

Meta-analysis of genome-wide association studies identifies two loci associated with circulating osteoprotegerin levels

Johnny S.H. Kwan^{1,2}, Yi-Hsiang Hsu^{4,5,6}, Ching-Lung Cheung¹, Josée Dupuis^{7,8}, Aude Saint-Pierre^{9,10}, Joel Eriksson¹¹, Samuel K. Handelman¹², Aaron Aragaki¹³, David Karasik⁴, Peter P. Pramstaller^{9,15,16}, Charles Kooperberg¹⁴, Andrea Z. Lacroix¹⁷, Martin G. Larson^{7,8}, Kam-Shing Lau¹, Mattias Lorentzon¹¹, Irene Pichler⁹, Pak C. Sham^{2,3}, Daniel Taliun⁹, Liesbeth Vandenput¹¹, Douglas P. Kiel^{4,6,19}, Andrew A. Hicks⁹, Rebecca D. Jackson¹³, Claes Ohlsson¹¹, Emelia J. Benjamin^{7,18} and Annie W.C. Kung^{1,*}

¹Department of Medicine, ²Department of Psychiatry and ³Centre for Genomic Sciences, University of Hong Kong, Pokfulam, Hong Kong, ⁴Institute for Aging Research, Hebrew SeniorLife, Boston, MA, USA, ⁵Program of Quantitative Genomics, Harvard School of Public Health, Boston, MA, USA, ⁶BROAD Institute of the MIT and Harvard, Cambridge, MA, USA, ⁷Framingham Heart Study of the National, Heart, Lung, and Blood Institute and Boston University, Framingham, MA, USA, ⁸Department of Biostatistics, Boston University School of Public Health, Boston, MA, USA, ⁹Center for Biomedicine, European Academy Bozen/Bolzano (EURAC), Bolzano, Italy – Affiliated Institute of the University of Lübeck, Lübeck, Germany, ¹⁰INSERM U1078, Etablissement Français du Sang, Brest, France, ¹¹Centre for Bone and Arthritis Research, Departments of Internal Medicine and Geriatrics, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, ¹²Department of Pharmacology and ¹³Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, The Ohio State University, Columbus, OH, USA, ¹⁴Fred Hutchinson Cancer Research Center, Seattle, WA, USA, ¹⁵Department of Neurology, General Central Hospital, Bolzano, Italy, ¹⁶Department of Neurology, University of Lübeck, Lübeck, Germany, ¹⁷Department of Preventive Medicine, University of California San Diego, San Diego, CA, USA, ¹⁸Department of Medicine, Boston University School of Medicine, Boston, MA, USA and ¹⁹Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA

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Osteoprotegerin (OPG) is involved in bone homeostasis and tumor cell survival. Circulating OPG levels are also important biomarkers of various clinical traits, such as cancers and atherosclerosis. OPG levels were measured in serum or in plasma. In a meta-analysis of genome-wide association studies in up to 10 336 individuals from European and Asian origin, we discovered that variants >100 kb upstream of the *TNFRSF11B* gene encoding OPG and another new locus on chromosome 17q11.2 were significantly associated with OPG variation. We also identified a suggestive locus on chromosome 14q21.2 associated with the trait. Moreover, we estimated that over half of the heritability of OPG levels could be explained by all variants examined in our study. Our findings provide further insight into the genetic regulation of circulating OPG levels.

Osteoprotegerin (OPG), a secreted member of the tumor necrosis factor receptor superfamily, is a decoy receptor for receptor activator of nuclear factor κ B ligand (RANKL) that inhibits osteoclastogenesis (1). OPG is found in multiple tissues,

including bone, lung, heart, kidney, intestine and vascular tissues (2–4); notably, OPG knockout mice develop severe osteoporosis (5). In addition to its well-established role in bone homeostasis (6,7), OPG is known to be involved in tumor cell

*To whom correspondence should be addressed at: Department of Medicine, University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong. Tel: +852 22554769; Fax: +852 28162187; Email: awckung@hkucc.hku.hk

survival (8–11), normal B-cell function (12) and vascular calcification (13–19). Circulating OPG levels are also markers of bone metastases in prostate cancer patients (20), systemic lupus erythematosus (21) and atherosclerosis (22), as well as potential markers of osteoporosis (23), Paget's disease (24), colorectal cancer recurrence (25), intestinal inflammation (26), incident atrial fibrillation (27) and vascular disease (23,28).

