## Articles

# Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study

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## Summary

**Background** Osteoporosis is diagnosed by the measurement of bone mineral density, which is a highly heritable and multifactorial trait. We aimed to identify genetic loci that are associated with bone mineral density.

**Methods** In this genome-wide association study, we identified the most promising of 314 075 single nucleotide polymorphisms (SNPs) in 2094 women in a UK study. We then tested these SNPs for replication in 6463 people from three other cohorts in western Europe. We also investigated allelic expression in lymphoblast cell lines. We tested the association between the replicated SNPs and osteoporotic fractures with data from two studies.

**Findings** We identified genome-wide evidence for an association between bone mineral density and two SNPs ($p<5\times10^{-8}$). The SNPs were rs4355801, on chromosome 8, near to the *TNFRSF11B* (osteoprotegerin) gene, and rs3736228, on chromosome 11 in the *LRP5* (lipoprotein-receptor-related protein) gene. A non-synonymous SNP in the *LRP5* gene was associated with decreased bone mineral density (rs3736228, $p=6\cdot3\times10^{-12}$ for lumbar spine and $p=1\cdot9\times10^{-4}$ for femoral neck) and an increased risk of both osteoporotic fractures (odds ratio [OR] $1\cdot3$, 95% CI $1\cdot09-1\cdot52$, $p=0\cdot002$) and osteoporosis (OR $1\cdot3$, $1\cdot08-1\cdot63$, $p=0\cdot008$). Three SNPs near the *TNFRSF11B* gene were associated with decreased bone mineral density (top SNP, rs4355801: $p=7\cdot6\times10^{-10}$ for lumbar spine and $p=3\cdot3\times10^{-8}$ for femoral neck) and increased risk of osteoporosis (OR $1\cdot2$, 95% CI $1\cdot01-1\cdot42$, $p=0\cdot038$). For carriers of the risk allele at rs4355801, expression of *TNFRSF11B* in lymphoblast cell lines was halved ($p=3\cdot0\times10^{-6}$). 1883 (22%) of 8557 people were at least heterozygous for these risk alleles, and these alleles had a cumulative association with bone mineral density (trend $p=2\cdot3\times10^{-17}$). The presence of both risk alleles increased the risk of osteoporotic fractures (OR $1\cdot3$, $1\cdot08-1\cdot63$, $p=0\cdot006$) and this effect was independent of bone mineral density.

**Interpretation** Two gene variants of key biological proteins increase the risk of osteoporosis and osteoporotic fracture. The combined effect of these risk alleles on fractures is similar to that of most well-replicated environmental risk factors, and they are present in more than one in five white people, suggesting a potential role in screening.

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## Introduction

Osteoporosis and its main complication, fragility fractures, incur substantial global morbidity and mortality.[1] The public-health burden of this disease is US$17 billion every year in direct expenditure—and this is expected to increase dramatically as populations age.[2] Osteoporosis is defined clinically through the measurement of bone mineral density, which remains the single best predictor of primary osteoporotic fractures.[3] Bone mineral density is highly heritable, with estimates from a cohort study in the UK of 78% heritability of density at lumbar spine and 84% at femoral neck;[4] these figures are consistent with other twin studies.[5] Only a few well-replicated studies of candidate genes for osteoporosis have so far emerged,[6–8] suggesting that bone mineral density is a complex polygenic trait.[5]

Genome-wide association studies have been facilitated by the HapMap project, and by recent advances in genome-wide genotyping arrays that provide a high degree of genome coverage.[9] We therefore undertook a genome-wide association study to identify genetic loci that influence bone mineral density.

## Methods

### Study participants and phenotypes

We did a genome-wide association study with 8557 participants, all of whom were of white European ancestry, unselected for any disease or trait, and aged 18 years and older. Baseline characteristics are in table 1 and the webappendix. The discovery cohort consisted of women from the TwinsUK cohort, which was a population-based sample from the UK.[4] The replication cohorts comprised men and women from a Dutch population-based study of unrelated and unselected elderly patients (the Rotterdam study),[10–12] a population-based sample of British women (the Chingford study),[13]

