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Polymorphisms in the Low-Density Lipoprotein Receptor–Related Protein 5 *(LRP5)* Gene Are Associated with Variation in Vertebral Bone Mass, Vertebral Bone Size, and Stature in Whites

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Stature, bone size, and bone mass are interrelated traits with high heritability, but the major genes that govern these phenotypes remain unknown. Independent genomewide quantitative-trait locus studies have suggested a locus for bone-mineral density and stature at chromosome 11q12-13, a region harboring the low-density lipoprotein receptor–related protein 5 (*LRP5*) gene. Mutations in the *LRP5* gene were recently implicated in osteoporosis-pseudoglioma and "high-bone-mass" syndromes. To test whether polymorphisms in the *LRP5* gene contribute to bone-mass determination in the general population, we studied a cross-sectional cohort of 889 healthy whites of both sexes. Significant associations were found for a missense substitution in exon 9 (c.2047G→A) with lumbar spine (LS)–bone-mineral content (BMC) ($P = .0032$), with bone area ($P = .0014$), and with stature ($P = .0062$). The associations were observed mainly in adult men, in whom *LRP5* polymorphisms accounted for ≤15% of the traits' variances. Results of haplotype analysis of five single-nucleotide polymorphisms in the *LRP5* region suggest that additional genetic variation within the locus might also contribute to bone-mass and size determination. To confirm our results, we investigated whether *LRP5* haplotypes were associated with 1-year gain in vertebral bone mass and size in 386 prepubertal children. Significant associations were observed for changes in BMC ($P = .0348$) and bone area ($P = .0286$) in males but not females, independently supporting our observations of a mostly male-specific effect, as seen in the adults. Together, these results suggest that *LRP5* variants significantly contribute to LS–bone-mass and size determination in men by influencing vertebral bone growth during childhood.

Introduction

The lifetime risk of suffering an osteoporosis-related fracture is >40% in women and >13% in men (Kanis 2002). This risk is largely influenced by the bone-mineral density and size achieved in young adults (Duan et al. 2001*a*, 2001*b*). Twin and parent-offspring studies have indicated that additive genetic effects account for 60%–80% of the population variance in areal bone-mineral density (aBMD), bone area, bone-mineral content (BMC), and stature (Ferrari et al. 1998*b*; Eisman 1999). As with other complex phenotypes, interindividual variation in these traits seems to be determined at multiple loci (Perola et al. 2001; Peacock et al. 2002). Many association studies with osteoporosis candidate genes have been performed to date, but, so far, only small and often inconsistent differences in aBMD between genotypes have been reported (Ferrari et al. 1999*b*; Peacock et al. 2002 [for review]). Therefore, allelic variants of genes with a major contribution to bone mass and size remain to be identified.

The low-density lipoprotein (LDL) receptor–related protein 5 *(LRP5)* gene, which, in humans, maps to chromosome 11q12-13, encodes a transmembrane protein of 1,615 amino acids that is a member of the LDL receptor–related family (Hey et al. 1998). These proteins mediate WNT signaling through the β-catenin pathway and are involved in *Drosophila* and mouse development (Wodarz and Nusse 1998; Tamai et al. 2000).

Recently, several lines of evidence have pointed to the *LRP5* gene as a candidate susceptibility factor for osteoporosis in the general population. (1) Loss-of-function mutations in *LRP5* are responsible for osteoporosis pseudoglioma (OPPG [MIM 259770]), a rare autosomal recessive disorder characterized by low bone mass, spontaneous fractures, and blindness (Gong et al. 2001), whereas *LRP5* gain-of-function mutations cause high-

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bone-mass syndromes (HBM [MIM 601884]) (Boyden et al. 2002; Little et al. 2002; Van Wesenbeeck et al. 2003). (2) Mice with targeted disruption of *Lrp5* have a deficit in bone formation and sustain spontaneous fractures (Kato et al. 2002). (3) A QTL for bone-mineral density in the general population was mapped to 11q12-13, the same chromosomal region where *LRP5* is located (Koller et al. 1998; Carn et al. 2002; Livshits et al. 2002). Moreover, a QTL for stature has also been identified in this region (Hirschhorn et al. 2001; Perola et al. 2001), suggesting that the two traits may be influenced by allelic variation of the same gene.

To investigate whether allelic variants of the *LRP5* gene contribute to bone-mass and stature determination in the general population (Patel and Karsenty 2002), we first studied the frequency and linkage disequilibrium (LD) of 13 previously reported SNPs in a subset of our sample. We then selected those polymorphisms that provided most of the genetic information to perform an association study in our entire cohort, comprising healthy adults, children, and adolescents of both sexes, for which detailed bone-mass and size measurements at the lumbar spine (LS) had been collected. To confirm and further dissect our results, we subsequently performed a 1-year longitudinal association study in a subgroup of 386 prepubertal children to assess the contribution of *LRP5* genotypes to vertebral bone growth.

