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Genetic Analyses in a Sample of Individuals With High or Low BMD Shows Association With Multiple Wnt Pathway Genes

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ABSTRACT: Using a moderate-sized cohort selected with extreme BMD (n = 344; absolute value BMD, 1.5–4.0), significant association of several members of the Wnt signaling pathway with bone densitometry measures was shown. This confirms that extreme truncate selection is a powerful design for quantitative trait association studies of bone phenotypes.

Introduction: Although the high heritability of BMD variation has long been established, few genes have been conclusively shown to affect the variation of BMD in the general population. Extreme truncate selection has been proposed as a more powerful alternative to unselected cohort designs in quantitative trait association studies. We sought to test these theoretical predictions in studies of the bone densitometry measures BMD, BMC, and femoral neck area, by investigating their association with members of the Wnt pathway, some of which have previously been shown to be associated with BMD in much larger cohorts, in a moderate-sized extreme truncate selected cohort (absolute value BMD Z-scores = 1.5–4.0; n = 344).

Materials and Methods: Ninety-six tag-single nucleotide polymorphism (SNPs) lying in 13 Wnt signaling pathway genes were selected to tag common genetic variation (minor allele frequency [MAF] > 5% with an $r^2 > 0.8$) within 5 kb of all exons of 13 Wnt signaling pathway genes. The genes studied included *LRP1*, *LRP5, LRP6*, *Wnt3a*, *Wnt7b, Wnt10b*, *SFRP1, SFRP2*, *DKK1, DKK2*, *FZD7*, *WISP3,* and *SOST*. Three hundred forty-four cases with either high or low BMD were genotyped by Illumina Goldengate microarray SNP genotyping methods. Association was tested either by Cochrane-Armitage test for dichotomous variables or by linear regression for quantitative traits.

Results: Strong association was shown with *LRP5*, polymorphisms of which have previously been shown to influence total hip BMD (minimum $p = 0.0006$). In addition, polymorphisms of the Wnt antagonist, *SFRP1*, were significantly associated with BMD and BMC (minimum $p = 0.00042$). Previously reported associations of *LRP1*, *LRP6,* and *SOST* with BMD were confirmed. Two other Wnt pathway genes, *Wnt3a* and *DKK2*, also showed nominal association with BMD.

Conclusions: This study shows that polymorphisms of multiple members of the Wnt pathway are associated with BMD variation. Furthermore, this study shows in a practical trial that study designs involving extreme truncate selection and moderate sample sizes can robustly identify genes of relevant effect sizes involved in BMD variation in the general population. This has implications for the design of future genome-wide studies of quantitative bone phenotypes relevant to osteoporosis.

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Key words: single nucleotide polymorphism, association, BMD, Wnt

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INTRODUCTION

THE IDENTIFICATION OF MUTATIONS in the *LRP5* gene as a cause of high bone mass and of the osteoporosis-pseudoglioma syndrome provided the first indications that the Wnt pathway was an important component of the control of skeletal development. Although in many cases genes implicated in monogenic disorders characterized by extreme phenotypes have not proven to be involved in less extreme variation in the general population, there have now been several studies indicating that *LRP5* variation influences BMD and fracture risk in the general population.[1–8] There have also been some reports suggesting that other Wnt pathway genes, including *SOST* (encoding sclerostin)[9] and *LRP6*,[3] also influence BMD in the general community. Suggestive evidence has been presented that *LRP5* variants in particular are associated with height and bone area in adolescents,[6] and lumbar spine bone area in adults.[3]

Theoretical studies have suggested that power to detect genetic associations with quantitative traits can be increased by selection of subjects from the extremes of the population distribution (extreme truncate selection).[10–12] This is particularly relevant in the era of genome-wide association studies, where the genotyping costs can be as high as $US1000 per individual. Study designs that optimize power while minimizing the number of subjects genotyped are thus essential for these studies to be affordable.