Due to the clinical interest in OPG, several studies have investigated genetic and environmental influences on circulating OPG levels (29–31). Abrahamsen *et al.* (31) reported no significant genetic influence on OPG levels in Danish female twins, whereas Livshits *et al.* (29), Kwan *et al.* (30) and we found that the trait is highly heritable in Chuvashians (heritability estimate, $h^2 = 46\%$), Southern Chinese (h^2 for age-adjusted OPG was 75% for females and 37% for males) and Framingham participants (h^2 for age-adjusted OPG was 23% for females and 24% for males) (see Supplementary Material, Methods and Table S5), respectively. In addition, Vistorovsky *et al.* (32) reported that an intronic single-nucleotide polymorphism rs875525 in the *ANKH* gene is significantly associated with OPG levels in a candidate gene study. But whether genes other than *ANKH* contribute to the variation in circulating OPG levels remains to be clarified. Genome-wide association studies (GWAS) are now a standard approach to investigate the genetic architecture of human traits and diseases (33–36). To gain more insight into the genetic regulation of OPG levels, we conducted a meta-analysis of GWAS data from 10 336 individuals from five cohorts. We found that variants > 100 kb upstream of the *TNFRSF11B* gene encoding OPG and a locus on chromosome 17q11.2 significantly contribute to OPG variation. We also identified a suggestive locus on chromosome 14q21.2 associated with OPG levels.

RESULTS

Figures 1 and 2 show, respectively, the Manhattan and QQ plots of association results from the fixed-effects inverse-variance weighted meta-analyses (37). The QQ plots of single-nucleotide polymorphisms (SNPs) for the sex-specific meta-analyses are provided in Supplementary Material, Figure S1. Summary association statistics of all SNPs are available at http://hpcf.cgs.hku.hk/gwas_pub/OPG. There was little evidence of inflation of the association results as the genomic inflation factors λ are 1.026, 1.023 and 1.018 for the sex-combined, female-specific and

male-specific meta-analyses, respectively. Therefore, genomic control correction was not applied to any of the meta-analysis results. A sample-size weighted meta-analysis gave essentially the same results and significant loci.

In the sex-combined meta-analysis, 72 SNPs in two loci had association P -values that reached genome-wide significance ($P < 5 \times 10^{-8}$): 8q23–q24.1 (smallest $P = 8.15 \times 10^{-15}$ near *MAL2*, *COLEC10* and *TNFRSF11B* encoding OPG), and 17q11.2 (smallest $P = 1.46 \times 10^{-9}$ within a gene-rich region) (Table 1 and Fig. 3). The degree of heterogeneity was low ($I^2 < 0.5$) across studies for the significant SNPs. In addition, four SNPs in a gene desert region at locus 14q21.2 almost reached genome-wide significance (smallest $P = 5.45 \times 10^{-8}$) (Table 1 and Fig. 3). In the female-specific meta-analysis, the chromosome 8 locus identified in the sex-combined meta-analysis remained significant (smallest $P = 8.46 \times 10^{-9}$) (Table 1; Supplementary Material, Fig. S2). None of the SNPs achieved genome-wide significance in the male-specific meta-analysis. Although we did not perform any replication study, the effect alleles of the top SNPs in the chromosome 8 and 17 loci showed consistent directions of effect in all participating studies (Table 1). Conditional analyses on the lead SNP in each significant locus found no strong evidence for additional independent signals reaching genome-wide significance. Pathway association analysis using a HYbrid Set-Based Test (HYST) (38) and hypergeometric test on 1320 canonical pathways revealed no significant pathways with genes moderately associated with OPG levels.

The most significant association signal in our study came from 8q23–q24.1, located > 100 kb upstream of the *TNFRSF11B* transcriptional start. The *TNFRSF11B* gene encodes OPG and so the region likely harbors important regulatory elements that control the expression of the OPG protein molecule. But our expression quantitative-trait loci (eQTL) analyses in various human tissues including bone cells found no association of the *TNFRSF11B* gene expression with the selected SNPs in the region (Table 2). Encyclopedia of DNA elements (ENCODE) annotation of the locus indicated that there are predicted insulators in 7 out of 9 cell lines examined within the *COLEC10* gene (Fig. 3). HaploReg v2 (39) and RegulomeDB (40) indicated that some of the proxies ($r^2 > 0.8$ in 1000 Genomes Pilot 1) of the top SNPs (i.e. rs1425053) lie at strong enhancers in the epithelial cell line HMEC (Supplementary Material, Table S6).

