Abstract

Osteoporotic fracture is a major cause of morbidity and mortality worldwide. Low bone mineral density (BMD) is a major predisposing factor to fracture and is known to be highly heritable. Site-, gender-, and age-specific genetic effects on BMD are thought to be significant, but have largely not been considered in the design of genome-wide association studies (GWAS) of BMD to date. We report here a GWAS using a novel study design focusing on women of a specific age (postmenopausal women, age 55–85 years), with either extreme high or low hip BMD (age- and gender-adjusted BMD z-scores of +1.5 to +4.0, n = 1055, or −4.0 to −1.5, n = 900), with replication in cohorts of women drawn from the general population (n = 20,898). The study replicates 21 of 26 known BMD-associated genes. Additionally, we report suggestive association of a further six new genetic associations in or around the genes CLCN7, GALNT3, IBSP, LTBP3, RSP03, and SOX4, with replication in two independent datasets. A novel mouse model with a loss-of-function mutation in GALNT3 is also reported, which has high bone mass, supporting the involvement of this gene in BMD determination. In addition to identifying further genes associated with BMD, this study confirms the efficiency of extreme-truncate selection designs for quantitative trait association studies.
Introduction

Osteoporotic fracture is a leading cause of morbidity and mortality in the community, particularly amongst the elderly. In 2004 ten million Americans were estimated to have osteoporosis, resulting in 1.5 million fractures per annum [1]. Hip fracture is associated with a one year mortality rate of 36% in men and 21% in women [2]; and the burden of disease of osteoporotic fractures overall is similar to that of colorectal cancer and greater than that of hypertension and breast cancer [3]. Bone mineral density (BMD) is strongly correlated with bone strength and fracture risk, and its measurement is widely used as a diagnostic tool in the assessment of fracture risk [4–6]. BMD is known to be highly heritable, with heritability assessed in both young and elderly twins, and in families, to be 60–90% [7–14]. Although the extent of covariance between BMD and fracture risk is uncertain, of the 26 genes associated with BMD at genome-wide significant levels to date, nine have been associated with fracture risk [reviewed in [15]], supporting the use of BMD as an intermediate phenotype in the search for genes associated with fracture risk.

There is considerable evidence from genetic studies in humans [12,16,17], and in mice [18], indicating that the genes that influence BMD at different sites, and in the different genders, overlap but are not identical. Thus far all genome-wide association studies (GWAS) of BMD have studied cohorts of a wide age range, and with one exception have included both men and women; when only women have been studied, both pre- and postmenopausal women have been included. Therefore, to identify genes involved in osteoporosis in the demographic at highest risk of osteoporotic fracture we have performed a GWAS in postmenopausal women selected on the basis of their hip BMD, and replicated the GWAS findings in a large cohort of adult women drawn from the general population.

Results

Considering markers previously reported as associated with BMD, our discovery dataset replicates previously associated SNPs in 21 of the 26 genes reported to date to have genome-wide significant associations (Table S6) (P<0.05, association in the same direction as initially reported, or, in the case of LRP5 and GPR177, with the next flanking SNP genotyped) [17,21,22,23,28,32,33,34]. Replicated genes include ARH GAP1, CTXNBI, ESR1, FAM5C, FLJ42280, FOXL1, GPR177, HDAC5, JAG1, LRP3, MARK3, MEF2C, MEPE, OPG, RANK, RANKL, SOST, SOX6, SP7 (Osterix), STARD3NL and ZBTB40. Considering the combined Anglo-Australasian Osteoporosis Genomics Consortium (AOGC) and deCODE/TwinsUK/Rotterdam cohorts, 97 SNPs from six loci achieved P<5×10−8 at the femoral neck (FN), of which four had previously been reported (FLJ42280, MEF2C, SOX6, ZBTB40). At the lumbar spine (LS), six SNPs from two known loci (RANKL, OPG) achieved P<5×10−8. No support was seen for previously reported associations involving SNPs in ADAMTS16, CRHR1, DCDG5, MHC, or SBTN1 (P>0.05).

This study also identifies and replicates two novel loci with confirmed association with BMD in GALNJ3 (MIM: 601756) and at chromosome 6q22 near RSPO3 (MIM: 610574), and provides strong evidence of a further four BMD-associated loci (LCN7 (MIM: 602727), IGBP (MIM: 147563), LTBP3 (MIM: 602090), SOX4 (MIM: 184430)) (Table 1). Although these did not achieve ‘genome-wide significance’ in the discovery set alone, they achieved P-values in the AOGC-discovery cohort of P<10−4, and support in the AOGC-replication cohort, TwinsUK, Rotterdam and deCODE cohorts; and all have additional evidence supporting their role in bone. Support was also seen for TGFBR3 (MIM: 600742), a gene previously reported to have suggestive association with BMD [33].
**Author Summary**

Osteoporotic fracture is a major cause of early mortality and morbidity in the community. To identify genes associated with osteoporosis, we have performed a genome-wide association study. In order to improve study power and to address the demographic group of highest risk from osteoporotic fracture, we have used a unique study design, studying 1,955 postmenopausal women with either extreme high or low hip bone mineral density. We then confirmed our findings in 20,898 women from the general population. Our study replicated 21 of 26 known osteoporosis genes, and it identified a further six novel loci (in or nearby CLCN7, GALNT3, IBSP, LTBP3, RSPO3, and SOX4). For one of these loci, GALNT3, we demonstrate in a mouse model that a loss-of-function genetic mutation in GALNT3 causes high bone mass. These findings report novel mechanisms by which osteoporosis can arise, and they significantly add to our understanding of the aetiology of the disease.