The principal aim of this study was to test the relative role of major genes in the Wnt signaling pathway on BMD variation. We also sought to test whether, in practice, genes of the effect size that influence population variation in BMD can be detected using moderate sample sizes of extreme truncate selected subjects.

MATERIALS AND METHODS

Cohorts studied

Subjects were recruited from four Australian population studies, including the Dubbo Osteoporosis Epidemiology Study,[13] Geelong Osteoporosis Study,[14] Tasmanian Older Adult Cohort,[15] and the Calcium Intake Fracture Outcome Study,[16] and from the Oxford Familial Osteoporosis Study,[17] a study of British families recruited with probands with low BMD (Z-score < –2). Subjects selected from these cohorts were unrelated white women >55 and <80 yr of age and >5 yr postmenopausal. Screening had been performed for secondary causes of osteoporosis, and subjects had not been taking osteoporosis agents before bone densitometry measures. In addition, subjects were selected by bone densitometry criteria (measured at the total hip), having either low BMD (Z-scores > –4 but < –1.5; n = 174) or high BMD (Z-scores > +1.5 but < +4; n = 170). Further details regarding the cohorts are provided in Table 1. All subjects gave written, informed consent, and each study had been approved by its ethics review committee.

Single nucleotide polymorphism selection and genotyping methods

Ninety-six single nucleotide polymorphisms were selected from International HapMap Project Data Release 20 (January 2006). SNPs (Table 2) were selected to tag common genetic variation (minor allele frequency [MAF] > 5% with an $r^2 > 0.8$) within 5 kb of all exons of 13 Wnt signaling pathway genes, using the Haploview program.[18] The genes studied included *LRP1*, *LRP5, LRP6*, *Wnt3a*, *Wnt7b, Wnt10b*, *SFRP1, SFRP2*, *DKK1, DKK2*, *FZD7*, *WISP3,* and *SOST*.

Genotyping was performed by Illumina Goldengate assay, a microarray chip SNP genotyping technology; five 96-sample chips were required for the study. For quality control purposes, three Centre d'Etude dU Polymorphisme Humain (CEPH) control samples and five samples (one from each recruitment center) were genotyped on each chip. Genotypes for the CEPH controls were already available from the International HapMap Study (www.hapmap.org) and could therefore be used for quality control purposes. Genotyping accuracy was also assessed by assessment of the genotype clustering accuracy using the Illumina Gencall score and by checking Hardy-Weinberg equilibrium. The Gencall score is a measure of the confidence of the clustering of individual SNP genotypes.

Statistical analysis

The primary analysis performed was a single marker analysis of allelic association in which data were analyzed as a dichotomous trait comparing high and low BMD subjects by the Cochrane-Armitage test. Quantitative trait analysis was performed for BMD, BMC, and femoral neck area using the program WHAP, which uses linear regression for quantitative traits and logistic regression for binary traits.[19] Single marker and two-marker sliding window haplotype analyses were performed. All BMC data presented were adjusted for age by linear regression.

BMD was measured using different densitometers in different centers, and no cross-calibration was performed. The quantitative analyses are thus potentially susceptible to bias caused by between-center measurement differences. To test whether this had a significant effect, association was tested with permutations clustered within centers and compared with permuted p values performed with no clustering, using the program PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/). Correlation between the clustered and unclustered analyses was performed by linear regression, and the paired t-test was used to investigate differences in findings for individual SNPs analyzed by either method. No bias was observed because of differences between centers in BMD measurements (see Quality control).

Gene–gene interaction was studied for associated SNPs ($p < 0.05$ for BMD analysis) in two-marker combinations with all other individual SNPs by logistic regression.