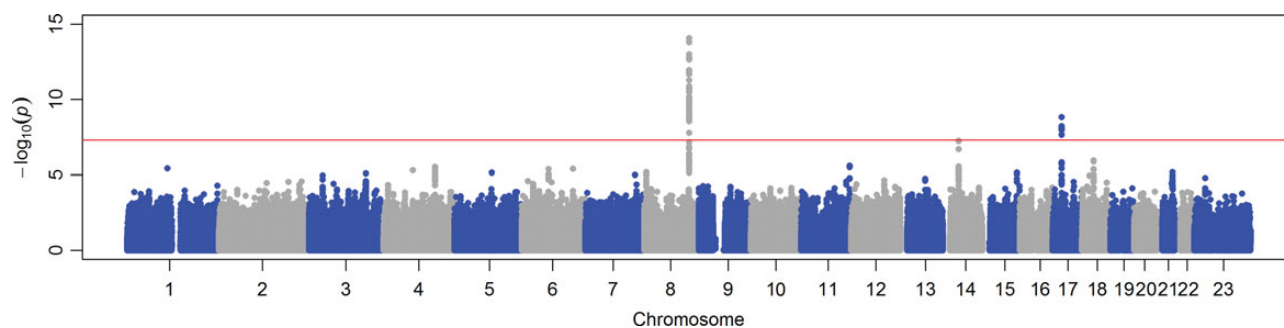


Figure 1. Manhattan plot of SNPs for the sex-combined meta-analysis. The X-axis indicates the chromosomal position of each SNP, whereas the Y-axis denotes the evidence of association shown as $-\log(P\text{-value})$. The red line indicates genome-wide significance of association ($P = 5 \times 10^{-8}$).

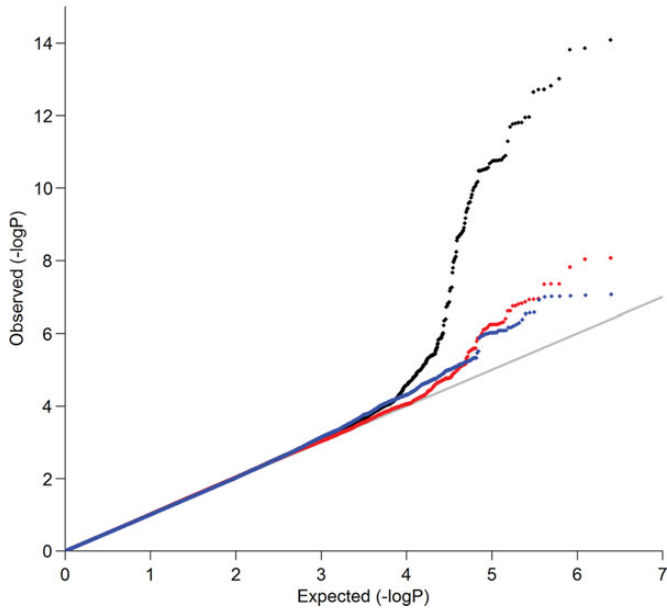


Figure 2. QQ plots of SNPs for sex-combined (in black), female-specific (in red) and male-specific (in blue) meta-analyses, respectively.

The chromosome 17 locus identified in the sex-combined meta-analysis encompasses multiple genes, including *FLJ40504*, *POLDIP2*, *TMEM97*, *MIR4723*, *VTN*, *IFT20*, *SARMI1*, *TNFAIP1*, *SLC46A1*, *TMEM199*, and *SEBOX*. The most significant SNP in this locus (rs704) encodes a possibly damaging (predicted by PolyPhen2) (41) missense mutation in the *VTN* gene. Moreover, eQTL analyses showed that the SNP was significantly associated with *TMEM199* expression in pancreas and whole bone, as well as *TMEM97* expression in whole blood (Table 2). The low RegulomeDB score of the SNP (i.e. 2b) also suggested that the SNP has important regulatory functions (Supplementary Material, Table S7).

Moreover, we examined the association of SNP rs875525 in the *ANKH* gene previously identified by Vistorovsky *et al.* (32) with OPG levels. However, it did not reach genome-wide significance in all three meta-analyses ($P = 0.58, 0.52$ and 0.88 in sex-combined, female-specific and male-specific meta-analyses, respectively). In addition, we interrogated published meta-analysis of BMD from the Genetic Factors for Osteoporosis (GEFOS) Consortium (42) to see whether the lead SNPs in our study overlapped with any BMD-associated loci. However, no significant association between BMD and our lead SNPs was found (all P -values > 0.05 after Bonferroni correction for multiple testing) (Table 2).

We also estimated the proportion of age-adjusted OPG variation explained by all SNPs studied in all 10 336 samples using a density estimation method proposed by So *et al.* (43). We found that the ~ 2.4 million SNPs examined in our study could explain $\sim 11\%$ of age-adjusted OPG variation. Given that h^2 for age-adjusted OPG levels was $\sim 20\%$ in Framingham participants, which constitute the majority of our study samples (Supplementary Material, Methods and Table S5), over half of the h^2 for age-adjusted OPG levels could be explained by all SNPs examined in our study alone.

