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Polymorphisms in the Sclerosteosis/van Buchem Disease Gene (*SOST*) Region Are Associated with Bone-Mineral Density in Elderly Whites

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Osteoporosis has a strong genetic component, but the genes involved are poorly defined. We studied whether the sclerosteosis/van Buchem disease gene (*SOST*) is an osteoporosis-risk gene by examining its association with bone-mineral density (BMD). Mutations in *SOST* result in sclerosteosis, and alterations in the *SOST* gene expression may be causal in the closely related van Buchem disease. We used a set of eight polymorphisms from the *SOST* gene region to genotype 1,939 elderly men and women from a large population-based prospective-cohort study of Dutch whites. A 3-bp insertion ($f = 0.38$) in the presumed *SOST* promoter region (SRP3) was associated with decreased BMD in women at the femoral neck (FN) ($P = .05$) and lumbar spine (LS) ($P = .01$), with evidence of an allele-dose effect in the oldest age group ($P = .006$). Similarly, a G variant ($f = 0.40$) in the van Buchem deletion region (SRP9) was associated with increased BMD in men at the FN ($P = .007$) and LS ($P = .02$). In both cases, differences between extreme genotypes reached 0.2 SD. We observed no genotype effects on fracture risk, for the 234 osteoporotic fractures validated during 8.2 years of follow-up and for the 146 vertebral prevalent fractures analyzed. We did not find association between any of several frequent haplotypes across the *SOST* gene region and BMD. We did find evidence of additive effects of SRP3 with the *COLIA1* Sp1 polymorphism but not with haplotypes of 3′ polymorphisms in the vitamin-D receptor gene. The *SOST-COLIA1* additive effect increased with age and reached 0.5 SD difference in BMD at LS in the oldest age group ($P = .02$). The molecular mechanism whereby these moderate *SOST* genotype effects are mediated remains to be elucidated, but it is likely to involve differences in regulation of *SOST* gene expression.

Introduction

Osteoporosis is a common disorder characterized by decreased bone-mineral density (BMD), degenerative microarchitectural changes in bone tissue, and an increased fracture risk. Certain aspects of the disorder show strong genetic influences. For example, the heritability of BMD has been estimated to be up to 80%, which suggests the existence of "bone density" genes (Smith et al. 1973; Pocock et al. 1987; Cummings et al. 1995; Flicker et al. 1995). Variance between individuals in the activity of these genes may be manifested in different ways—for example, by differences in peak bone mass or rates of bone loss with increasing age. Although fracture risk is also strongly inherited, it does not correlate absolutely with BMD, which suggests that bone density and bone architecture and/or strength probably are influenced by both shared and distinct genetic factors (Cummings et al. 1995; Flicker et al. 1996). In addition to genetic traits, the expression of osteoporosis is influenced by environmental factors, such as diet, exercise, and exposure to tobacco, alcohol, and sunlight. This multifactorial and polygenetic character makes osteoporosis a so-called complex disease.

A number of approaches have been taken to try to understand the genetic basis of osteoporosis (Uitterlinden et al. 2001; Ralston 2002). Although genome scans in large collections of related individuals have identified a number of potentially interesting chromosomal loci (Devoto et al. 1998, 2001; Duncan et al. 1999; Koller et al. 2000), significant linkage to the same loci is rarely observed in more than one study. Furthermore, such relatively large chromosomal areas still have to be searched for candidate genes, variants of which could explain the original linkage found.