Nominal significance was defined as uncorrected $p < 0.05$; all p values presented are uncorrected. To estimate the appropriate level of correction for the number of SNPs analyzed while taking into account the linkage disequilibrium between SNPs, the required level of Bonferroni correction was determined using the program SNPSpD.[20] Power calculations for BMD and area studies were performed using Genetic Power Calculator.[21] The presence of population stratification was studied using the program Structure, version 2.2.[22] Population attributable risk for

Table 1. Age and Bone Densitometry Characteristics of Cohort Studied

	Age (yr)	*BMD (Z-score)*	*BMC (g)*	*Area (cm^2)*
High BMD group (n = 170)	67.7 (55–84)	1.99 (1.51–3.97)	4.92 (2.78–7.37)	5.01 (3.98–6.79)
Low BMD group (n = 174)	68.7 (57–85)	–1.94 (–1.5 to –3.33)	2.88 (1.44–4.5)	4.93 (3.5–6.35)

Table 2. Characteristics of Genes Studied and SNPs Genotyped

Gene symbol	*Region*	*Length (kb)*	*SNPs used*
DKK1	10q21.1	3.5	1
DKK2	4q25	114.5	12
FZD7	2q33.1	3.9	2
LRP1	12q13.3	84.9	11
LRP5	11q13.2	136.6	12
LRP6	12q13.2	146.1	12
SFRP1	8p11.21	47.5	9
SFRP2	4q31.3	8.5	4
SOST	17q21.31	5.1	3
Wisp3	6q21	16.9	4
Wnt3a	1q42.13	54.2	3
Wnt7b	22q13.31	55.1	8
Wnt10b	12q13.12	6.4	1

specific markers was calculated using the dichotomized BMD data using the method of Miettenin.[23]

RESULTS

Quality control

Excluding markers whose Genecall scores indicated unreliable genotype clustering, 88 SNPs were successfully genotyped, of which 82 were polymorphic in this population. All duplicate samples gave identical genotypes, and CEPH control genotypes matched the listed genotypes on the HapMap database. All markers were in Hardy-Weinberg equilibrium. Excluding failed and monomorphic SNPs, the overall genotyping success rate was 97.9% (52,472/53,604 alleles called). Structure analysis identified just one cluster, with no evidence of unidentified population stratification.

Significance levels and comparison of analysis methods

Spectral decomposition analysis indicated that the 82 SNPs genotyped were equivalent to 60.5 independent observations and that the equivalent uncorrected p value to a corrected p value of 0.05 was 0.00085.

Findings for BMD analyzed as a dichotomous trait were virtually identical with analysis as a quantitative variable (Z-scores). The correlation between p values by these two methods was high (r = 0.95), and comparing p values for the same SNPs, no significant difference was observed (paired t-test, p = 0.4). Potential bias because of center BMD instrument differences was analyzed by comparison of p values determined either by permutations clustered within centers or with no clustering. Clustered and nonclustered analyses producing very similar results (r = 0.996) and with no significant difference observed comparing p values for the same SNPs analyzed by either method (paired t-test, p = 0.8). Thus, we concluded that there was no bias because of center BMD instrument differences.

Associations with BMD, BMC, and area

BMD was associated with 19 SNPs with uncorrected $p < 0.05$ (Table 3); the probability of obtaining this many significant findings by chance having genotyped 82 SNPs is 10^{-8}. The strongest association observed was with *LRP5*, in which 5 of the 12 SNPs genotyped achieved nominal significance, the most strongly associated SNP being rs3781590 (p = 0.0006, BMD Z-score = –0.57 lower in minor allele carriers; n = 213). Haplotypic findings globally were less significant than for this individual SNP, suggesting that this marker is the primary associated marker. However, regression analysis, in which the presence of association with one SNP is tested controlling for the association tagged by another marker, showed that associations of each of the associated *LRP5* markers were interdependent. Having controlled for the association tagged by any individual associated marker, no other marker remained associated with BMD. Thus, the study is not able to differentiate association caused by linkage disequilibrium from true association with a functionally relevant polymorphism.

Other genes with two or more SNPs associated with BMD included *LRP1* (4 nominally significant SNPs of 11 studied), *LRP6* (2 significant SNPs of 12 studied), *SOST* (2 significant SNPs of 3 studied), *Wnt3a* (2 significant SNPs of 3 studied), and *DKK2* (2 significant SNPs of 12 studied).