Table 1. Meta-analysis results for loci that reached or marginally reached genome-wide significance ($P < 5 \times 10^{-8}$)

Chr	Nearest gene(s)	Lead SNP	Position ^a	EAF ^b	Effect allele	Other allele	β^c in individual study	FHS	GOOD	HKOS	MICROS	WHI	β^c	SE	P	% Variance explained	r^2	Cochran's $Q(P)$	P for association with FNBMD ^d	P for association with LSBMD ^d
Sex-combined meta-analysis																				
8	<i>COLLEC10</i> , <i>MAL2</i> , <i>TNFRSF11B</i>	rs1425053	120 282 171	0.607 (0.713)	A	T	0.039	0.034	0.045	0.045	0.033	0.016	0.037	0.0048	8.15×10^{-15}	0.58	0	0.80	0.047	0.24
17	<i>FLJ40504</i> , <i>POLDIP2</i> , <i>TMEM97</i> , <i>MIR4723</i> , <i>VTN</i> , <i>IFT20</i> , <i>SARMI1</i> , <i>TNFAIP1</i> , <i>SLC46A1</i> , <i>TMEM199</i> , <i>SEBOX</i>	rs704	23 718 988	0.494 (0.701)	A	G	-0.027	-0.027	-0.034	-0.051	-0.045	-0.045	-0.030	0.0049	1.46×10^{-9}	0.35	0	0.74	7.07×10^{-3}	0.025
14	—	rs2488856	42 601 152	0.898 (NA)	A	C	-0.048	-0.051	NA	-0.027	0.023	-0.044	0.0080	5.45×10^{-8}	0.28	33	0.21	0.39	0.55	
Female-specific meta-analysis																				
8	<i>COLLEC10</i> , <i>MAL2</i> , <i>TNFRSF11B</i>	rs2468186	120 269 883	0.623 (0.698)	T	C	0.045	—	0.047	0.027	0.019	0.040	0.0070	8.46×10^{-9}	0.59	0	0.56	0.45	0.45	

Note: the LD r^2 between rs1425053 and rs2468186 is ≥ 0.9 in both European and Chinese populations in HapMap.

^aChr, chromosome.
^bPositions are according to NCBI Build 36.
^cEffect allele frequency (EAF) averaged from the samples, with the Chinese EAF specifically indicated in brackets.
^d β represents the change in the natural log-transformed serum OPG levels (pmol/l) per additional copy of the effect allele.
^e P -value for association with BMD from a published meta-analysis (42).

DISCUSSION

Using a meta-analysis of GWAS from FIVE studies comprising > 10 000 individuals, we identified two genome-wide significant loci (8q23-q24.1 and 17q11.2) and one locus on chromosome 14 associated with OPG levels with near genome-wide significance.

The association signal in the chromosome 8 resides in a linkage disequilibrium (LD) block containing the *COLEC10* gene, whose functional relationship to OPG levels is unknown. Also, based on the GWAS catalog data (<http://www.genome.gov/gwastudies/>, accessed 7 January 2014) (36), no SNP in the LD block has been reported to be associated with any trait or disease. Although there existed predicted insulators generated by the ENCODE project in 7 out of 9 cell lines, the function of an insulator is to block the interaction between enhancers and promoters, and so the enhancer affected remains to be identified. Moreover, our eQTL analyses found no association of gene

expression of *TNFRSF11B*, *MAL2*, *VTN*, *TMEM199* and *TMEM97* with the top SNPs rs1425053 and rs2468186 or their proxies in strong LD. We acknowledge that the lack of eQTL associations may be due to small sample sizes except whole blood. So the functional roles of the genetic variants in the region remain to be elucidated. However, it is worth noting that HaploReg v2 (39) and RegulomeDB (40) indicated that some of the proxies ($r^2 > 0.8$ in 1000 Genomes Pilot 1) of the top SNPs (i.e. rs1425053) lie at strong enhancers in the epithelial cell line HMEC and multiple studies suggested that OPG may promote the survival of endothelial cells (14,16,44).

The most significant SNP in the chromosome 17 hit (i.e. rs704) encodes a possibly damaging missense mutation in the *VTN* gene. The *VTN* protein encoded by the gene was previously found to bind to OPG (13), as well as to regulate osteogenesis in mesenchymal stem cells (45). Also, we found that the SNP was associated with *TMEM199* expression in pancreas and