Subjects and Methods

Cohort

For the association study, 889 healthy children, adolescents, and adults of European descent were recruited from among volunteers drawn from the population living in Geneva, Switzerland. This cohort comprised 149 and 240 prepubertal girls and boys, respectively (Tanner's pubertal stage 1), of whom 148 girls and 238 boys were re-evaluated after 1 year for the longitudinal study (Bonjour et al. 1997, 2001); 74 female and 68 male adolescents in Tanner's pubertal stages 2–5 (Bonjour et al. 1991; Theintz et al. 1992), of whom 15 female and 5 male adolescents had reached peak bone mass and were thereafter classed as adults; 100 young men, students at Geneva University (Ferrari et al. 1999*a*); and 186 pre- and perimenopausal women and 72 men who were parents of some of the children and adolescents included in the study cohort (Ferrari et al. 1998*b*, 1998*c*). Most of these subjects have participated in a number of previous studies of bone mass at our institution, and their detailed inclusion/exclusion criteria have been reported elsewhere (see references above).

Clinical Measurements

The aBMD (g/cm^2), BMC (g), and projected bone area (cm^2) were measured at the LS (L2-L4 vertebrae) in anteroposterior view by dual X-ray absorptiometry (DXA) with Hologic QDR-1000, -2000, and -4500 instruments, as reported elsewhere (see references above). The coefficient of variation at L2-L4 was 1% for all measurements. Stature was evaluated by measuring standing height (without shoes) with a stapediometer (Holtain). Calcium intake was evaluated using validated food-frequency questionnaires completed under supervision of a trained dietician (Bonjour et al. 1997; Ferrari et al. 1998*c,* 1999*a*). The study was approved by the ethics committee of the University Hospitals of Geneva, and informed consent for genotyping of osteoporosis candidate genes was obtained from all participants and/or their parents.

SNP Genotyping

Genomic DNA was extracted from blood lymphocytes or mouth epithelial cells by use of the QIAmp DNA blood kit (Qiagen). SNPs were genotyped by the pyrosequencing method (Pyrosequencing), in which short regions including the SNP are automatically sequenced through a series of four enzymatic steps (Ronaghi 2001). We designed specific PCR primers for each SNP so that regions of ~200 bp spanning the polymorphism were amplified (dbSNP Database).

Data Analysis

Hardy-Weinberg equilibrium was tested for each SNP by use of the HW Exact Test, as implemented in the Genepop software (Garnier-Gere and Dillmann 1992). To assign inferred haplotypes to each individual, we used the HAPLOTYPER program and default parameters (Niu et al. 2002). Haplotype frequencies were confirmed by the Arlequin program (Excoffier and Slatkin 1995). LD for all SNP-pair combinations was calculated using the DnaSP software (Rozas and Rozas 1995). For LD measurement, we used the R^2 statistic. To determine whether there was underlying population structure in our cohort that could potentially generate false-positive associations, we ran the program STRUCTURE (Pritchard et al. 2000), using genotype data of five markers located in different genomic regions (*LRP5, IL-6, VDR, LEPR,* and *LEP*).

For association analyses, stature, aBMD, BMC, and projected bone area were adjusted for age and sex and were expressed as standardized *Z* scores, by use of the study population mean and SD for age and sex as reference (at 1-year intervals for children and at 5-year intervals for adults). *Z*-score differences between *LRP5* polymorphisms were tested by analysis of covariance (ANOVA), with weight (*Z* scores) as a continuous vari-

