	$\mathbf{2.37\times10^{-6}}$	2.61×10^{-1}	2.50×10^{-1}	7.03×10^{-1}	8.57×10^{-1}	7.46×10^{-1}	8.92×10^{-2}
13q31.1	rs9574655	*SPRY2*	Downstream	2.58×10^{-1}	1.38×10^{-1}	$\mathbf{9.09\times10^{-8}}$	8.59×10^{-1}	6.43×10^{-1}	8.67×10^{-2}	5.81×10^{-1}	4.72×10^{-1}	3.50×10^{-1}
16q12.2	rs923220	*IRX5*	Upstream	1.24×10^{-3}	7.98×10^{-3}	$\mathbf{6.05\times10^{-7}}$	7.85×10^{-1}	7.28×10^{-1}	9.34×10^{-1}	2.58×10^{-2}	3.95×10^{-2}	1.56×10^{-2}
20q11.22	rs3746429	*EDEM2*	Missense variant	4.42×10^{-1}	8.27×10^{-1}	$\mathbf{3.80\times10^{-7}}$	2.07×10^{-1}	9.14×10^{-2}	3.35×10^{-1}	7.23×10^{-1}	3.93×10^{-1}	4.35×10^{-4}
21q22.2	rs2836789	*FLJ45139*	Upstream	1.56×10^{-1}	1.36×10^{-2}	$\mathbf{1.51\times10^{-6}}$	2.09×10^{-3}	4.09×10^{-2}	5.07×10^{-1}	3.77×10^{-3}	2.27×10^{-3}	1.57×10^{-3}

P-values smaller than the genome-wide significance threshold ($P < 5 \times 10^{-8}$) or suggestive significance threshold ($P < 5 \times 10^{-6}$) are indicated in bold typeface[a].
The association statistics for a new locus at chr 11q14.2 are italicized.
[a]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.
[b]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 *de novo* genotyping of the particular SNP. Figure 1 and Supplementary Material, Figure S3 show the exact numbers that were available for each SNP at each stage for the confirmed loci.
[c]Secondary signals at the discovery phase following conditional analyses within the region (see Supplementary Material, Fig. S6 for the regional association plots).