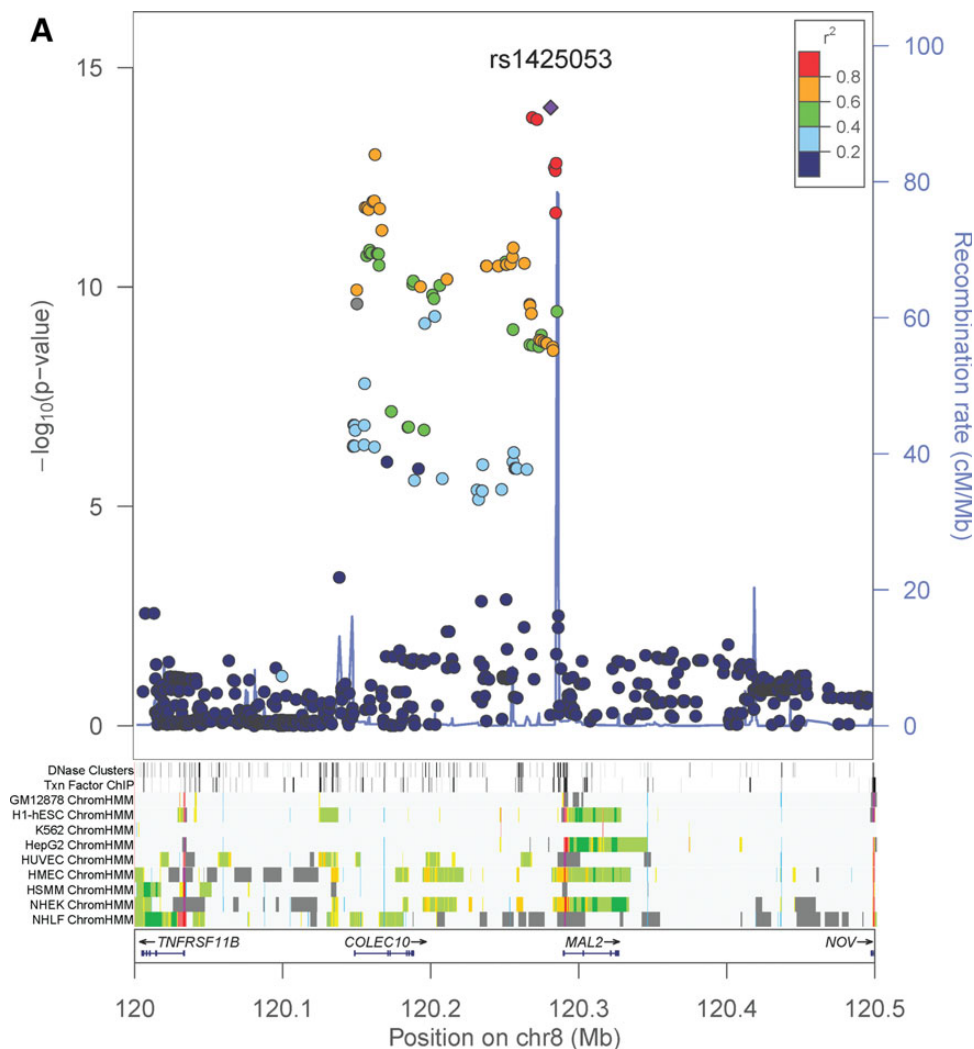


Figure 3. Regional association plots and ENCODE annotation of the loci that reached or marginally reached genome-wide significance ($P < 5 \times 10^{-8}$) in the sex-combined meta-analysis. The X-axis indicates the physical position of each SNP on the chromosome specified, whereas the Y-axis denotes the evidence of association shown as $-\log(P\text{-value})$. ENCODE annotation are also provided, including transcription factor-binding sites (TFBS) from 95 cell lines, DNase I hypersensitivity sites (DNase HS) from 125 cell lines, and chromatin states in nine ENCODE cell lines (GM12878, H1-hESC, K562, HepG2, HUVEC, HMEC, HSMM, NHEK and NHLF). The chromatin states are annotated as follows: strong enhancer (orange), weak enhancer (yellow), active promoter (red), poised promoter (pink), insulator (blue), transcribed (pale green), transcription transition (dark green), repressed (dark gray) and heterochromatin (pale gray).