**GALNT3**

SNPs at chromosome 2q24, in and around GALNT3, achieved near genome-wide significance in our discovery cohort (peak P-value rs1863196, total hip (TH) P = 2.3 × 10^{-5}; LS P = 0.037) (Figure 1A). This SNP was not typed or imputed by either the Rotterdam or the TwinsUK cohorts, but a nearby SNP showed strong association in both AOGC and the combined replication cohorts (rs6710518; AOGC discovery, TH P = 6.9 × 10^{-5}; combined replication sets, FN P = 2.7 × 10^{-5}). In the combined datasets the finding achieved genome-wide significance at the FN (P = 1.7 × 10^{-5}). Strong association was also seen with this SNP at LS (P = 7.5 × 10^{-5}). Another marker within GALNT3, rs4667492, was also associated with fracture risk, including vertebral fractures (OR = 0.89; 95%CI = 0.80–0.99; P = 0.032) and overall low trauma fractures (OR = 0.92; 95%CI = 0.85–0.99; P = 0.024).

We have recently identified a mouse with an N-ethyl-N-nitrosourea induced loss-of-function GALNT3 mutation (Trp389Arg), that develops hyperphosphataemia with extraskeletal calcium deposition, and hence represents a model for FTC [35]. To establish further the association of GALNT3 and BMD, we determined BMD in these GALNT3 mutant mice. This revealed that homozygous (+/−) GALNT3 mutant male and female adult mice had a higher areal BMD than their wild-type (+/+), litter mates, with heterozygous (+/−) mice having intermediate BMD (Figure 2). This loss-of-function GALNT3 mutation is predicted to lead to a reduced glycosylation of FGFB3, which increases its breakdown and leads to reduced serum FGFB3 concentrations [35].

**RSPO3**

A novel genome-wide significant association was also seen at markers on chromosome 6q22-23 (Figure 1B). In the combined dataset, marker rs13204965 achieved genome-wide significance at this locus at the FN (P = 2.2 × 10^{-5}), with strong support in both the AOGC discovery set, and the combined replication sets (AOGC-discovery, TH P = 2.1 × 10^{-5}; combined replication P = 3.5 × 10^{-5}). Strong association was also seen with LS BMD (rs13204965 P = 0.00067). The peak of association at this locus lies within a cDNA fragment, AK127472. The nearest gene, RSPO3 (R-spondin-3), is 273 kb telomeric of the strongest associated SNP, but is within the associated linkage disequilibrium region (Figure 1B).

**CLCN7**

Association was observed at chromosome 16p13 with SNPs in and around CLCN7, which encodes a Cl^{-}/H^{+} antipporter expressed primarily in osteoclasts, and critical to lysosomal acidification, an essential process in bone resorption. Peak association at this locus was seen with SNP rs13336426 in the discovery set (TH P = 7.0 × 10^{-5}; LS P = 0.028) (Figure S3A), which was confirmed in the replication set (FN P = 3.6 × 10^{-5}; LS P = 0.00012), achieving P = 1.7 × 10^{-6} at the FN and 1.2 × 10^{-5} at LS in the overall cohort. Association has previously been reported between two SNPs in exon 15 of CLCN7 (rs12926089, rs12926669) and FN BMD (P = 0.001–0.005) [36]; no association was seen with either of those SNPs in the current study (P > 0.4 at FN and LS).

**IBSP**

Association was observed with SNPs in IBSP (integrin-binding bone sialoprotein) (Figure S3B), encoded at chromosome 4q22, a gene which has previously had suggestive association reported with BMD in two studies (rs1054627, Styrkarrsott et al P = 4.6 × 10^{-5} [22]; Koller et al P = 1.3 × 10^{-4} [37]). In the current study, moderate association was observed in the discovery set with the same SNP as previously reported (rs1054627, AOGC discovery TH, P = 6.6 × 10^{-5}), with support in the replication set and strong association overall (FN combined replication P = 9.2 × 10^{-5}; FN overall association P = 7.6 × 10^{-5}). Nominal association was observed at LS (rs1054627, P = 0.019).

**LTBP3**

Association with BMD was also seen at chromosome 11p13, with SNP rs1152620 achieving P = 4.4 × 10^{-5} (TH) in the discovery set, P = 0.0051 (FN) in the replication set, and P = 3.6 × 10^{-4} overall (Figure S3C). This SNP was also nominally associated with LS BMD in the discovery set (P = 0.041). The nearest gene to this locus is LTBP3 (latent transforming growth factor beta binding protein 3), which is located 292 kb q-telomeric of rs1152620.

**SOX4**

At chromosome 6p22, SNPs in and around SOX4 (Sex determining region Y box 4) were moderately associated with BMD in our discovery set (most significant association rs4966056, TH P = 5.3 × 10^{-4}; LS P = 0.0030) (Figure S3D), with support at the hip and LS in the replication set (FN P = 0.00013, LS P = 0.013), achieving association overall with P = 2.6 × 10^{-7} (FN) and P = 0.00081 (LS).