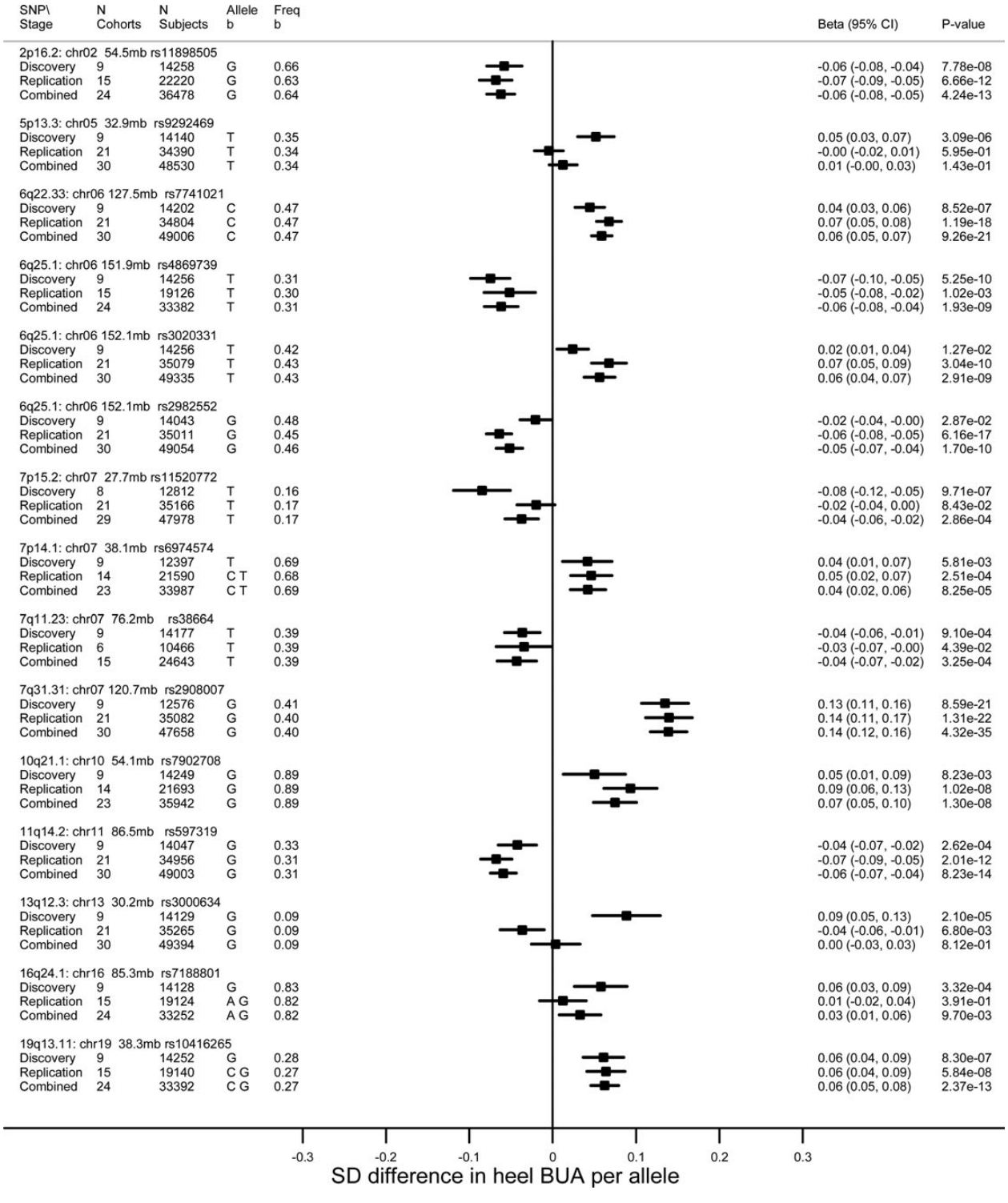

Figure 2. Summary of SNP associations with heel BUA or VOS in GWAS discovery meta-analysis and replication in independent samples of participants. The pooled estimates in the GWAS discovery are based on a fixed-effect meta-analysis model, while those in the replication and combined analyses are based 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.

suggested that it is critically involved in the process of osteoblastogenesis from human multipotent 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 neighboring cell types (25). In depth protein sequence analysis showed that *TMEM135* is a multi-transmembrane protein with seven