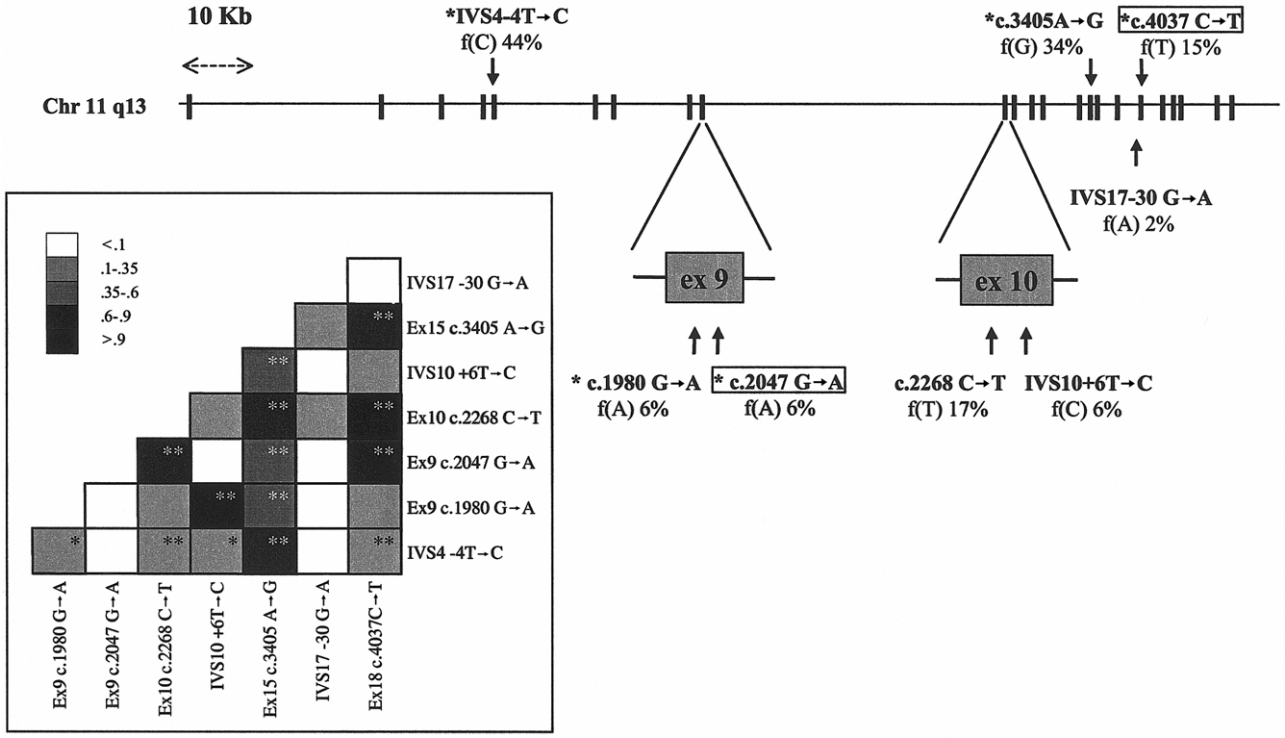

Figure 1 Schematic diagram of SNP localization in *LRP5*. Vertical bars represent the 23 exons of *LRP5*, and arrows indicate the positions of the eight validated SNPs with a minimum allele frequency of 2%. SNPs encoding missense substitutions are boxed. Leading asterisks (*) indicate SNPs used in the association study. Percentages indicate the frequency of the rare allele. The insert shows LD for all SNP pairs, calculated as R^2 values. Gray-shaded coding represents the strength of LD (R^2 values), according to the scale shown on the left. Leading asterisks (*) inside the boxes indicate the level of significance for LD; one asterisk (*) denotes $P = .01$ to .001, and two asterisks (**) denotes $P < .001$.

able and with genotypes and sex as categorical variables. Sex was included as a covariate to account for potential interactions between sex and genotypes, since both genomewide screening in mice and association studies in humans indicate that some genes may govern bone mass in a sex-specific manner (Orwoll et al. 2001; Ferrari et al. 2004). To avoid biases, only unrelated individuals were analyzed at one time; that is, adults and children were analyzed separately. To correct for multiple comparisons, we used a modified Bonferroni method, as described by Sankoh et al. (1997), which takes into consideration the level of correlation between linked markers. For the haplotype associations, the classical Bonferroni correction was applied (hence P values $\leqslant .0125$ were considered significant at $\alpha = 0.05$).

In prepubertal children, association of *LRP5* polymorphisms with longitudinal changes in bone-mass measurements, calculated as the difference between values obtained at baseline and after 1 year, were tested by two-factor ANOVA, with genotypes and sex as independent variables. Subsequently, differences between *LRP5* genotypes (and haplotypes) were tested by ANOVA within sexes. The percentage of the population variance for each trait explained by *LRP5* genetic variation was estimated as the R^2 value from multiple regression analysis. This included five *LRP5* SNPs transformed into dummy variables (two homozygous for the rare allele, one heterozygous, and zero homozygous for the common allele) as independent and residuals of bone measurements—adjusted by age, sex, and weight—as dependent variables.

Results

Allele Frequencies and Haplotype Structure

We initially studied 13 SNPs, 10 of which were reported elsewhere (Okubo et al. 2002; Van Wesenbeeck et al. 2003) (table A [online only]), that were located in the coding region or intron/exon junctions of the *LRP5* gene. As a first step, SNPs were genotyped for a sample of 88 unrelated individuals randomly selected from among 889 subjects in our cohort. Eight of the 13 SNPs were found to have a minor-allele frequency of $\geqslant 2\%$ in our population (fig. 1), and the genotype frequencies of these 8 SNPs did not significantly deviate from Hardy-Weinberg equilibrium. On the basis of these polymorphisms, we inferred that 11 different haplotypes were present in our study population, using a likelihood

method based on a Baysesian algorithm (Niu et al. 2002). The most common haplotype had a frequency of 50%, and four haplotypes accounted for 85% of the total sample (table B [online only]). Significant LD was observed across the gene (fig. 1), although LD was not correlated with intermarker distance ($R^2 = 0.11$; $P = .588$).