**Discussion**

This study demonstrates convincing evidence of association with six genes with BMD variation, GALNT3, RSPO3, CLCN7, IBSP, LTBP3 and SOX4. Using a moderate sample size, the use of a novel study design also led to the confirmation of 21 of 26 known BMD-associations. This study thus demonstrates the power of extreme-truncate selection designs for association studies of quantitative traits.

GALNT3 encodes N-acetylgalactosaminyltransferase 3, an enzyme involved in 0-glycosylation of serine and threonine residues. Mutations of GALNT3 are known to cause familial tumoral calcinosis (FTC, OMIM 211190) [38] and hyperostosis-hyperphosphataemia syndrome (HOHP, OMIM 610233) [39]. FTC is characterised by hyperphosphataemia in association with the deposition of calcium phosphate crystals in extraskeletal tissues; whereas in HOHP, hyperphosphataemia is associated with recurrent painful long bone swelling and radiographic evidence of...
Table 1. Findings for novel replicated associations.

| LOCUS | SNP | A1/A2 | GENe | Beta | P-VALUE | Beta | P-VALUE | Beta | P-VALUE | Beta | P-VALUE | Beta | P-VALUE |
|-------|-----|-------|------|------|---------|------|---------|------|---------|------|---------|------|---------|
| 6p22  | rs9466056 A/G | 0.237 5.3 | 6 | 0.060 0.020 | 0.080 0.041 | 0.039 | 0.107 0.034 | 0.062 0.025 | 0.056 0.00067 |

GALNT3

6q22 rs13204965 A/C

RSPO3

6p22 rs13336428 A/G

LTP3

R-spondin-4 mutations cause anhydrosis (absence or severe hypoplasia of all fingernails and toenails, OMIM 206800) [49]. No human disease has been associated with R-spondin-3, and knockout of R-spondin-3 in mice is embryonically lethal due to defective placental development [50].

Mutations of CLCN7 cause a family of osteopetroses of differing age of presentation and severity, including infantile malignant CLCN7-related recessive osteopetrosis (ARO), intermediate autosomal osteopetrosis (IAO), and autosomal dominant osteopetrosis type II (ADOII, Albers-Schoenberg disease). These conditions are characterized by expanded, dense bones, with markedly reduced bone resorption. Our data support associations of polymorphisms at this locus with BMD variation in the population.

IBSP is a major non-collagenous bone matrix protein involved in calcium and hydroxyapatite binding, and is thought to play a role in cell-matrix interactions through RGD motifs in its amino acid sequence. IBSP is expressed in all major bone cells including osteoblasts, osteocytes and osteoclasts; and its expression is upregulated in osteoporotic bone [51]. IBSP knockout mice have low cortical but high trabecular bone volume, with impaired bone formation, resorption, and mineralization [52]. IBSP lies within a cluster of genes including DMP1, MEPE, and SPP1, all of which have known roles in bone and are strong candidate genes for association with BMD. MEPE has previously been associated with BMD at genome-wide significance [17]. In the current study the strongest association was seen with an SNP in IBSP, rs1054627, as was the case with two previous studies [22,37]. Linkage disequilibrium between this SNP, and the previously reported BMD-associated SNP rs1471403 in MEPE, is modest ($r^2 = 0.16$). Whilst out study supports the association of common variants in IBSP in particular with BMD, further studies will be required to determine if more than one of these genes is BMD-associated.

Recessive mutations of LTP3 have been identified as the cause of dental agenesis in a consanguineous Pakistani family (OMIM 613097) [53]. Affected family members had base of skull thickening, and elevated axial but not hip BMD. LTP3−/− mice develop axial osteosclerosis with increased trabecular bone thickness, as well as craniosynostosis [54]. LTP3 is known to bind periostial reaction and cortical hyperostosis. FGF23 mutations associated with FTC cause hyperphosphataemia through effects on expression of the sodium-phosphate co-transporter in the kidney and small intestine, and through increased activation of vitamin D due to increased renal expression of CYP27B1 (25-hydroxyvitamin-D 1 alpha hydroxylase) [40]. It is unclear whether FGF23 has direct effects on the skeleton or if its effects are mediated through its effects on serum phosphate and vitamin D levels. FGF23 signals via a complex of an FGF receptor (FGFR1/[IIIc]) and Klotho [41]; mice with a loss-of-function mutation in Klotho develop osteoporosis amongst other abnormalities, and modest evidence of association of Klotho with BMD has been reported in several studies [42,43,44,45]. We saw no association with polymorphisms in Klotho and BMD in the current study (P > 0.05 for all SNPs in and surrounding Klotho). To our knowledge, this finding is the first demonstration in humans that genetic variants in the FGF23 pathway are associated with any common human disease.
Figure 1. SNP association plots for BMD-associated regions. Discovery cohort association significance level is plotted against the left hand y-axis as -log10(P-values). Genetic coordinates are as per NCBI build 36.1. Filled circles represent genotyped SNPs, and outlined diamonds represent imputed SNPs. The recombination rate (cM/Mb as per HapMap data) is indicated by the purple dotted line and right hand y-axis. Genes and ESTs are indicated with their approximate sizes and direction of translation. (A) Chromosome 2q24 - GALNT3 region. SNP association plot of findings from TH case-control analysis of AOGC discovery set for a 500 kb region (166,100 kb to 166,600 kb) of chromosome 2. LD is indicated by colour scale in...
TGFβ1, -β2 and -β3, and may influence chondrocyte maturation and enchondral ossification by effects on their bioavailability [54].