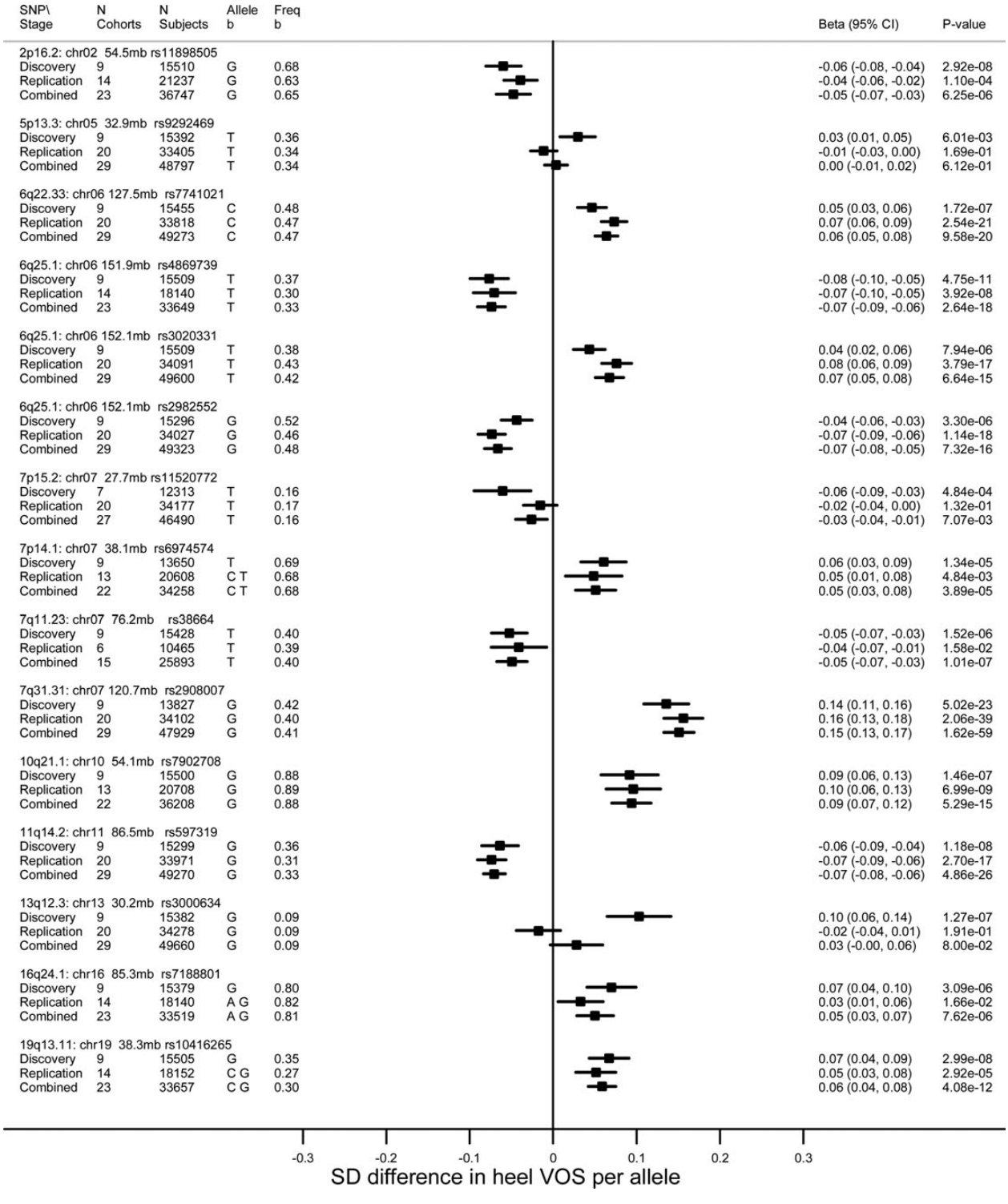

Fig. 2 *Continued*

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 show that two SNPs in the intronic region of *TMEM135* and close to our lead signal (rs502580 and rs603140, both with high linkage disequilibrium with rs597319 [$r^2 > 0.92$], and both highly associated with QUS outcomes in our discovery cohorts [$P \sim 1.3 \times 10^{-7}$ 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