To reduce the number of tests for association, we discarded SNPs with a minor-allele frequency <5% and selected only one SNP for each pair of markers in nearly complete LD ($R^2 \geq 0.9$). Five SNPs, which we call the "informative SNPs," fulfilled these criteria; two of them alter the amino acid sequence of the protein: missense substitutions exon 9, c.2047G→A (p.V667M), and exon 18, c.4037C→T (p.A1330V) (fig. 1).

To rule out potential population stratification in our study cohort, we ran the program STRUCTURE (Pritchard et al. 2000), using data from markers on five different independent loci that had been genotyped in 221 individuals from our sample (Ferrari et al. 1998*a*, 2003; Quinton et al. 2001; Nieters et al. 2002). The highest likelihood was obtained under the assumption of one population ($K = 1$), and, for higher values of K, none of the individuals was assigned to a particular population, thereby indicating that an unknown population substructure was unlikely to be present in our cohort.

Cross-Sectional, Population-Based Association Study in Adults

Among 889 subjects who were genotyped for these five SNPs, 877 with complete data sets could eventually be analyzed. To account for the age distribution of the cohort, subjects were subdivided into "adults," whom we assumed had reached peak bone mass and size—that is, the maximal amount of bone accumulated by the end of the pubertal growth period (in this case, by the age of 17.0 years for females and 18.0 years for males) (Rizzoli and Bonjour 1999)—and growing "children/adolescents" (table 1). Association analyses were performed using bone measurements and stature adjusted for age and sex (Z scores) within these groups. Because of the small number of genotype AA at c.1980 and c.2047 and genotype TT at c.4037 ($n = 3$, 4, and 13, respectively), these subjects were grouped with heterozygotes for each specific SNP.

In adults, the most significant and consistent associations with all LS bone measurements, as well as with stature, occurred with the missense SNP in exon 9, c.2047G→A (p.V667M) (table 2). After adjustment for multiple comparisons, associations remained significant for LS BMC and area and for stature (P values ≤.006 are significant at $\alpha = 0.05$ after correction, given that 20 tests were performed with an average marker $R^2 = 0.3$). Associations with vertebral BMC and bone area were driven mainly by men, in whom average differences between carriers and noncarriers of the exon 9 c.2047A rare allele were ≤0.67 Z scores (fig. 2). In contrast, differences in stature between exon 9 c.2047G→A genotypes were similar for both sexes (fig. 2), corresponding to an average 2.0 cm lower adult height in carriers of the rare A allele. To our knowledge, this finding constitutes the first genetic variant to be linked to stature in humans, although it explained <5% of the population-based variance for the trait.

Although no significant associations were observed with the other four "informative SNPs" (table 2), single SNPs may fail to capture all of the contribution of a locus to a particular trait. We therefore tested association, using inferred haplotypes assigned to each individual. Reconstruction of haplotypes with the five informative SNPs resulted in a number of haplotypes with low probability of correct assignment. Hence, we built haplotypes using four informative SNPs (c.1980G→A, c.2047G→A, c.3405A→G, and c.4037C→T), which yielded all haplotypes with >.98 probability of correct assignment (table 3).

The results of the haplotype association revealed significant Z-score differences in LS BMC and area among

Table 1

Characteristics of Subjects in Cross-Sectional Study

	Findings in Children and Adolescents		Findings in Adults	
Characteristic	Male	Female	Male	Female
No. of subjects	305	208	164	200
Age[a] (years)	9.1 ± 3.0	9.9 ± 3.0	34.4 ± 12.9	40.2 ± 8.0
Age range (years)	6.6–17.9	6.6–16.9	17.0–57.0	17.0–56.0
Height[a] (cm)	135.0 ± 17.7	137.7 ± 15.9	178.0 ± 6.5	164.0 ± 6.3
Weight[a] (kg)	31.6 ± 12.9	33.4 ± 11.9	74.3 ± 9.4	61.7 ± 9.2
LS aBMD[a] (g/cm^2)	.629 ± .144	.706 ± .167	1.051 ± .141	1.059 ± .121
LS BMC[a] (g)	20.64 ± 9.94	21.63 ± 11.13	56.26 ± 10.24	48.13 ± 7.84
LS area[a] (cm^2)	31.61 ± 6.48	29.11 ± 7.22	53.28 ± 4.42	45.29 ± 3.94

[a] Mean ± SD.