Our study also confirms the previously reported association of another TGF pathway gene, TGFBR3, encoded at chromosome 1p22, with BMD [33] (Figure S3E). In that study, association was observed in four independent datasets, but overall the findings did not achieve genome-wide significance at any individual SNP (most significant SNP rs17131547, \( P = 1.5 \times 10^{-6} \)). In our discovery set, peak association was seen at this locus with SNP rs7550034 (TH \( P = 1.5 \times 10^{-4} \)), which lies 154 kb q-telomeric of rs17131547, but still within TGFBR3 (rs17131547 was not typed or imputed in our dataset) (Figure S3E). This supports TGFBR3 as a true BMD-associated gene.

This study also demonstrated that SOX4 polymorphisms are associated with BMD variation. Both SOX4 and SOX6 are cartilage-expressed transcription factors known to play essential roles in chondrocyte differentiation and cartilage formation, and hence enchondral bone formation. SOX6 has previously been reported to be BMD-associated at genome-wide significant levels [17]. Whilst SOX4−/− mice develop severe cardiac abnormalities and are non-viable, SOX4+/− mice have osteopaenia with decreased bone formation but normal resorption rates, and diminished cortical and trabecular bone volume [55]. Our data suggest that SOX4 polymorphisms contribute to the variation in BMD in humans.

This study has a unique design amongst GWAS of BMD reported to date, using an extreme-truncate ascertainment scheme, focusing on a specific skeletal site (TH), and with recruitment of a narrow age- and gender-group (post-menopausal women age 55–85 years). Our goal in employing this scheme was...
to increase the study power by reducing heterogeneity due to age-, gender- and skeletal site-specific effects. Whilst osteoporotic fracture can occur at a wide range of skeletal sites, hip fracture in postmenopausal women is the major cause of morbidity and mortality due to osteoporosis. To date, with only one exception, all GWAS of BMD have studied cohorts unselected for BMD [20], and no study has restricted its participants to postmenopausal women ascertainment purely on the basis of hip BMD. Assuming marker-disease-associated allele linkage disequilibrium of $r^2 = 0.9$, for alpha = $5 \times 10^{-8}$ our study has 80% power to detect variants contributing 0.3% of the additive genetic variance of BMD. An equivalent-powered cohort study would require ~16,000 unselected cases.

Considering the 26 known genes (or genomic areas) associated with BMD, $P$-values less than <0.05 were seen in our discovery for 21 of the BMD-associated SNPs. Of the 26 known BMD genes, 16 would have been included in our replication study on the basis of the strength of their BMD association in our discovery cohort, but were not further genotyped as they were known already to be BMD-associated. Had these 16 genes replicated, 22 genes would have been identified in this single study, demonstrating the power of the design of the current study.

A potential criticism of studies of highly selected cohorts, such as the AOGC-discovery cohort, is that the associations identified may not be relevant in the general population. However, the confirmation of our findings in replication cohorts of women unselected for BMD confirms that our findings are of broad relevance.

In summary, our study design therefore represents a highly efficient model for future studies of quantitative traits and is one of the first reported studies using an extreme truncate design in any disease. We have identified two new BMD loci at genome-wide significance ($GALNT3$, $RSPO3$), with $GALNT3$ SNPs also associated with fracture. Strong evidence was also demonstrated for four novel loci ($CLCN7$, $IBSP$, $LTBP3$, $SOX4$). Further support was also provided that $TGFBR3$ is a true BMD-associated locus. Our discovery cohort replicated 21 of 26 previously identified BMD-associated loci. Our novel findings further advance our understanding of the aetiology of osteoporosis, and highlight new genes and pathways not previously considered important in BMD variation and fracture risk in the general population. Our study also provides strong support that the use of extreme truncate selection is an efficient and powerful approach for the study of quantitative traits.

Materials and Methods

Ethics statement

All participants gave written, informed consent, and the study was approved by the relevant research ethics authorities at each participating centre.

Subjects and phenotypes

The discovery sample population included 1128 Australian, 74 New Zealand and 753 British women, between 55–85 years of age, five or more years postmenopausal, with either high BMD (age- and gender-adjusted BMD $z$-scores of $+1.5$ to $+4.0$, $n = 1055$) or low BMD (age- and gender-adjusted BMD $z$-scores of $-4.0$ to $-1.5$, $n = 900$) (Tables S1 and S2). BMD $z$-scores were determined according to the Geelong Osteoporosis Study normative range [19]. Low BMD cases were excluded if they had secondary causes of osteoporosis, including corticosteroid usage at doses equivalent to prednisolone $\geq 7.5$ mg/day for $\geq 6$ months, past or current anticonvulsant usage, previous strontium
A total of 124 SNPs were successfully genotyped in the AOGC replication cohort. These replication study SNPs were selected from the findings of the discovery cohort, either based on the strength of association (P-value) or following analysis with GRAIL (n = 45) [25], using as seed data all SNPs previously reported to be associated with BMD at GWAS significant levels (results for all replication SNPs presented in Table S3). GRAIL is a bioinformatic program that assesses the strength of relationships between genes in regions surrounding input SNPs (usually derived from genetic association studies) and other SNPs or genes associated with the trait of interest, by assessing their co-occurrence in PubMed abstracts. Where genes surrounding input SNPs occur more frequently in abstracts with known associated genes, these SNPs are more likely themselves also to be associated, and can thus be prioritized for inclusion in replication studies.