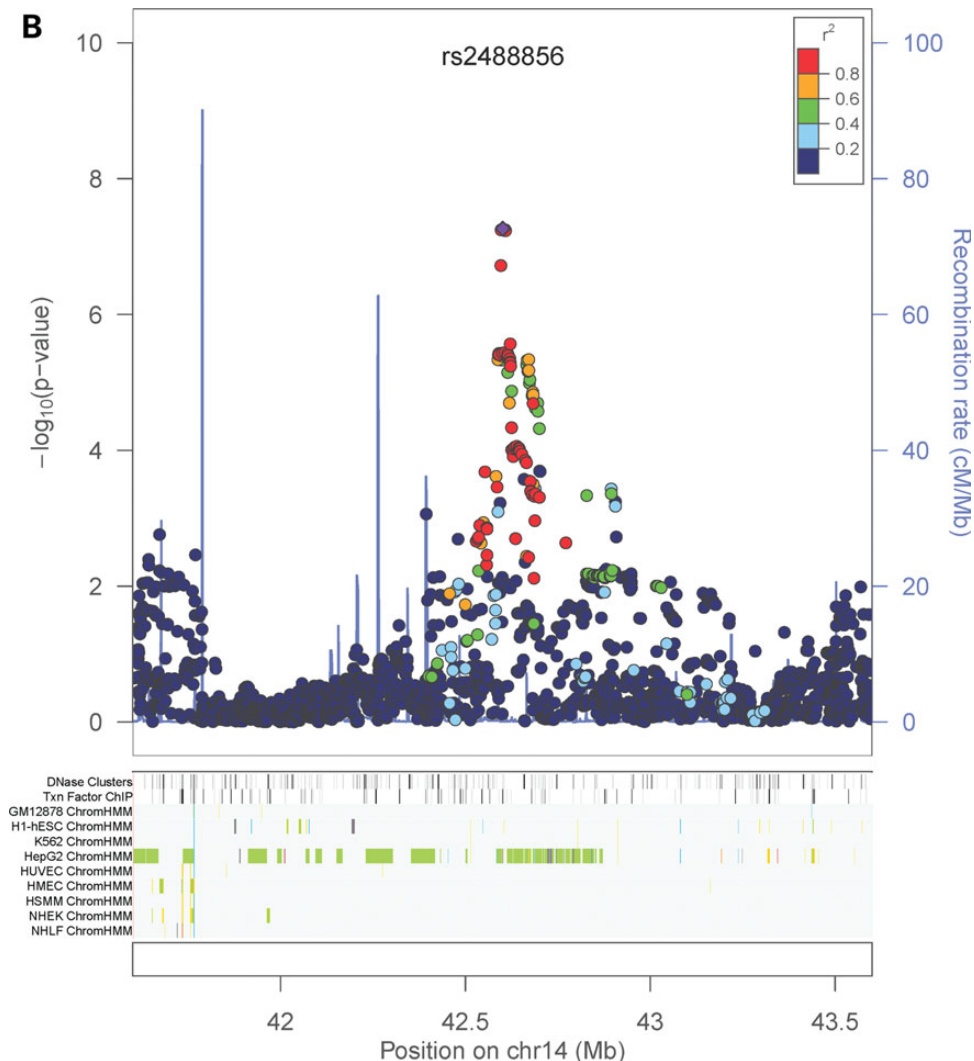


Figure 3. Continued

whole bone as well as *TMEM97* expression in whole blood. The biological function of *TMEM199* in pancreas and whole bone tissues has not yet been established, but the gene was associated with cancer progression in breast tissues (46). *TMEM97* plays a role in cholesterol and lipid metabolism (47) and is also involved in cancer development (48,49). But as pointed out by Edwards *et al.* (50), experiments are needed to elucidate the molecular mechanisms in detail.

We only identified two statistically significant regions and one suggestive region in which the potentially causal variants (i.e. the ones shown to be causal in influencing OPG levels that are in LD with other variants associated with OPG levels) reside. The next steps should be to fine-map these regions with a dense marker panel and a large sample size to identify candidate causal variants. Since the majority of participating studies in our meta-analysis were primarily of European ancestry, we recommend that fine-mapping studies be performed in populations of multiple ethnicities. First, the LD patterns differ between the different ethnic groups (Supplementary Material, Fig. S3), which may be able to reduce the number of candidate causal variants

in the fine-mapping studies. Second, fine-mapping other racial groups may answer the question of whether the associations we found generalize to populations of non-European ancestry (51,52).

The results of our meta-analysis failed to confirm an association of a previously identified OPG-associated SNP rs875525 (32). As the study of Vistoropsky *et al.* was performed in Chuvashians, LD patterns between their subjects and ours (which were mostly European ancestry) should be similar. So the absence of such association in our study indicates either their association was a false positive or we had poor power to detect such association. Since Vistoropsky *et al.* did not provide the effect size of rs875525, we cannot estimate our power to discover such an effect.

The current study was limited by the measurement of circulating OPG. In addition, the studies varied by whether they measured OPG in serum versus plasma; however, we note that the β s and directions of association were similar across studies. Although monoclonal antibody was used, it is unclear whether the mono- or homodimeric form should be measured. The