Table 2

***P* Values for Association of *LRP5* Polymorphisms with Vertebral Bone Mass and Stature in Adults**

	P Value for Genotype[a]					
Variable	IVS4-4 T→C	Exon 9 c.1980 G→A	Exon 9 c.2047 G→A	Exon 15 c.3405 A→G	Exon 18 c.4037 C→T	Haplotype[b]
aBMD	.820	.087	.041	.551	.413	.062
BMC	.716	.213	***.003***	.294	.182	***.009***
Area	.531	.612	***.001***	.190	.092	***.011***
Stature	.569	.852	***.006***	.733	.159	.447

Note.—*P* values for association with genotypes (and haplotypes) were calculated using ANCOVA, with weight and sex as independent covariates and aBMD, BMC, bone area of the L2–L4 vertebrae, and stature standardized for age and sex (*Z* scores) as dependent variable. Significant *P* values, after correction for multiple comparisons, are in bold italics.

[a] Subjects homozygous for the rare alleles c.1980A, c.2047A, and c4037T were grouped with heterozygous carriers of the respective genotypes.

[b] Haplotypes were built using the four exonic SNPs, as described in detail in table 3, and subjects were divided into 13 groups reflecting all haplotype combinations that were present in the cohort.

adults of different haplotype groups, which remained statistically significant after correction for multiple comparisons (table 2; fig. 3*A*). Stature, on the other hand, showed no significant differences with the haplotype analysis, suggesting that only the c.2047 variant is likely to play a role in the determination of this trait.

For all three LS bone parameters (aBMD, BMC, and area) there was a marked trend for lower *Z* scores in individuals carrying haplotype 4. Since haplotype 4 is the sole haplotype that carries the c.2047A allele, which was strongly associated with lower bone mass and size when analyzed independently, this finding is consistent with the genotype data. In addition, there was a trend for higher *Z* scores in individuals with haplotype combinations 3,1; 3,2; and 3,3. However, since these groups are scarcely populated ($n \leqslant 5$ subjects/group), and the 0,3 group ($n = 39$) has *Z* scores close to zero, this analysis did not support a contribution of haplotype 3 to vertebral bone mass and size phenotypes.

To refine the haplotype analysis, we (1) performed a sex-specific comparison, since the genotype data had shown a mostly male-driven association, and (2) reduced the number of classes by considering only SNPs at positions c.2047 and c.4037, the two missense polymorphisms, which are sufficient to define the most interesting haplotypes. This resulted in only three haplotypes: haplotype 0′—which combines haplotypes 0, 1, and 2 (c.2047G-c.4037C)—and haplotypes 3 and 4, which are the same as those defined in table 3.

The results of this analysis (fig. 3*B*) confirm a strong sex-specific effect (as with the genotype data), since, whereas in the women, there are no significant differences between the haplotypic groups, in men, significant differences are present ($\leqslant$0.8 *Z* scores), mainly driven by haplotype 4. The increased *Z* scores observed for individuals carrying haplotype 3 suggest that this haplotype could also contribute to the vertebral bone traits in men and deserves further investigation.

Altogether, *LRP5* polymorphisms accounted for 4.0% and 3.8% of the adult population variance in LS BMC and area (independent of age and weight), respectively. It is remarkable that, in men, the contribution of *LRP5* alleles reached 15.4% and 12.7% for BMC and area, respectively.

Cross-Sectional and Longitudinal Association Study in Childhood

Similar association studies with genotypes and haplotypes were performed independently in the children and adolescent group of our cohort, but only marginal associations with aBMD and BMC were observed for the exon 9 c.2047 SNP (data not shown), and no associations were observed with the haplotypes (table C [online only]).

These observations led us to hypothesize that *LRP5* variants might influence vertebral bone size and mass gain during growth, in which case, association of *LRP5* alleles with the cross-sectional bone measurements would not be fully expressed in children. To test this hypothesis, we studied the association of *LRP5* variants with longitudinal changes in LS parameters in 386 children from the original cohort who were remeasured after 1 year (148 girls and 238 boys). Since the polymorphism at position c.2047 and the two-SNP haplotypes (c.2047 and c.4037) were the most informative in the adults, we focused on those markers for the longitudinal analysis. As shown in figure 4*A,* the GA/AA genotypes were indeed associated with smaller gain in bone area in the boys. This was not observed in the growing girls (see below), consistent with the adult data, in which most of the association with vertebral *Z* scores was driven by men (fig. 2).