One *LRP1* SNP was associated with bone area (rs4759044), with a stronger association noted with the two marker combinations of this SNP with the neighboring SNP (rs11172113; global p = 0.0018). The haplotype of the common allele at rs11172113 and rare allele at rs4759044, which made up 8.7% of haplotype combinations for this pair of SNPs, was associated with bone area (p = 0.00014, area 0.31 cm^2 lower in carriers of this haplotype). This haplotype was also associated with BMC (p = 0.015, BMC 0.556 g lower in carriers).

SFRP1 was associated with BMC with four markers; two of which (rs921142 and rs4736965) were also associated either individually or as part of a two-marker haplotype with BMD. The strength of association of the two-marker haplotype was particularly strong (p = 0.00042 for BMC and p = 0.0046 for BMD). This association is primarily caused by association of a haplotype of the common allele at rs921142 and rare allele at rs4736965 (p = 0.00047 for BMC, p = 0.0014 for BMD, making up 22% of observed haplotypes; BMC was 0.423 g lower in carriers).

The study power for BMD and area are given in Fig. 1. Power was investigated for each model with different minor allele frequencies, with findings being very similar for marker and disease-associated allele frequencies ranging

Table 3. Single Marker or Two-Marker Haplotype Associations With BMD, BMC, and Femoral Area