In contrast, the candidate-gene approach builds on biological knowledge to implicate particular genes in bone metabolism. Evidence that would implicate certain genes can include specific expression and function in bone cells, genetically manipulated animal models with a bone phenotype, and human Mendelian disease that expresses

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a bone phenotype. The contribution of genetic variations in candidate genes involved in bone metabolism (formation, remodeling, microarchitecture, etc.) and bone density or fracture risk has been investigated in population-based association studies. So far, polymorphisms in several candidate genes have been investigated, including those encoding collagen type I α1 (Grant et al. 1996; Uitterlinden et al. 1998), vitamin-D receptor (*VDR*) (Morrison et al. 1994; Uitterlinden et al. 1996), and estrogen receptor (Kobayashi et al. 1996; Becherini et al. 2000; van Meurs et al. 2003). "Risk alleles" for some of these genes have been observed in some populations, but the polymorphisms are generally associated with modest effects on the phenotypes of interest. Of these, the collagen type I α1 Sp1 polymorphism has been found, in a number of studies, to be consistently associated with differences in bone density and fracture risk, as demonstrated by two meta-analyses (Efstathiadou et al. 2001; Mann et al. 2001).

The gene that encodes sclerostin, or *SOST,* is mutated in the disorder sclerosteosis (SCL [MIM 269500]), which is characterized by massive, progressive bone overgrowth throughout life (Hansen 1967; Beighton 1988; Balemans et al. 2001; Brunkow et al. 2001). Along with thicker-than-normal trabeculae and cortices, the BMD in affected individuals can be up to twice that of unaffected individuals (Epstein et al. 1979; Stein et al. 1983). In contrast to conditions that result in osteopetrosis, SCL is most likely a defect in osteoblast function, as evidenced by elevated alkaline phosphatase, increased rates of bone formation, and generally normal numbers and activity of osteoclasts. No obvious endocrinological abnormalities have been reported. van Buchem disease (VBD [MIM 239100]) is a similar disorder, with generally less-severe clinical outcomes (van Buchem et al. 1955, 1976; Beighton et al. 1984). Although the exact molecular mechanism for its manifestation is unknown, a 52-kb deletion located 35 kb downstream of the *SOST* gene was suggested to play some role, perhaps by affecting expression of *SOST* and/or other tightly linked gene(s) (Balemans et al. 2002*b*; Staehling-Hampton et al. 2002).

Sclerostin is a cystine knot–containing, secreted glycoprotein most similar in sequence to the DAN (differential screening-selected gene aberrative in neuroblastoma) family of bone-morphogenetic-protein (BMP) antagonists (Brunkow et al. 2001) and more distantly related to the BMP antagonists noggin and chordin. Members of the DAN family—including DAN, Cerberus/CER1, Gremlin/DRM/CKTSF1B1, PRDC, Caronte, and Dante (Hsu et al. 1998; Pearce et al. 1999)—exert their effects by negatively regulating the activity of specific BMPs in a wide variety of processes, such as differentiation, mitogenesis, chemotaxis, and apoptosis. The *SOST* gene is expressed most highly in cells involved in osteogenesis—specifically differentiated osteoblasts and osteocytes—and, like its related family members, has been shown to act via specific BMPs (Winkler et al. 2003).

This evidence, together with the very specific effect on bone mass seen in the autosomal recessive disorder SCL, led us to ask whether the *SOST* gene plays a role in the genetic control of bone mass and/or predisposition to bone fracture in elderly individuals. We report on the relationship of eight polymorphisms across the *SOST* gene region to bone density and to the occurrence of osteoporotic fractures in 1,939 elderly individuals from a population-based prospective-cohort study of diseases in the elderly in the Netherlands—the Rotterdam Study (Hofman et al. 1991). Furthermore, we studied the interaction between polymorphisms in the gene that encodes sclerostin and polymorphisms in two genes previously implicated by us and others to play a role in the genetic control of bone density and fractures—that is, *VDR* and the collagen type I α1 gene (*COLIA1*).

Subjects and Methods

Study Subjects

Subjects were participants of the Rotterdam Study, a population-based cohort study of subjects aged $\geqslant$55 years who live in the Ommoord district of Rotterdam. The study was designed to investigate the incidence and determinants of chronic disabling diseases in the elderly. The rationale and design have been described elsewhere (Hofman et al. 1991). All 10,275 Rotterdam inhabitants who were aged $\geqslant$55 years were invited for baseline examination, between August 1990 and June 1993. Of those, 7,983 participated. Among the subjects living independently, the overall response rate was 77% for home interview and 71% for examination at a research center, where anthropometric characteristics and BMD were measured and blood samples were taken. The Rotterdam Study was approved by the medical ethics committee of the Erasmus University Medical School, and written informed consent was obtained from each subject.