We then looked at haplotype association with changes in vertebral bone mass and size, using the reduced number of haplotypes already defined in the adults, namely 0′, 3, and 4. Age, weight, and calcium intake at baseline and during follow-up, all factors known to influence

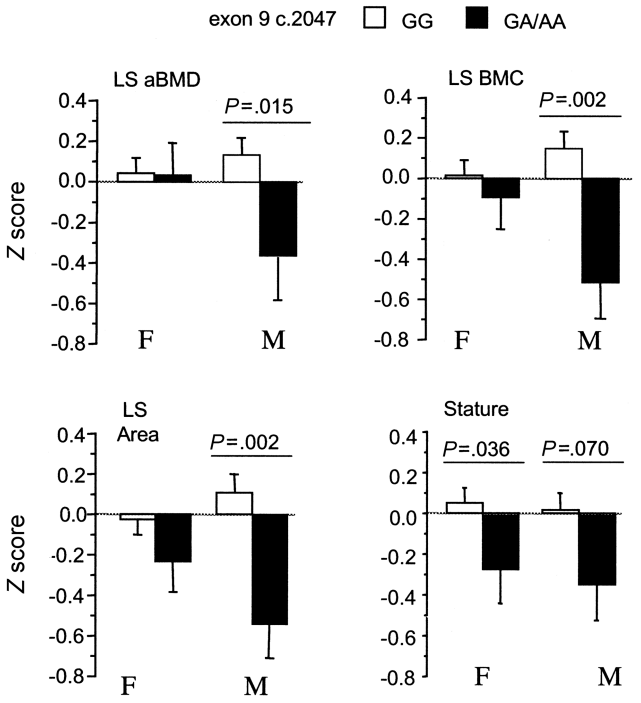

Figure 2 Differences in adult LS bone measurements and stature, according to *LRP5* exon 9 missense SNP and sex. Stature and aBMD, BMC, and bone area at the LS were measured cross-sectionally in 364 adult subjects of both sexes. Results are mean *Z* scores ± SEM adjusted for age and sex. Only significant *P* values (measured by ANCOVA), with weight as covariate, within sexes are shown.

bone-mass gain in childhood, were similar among haplotypic groups (table C [online only]).

Significant differences between the haplotype groups were observed only in the growing boys (fig. 4*B*), with carriers of haplotype 4 having lower BMC and area gains, particularly when compared with carriers of haplotype 3, who showed a slight trend for higher gains. The pattern in the growing boys resembled that of the adults (fig. 3*B*), suggesting a consistent sex-related effect of *LRP5* to LS bone-mass and size acquisition.

In the girls, a nonsignificant trend for higher gains was observed for carriers of the c.2047A variant and haplotype 4, opposite to what was seen in the boys. But since this effect was not present in the adults, the biological relevance of this observation is uncertain.

Discussion

We performed a cross-sectional association study to analyze the contribution of *LRP5* polymorphisms to variation in LS bone mass and size in a large sample of white Europeans. To this end, we studied the LD structure of the gene and selected five SNPs that provide most of the genetic information for this locus, which we genotyped in 364 healthy adults and 513 children and adolescents. We identified a missense substitution in exon 9 of the gene (c.2047G→A) that, in adults, is significantly associated with LS bone mass (BMC), projected bone area, and stature and is marginally associated with LS aBMD. Our findings are therefore consistent with previous reports of QTLs for bone-mineral density and stature in humans, both mapping to 11q12-13 (Koller et al. 1998; Hirschhorn et al. 2001; Perola et al. 2001; Carn et al. 2002; Livshits et al. 2002), the genomic region where *LRP5* is located. Together with variants in the vitamin D–receptor gene (van der Sluis et al. 2003), *LRP5* genetic variation is one of the first identified common genetic determinants of vertebral bone size and stature in whites.

To better describe the contribution of the locus to the determination of LS bone phenotypes, we studied the *LRP5* genetic variants in the context of their haplotypes. Recent publications involving large cohorts, multiple SNPs, and haplotype analysis, such as for *TNFRSF1B* and *ESR1* (Albagha et al. 2002; van Meurs et al. 2003), have indeed shown robust associations between haplotypes and bone phenotypes.

The haplotype associations revealed large differences in bone parameters between the different groups, which were as much as 2 SDs in some cases (fig. 3*A*). The main genetic determinant behind the genotype and haplotype associations was the c.2047G→A variant that defines haplotype 4. Nevertheless, the increase in *Z* scores observed for carriers of haplotype 3 in the males (fig. 3*B*) suggests that additional polymorphic sequences within this locus might contribute to the vertebral bone traits.

The associations observed in the adult group were clearly driven by the men, as seen in figures 2 and 3*B*, suggesting that some sex-specific factors, such as gonadal steroids (which are known to play a major role in bone homeostasis [Rizzoli and Bonjour 1997; Khosla et al. 2001]) or sequences in the X or Y chromosomes, directly or indirectly affect the action of *LRP5* on bone phenotypes. Similar findings have recently been reported

Table 3

Haplotypes of "Informative SNPs" in the Cross-Sectional Cohort

	Nucleotides for Haplotype (n = 889)				
SNP	0[a]	1[b]	2[c]	3[d]	4[e]
Exon 9 c.1980 G→A	G	A	G	G	G
Exon 9 c.2047 G→A	G	G	G	G	A
Exon 15 c.3405 A→G	A	G	G	G	G
Exon 18 c.4037C→T	C	C	C	T	T

[a] Frequency (%) = 65.8.
[b] Frequency (%) = 6.52.
[c] Frequency (%) = 11.7.
[d] Frequency (%) = 8.44.
[e] Frequency (%) = 7.4.