				BMD dichotomous			*BMD Z-score*		*Age-adjusted BMC*		*Femoral area*	
Chromosome	*Gene*	*SNP*	*MAF*	*OR*	*Single*	*Two sliding*	*Single*	*Two sliding*	*Single*	*Two sliding*	*Single*	*Two sliding*
1	*WNT7b*	rs12756110	0.46	0.9	NS	0.095	NS	NS	NS	NS	NS	NS
1	*WNT7b*	rs12131703	0.07	0.6	0.036	0.033	0.046	0.046	NS	NS	NS	NS
1	*WNT7b*	rs7526484	0.12	0.8	NS	NS	NS	NS	NS	NS	NS	NS
1	*WNT3a*	rs708114	0.25	0.8	NS	0.028	0.1	0.017	NS	0.059	NS	NS
1	*WNT3a*	rs4653533	0.05	0.6	0.057	0.045	0.04	0.022	0.068	0.071	NS	NS
1	*WNT3a*	rs752107	0.19	1.4	0.043	NS	0.021	—	0.08	NS	NS	NS
4	*DKK2*	rs17037083	0.03	1.3	NS	NS	NS	NS	NS	NS	0.05	NS
4	*DKK2*	rs13103371	0.49	0.9	NS	NS	NS	NS	NS	NS	NS	NS
4	*DKK2*	rs419764	0.16	1.0	NS	0.066	NS	0.072	NS	NS	0.053	NS
4	*DKK2*	rs17037102	0.04	0.5	0.027	—	0.045	—	0.086	—	NS	—
4	*DKK2*	rs6827902	0.39	0.8	NS	0.046	NS	0.032	NS	0.0204	NS	NS
4	*DKK2*	rs17037297	0.01	0.3	0.023	0.06	0.017	0.042	0.026	0.0465	NS	NS
6	*WISP3*	rs4947163	0.13	1.0	NS	NS	NS	NS	NS	NS	NS	NS
6	*WISP3*	rs1230348	0.15	1.2	NS	NS	NS	NS	—	NS	NS	NS
6	*WISP3*	rs17219737	0.47	0.4	0.019	—	0.011	—	NS	—	NS	—
8	*SFRP1*	rs7832749	0.21	0.8	NS	NS	0.097	NS	0.037	0.027	NS	NS
8	*SFRP1*	rs10106678	0.25	0.8	NS	NS	NS	NS	0.0052	0.013	NS	NS
8	*SFRP1*	rs7832767	0.02	1.8	NS	NS	NS	NS	NS	NS	NS	NS
8	*SFRP1*	rs9694405	0.25	1.1	NS	NS	NS	NS	NS	NS	NS	NS
8	*SFRP1*	rs968427	0.28	1.0	NS	NS	NS	NS	NS	NS	NS	—
8	*SFRP1*	rs921142	0.32	1.0	NS	0.0014	NS	0.0046	0.014	0.00042	NS	NS
8	*SFRP1*	rs4736965	0.39	1.8	0.0011	—	0.0029	—	0.001	—	NS	—
11	*LRP5*	rs7116604	0.06	1.0	NS	NS	NS	NS	NS	NS	NS	NS
11	*LRP5*	rs314756	0.47	0.6	0.092	0.058	NS	0.084	NS	NS	NS	NS
11	*LRP5*	rs729635	0.43	1.6	0.083	0.033	0.098	0.013	NS	NS	NS	NS
11	*LRP5*	rs312786	0.14	1.3	0.084	0.04	0.034	0.015	NS	NS	NS	NS
11	*LRP5*	rs160607	0.21	1.5	0.012	0.0041	0.0038	0.0026	0.071	0.067	NS	NS
11	*LRP5*	rs3781590	0.18	1.7	0.0012	0.019	0.0006	0.0097	0.01	NS	0.0939	NS
11	*LRP5*	rs314750	0.34	1.6	0.018	0.0031	0.019	0.0065	0.039	0.026	NS	NS
11	*LRP5*	rs1784235	0.38	1.1	NS	0.0072	NS	0.011	NS	0.018	NS	NS
11	*LRP5*	rs7125942	0.46	2.4	0.0036	0.0079	0.0063	0.012	0.0091	0.019	NS	NS
11	*LRP5*	rs676318	0.46	0.7	NS	NS	NS	NS	NS	NS	NS	NS
12	*LRP6*	rs2075241	0.41	1.2	NS	0.049	NS	0.082	0.045	0.032	NS	NS
12	*LRP6*	rs11054704	0.07	0.5	0.019	0.052	0.041	0.089	NS	0.04	NS	NS
12	*LRP6*	rs12320259	0.07	0.9	NS	NS	NS	NS	NS	0.09	NS	NS
12	*LRP6*	rs2302685	0.41	0.7	NS	NS	NS	NS	NS	NS	NS	NS
12	*LRP6*	rs2417085	0.28	0.8	NS	NS	NS	NS	NS	NS	NS	NS
12	*LRP6*	rs10845493	0.06	0.6	0.056	NS	0.075	NS	0.083	0.042	NS	NS
12	*LRP6*	rs10492120	0.31	0.9	NS	NS	NS	NS	NS	NS	NS	NS
12	*LRP1*	rs11172113	0.28	0.9	NS	NS	NS	NS	NS	0.046	0.066	0.0018
12	*LRP1*	rs4759044	0.28	1.0	NS	NS	NS	NS	NS	NS	0.046	NS
12	*LRP1*	rs715948	0.15	0.8	NS	NS	NS	NS	NS	0.051	NS	NS
12	*LRP1*	rs4759277	0.18	0.9	NS	NS	NS	NS	NS	NS	NS	NS
12	*LRP1*	rs7975818	0.18	1.3	0.034	0.034	0.047	0.043	NS	NS	NS	NS
12	*LRP1*	rs1800175	0.17	1.3	0.027	0.034	0.034	0.043	NS	NS	NS	NS
12	*LRP1*	rs6581128	0.18	1.4	0.021	0.029	0.037	0.04	NS	0.079	NS	NS
12	*LRP1*	rs1800154	0.17	1.4	0.029	NS	0.038	NS	NS	NS	NS	NS
12	*LRP1*	rs1800159	0.17	1.3	0.062	NS	0.069	NS	NS	NS	NS	NS
12	*LRP1*	rs7956957	0.32	1.2	NS	0.0068	NS	NS	NS	NS	0.024	0.024
17	*SOST*	rs851054	0.33	1.5	0.007	0.0078	0.0035	0.0041	NS	NS	NS	NS
17	*SOST*	rs851056	0.33	1.4	0.0084	0.027	0.0045	0.016	NS	NS	NS	NS
17	*SOST*	rs2023794	0.47	0.7	NS	—	NS	—	0.046	—	NS	—

Two-marker haplotypes are combinations of the listed marker and the next most 3′ SNP. p = NS indicates $p \geq 0.1$.