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| | TwinsUK discovery cohort | Rotterdam cohort | TwinsUK replication cohort | Chingford cohort | Total sample |
|---|---|---|---|---|---|
| Number assessed for bone mineral density and osteoporosis | 2094 | 4081 | 1692 | 690 | 8557 |
| Age (years) | 49·7 (13·1) | 68·9 (8·8) | 49·7 (14·1) | 62·1 (6·0) | 59·7 (14·1) |
| Sex (men) | .. | 784 (19%) | .. | .. | 784 (9·2%) |
| Lumbar spine (g/cm²) | 1·00 (0·14) | 1·06 (0·19) | 0·97 (0·14) | 0·95 (0·15) | 1·03 (0·18) |
| Femoral neck (g/cm²) | 0·80 (0·13) | 0·85 (0·14) | 0·80 (0·13) | 0·75 (0·12) | 0·83 (0·14) |
| Osteoporosis | 116 (5·6%) | 111 (3·0%) | 98 (5·9%) | 70 (10·2%) | 395 (4·6%) |

Data are mean (SD) or number (%).

*Table 1:* Characteristics of 8557 genotyped individuals assessed for bone mineral density and osteoporosis

| | Rotterdam fracture cohort | Chingford fracture cohort |
|---|---|---|
| Number followed up for fracture* | 5921 | 718 |
| Number who had an osteoporotic fracture† | 526 (6·6%) | 134 (18·7%) |
| Age (years) | 68·5 (8·5) | 62·1 (5·9) |
| Men | 1581 (26·7%) | .. |

Data are mean (SD) or number (%). *Osteoporotic fractures were defined as site-specific, validated, clinical, or low-trauma fractures. †Not all people followed up for fracture had measurements of bone mineral density.

*Table 2:* Characteristics of 6639 genotyped individuals assessed for osteoporotic fractures

See **Online** for webappendix

and the TwinsUK replication cohort, with individuals who were not included in the TwinsUK discovery cohort. Participants were excluded if they reported use of bisphosphonate medications or corticosteroids. All studies were approved by institutional ethics review committees at the relevant organisations, and all participants provided written informed consent.

We measured bone mineral density in all cohorts at the lumbar spine (L1–4) and femoral neck by dual energy radiograph absorptiometry with standard protocols. In the TwinsUK cohorts, bone mineral density was measured at several time points, so the most recent measurement was used to better match the age range of the other two cohorts. Osteoporosis was defined as a T-score of less than or equal to 2·5 SD at either the femoral neck or lumbar spine site (derived from peak female bone mass).[14]

Osteoporotic fractures were defined as site-specific, validated, clinical, low-trauma fractures. All non-osteoporotic site fractures (nose, toe, head, jaw, skull, and hands) were excluded. In the Chingford study, fractures were assessed prospectively by interview at each annual visit and by phone calls every 6 months to reduce information bias.[13] We validated reports of fractures from general practitioners' notes with a standardised protocol.[13] Fractures were excluded if no radiograph report, hospital letter, or clinical examination was available to confirm the fracture. If notes were missing, all self-reported fractures were coded as missing. The methods used for identification of fractures in the Rotterdam cohort have been described previously.[15] In brief, the computerised

records of general practitioners and hospital registries were regularly checked by research physicians who reviewed and coded the information (table 2).

## Genotyping and quality control

We genotyped samples from the TwinsUK discovery cohort with the Infinium assay (Illumina, San Diego, USA) across three genome-wide SNP sets, after strict quality-control criteria were applied (see webappendix). We retained 314 075 (98·7%) SNPs for analysis. 2704 SNPs were excluded because they violated the Hardy–Weinberg equilibrium ($p < 1·0 \times 10^{-4}$); 733 SNPs were excluded because they had genotype call rates of 90% or less; and 725 SNPs were excluded because they had a minor allele frequency of less than 0·01. We then assayed the 314 075 SNPs in the Rotterdam cohort, with the HumanHap 550 v3.0 array (Illumina, San Diego, USA), which contained the same SNPs as the TwinsUK cohort, after applying the same quality-control criteria. The Rotterdam cohort was genotyped in two phases: first with a subsample of 1586 women who were randomly selected from the cohort, and then with the remainder of individuals in the cohort. We genotyped the Chingford and TwinsUK replication cohorts with the Taqman system (Applied Biosystems, Foster City, CA, USA), as described earlier.[16,17]