For the replication study, genotyping was performed either by Applied Biosystems OpenArray (n = 113) or Taqman technology (n = 11) [Applied Biosystems, Foster City, CA, USA], according to the manufacturer’s protocol.

**Statistical methods**

Eleven individuals were removed because of abnormal X-chromosome homozygosity (X-chromosome homozygosity either <−0.14, or >+0.14). Outliers with regard to autosomal homozygosity (either <0.34225 or >0.357, n = 40) and missingness (>3%, n = 4) were removed. Using an IBS/IBD analysis in PLINK to detect cryptic relatedness, one individual from 35 pairs of individuals with pi-hat >0.12 (equivalent to being 3rd degree relatives or closer) were removed. SNPs with minor allele frequency <1% (n = 561), and those not in Hardy-Weinberg equilibrium (P < 10⁻⁷, n = 170) were then removed, leaving 288,768 SNPs in total. Nine replication SNPs were removed because of excess missingness (>10%) or because they failed tests of Hardy-Weinberg equilibrium (P < 0.001).

To detect and correct for population stratification EIGENSTRAT software was used. We first excluded the 24 regions of long range LD including the MHC identified in Price et al. before running the principal components analysis, as suggested by the authors [26]. Sixteen individuals were removed as ethnic outliers, leaving 1955 individuals in the final discovery dataset.

Imputation analyses were carried out using Markov Chain Haplotyping software (MaCH; http://www.sph.umich.edu/egs/abcasis/MACH/) using phased data from CEU individuals from release 22 of the HapMap project as the reference set of haplotypes. We only analyzed SNPs surrounding disease-associated SNPs that were either genotyped or could be imputed with their approximate sizes and direction of translation. (A) Chromosome 1p22 - CLCN7 region. SNP association plot of findings from TH case-control analysis of AOGC discovery set for a 100 kb region (1,420 kb to 1,520 kb) of chromosome 16. LD is indicated by colour scale in relationship to marker rs13336428. (B) Chromosome 4q22 - IBSP region. SNP association plot of findings from TH case-control analysis of AOGC discovery set for a 500 kb region (88,700 kb to 89,200 kb) of chromosome 4. LD is indicated by colour scale in relationship to marker rs1054627. (C) Chromosome 11p13 - LTBP3 region. SNP association plot of findings from TH case-control analysis of AOGC discovery set for a 300 kb region (64,950 kb to 65,250 kb) of chromosome 11. LD is indicated by colour scale in relationship to marker rs13336428. (D) Chromosome 6p22 - SOX4 region. SNP association plot of findings from TH case-control analysis of AOGC discovery set for a 2 Mb region (20,500 kb to 22,500 kb) of chromosome 6. LD is indicated by colour scale in relationship to marker rs128260. (E) Chromosome 1p22 - TGFBR3 region. SNP association plot of findings from TH case-control analysis of AOGC discovery set for

**Mouse BMD analysis**

All animal studies were approved by the MRC Harwell Unit Ethical Review Committee and are licensed under the Animal (Scientific Procedures) Act 1986, issued by the UK Government Home Office Department. Dual-energy X-ray absorptiometry (DEXA) was performed using a Lunar Piximus densitometer (GE Medical Systems) and analysed using the Piximus software.

**Data availability**

Data related to this study will be available to research projects approved by a Data Access Committee including representatives of the University of Queensland Research Ethics Committee. For enquiries regarding access please contact the corresponding author, MAB (matt.brown@uq.edu.au).

**Supporting Information**

Figure S1 Manhattan plot of discovery genome-wide association study findings for BMD at total hip. P = 10⁻³ is indicated by a blue horizontal line. Found at: doi:10.1371/journal.pgen.1001372.s001 (0.51 MB TIF)

Figure S2 Genomic control findings. The genomic inflation factor (λ) when reported as the median $\chi^2$ was 1.0282. Found at: doi:10.1371/journal.pgen.1001372.s002 (0.36 MB TIF)

Figure S3 SNP association plots for OP-associated regions. Discovery cohort association significance level is plotted against the left hand y-axis as -log10(P-values). Genetic coordinates are as per NCBI build 36.1. Filled circles represent genotyped SNPs, and outlined diamonds represent imputed SNPs. The recombination rate (cM/Mb as per HapMap data) is indicated by the purple dotted line and right hand y-axis. Genes and ESTs are indicated with their approximate sizes and direction of translation. (A) Chromosome 16p13 - CLCN7 region. SNP association plot of findings from TH case-control analysis of AOGC discovery set for a 100 kb region (1,420 kb to 1,520 kb) of chromosome 16. LD is indicated by colour scale in relationship to marker rs13336428. (B) Chromosome 4q22 - IBSP region. SNP association plot of findings from TH case-control analysis of AOGC discovery set for a 500 kb region (88,700 kb to 89,200 kb) of chromosome 4. LD is indicated by colour scale in relationship to marker rs1054627. (C) Chromosome 11p13 - LTBP3 region. SNP association plot of findings from TH case-control analysis of AOGC discovery set for a 300 kb region (64,950 kb to 65,250 kb) of chromosome 11. LD is indicated by colour scale in relationship to marker rs13336428. (D) Chromosome 6p22 - SOX4 region. SNP association plot of findings from TH case-control analysis of AOGC discovery set for a 2 Mb region (20,500 kb to 22,500 kb) of chromosome 6. LD is indicated by colour scale in relationship to marker rs128260. (E) Chromosome 1p22 - TGFBR3 region. SNP association plot of findings from TH case-control analysis of AOGC discovery set for