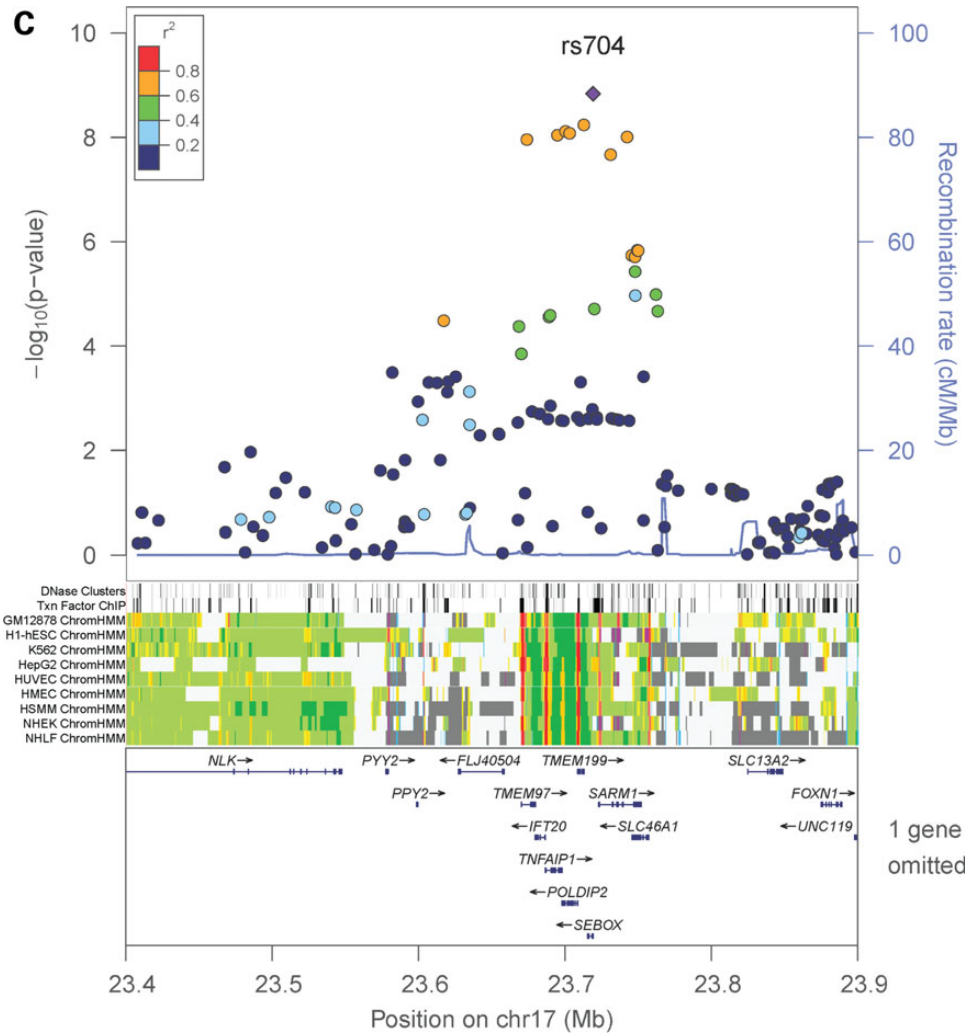


Figure 3. Continued

Table 2. Results for e-QTL analyses

Chr.	SNP	Transcript	GTEx whole blood (n = 144)	GTEx subcutaneous adipose (n = 111)	GTEx skeletal muscle (n = 138)	GTEx pancreas (n = 67)	Whole bone (n = 54)	Primary osteoblasts (n = 95)	Whole blood (n = 5311)
8	rs1425053	<i>TNFRSF11B</i>	>0.1	0.05	0.03	>0.1	>0.1	>0.1	>0.1
		<i>MAL2</i>	0.002	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
8	rs2468186	<i>TNFRSF11B</i>	>0.1	>0.1	0.02	>0.1	>0.1	>0.1	>0.1
		<i>MAL2</i>	0.002	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
17	rs704	<i>VTN</i>	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
		<i>TMEM199</i>	>0.1	0.03	>0.1	$4 \times 10^{-4*}$	0.004*	>0.1	>0.1
		<i>TMEM97</i>	>0.1	0.02	>0.1	>0.1	>0.1	>0.1	$1.08 \times 10^{-51*}$

Significant *P*-values for association between the SNP and expression of the selected transcripts are marked with an asterisk.

assay used by all studies detected both forms of OPG, and the RANKL-bound form. Also, one target action point of OPG is skeletal and there is no evidence that circulating OPG level reflects the amount of bioavailable OPG in the skeletal micro-environment. As OPG is produced locally in bone tissue by osteoblasts, another study design would be to examine genome-

wide association of bone-specific OPG. A bone biopsy study in a large number of individuals is nonetheless impractical to resolve the matter.

In conclusion, we discovered that variants > 100 kb upstream of the *TNFRSF11B* gene encoding OPG are associated with variation in circulating OPG levels and identified another new

significant locus on chromosome 17q11.2 as well as a suggestive locus on chromosome 14q21.2 associated with the trait. Moreover, we estimated that over half of the heritability of age-adjusted OPG levels could be explained by all SNPs studied. Further work is needed to identify the causal variants and to understand how they influence the circulating OPG levels.