—, no analysis was performed because this was the most q-telomeric SNP in the gene; MAF, marker minor allele frequency; OR, odds ratio of the individual SNP.

from 0.05 to 0.95 (data not shown). The sample size of unselected cases that would be required to have equal power to the 344 extreme truncate selected cases studied here was calculated for different values of D′ and marker and disease-associated allele frequencies. For D′ ranging from 0.4 to 0.8 and allele frequencies from 0.05 to 0.95, the

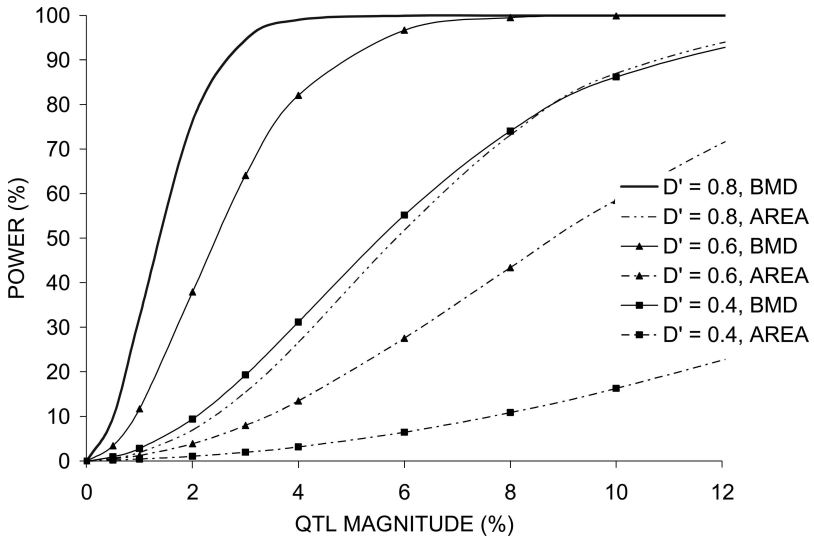

FIG. 1. Study power to detect associations with BMD and area, for significance level of $p = 0.00085$, assuming a disease and marker minor allele frequency of 0.25, with varying linkage disequilibrium (D′) between marker and disease-associated alleles.

equivalent required sample size ranged from 1207 to 1286, being 3.51–3.74 times greater than required for the extreme truncate selected cohort. The bone area measures are normally distributed (Kolmogorov-Smirnov test, $p = 0.8$), whereas the BMC and BMD data are not (Kolmogorov-Smirnov test, $p < 10^{-4}$), reflecting the ascertainment scheme.

DISCUSSION

This study confirmed the significant association of BMD with *LRP5* polymorphisms in particular and supports the association of polymorphisms of other genes in the Wnt signaling pathway with BMD variation. The study also shows that extreme truncate selection designs can, in practice, detect genes of relevant effect sizes influencing BMD variation, using moderate sample sizes.

LRP5 mutations have been shown to cause high or low bone mass syndromes, and several studies have now shown effects of common polymorphisms on BMD. Association of *LRP5* polymorphisms with BMC has been reported in adolescents,[6] and our study showed that this association is strongly observed in late adulthood. Furthermore, early studies suggested that the association of *LRP5* polymorphisms with BMD variation was stronger in men than in women.[3,4] This study, showing a strong association in postmenopausal women and in combination with previous studies, indicates that *LRP5* polymorphisms are associated with both male and female BMD variation, consistent with mouse models. The study was not able to determine the primary disease-associated variant, which may require either larger sample sizes and/or studies in different ethnic groups, where difference in the linkage disequilibrium structure may lead to greater genetic resolution.