## Statistical analysis

We assessed possible confounding bias arising from population substructure by use of the program STRUCTURE in the TwinsUK cohort.[18] We assessed bias from identity by state clustering analysis in the Rotterdam study,[19] excluding people who were not of European ancestry, and by applying genomic control. Such effects were not evident after exclusions, with genomic inflation factors of 1·04 or less for both phenotypes in both cohorts. Bone mineral density was adjusted for age in all cohorts. We then undertook a genome-wide association scan, accounting for family structure in the discovery population, as implemented in the PLINK software package (version 1.01).[19]

We planned a two-stage replication strategy (figure 1). In the first stage we did a genome-wide association scan to select SNPs, based on p values and linkage

disequilibrium, from the TwinsUK discovery cohort (webfigure 1). We tested these SNPs for replication in the subsample of 1586 women from the Rotterdam cohort, who had previously been genotyped. In this first stage, we tested the SNPs from the genome-wide association scan that had a false-positive report probability of less than 0·5 (corresponding to a p value threshold of $1·6\times10^{-4}$ or less; webappendix and webtable 1).[20] We also tested SNPs which were in close linkage disequilibrium ($r^2\geq0·7$) with one of the selected SNPs, and for which the p value was 0·001 or lower, for replication in this stage. 39 SNPs for lumbar spine and 60 for femoral neck met these criteria and are displayed in webtable 1.

In the second stage of replication testing, we considered at least one SNP from each locus that had a p value of less than 0·05 in the Rotterdam subsample for replication in stage two. Six SNPs (rs2445803, rs3736228, rs6469792, rs6469804, rs11099284, and rs4355801) met these criteria and were tested for their association with bone mineral density in stage two, which incorporated the remaining participants from the Rotterdam cohort, with the Chingford cohort and the Twins UK replication cohort.

Many different replication strategies have been used in genome-wide association scans.[21–23] We used $5\times10^{-8}$ as a threshold for p values (Bonferroni correction, based on 0·05 multiplied by 1 million independent SNPs in the CEU population of the HapMap Phase II).[9] This threshold should not only account for the multiple tests required for a typical genome-wide association scan, but also for the additional tests required for replication and functional studies.[24] Effect sizes were estimated from the replication cohorts only. p values from each replication phase of the study and each cohort are in webtable 1. The p value for the association between the number of risk alleles and bone mineral density was derived from a non-parametric trend test.[25] The p value for the relationship between both risk alleles and bone mineral density is derived from a two-sided Student's $t$ test. All analyses of bone mineral density, unless otherwise stated, were from the combined analysis of the men and women from all cohorts. We used cohort-specific, age-residualised, standardised bone mineral density $Z$ scores (mean 0, SD 1) that were based on the assumption of an additive effect of the risk alleles. Adjustment for age did not modify the association between the replicated SNPs and a diagnosis of osteoporosis (results not shown).

### Expression analysis

We used *cis*-associated allelic expression studies to ascertain whether the risk alleles associated with low bone mineral density affected expression of their nearest transcript in lymphoblast cell lines.[26] Only *TNFRSF11B* was informative in the HapMap CEU lymphoblast cell lines[27] since *LRP5* was not expressed in lymphoblast cell lines. To create a reference for allelic expression in *TNFRSF11B* we used five intragenic SNPs (rs11573829, rs3134063, rs3548440, rs6469788, and rs10505346) in RNA and DNA samples of 55 unrelated HapMap parents (see webappendix).[28]

### Role of the funding source

The sponsors of this study had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the paper for publication. JBR had full access to all data and JBR and TDS were jointly responsible for the decision to submit for publication.

### Results

We identified two SNPs (rs4355801 on chromosome 8 close to the *TNFRSF11B* [osteoprotegerin] gene and rs3736228 on chromosome 11 in the *LRP5* [low density lipoprotein-receptor-related protein] gene) for which there was genome-wide evidence for association with bone mineral density or osteoporotic fracture ($p<5\times10^{-8}$).

Several SNPs within, and in proximity to, the *LRP5* gene showed strong associations with bone mineral density at lumbar spine (figure 2 and table 3). The highest ranked SNP within the locus (rs3736228) was a non-synonymous base-pair change: Ala1330Val ($p=6·3\times10^{-12}$ for lumbar spine, webtable 2). This SNP had the largest effect on bone mineral density, since each risk allele, T, decreased bone mineral density by 0·13 SD in the replication cohorts. 2397 (28%) of the 8557 participants had this risk allele. The T allele at rs3736228 was associated with decreased bone mineral density at the femoral neck ($p=1·9\times10^{-4}$). The variance in bone mineral density explained by allelic variance at rs3736228 was 0·6% for lumbar spine and 0·2% for femoral neck.