For the present study, a subgroup of men and women was analyzed (see also Uitterlinden et al. [1998]). Baseline measurements of BMD were available for 5,931 subjects who were living independently. Of those, 1,453 were excluded because they were aged >80 years, used a walking aid, had diabetes mellitus, or were taking drugs known to affect bone metabolism (diuretics, estrogen, thyroid hormone, or cytostatic drugs). Of the 4,478 remaining subjects, we studied a random sample of 1,939 subjects comprising 1,016 women and 923 men.

Clinical Examination and Measurements

Height and weight were measured at baseline examination, with the subject in a standing position and wearing indoor clothing but not shoes. BMD (in g/cm^2) was

determined by dual-energy X-ray absorptiometry (DEXA) (DPX-L densitometer [Lunar]) at the femoral neck (FN) and lumbar spine (LS) (vertebrae L2, L3, and L4), as described elsewhere (Burger et al. 1994). Dietary intake of calcium (in mg/d) during the preceding year was assessed by a food-frequency questionnaire and was adjusted for energy intake. Age at menopause and current use of cigarettes were assessed by a questionnaire.

Assessment of Incident Nonvertebral Fracture

Follow-up started either January 1, 1991, or at the time of inclusion in the study, if later. For this analysis, follow-up ended either by January 1, 2000, or at the participant's death, whichever came first. The general practitioners of the participants provided data on morbidity, including nonvertebral fractures, and mortality. For ~80% of the study population, medical events were reported through computerized registers of general-practitioner diagnosis. For the remaining 20%, research physicians collected data from the general practitioners' medical records of the study participants. All collected data on fractures were verified by review of discharge reports and letters from medical specialists. Fracture events were coded independently by two research physicians, in accordance with the International Classification of Diseases, 10th revision. In case of discrepancy, consensus was attained in a separate session. A medical expert in the field reviewed all coded events for final classification.

Assessment of Vertebral Fracture

Both at baseline and at follow-up visits, between 1997 and 1999, thoracolumbar radiographs of the spine were obtained. The follow-up radiographs were available for the 1,184 individuals who survived an average of 7.4 years after the center visit for baseline assessment and who were still able to come to our research center. All follow-up radiographs were scored for the presence of vertebral fracture by the McCloskey/Kanis method (McCloskey et al. 1993), as described elsewhere (Van der Klift et al. 2002; Schuit et al. 2004). If a vertebral fracture was detected, the baseline radiograph was evaluated as well. If the vertebral fracture was already present at baseline, it was considered a baseline-prevalent fracture. If it was not present at baseline, the fracture was defined to be an incident. For 600 women and 519 men, lateral radiographs of the spine from the 4th thoracic to the 5th lumbar vertebra were measured at follow-up and were examined for the presence of prevalent vertebral fractures by morphometric analysis, as described above.

Identification of SOST*-Region Polymorphisms (SRPs)*

We used a number of different approaches to search for polymorphisms in the *SOST* gene region (see Brunkow et al. [2001] and GenBank [accession number AF326736] for complete description of the *SOST* gene structure): (1) a 21.5-kb genomic fragment—including both coding exons, the ~2.8-kb intron, and ~12 kb of 5′ and ~4.5 kb of 3′ flanking regions—was sequenced to completion from four sources (Afrikaner, Dutch white, and Senegalese individuals and human BAC clone 202l9 from California Institute of Technology BAC B and C libraries); (2) the two coding exons were sequenced to completion in a set of 90 ethnically diverse individuals from the National Institute of General Medical Sciences (NIGMS) Human Diversity Panel (panels HD01–HD09); (3) an additional 87.6-kb region 3′ of the gene was sample sequenced in the four sources of (1) above, by use of PCR primer pairs distributed across the region; and (4) a sequence from the *SOST* gene region found in National Center for Biotechnology Information (NCBI) databases was examined *in silico*. The examined *SOST*-gene flanking regions correspond to the *DUSP-SOST* and *SOST-MEOX1* intergenic intervals (GenBank accession numbers AF326736 and AF397423). All polymorphisms found by these approaches were also compared with those reported in a study by Balemans et al. (2002*b*).