**Note:** The image contains diagrams illustrating genomic regions and association plots.
a 1 Mb region (91,800 kb to 92,800 kb) of chromosome 1, LD is indicated by colour scale in relationship to marker rs7550034.

Found at: doi:10.1371/journal.pgen.1001372.s003 (5.13 MB TIF)

**Table S1** Case numbers for the discovery cohort, with BMD affection status and fracture history.

Found at: doi:10.1371/journal.pgen.1001372.s004 (5.3 MB DOC)

**Table S2** Descriptive statistics for discovery cohort.

Found at: doi:10.1371/journal.pgen.1001372.s005 (0.06 MB DOC)

**Table S3** Replication cohort details.

Found at: doi:10.1371/journal.pgen.1001372.s006 (0.04 MB DOC)

**Table S4** Replication cohort fracture data.

Found at: doi:10.1371/journal.pgen.1001372.s007 (0.04 MB DOC)

**Table S5** Replication study SNPs, beta coefficients and P-values for analysis of TH, FN and LS. The regression coefficient in the case-control analysis of TH in the discovery set shows the expected affection status and fracture history.

Found at: doi:10.1371/journal.pgen.1001372.s008 (0.22 MB DOC)

**Table S6** Association findings in AOGC discovery set for markers achieving genome-wide significant association with BMD in previous studies. The regression coefficient in the TH analysis shows the expected increase in the log odds ratio of low BMD per addition of allele A2. The regression coefficients in the TH, FN and LS analyses refer to the expected increase in standardized BMD per addition of allele A2 in the discovery set.

Found at: doi:10.1371/journal.pgen.1001372.s009 (12.0 MB DOC)

**References**

1. US Department of Health and Human Services (2004) Bone health and Osteoporosis: a report of the surgeon general. Rockville, MD, USA.

2. US Department of Commerce (1993) Hip fracture rates in people aged fifty years and over: mortality, service use, expenditures, and long-term functional impairment. Washington, DC.

3. Johnell O, Kanis JA (2006) An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. Osteoporos Int 17: 1726–1733.

4. Kanis JA, Johnell O, Odén A, Johansson H, McCloskey E (2008) FRAX: an assessment of fracture probability in men and women from the UK. Osteoporos Int 19: 385–397.

5. Henry MJ, Pasco JA, Seeman E, Nicholson GC, Sanders KM, et al. (2001) Assessment of fracture risk value of random population-based samples—the Geelong Osteoporosis Study. J Clin Densitom 4: 283–288.

6. Nguyen ND, Pongchaiyakul C, Center JR, Eisman JA, Nguyen TV (2005) Identification of high-risk individuals for hip fracture: a 14-year prospective study. J Bone Miner Res 20: 1921–1929.

7. Arden NK, Baker J, Hong C, Baan K, Spector TD (1996) The heritability of bone mineral density, ultrasound of the calcaneus and hip axis length: a study of postmenopausal twins. J Bone Miner Res 11: 530–534.

8. Arden NK, Pongchaiyakul C, Center JR, Eisman JA, Nguyen TV (2005) Genetic determinants of bone mineral content, ultrasound of the calcaneus and hip axis length: a study of postmenopausal twins. J Bone Miner Res 11: 530–534.

9. Arden NK, Spector TD (1997) Genetic influences on muscle strength, lean body mass, and bone mineral density: a twin study. J Bone Miner Res 12: 2076–2081.

10. Dequeker J, Nijs J, Verstraeten A, Greaves P, Govers G (1987). Genetic and environmental correlations between bone formation and bone mineral density: a twin study. Bone 8: 207–209.

11. Harris M, Nguyen TV, Howard GM, Kelly PJ, Eisman JA (1998) Genetic and environmental factors in bone mass: same genes or same environments? Am J Epidemiol 147: 3–16.

12. Duncan E, Cardon L, Sincler J, Waj J, Brown M (2003) Site and Gender Specificity of Inheritance of Bone Mineral Density. J Bone Miner Res 18: 1531–1538.

13. Sigurdsson G, Halldorsson BV, Styrkarsdottir U, Kristjansson K, Stefansson K (2008) Impact of genetics on low bone mass in adults. J Bone Miner Res 23: 1594–1599.

14. Flicker L, Hopper J, Rodgers L, Kaymakci B, Green R, et al. (1995) Bone density determinants in elderly women: a twin study. J Bone Miner Res 10: 1607–1613.

15. Duncan EL, Brown MA (2010) Clinical review 2: Genetic determinants of bone density and fracture risk—state of the art and future directions. J Clin Endocrinol Metab 95: 2576–2587.

16. Nagrangthan V, Macgregor A, Sturier H, Nguyen T, Spector T, et al. (2002) Gender differences in the genetic factors responsible for variation in bone density and ultrasound. J Bone Miner Res 17: 725–733.

17. Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, et al. (2009) Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet 41: 1199–1206.

18. Orwell ES, Belpaeme K, Klein RF (2001) Gender specificity in the genetic determinants of peak bone mass. J Bone Miner Res 16: 1962–1971.

19. Henry MJ, Pasco JA, Nicholson GC, Seeman E, Kotowicz MA (2000) Prevalence of osteoporosis in Australian women: Geelong Osteoporosis Study. J Clin Densitom 3: 261–268.

20. Ballo M, Stanek J, Pasco J, Hickey P, Taylor BV, et al. (2010) Saliva-Derived Microarray Studies. Cancer Epidemiol Biomarkers Prev 19: 794-798.

21. Richard JB, Rivadeneira F, Inouye M, Pastinen T, Soranzo N, et al. (2008) Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet 371: 1505–1512.

**Acknowledgments**

We would like to thank all participants who provided the DNA and clinical information necessary for this study. We would like to gratefully acknowledge the contributions of Prof. Gunnar Sigurdsson and Dr. Unnur Thorsteinsdottir (Iceland) for their valuable contribution to the study. We thank Pascal Arp, Mila Jhannai, Dr Michael Moorhouse, Marijn Verkerk, and Sander Bervoets for their help in creating the Rotterdam GWAS database. The authors are grateful to the Rotterdam study participants, the staff from the Rotterdam Study, and the participating general practitioners and pharmacists. We thank Barbara Mason and Amanda Horne (Auckland) for patient recruitment; Judith Finigan (Sheffield) for laboratory support and database support; Selina Simpson (Sheffield) for DNA handling; Fatma Gossiel (Sheffield) for DNA handling; Alison Steward and Lana Gibson (Aberdeen) for patient recruitment; Katherine Kolk (Geelong); Janelle Rampellini (Perth) for patient recruitment; Jemima Christie (Melbourne) for patient recruitment; Helen Steane (Hobart) for patient recruitment; Denia Mang and Ruth Toppler for DNA extraction, DNA handling, and database support (Dubbo/Sydney); Kate Lowings (Brisbane) for patient recruitment; and Marieke Brumagm and Leanne Brookes (Brisbane) for DNA preparation and genotyping.