The risk allele at rs3736228 was also associated with an increased risk of osteoporotic fracture (OR 1·30, 95% CI 1·09–1·52, p=0·002 for both fracture cohorts; OR 1·59,
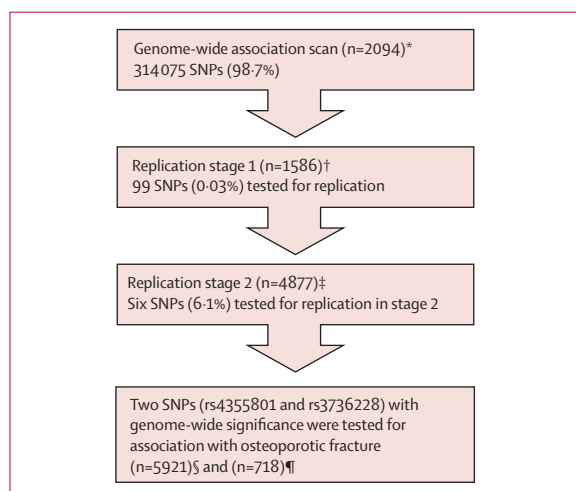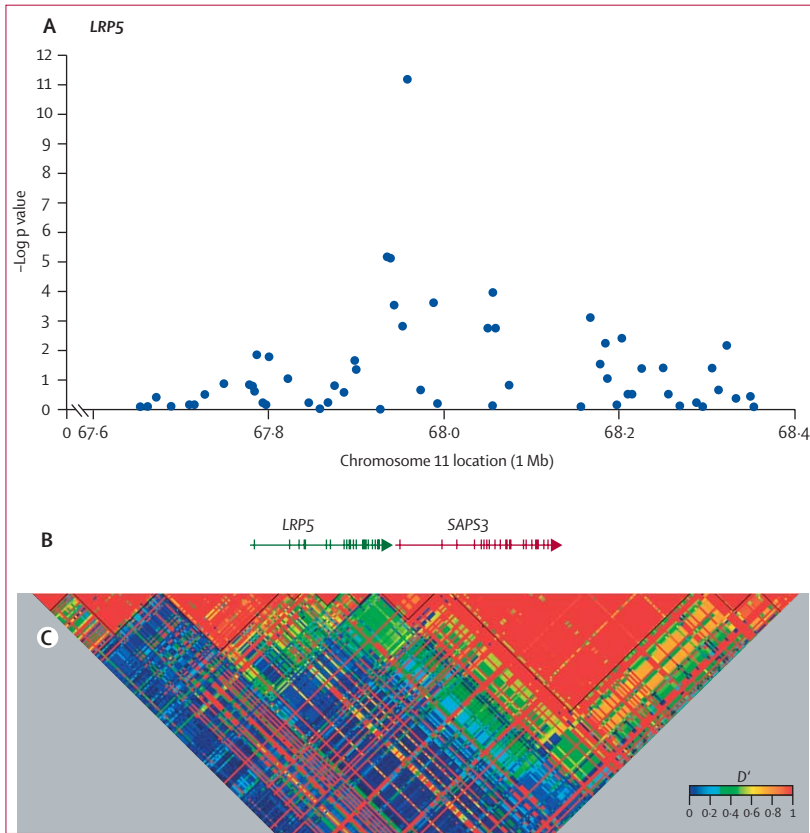
*Figure 1:* **Study design**
SNP=single nucleotide polymorphism. *TwinsUK Discovery cohort. †Subsample of the Rotterdam cohort. ‡TwinsUK replication cohort, Chingford cohort, and the remainder of the Rotterdam cohort. §Rotterdam fracture cohort. ¶Chingford fracture cohort.

**Figure 2:** Association of single nucleotide polymorphisms at *LRP5* with bone mineral density in the lumbar spine
(A) Negative log p values for associations between the SNP, rs3736228, and bone mineral density are plotted against chromosomal position. (B) Annotated reference sequence genes by chromosomal position (according to the US National Centre for Biotechnology Information). (C) Linkage disequilibrium in GOLD heatmap Haploview 4.0 colour scheme.[29] SNP rs3736228 represents data from all cohorts. Details of all SNPs are in webtable 3.