The magnitudes of effect of the observed *LRP5* associations are not great, as expected for any relatively commonly polymorphic locus. Comparing allele frequencies in our high and low BMD groups, the ORs for associated *LRP5* polymorphisms were 1.3–2.4. Considering BMD as a dichotomous trait using the thresholds used in this study (absolute value BMD Z-score >1.5 and <4), the association of the *LRP5* SNP rs3781590 contributes a remarkably high 17% of the population attributable risk. This value likely over-represents the contribution in unselected populations. Given that *LRP5* seems to be a major gene for affecting BMD variation in the general population, it is likely that most BMD-associated genes would have lower effect sizes.

The association with *LRP5* was primarily with BMC, and no significant association of this gene was observed with area. In this cohort, BMC was a much greater determinant of BMD than was area. However, this observation should be interpreted with caution. The projected area of the region of interest has a fixed scan length, so differences in area reflect differences in femoral neck width. Consequently, our study has much less power to identify effects on bone area than BMD or BMC (see Fig. 1). Thus, although we can say that these findings indicate that *LRP5* is associated with BMD in this dataset because of effects on BMC, whether it also has effects on bone area in the general population is unclear.

SFRP1 is a well-characterized soluble Wnt antagonist that has not previously been studied for genetic association with osteoporosis. Our study identified association of four SNPs in *SFRP1* with BMC, and two of these were also associated with BMD (rs921142, rs4736965). The association of the haplotype of these two markers was particularly strong ($p = 0.00042$ for BMC). These two markers lie in a single haplotype block in the 3′ UTR of *SFPR1*. No association was observed with bone area. *SFRP1*$^{-/-}$ mice have increased trabecular bone mass,[24] with minimal effect noted on cortical bone. This is consistent with the findings of this study, because increases in trabecular bone produces increased BMC/BMD without affecting bone area. Our observation suggests that genetic variation in this region influences *SFRP1* function in some manner, perhaps through effects on RNA stability.

LRP6 bears 71% amino acid identity with LRP5,[25] and murine models suggest incomplete functional redundancy between the two receptors.[26] Heterozygous or homozy-

gous deletions at either the *LRP5* or *LRP6* locus result in decreased trabecular bone volume fraction compared with wildtype controls,[27] and *LRP6* haploinsufficiency exacerbates the low BMD phenotype of *LRP5*$^{-/-}$ mice.[27] Furthermore, spontaneous loss-of-function mutation of *LRP6* in mice leads to dysmorphologies of the axial skeleton and digits, as well as the neural tube.[28] In humans, a missense mutation has been linked to early coronary disease and severe osteoporosis.[29] Association of a coding *LRP6* SNP with BMD (rs2302685, Ile1062Val) has also been reported.[3] The two markers showing association in our dataset with BMD and/or BMC in our dataset lie just 2129 (rs11054704) and 10,419 bp (rs2075241) away from that SNP, and HapMap data indicate that they are part of the same linkage disequilibrium block, in strong linkage disequilibrium with rs2302685 ($D' = 1$ for both markers). The data are therefore supportive of the earlier association reported but extend their single marker study, showing that the association is with a particular haplotype block, which also encodes the Dickkopf binding region.[30] The association of *LRP6* genetic variation with both coronary artery disease and BMD may partially explain their clinical association; if confirmed, this finding would be of obvious major public health significance.

Four SNPs in *LRP1* were associated with both BMD and BMC and, although none of these reached experiment-wise significance, the probability of this many positive associations by chance of 11 SNPs studied is extremely low ($p = 0.001$). Furthermore, a haplotype of the neighboring SNPs rs4759044 and rs11172113 was associated with lower femoral area and lower BMC. This suggests that this haplotype contains a genetic variant associated with reduced bone formation. These two SNPs, both located in intron 1, lie in a haplotype block that extends as far as intron 6 and includes two nonsynonymous SNPs (rs2306691, Asp166Asn; rs1800127, Val217Ala) in exon 6. Further fine-mapping and confirmation studies of this region are needed to determine the principal associated variants. Because the haplotype was associated with reduction in both BMC and area, no effect was seen on BMD. This emphasizes the value of studying these measures separately, because genetic effects that influence BMC and area in parallel are not expected to be observed by studies of BMD alone.