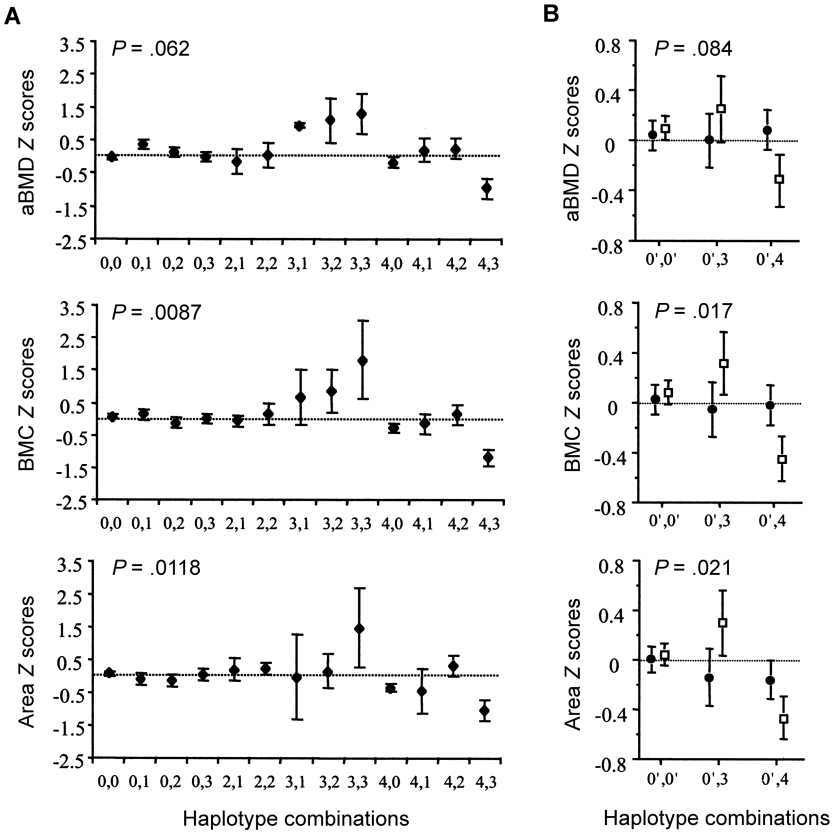

Figure 3 Differences in adult LS measurements, according to *LRP5* haplotypes. aBMD, BMC, and bone area at the LS were measured cross-sectionally in men and women and were expressed as standardized *Z* scores (±SE). Haplotypes were based on SNPs c.1980, c.2047, c.3405, and c.4037, as detailed in table 3. Sizes for haplotype groups in adults of both sexes (*panel A*) are as follows: 0,0 $n = 156$; 0,1 $n = 21$; 0,2 $n = 50$; 0,3 $n = 39$; 2,1 $n = 4$; 2,2 $n = 5$; 3,1 $n = 2$; 3,2 $n = 5$; 3,3 $n = 2$; 4,0 $n = 44$; 4,1 $n = 5$; 4,2 $n = 7$; 4,3 $n = 4$. Group sizes for the reduced haplotype combinations in female/male (females indicated by blackened circles; males indicated by unblackened squares) (*panel B*) are as follows: 0′,0′ (0′=2047G-4037C), $n = 133/112$; 0′,3 (3=2047G-4037T), $n = 29/19$; 0′,4 (4=2047A-4037T), $n = 31/27$. *P* values for overall differences between haplotype groups were calculated by use of ANCOVA, with weight as a covariate.

for haplotypes in the *ESR1* gene, which were found to be associated with vertebral bone area and fracture risk in females but not in males (van Meurs et al. 2003). These data support the notion that loci conferring risk for spine osteoporosis frequently do so in a sex-specific manner.

To confirm and further dissect the associations observed in the adults, we performed similar cross-sectional studies in the children, but no significant differences were observed at that stage of development. This might be explained by the fact that *LRP5* gene variants influence the bone phenotypes during growth; hence, children have not had time to express these differences. To directly test this hypothesis, we performed a 1-year longitudinal study of 386 children from the original cohort.

We observed significant differences in bone-mass and size gain in growing boys that were consistent with the pattern of *Z*-score distribution observed in the men, in that boys carrying haplotype 4 had significantly smaller gains when compared with the other groups, in particular with those individuals carrying haplotype 3 (fig. 4*B*). As with the adults, the significant *P* value was mainly driven by haplotype 4; however, the fact that haplotype 3 carriers also showed a mild effect is interesting. In contrast, growing girls carrying haplotype 4 appeared to have a marginally higher bone-mass acquisition compared with the other groups. Since these differences did not translate into significant *Z*-score differences in women, they are unlikely to be biologically relevant.