1·11–2·25, p=0·01 for the Chingford cohort; and OR 1·25, 1·04–1·51, p=0·02 for the Rotterdam cohort; table 3). The fracture risk imparted by rs3736228 persisted, but was partly attenuated by adjustment for bone mineral density (OR 1·22, 1·02–1·45, p=0·03). The presence of this same risk allele was associated with a greater risk of osteoporosis in the replication cohorts (OR for osteoporosis 1·33, 1·08–1·63, p=0·008). Since *LRP5* is not expressed in lymphoblast cell lines we could not assess the association between this SNP and *LRP5* expression.

Three SNPs, rs4355801, rs6469792, and rs6469804, which are in close proximity to the *TNFRSF11B* gene,

were associated with bone mineral density. The lead SNP, rs4355801, which is located in the 3′ untranslated region of the gene, remained associated with bone mineral density at lumbar spine (p=7·6×10⁻¹⁰) and femoral neck (p=3·3×10⁻⁸), with genome-wide significance. The other two SNPs at this locus were less strongly associated with bone mineral density (p=1·1×10⁻⁶ for rs6469792 and p=6·7×10⁻⁶ for rs6469804, for lumbar spine). Many SNPs within or near this gene were associated with bone mineral density in both cohorts (figure 3). The variant with the greatest association (rs4355801) was common, and 6743 (79%) of the study population had this risk allele A. Each copy of the A risk allele was associated with a decrease in lumbar spine bone mineral density by 0·09 SD in the replication cohorts (table 3). The variance in bone mineral density explained by allelic variance at rs4355801 was 0·4% at both lumbar spine and femoral neck.

Functional genomics investigations into the allele-specific expression patterns of the *TNFRSF11B* transcript in lymphoblast cell lines of HapMap individuals confirmed the relevance of the association data (webfigure 2). A clear spatial link was shown, such that the region with the strongest association with bone mineral density also most strongly affected expression of *TNFRSF11B*.

The strongest association was recorded for rs4355801 (p=8·3×10⁻⁶, for allelic expression association test, webtable 4).[28] In quantitative analysis of 27 samples with G alleles at rs4355801, we noted a two-fold overexpression of the G allele (G:A allele ratio 2·16, 95% CI 1·4–2·9). Finally, we recorded corroborating evidence for this allelic expression association in two published datasets.[30,31] In summary, the G allele at rs4355801, which was associated with higher bone mineral density, was also associated with higher expression of *TNFRSF11B*.

The risk allele A at rs4355801 was associated with an increased risk of osteoporosis (OR for osteoporosis 1·20, 95% CI 1·01–1·42, p=0·038) in the replication cohorts, but no change in the risk of osteoporotic fracture (OR 1·1, 0·94–1·22 for both fracture cohorts, OR 1·1, 0·85–1·46 for the Chingford fracture cohort, OR 1·04, 0·90–1·21 for the Rotterdam fracture cohort).

1883 (22%) of the overall study population was homozygous or heterozygous for the risk alleles at *LRP5* (rs3736228) and *TNFRSF11B* (rs4355801). The presence of both risk alleles was associated with a decrease in bone

| Single nucleotide polymorphism | Closest gene | Minor allele | Risk allele | Risk allele frequency | | Population with at least one risk allele | Effect* of each risk allele on bone mineral density (SD) | Combined p value |
|---|---|---|---|---|---|---|---|---|
| | | | | TwinsUK discovery cohort | Replication cohorts | | | |
| rs3736228 | *LRP5* | T | T | 0·15 | 0·14 | 28 | −0·13 | 6·3×10⁻¹² |
| rs4355801 | *TNFRSF11B* | G | A | 0·54 | 0·53 | 79 | −0·09 | 7·6×10⁻¹⁰ |

*Effect is the additive effect of each risk allele on the standardised residual for bone mineral density (residualised for age) in the replication cohorts.

**Table 2:** Association between two single nucleotide polymorphisms and bone mineral density at lumbar spine

mineral density at lumbar spine by 0·18 SD (p=2·5×10⁻⁹) and 0·1 SD (p=7·0×10⁻⁴) at femoral neck in the replication cohorts (table 4). Figure 4 shows the cumulative effect of these risk alleles on bone mineral density at lumbar spine (p for trend=2·3×10⁻¹⁷) and femoral neck (p for trend=7·0×10⁻¹¹). The presence of both risk alleles was associated with an increased risk of osteoporosis in the replication cohorts (OR for osteoporosis 1·5, 95% CI 1·2–2·0, p=0·0026).