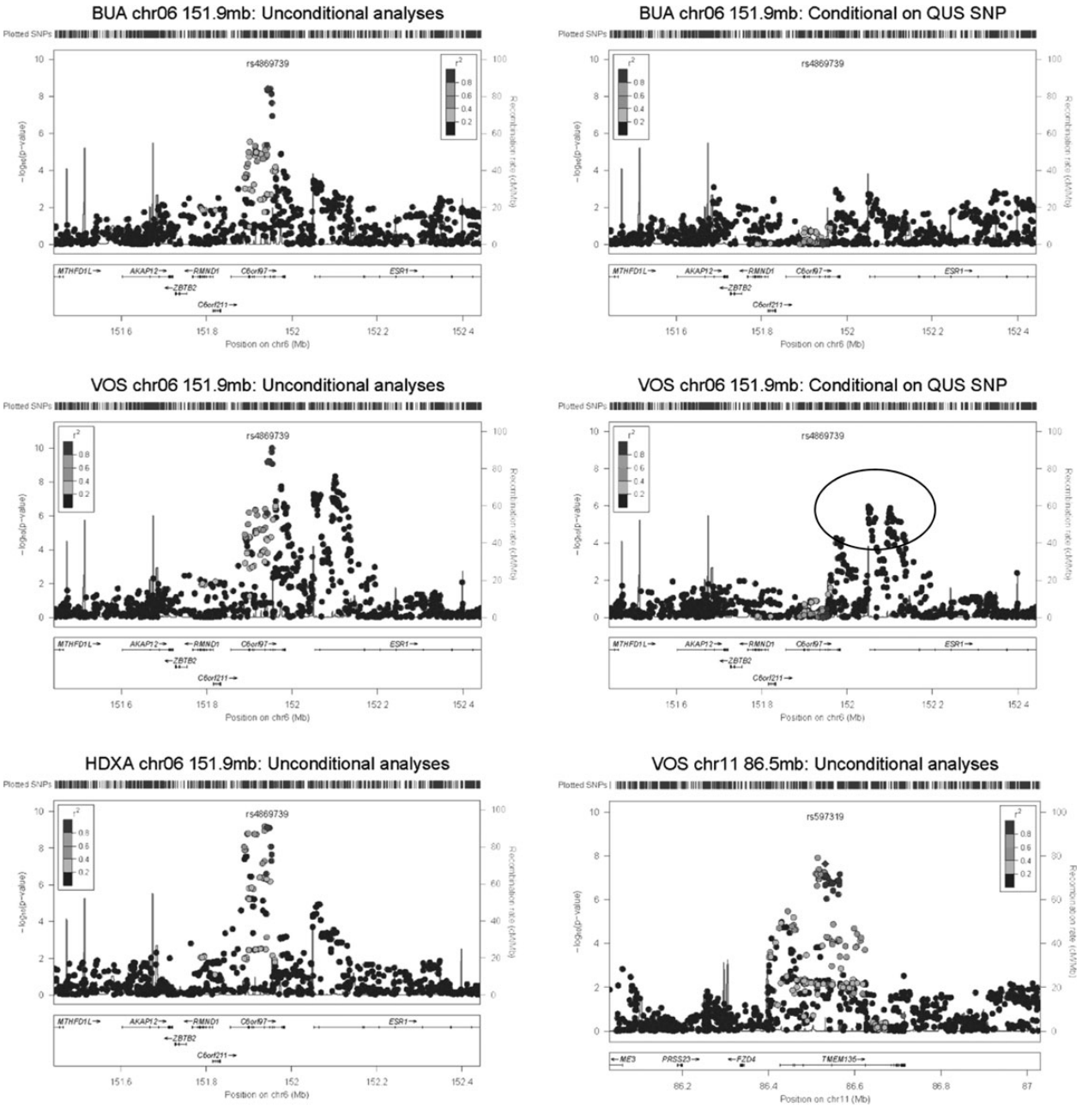

Figure 3. Association of SNPs at chromosome 6q25.1 region with heel BUA, VOS, and heel DXA BMD in meta-analysis of discovery cohorts before (left column) and after (right column) adjusting for the most significant SNP in the region (i.e. unconditional and conditional analyses respectively); as well as the unconditional results for a novel locus for heel bone properties at chromosome 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 three relevant discovery cohorts. Color versions of the above figures have been made available in Supplementary Material, Fig. S6.).

previously reported to be associated with longevity in *Caenorhabditis 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