MATERIALS AND METHODS

Study populations

The meta-analysis comprised 10 336 individuals from five GWAS: Framingham Heart Study (FHS)—3223 men and 3685 women of European ancestry; Gothenburg Osteoporosis and Obesity Determinants (GOOD) study—935 men of European ancestry; Hong Kong Osteoporosis Study (HKOS)—702 women of Chinese ancestry; Microisolates in South Tyrol (MICROS) study—531 men and 692 women of European ancestry; and Women's Health Initiative (WHI)—568 women of European ancestry. Each participating study was approved by a research ethics committee and all participants provided written informed consent. OPG levels were measured in serum (except for FHS, which measured in plasma) by either ELISA or the Fluorescent Bead Immunoassay method. More details of all participating studies are provided in Supplementary Material, Tables S1–S3.

GWAS analysis

Genome-wide genotyping for SNPs and quality control were done independently by each study following standard protocols. Each study performed genotype imputation with reference to HapMap Phase II of the corresponding population. Detailed descriptions of the genotyping platforms, quality control and imputation procedures for each study are provided in the Supplementary Material, Table S4. Each study performed GWAS tests for natural log-transformed OPG level (in pmol/l) under an additive (per allele) genetic model (53), using either linear regression (for unrelated samples) or linear-mixed models (for related samples). Associations were adjusted for sex (if not sex-specific), age, age-squared, principal components of genetic ancestry as well as other study-specific covariates. Before meta-analysis, SNPs were removed in each study if they had a minor allele frequency <1%, an extreme deviation from Hardy–Weinberg equilibrium ($P < 1 \times 10^{-6}$), and/or an imputation quality score <0.3. In each study, we also ensured that the allele frequencies of the SNPs observed matched closely with those in the corresponding HapMap reference panel. We tested for population stratification of the observed associations using the genomic inflation (λ) method (54).

Meta-analysis

Summary statistics from the individual studies were combined using fixed-effects inverse-variance weighted meta-analysis using the METAL software (37). Since the approach requires the trait distribution to be identical across individual studies, we also combined the summary statistics using the sample-size weighted method implemented in the METAL software and compared the association results given by both methods. SNPs

with missing data in more than one study were excluded. SNPs with $P < 5 \times 10^{-8}$ were considered genome-wide significant. We also performed a meta-analysis of sex-specific GWAS results. We examined between-study heterogeneity using I^2 and Cochran's Q test. An association analysis conditioned on the lead SNP was also performed in each significant locus to search for additional independent signals (55).

Functional annotation

Significant loci found and their proxies ($r^2 > 0.8$ in 1000 Genomes Pilot 1) were examined for regulatory functions generated by the ENCODE Project (56) using HaploReg v2 (39) (<http://www.broadinstitute.org/mammals/haploreg/haploreg.php>, accessed 14 July 2014) and RegulomeDB (40) (<http://regulomedb.org>, accessed 14 July 2014).

Pathway analysis

To examine whether any known biological pathway is associated with OPG levels, we applied HYST for GWAS (38). Briefly, each SNP was mapped to genes (including 10 kb upstream and downstream regions) according to hg18. An extended Simes' test was then applied to compute association P -value for each gene (57) and the P -values for the genes in a pathway were combined using a scaled χ^2 test to yield an association P -value of the pathway (38). We analyzed 1320 canonical pathways from the pathway databases available in MSigDb v4.0 (58) and a pathway with P -value < 0.05/1320 was declared significant after Bonferroni correction for multiple testing. In order to remove pathways with statistical significance caused by only a few extreme gene-based P -values, we defined a set of candidate genes with a false discovery rate < 0.05 and tested for enrichment of this candidate gene set using a hypergeometric test. Any significant pathway with a nominal P -value < 0.001 in the hypergeometric test was filtered out.

eQTL analysis

We performed *cis*-eQTL analyses on the three lead SNPs (rs1425053, rs2468186 and rs704) or their proxies in strong LD ($r^2 \geq 0.8$) with selected transcripts within 500 kb of the SNP position. In each locus, linear regression was used to examine the association between the top-associated SNP and expression of the selected genes. The eQTL analyses were performed in several human tissues, including whole blood ($n = 144$), subcutaneous adipose ($n = 111$), skeletal muscle ($n = 138$) and pancreas tissues ($n = 67$) from postmortem donors in the Genotype Tissue Expression program (GTEx) (59) as well as transiliac bone biopsies (60), human primary osteoblast samples (obtained from 95 bone biopsies) (61) and peripheral blood samples ($n = 5311$) (62). The detailed methods of sample collection, DNA and RNA extraction, GWAS genotyping, gene expression profiling and RNA-sequence are described in the corresponding papers. Results were declared significant at $P \leq 0.05/2/5 = 0.005$ after Bonferroni correction of the number of loci (i.e. two) and different tissues (i.e. five) tested.

Estimation of variance explained by all SNPs examined

To estimate the joint effect of all SNPs studied in explaining the variance of age-adjusted OPG levels, we applied the density estimation method proposed by So *et al.* (43). It required only summary association statistics and sample size for computation and was demonstrated to give estimates similar to those by GCTA (63).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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