The presence of both risk alleles was associated with an increased risk of osteoporotic fracture (OR 1·33, 95% CI 1·08–1·63, p=0·006) in both the Chingford fracture cohort (OR 1·60, 1·04–2·48, p=0·03) and the Rotterdam fracture cohort (OR 1·30, 1·03–1·64, p=0·03). The association with an increased risk of fractures remained after adjustment for bone mineral density (OR 1·29, 1·05–1·59, p=0·014).

Signals that did not reach genome-wide significance were found in other regions. The C allele at SNP rs11099284 was associated with 0·09 SD lower bone mineral density at lumbar spine (p=0·00001), and 0·07 SD at femoral neck (p=0·001). The C allele at SNP rs2445803 was associated with a decrease in femoral neck bone mineral density of 0·09 SD (p<0·0001), but had little effect on lumbar spine (0·04 SD, p=0·02). These loci could possibly affect bone mineral density, but further investigation will be needed.

We did subgroup analysis on the 784 men in the Rotterdam cohort. Although the magnitude and direction of effects of these risk alleles (rs3736228 and rs4355801) was similar to that of the overall cohorts, the confidence intervals often included the null value in this subgroup analysis. The risk allele at rs3736228 decreased bone mineral density at lumbar spine by 0·11 SD (p=0·04) but not at femoral neck (0·04 SD, p=0·42); its effect on the risk of fractures was not significant (OR 1·34, 95% CI 0·96–1·86, p=0·10). The risk allele at rs4355801 decreased bone mineral density at femoral neck by 0·10 SD (p=0·03), but did not change bone mineral density at lumbar spine (0·07 SD, p=0·10) or fracture risk (OR 1·1, 95% CI 0·86–1·4, p=0·43).

## Discussion

We have identified genetic variants that decrease bone mineral density and predispose people to osteoporosis and osteoporotic fracture. The increased risk of osteoporotic fracture in people who had both risk alleles was independent of the effect of these alleles on bone mineral density. In combination, the risk variants at osteoprotegerin and *LRP5* were common. However, since the replicated SNPs explain only a small amount of variance in bone mineral density, our findings also suggest that single common genetic variants are unlikely to have a large effect on bone mineral density.

Physicians need the ability to assess risk factors that are prevalent, potent, and accurately quantifiable. A recent WHO meta-analysis of 60 000 individuals who
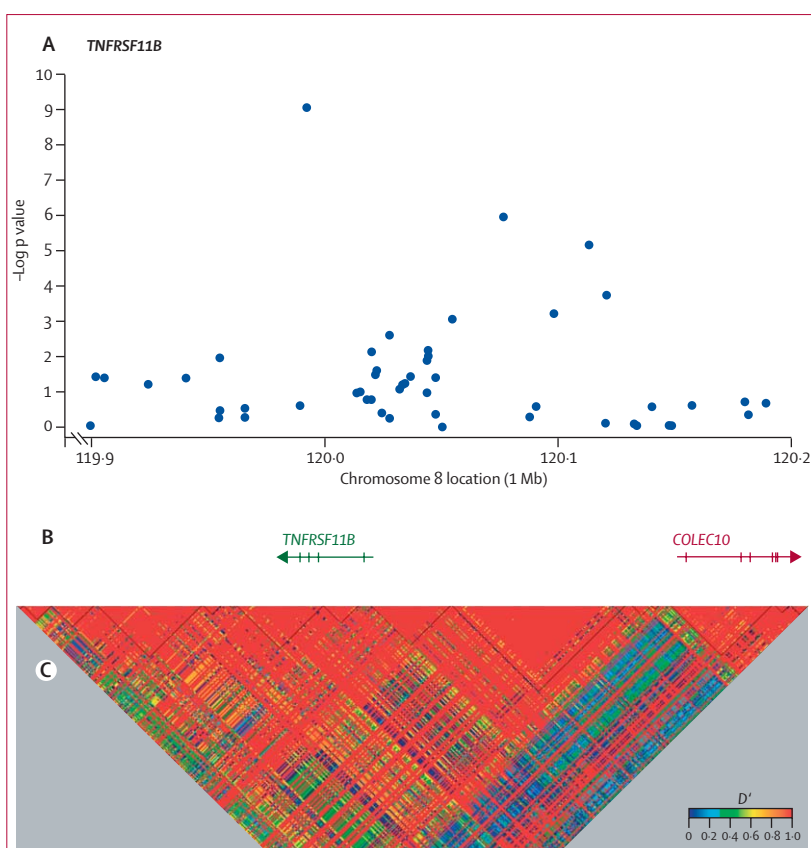