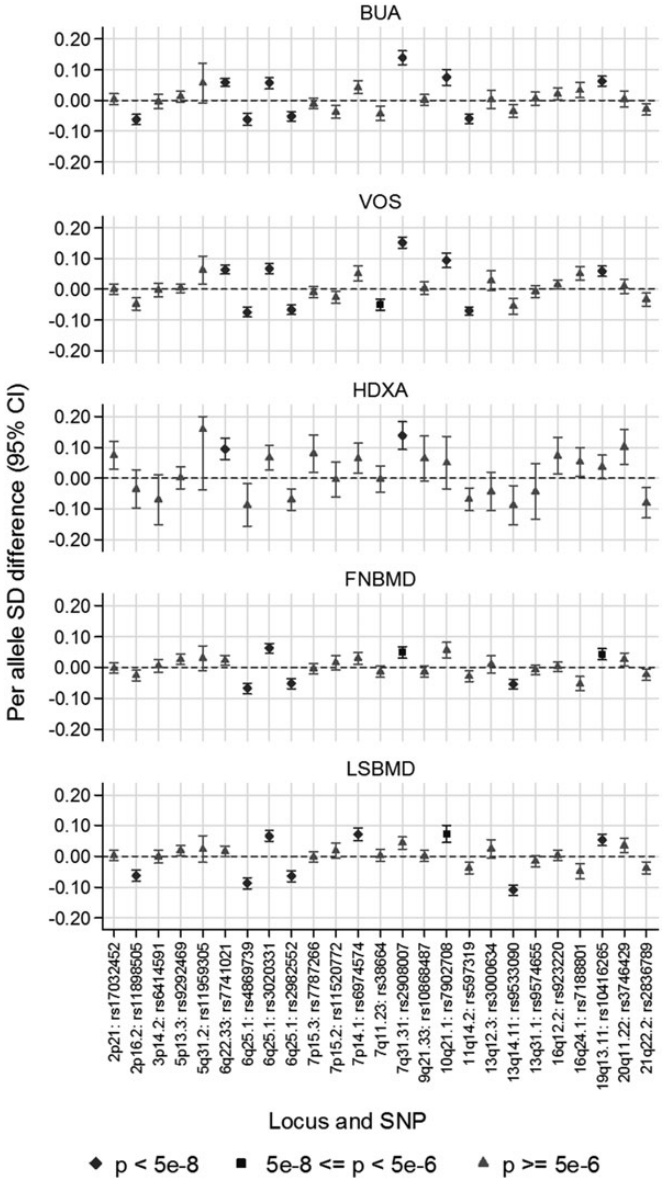

Figure 4. Comparison of magnitudes of associations of 25 SNPs with heel bone properties and central DXA BMD. The SNP associations with central DXA BMD are based on lookup of previously published results from GEFOS.

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 transmembrane 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 signaling 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 signaling 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 less than 7000 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