**Author Contributions**

Conceived and designed the experiments: EL Duncan, P Danoy, E McCloskey, GC Nicholson, R Eastell, RL Prince, JA Eisman, G Jones, JB Richards, AG Uitterlinden, TD Spector, C Esapa, RD Cox, SDM Brown, RV Thakker, K Estrada, F Rivadeneira, K Stafansson, U Strykarsdottir, G Thorleifsson, MA Brown. Performed the experiments: EL Duncan, P Danoy, C Esapa, RD Cox, KA Addison, LA Bradbury, C Cremin, K Estrada, CC Glueir, J Hadler, K Pryce. Analyzed the data: EL Duncan, P Danoy, JP Kemp, PJ Leo, JB Richards, AG Uitterlinden, TD Spector, C Esapa, RD Cox, SDM Brown, RV Thakker, K Estrada, CC Glueir, J Hadler, F Rivadeneira, K Stafansson, U Strykarsdottir, G Thorleifsson, DM Evans, MA Brown. Contributed reagents/materials/analysis tools: EL Duncan, P Danoy, JP Kemp, PJ Leo, E McCloskey, GC Nicholson, R Eastell, RL Prince, JA Eisman, G Jones, PN Sambrook, IR Reid, EM Dennison, JW Wark, JB Richards, AG Uitterlinden, TD Spector, C Esapa, SDM Brown, RV Thakker, LA Bradbury, JR Center, C Cooper, K Estrada, D Felsenberg, CC Glueir, MJ Henry, A Hofman, MA Kotowicz, J Makovey, SC Nguyen, TV Nguyen, JA Pasco, DM Reid, F Rivadeneira, C Roux, K Stafansson, U Strykarsdottir, G Thorleifsson, R Tchawangana, DM Evans, MA Brown. Wrote the paper: EL Duncan, PJ Leo, DM Evans, MA Brown.
22. Styrkarsdottir U, Halldorsdottir BV, Gretarsdottir S, Guðbjartsson DF, Walters GB, et al. (2009) New sequence variants associated with bone mineral density. Nat Genet 41: 15–17.
23. Styrkarsdottir U, Halldorsdottir BV, Gretarsdottir S, Guðbjartsson DF, Walters GB, et al. (2008) Multiple genetic loci for bone mineral density and fractures. N Engl J Med 358: 2355–2365.
24. Shepherd JA, Fan B, Lu Y, Lewiecki EM, Miller P, et al. (2006) Comparison of BMD precision for Prodigy and Delphi spine and femur scans. Osteoporos Int 17: 130–135.
25. Raychaudhuri S, Plenge RM, Rossin EJ, Ng AC, Purcell SM, et al. (2009) Identifying relationships among genomic disease regions: predicting genes at pathogenic SNP associations and rare deletions. PLoS Genet 5: e1000534. doi:10.1371/journal.pgen.1000534.
26. Price AL, Weale ME, Patterson N, Myers SR, Need AC, et al. (2006) Long-range LD can confound genome scans in admixed populations. Am J Hum Genet 83: 367–380.
27. Li Y, Willer CJ, Sanna S, Abecasis GR (2009) Genotype imputation. Annu Rev Genomics Hum Genet 10: 367–386.
28. Kung AWC, Xiao S-M, Cheny S, Li GHY, Gao Y, et al. (2010) Association of JAG1 with bone mineral density and osteoporotic fractures: a genome-wide association study and follow-up replication studies. Am J Hum Genet 86: 1–11.
29. Estrada K, Abuseiris A, Grosveld FG, Uitterlinden AG, Knoch TA, et al. (2009) GRIMP: a web- and grid-based tool for high-speed analysis of large-scale genome-wide association using imputed data. Bioinformatics 25: 2750–2752.
30. Willer CJ, Sanna S, Jackson AU, Scuteri A, Bonnycastle LL, et al. (2008) Newly identified loci that influence lipid concentrations and risk of coronary artery disease. Nat Genet 40: 161–169.
31. Purcell S, Cherny SS, Sham PC (2003) Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics 19: 149–150.
32. Cho YS, Go MJ, Kim YJ, Heo JY, Oh JH, et al. (2009) A large-scale genome-wide association study of Asian populations uncovers genetic factors influencing eight quantitative traits. Nat Genet 41: 527–534.
33. Xiong DH, Liu XG, Guo YF, Tan LJ, Wang L, et al. (2009) Genome-wide association and follow-up replication studies identified ADAMTS18 and TGFBR3 as bone mass candidate genes in different ethnic groups. Am J Hum Genet 84: 388–398.
34. Timpson NJ, Tobias JH, Richards JB, Soranzo N, Duncan EL, et al. (2009) Common variants in the region around Osterix are associated with bone mineral density and growth in childhood. Hum Mol Genet 18: 1510–1517.
35. Esapa C, Head R, Chan E, Crane M, Cheeseman M, et al. (2009) A mouse with abnormalities in latent TGF-beta binding protein (Ltbp)-3 null mice indicate a role for Ltbp-3 in modulating TGF-beta bioavailability. J Cell Biol 19: 2588–2596.
36. Dabovic B, Chen Y, Colarossi C, Obata H, Zambuto L, et al. (2002) Bone sialoprotein plays a functional role in bone formation and osteoclastogenesis. J Exp Med 205: 1145–1153.
37. Blaydon DC, Ishii Y, O'Toole EA, Unsworth HC, Teh MT, et al. (2006) The gene encoding R-spondin 4 (RSPO4), a secreted protein implicated in Wnt signaling, is mutated in inherited anonychia. Nat Genet 38: 1243–1247.
38. Frishberg Y, Topaz O, Bergman R, Behar D, Fisher D, et al. (2005) Identification of a recurrent mutation in GALNT3 demonstrates that hyperostosis-hyperphosphatemia syndrome and familial tumoral calcinosis are allelic disorders. J Mol Med 83: 35–38.
39. Larson T, Davis SL, Garringer HJ, Mooreney SP, Drinan MS, et al. (2005) Fibroblast growth factor-23 mutants causing familial tumoral calcinosis are differentially processed. Endocrinology 146: 3883–3891.
40. Frishberg Y, Topaz O, Behar D, Fisher D, et al. (2005) Identification of a recurrent mutation in GALNT3 demonstrates that hyperostosis-hyperphosphatemia syndrome and familial tumoral calcinosis are allelic disorders. J Mol Med 83: 35–38.
Author/s: Duncan, EL; Danoy, P; Kemp, JP; Leo, PJ; McCloskey, E; Nicholson, GC; Eastell, R; Prince, RL; Eisman, JA; Jones, G; Sambrook, PN; Reid, IR; Dennison, EM; Wark, J; Richards, JB; Uitterlinden, AG; Spector, TD; Esapa, C; Cox, RD; Brown, SDM; Thakker, RV; Addison, KA; Bradbury, LA; Center, JR; Cooper, C; Cremin, C; Estrada, K; Felsenberg, D; Glueer, C-C; Hadler, J; Henry, MJ; Hofman, A; Kotowicz, MA; Makovey, J; Nguyen, SC; Nguyen, TV; Pasco, JA; Pryce, K; Reid, DM; Rivadeneira, F; Roux, C; Stefansson, K; Styrkarsdottir, U; Thorleifsson, G; Tichawangana, R; Evans, DM; Brown, MA

Title: Genome-Wide Association Study Using Extreme Truncate Selection Identifies Novel Genes Affecting Bone Mineral Density and Fracture Risk

Date: 2011-04-01

Citation: Duncan, E. L., Danoy, P., Kemp, J. P., Leo, P. J., McCloskey, E., Nicholson, G. C., Eastell, R., Prince, R. L., Eisman, J. A., Jones, G., Sambrook, P. N., Reid, I. R., Dennison, E. M., Wark, J., Richards, J. B., Uitterlinden, A. G., Spector, T. D., Esapa, C., Cox, R. D., ... Brown, M. A. (2011). Genome-Wide Association Study Using Extreme Truncate Selection Identifies Novel Genes Affecting Bone Mineral Density and Fracture Risk. PLOS GENETICS, 7 (4), https://doi.org/10.1371/journal.pgen.1001372.

Persistent Link: http://hdl.handle.net/11343/264007

File Description: Published version

License: CC BY