*Figure 3:* Association of single nucleotide polymorphisms near the osteoprotegerin gene (*TNFRSF11B*) and bone mineral density at lumbar spine

(A) Negative log p values for associations between the SNP, rs4355801, and bone mineral density are plotted against chromosomal position. (B) Annotated reference sequence genes by chromosomal position (according to the US National Center for Biotechnology Information). (C) Linkage disequilibrium in GOLD heatmap Haploview 4.0 colour scheme.[29] SNPs rs4355801, rs6469792, and rs6469804 represent data from all cohorts. Details of all SNPs in this figure are in webtable 3.

were followed up for 250 000 patients-years identified bone mineral density, low body-mass index, previous fragility fractures, glucocorticoid exposure, parental history of fracture, smoking, excessive alcohol intake, and rheumatoid arthritis as common, well-replicated environmental risk factors for osteoporotic fractures.[32] Although this meta-analysis was unable to ascertain the risk of osteoporotic fracture from current use of glucocorticoids, a large case–control study estimated that the relative risk was 1·33.[33]

In our findings, the risk attributed to the presence of both genetic risk alleles was similar to or greater than all of these risk factors except prior fragility fractures and bone mineral density.[34–38] However, the prevalence of both risk alleles (22%) was ten-fold higher than use of corticosteroids, and was equivalent to or exceeded all other risk factors,[34–40] including osteoporosis as defined by bone mineral density.[41] These alleles can be measured with near-perfect precision and without bias years before the age at which fractures tend to occur—which could provide ample lead-time for preventive measures. Eventually, a

|  | Effect* of both risk alleles | Combined† p value for these two risk alleles |
|---|---|---|
| Bone mineral density (SD) | –0·18 | $2·5×10^{-9}$ |
| Osteoporotic fracture (OR) | 1·3 | $6·0×10^{-3}$ |
| Osteoporosis (OR) | 1·5 | $2·6×10^{-3}$ |

*Effect in the 22% of individuals with both risk alleles. †Combined p value is for the discovery, stage 1, and stage 2 replication samples.

***Table 4:*** **Effect of both single nucleotide polymorphisms on bone mineral density at lumbar spine, osteoporotic fracture, and osteoporosis**
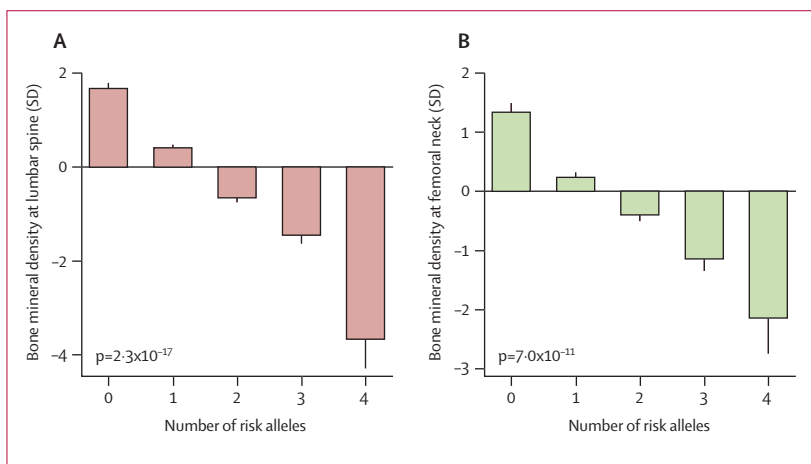


***Figure 4:*** **Bone mineral density and number of risk alleles at *TNFRSF11B* (rs6469792) and *LRP5* (rs3736228)**
Bone mineral density at (A) lumbar spine and (B) femoral neck. Vertical bars represent standard errors. p value is from the non-parametric trend test. The data represent all cohorts studied.

panel of genetic markers could be used in addition to environmental risk factors to identify individuals who are most at risk for osteoporotic fractures.