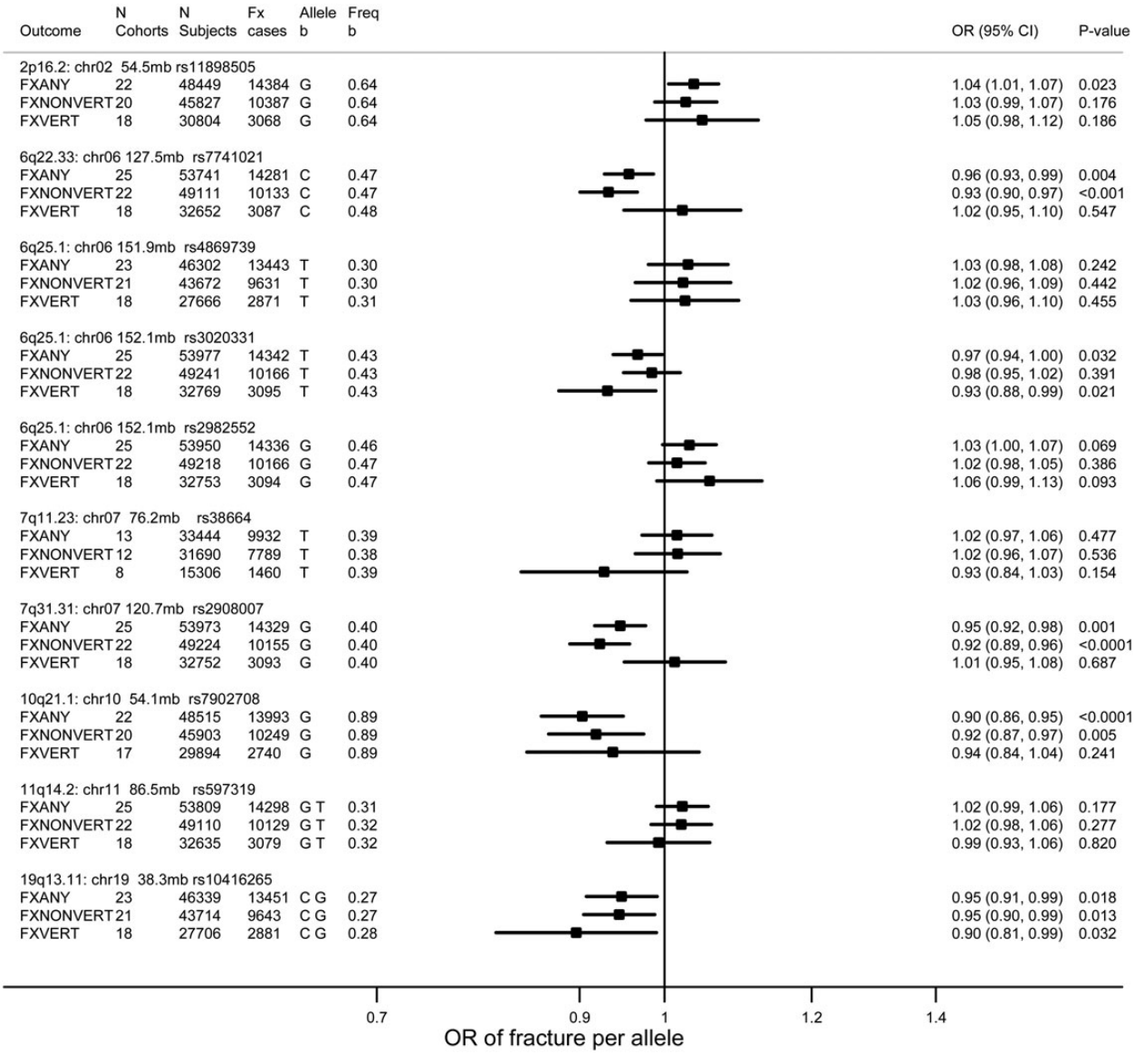

Figure 5. Per-allele odds ratios for association with fracture risk for 10 SNPs that were associated with heel BUA, VOS or heel DXA BMD at $P < 5 \times 10^{-6}$ in combined meta-analyses using a random-effects model. The pooled estimates are based on a random-effects meta-analysis model. FXANY = any fracture; FXNONVERT = non-vertebral fracture; FXVERT = vertebral fracture. Allele b indicates the effect of 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.

significant and expected directions of associations with fractures for six SNPs associated with heel bone measures, including three 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 standardized 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: BUA and VOS) and DXA (measure: heel BMD).

The discovery phase comprises 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 4556 participants from 3 cohorts) arising from populations across North America, Europe and East Asia. Independent replication was performed using summary results from seven 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/participants 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 prespecified 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 Center 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 centers (Cambridge, UK and Boston, USA). Because of potentially limited power to detect sex-specific associations, we prespecified 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 of <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 $P < 5 \times 10^{-8}$ and suggestive level of significance at $5 \times 10^{-8} \leq P < 5 \times 10^{-6}$. There were no extreme genomic inflation factors noted in the discovery phase studies or in the GWA meta-analysis (Supplementary Material, Table S1). QQ plots for the combined GWAS meta-analysis results are provided in Supplementary Material, Figure S1.

To help refine the choice of SNPs to be taken forward for replication, conditional analyses were conducted within a 1 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.

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Conflict of Interest statement. None declared.

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