No previous reports of association studies of *LRP1* have been published. LRP1 is known to be expressed by osteoblasts and is involved in uptake of vitamin K in chylomicron remnants, in turn leading to increased osteocalcin γ-carboxylation.[31] Vitamin K has also been show to stimulate osteogenic and inhibit adipogenic marrow stromal differentiation. *LRP1* knockout mice are not viable,[32] and no murine models are yet available to study the role of *LRP1* genetic variants on bone. However, our data suggest that *LRP1* polymorphisms influence BMD and BMC variation and may also influence bone area.

Mutations of *SOST* are known to cause the high bone mass syndrome van Buchem disease.[33,34] *SOST* polymorphisms have been shown to be associated with BMD in the Rotterdam Study.[9] Two of the three SNPs studied in *SOST* were BMD associated ($p = 0.004$–0.008). SOST is a known Wnt signaling antagonist expressed primarily in osteocytes and thought to be involved in osteocytic control of bone deposition. The associated SNPs lie in the 5′ region of the gene, distant from the region deleted in van Buchem disease but close to the deletion reported to be associated with BMD by Uitterlinden et al.,[9] lying 303 and 904 bp away, and thus likely to tag the deletion.

Two other genes in the same signaling pathway, *Wnt3a* and *DKK2*, showed nominally significant association with BMD involving more than one SNP. We are not aware of any previous association studies of these genes in osteoporosis. In both cases, the strength of the observed association was not definitive, and further studies in other cohorts and higher density mapping will be needed to confirm these findings and identify true condition-associated variants.

A potential weakness of any association study design using unrelated individuals is unaccounted population stratification, where differences in ethnicity between individuals can inflate the type 1 and type 2 error rates. Unrecognized population stratification is difficult to exclude in any study of unrelated individuals, and it is not impossible that it may have affected our findings. In British whites, analysis of the healthy control cohort in the Wellcome Trust Case Control Consortium has identified 13 regions with significant genotype frequency variation in British whites.[35] None of the loci investigated in this study lie within these 13 regions. Furthermore, analysis of ethnic structure within this study indicated that all cases belonged to the one cluster. This suggests that our findings are not significantly influenced by population stratification.

This study used a novel design, with extreme truncate selection of samples, aiming to improve power. The approach of studying cohorts drawn from the extremes of the population distribution of BMD has been used in several linkage studies of BMD variation,[17,36–38] but rarely in association studies.[17,39] The power calculations presented in Fig. 1 show that for BMD (studied by extreme truncate selection), the power was substantially greater than for area (where values were normally distributed) across a wide range of levels of linkage disequilibrium and also marker and disease-associated allele frequencies. Using a relatively small sample size, we showed statistically robust associations, indicating that this is a good model for future study designs in osteoporosis genetics. To achieve the same power using an unselected population sample would require studying 1207–1286 individuals depending on the genetic model, a sample size 3.51–3.74 times larger than studied here. This study does not formally compare extreme truncate selection with unselected cohort designs, but the strong positive findings produced with the moderate sample size studied would be quite unexpected in unselected cohorts of this size. Our findings support the conclusion of previous theoretical analyses, that extreme truncate selection is an efficient study design, providing optimum power and minimizing genotyping requirements, and is well suited to genome-wide association analysis to minimize genotyping costs.

In conclusion, this study confirmed the major role played by *LRP5* polymorphisms in determining population variation in BMD and BMC and supports a role for several other Wnt pathway genes including *LRP1*, *LRP6*, *SFRP1, SOST*,

Wnt3a, and *DKK2*, in which more than one SNP showed nominal association. This study also provides a practical demonstration of the power of extreme truncate selection methods in quantitative trait genetics.

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