We identified a novel SNP (rs4355801) in the vicinity of the osteoprotegerin gene, *TNFRSF11B*, which was associated with bone mineral density, osteoporosis, and expression of osteoprotegerin. This implicates osteoprotegerin as a genetic determinant of bone mineral density and osteoporosis. A previous non-replicated study of 595 individuals reported an association between SNPs in osteoprotegerin and fragility fractures, although the SNPs tested were different.[42] However, data for any association with bone mineral density conflict, because of insufficiently powered study designs.[43,44] We have identified a definitive genetic variant at this locus which affects osteoporosis outcomes.

We used allelic expression techniques to show a possible functional role for this SNP. Osteoprotegerin plays a fundamental part in bone biology through the regulation of osteoclastogenesis, and is the target of several novel therapeutic agents.[45] However, the *TNFRSF11B* gene has not previously been convincingly associated with bone mineral density. Osteoprotegerin acts as the natural decoy receptor to the osteoclast-derived receptor activator of nuclear factor-κB ligand (known as RANKL).[45] Denosumab,

a monoclonal antibody which mimics the action of osteoprotegerin, prevents bone loss in postmenopausal women,[46] and clinical trials are underway. Furthermore, the identification of genetic variants which decrease both bone mineral density and expression of osteoprotegerin suggests that people with these variants might respond best to medications which increase osteoprotegerin concentrations. The existence of such medications[46] allows the opportunity for personalised medicine on the basis of these genetic markers.

The lead SNP in the *LRP5* gene (rs3736228) was associated with decreased bone mineral density, osteoporosis, and an increased risk of osteoporotic fracture, independently of its effect on bone mineral density, which suggests that the effects of this variant on the risk of fracture can be either dependent on or independent of bone mineral density. The discovery that *LRP5* affects bone mass through regulation of Wnt signalling[47] was the culmination of many years of investigation, which began with the fine mapping of unusually high and low bone mineral density in some families with *LRP5* mutations.[48,49] SNPs in this gene have recently been shown to be related to bone mineral density[50,51] and fracture.[50,52] However, we used a definition of fracture that was limited to site-specific, validated, clinical, low-trauma fractures, whereas van Meurs and colleagues[50] used all fractures, irrespective of trauma, validation, or site. Another SNP, rs4988300, was shown to be associated with bone mineral density at femoral neck in women (p=0·025) in a genome-wide association study for bone related traits, which assessed 71000 SNPs in 495 men and 646 women.[53] However, this study used a sample that was half the size of the TwinsUK discovery cohort; it did not replicate results; and genome coverage was four-fold lower than that in our study.

Our study has several strengths and limitations. Although we used a hypothesis-free study design, our results are unlikely to be artifacts because the identified SNPs had a strong statistical association with bone mineral density and effects on osteoporosis and osteo-porotic fracture; the identified genes had biological relevance; we used large, international, population-based cohorts; we had evidence from expression studies; and different centres used different genotyping technologies.

Although we have produced genome-wide association data for more than 300 000 SNPs, we applied conservative criteria to claim genome-wide significance. This limitation can be overcome by increased study power. Therefore, discovery cohorts with sample sizes of tens of thousands from multiple genome-wide association studies should be used to identify additional loci which could confer smaller effects. Although we used lymphoblast cell lines, rather than osteoblasts, for functional studies, lymphocytes are the main source of osteoprotegerin expression in bone marrow and the absence of B-cells causes osteoporosis.[54] The identified SNPs might not be causal; however, our data for expression of osteoprotegerin do

suggest a direct mechanism. Moreover, the non-synonymous *LRP5* variant resides in a highly conserved domain, and similar domains in the LDL receptor are involved in binding to the receptor.[55] The variance in bone mineral density explained by variance in the two identified risk alleles was small, which is similar to other genome-wide association studies,[56–58] and again supports the contention that many other loci impart smaller effects on bone mineral density.

In conclusion, the risk alleles we have identified justify further clinical and biological investigations. These SNPs alone are unlikely to change current clinical practice, but as has been shown for other diseases,[59] extended panels of several SNP markers could be used in the future, in addition to traditional risk factors, to better identify populations who are at high risk for osteoporotic fractures.

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