It is also interesting to compare haplotype-specific gain in bone mass across sexes, since, although the overall amount of gain (for both phenotypes) is roughly the same in both sexes, its distribution among haplotype groups is very different. Thus, there would seem to be clear interactions between haplotypes 3 and 4 and sex (*P* interaction = .0008 for BMC and .026 for area).

Overall, the results of the longitudinal study indepen-

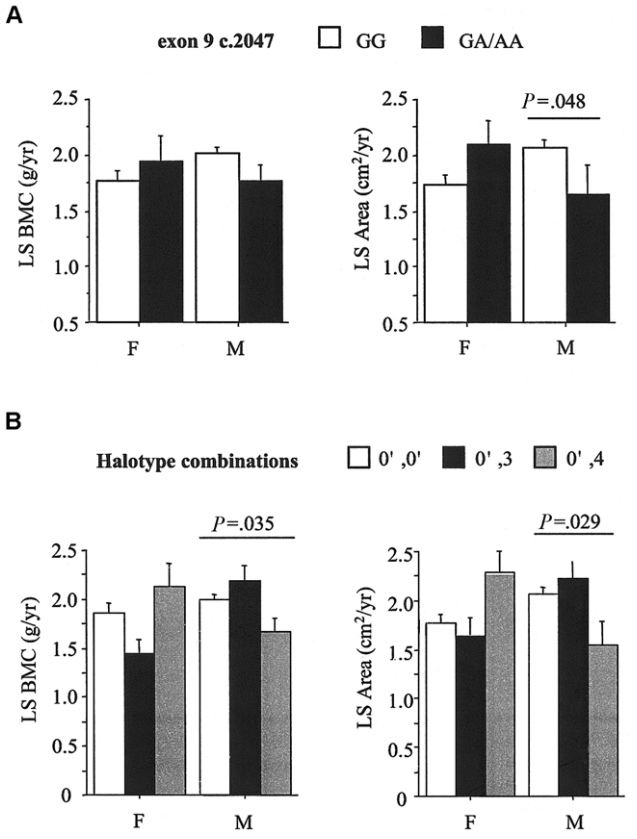

Figure 4 Changes in LS bone size and mass during childhood, according to *LRP5* exon 9 c.2047 genotypes and haplotypes. Mean changes (±SEM) in LS BMC and projected area were evaluated between baseline and 1 year in prepubertal males (M) and females (F). Panel A shows mean changes according to c.2047 genotypes, panel B according to reduced haplotype combinations, as in figure 3*B*. The number of females/males in each group is as follows: 0′,0′, n = 109/168; 0′,3, n = 22/38; 0′,4, n = 16/29. Significant P values for differences between exon 9 genotypes and between haplotypes 3 and 4 are shown.

dently confirmed the association of haplotype 4 to lower Z scores (for BMC and area) in the men and suggest that the main effect of *LRP5* on bone-phenotype variation is during growth, when it affects bone mass at the spine, mainly through the determination of vertebral-bone size (Seeman 2002).

An important aspect of our study that deserves further investigation concerns the functional role of the c.2407G→A SNP that was found to be associated with LS bone traits. Although it results in an amino acid substitution (p.V667M), it is not clear whether and how it affects the function or expression of the LRP5 protein. The localization of the p.V667M substitution at the top of the third propeller module in the receptor extracellular domain, similar to most missense mutations causing the OPPG and HBM syndromes, is intriguing (Gong et al. 2001; Boyden et al. 2002; Little et al. 2002). However, in vitro assays to functionally test the consequences of this polymorphism are necessary to understand whether this SNP is causative or is in LD with some other genetic variation, outside the coding sequence, that might have a regulatory effect (Twells et al. 2003). In addition, further characterization of haplotype 3, either by functional tests or by additional population- or family-based studies, should help clarify that haplotype's potential functional involvement.

In summary, our findings indicate that *LRP5* allelic variation contributes significantly to the determination of vertebral bone mass and size, mostly in white males. Taken together with the accumulating strong evidence of a major role of *LRP5* in bone metabolism, *LRP5* variants appear as potentially important genetic susceptibility factors for osteoporosis and vertebral fractures, particularly in men.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

dbSNP Database, http://www.ncbi.nlm.nih.gov/SNP/ (for SNPs IVS4-4T→C [accession number rs314776], exon 9 c.1980 G→A [accession number rs2277268], exon 9 c.2047 G→A [accession number rs4988321], exon 10 c.2268 C→T [accession number rs2306862], IVS10 +6T→C [accession number rs4988322], exon 15 c.3405 A→G [accession number rs556442], exon 18 c.4037 C→T [accession number rs3736228], and exon 19 c.4137 C→T [accession number rs3736229])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for OPPG and HBM)

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