Determination of Genotypes

Genomic DNA was extracted from samples of peripheral venous blood in accordance with standard procedures. Polymorphism-containing regions in the *SOST* gene locus were amplified from genomic DNA with PCR. Each PCR was performed in a 10-μl reaction volume that contained 5 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM deoxy-NTP, 2 pmol of each primer (see table 1), 0.2 units of *Taq* polymerase (Promega), and 10 × PCR buffer (Promega) that contained 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DDT, 50% glycerol, 0.5% Nonidet-P40, and 0.5% Tween20. The reactions were performed in a 384-well thermocycler (MJ Research Tetrad), with different cycling protocols for each amplicon (table 1). The genotypes were detected with the single-base extension (SBE) procedure by use of SBE primers of different lengths (table 1). The SBE reactions were performed in accordance with details provided by the manufacturer (ABI Prism SNaPshot Multiplex Kit), with slight modifications. The genotypes thus generated were analyzed with software programs Gene Mapper 1.1 and Genotyper 3.7 (both from Applied Biosystems) and were checked visually. To confirm the accuracy of the genotyping, 150 randomly selected samples were genotyped for a second time with the same method. No discrepancies were found.

Three anonymous polymorphic restriction-enzyme-recognition sites at the 3′ end of the gene encoding *VDR*—that is, for *Bsm*I, *Apa*I and *Taq*I—were assessed in relation to each other by a direct molecular haplotyping PCR procedure, as described elsewhere (Uitterlinden et

Table 1

Rotterdam Study SRPs and Primers for SBE Genotyping

Polymorphism ID, Position[a], and Alleles	Frequencies (N = 1,939)	HWE P	PCR Primers (5′→3′)	PCR T_M[b] (°C)	SBE Primer (5′→3′)
SRP1:					
4102[c] (−7859):					
C	.72	.18	F: CTTTCCACAGGCTCGTCT; R: CTCTCAACCGGAAATGTCT	55	$(T)_4$GAAGGTAGCGCCACCTGCTG
G	.28				
SRP2:					
10356[c] (−1605):					
C	.60	.02	F: AGGTGGGGCTATAAGCATCCATCC; R: GTCCTTTGCCACCTGCCTCAACTT	55	$(T)_{10}$TTGTGAGAAGCTGGCCCTCC
T	.40				
SRP3:					
10565[c] (−1396):					
DEL	.62	.17	F: AGGTGGGGCTATAAGCATCCATCC; R: GTCCTTTGCCACCTGCCTCAACTT	55	$(T)_{16}$ATGATGGATGATGGAAAGGA
GGA	.38				
SRP4:					
11988[c] (+27):					
G					
A	NA[e]	...	F: TGCCCCAGCCTGTCCTCATT; R: GGCCGCCCTCCGTTCTC	55	NA[e]
SRP5:					
17965[c] (+6004):					
C	.95	.89	F: TAAGGTGGGATGGTCAACTGG; R: CAGGAGAATCACTTGAATCCG	60	$(T)_{22}$TAGTGGTAGTTAAACTGACAA
G	.05				
SRP6:					
18292[c] (+6331):					
A	.42	.15	F: TAAGGTGGGATGGTCAACTGG; R: CAGGAGAATCACTTGAATCCG	60	$(T)_{28}$AAGTTGCAGTAAGCCGAGAT
G	.58				
SRP7:					
42722[d] (+46121):					
T	.65	.93	F: CCCTACCTTACTGTCCGCCTCTCA; R: GTGCTACCTCTCGGGAAAACATAA	55	$(T)_{34}$TTTAGTATAAAAGCTGGCTC
C	.35				
SRP8:					
58874[d] (+62273):					
A	.65	.42	F: GAGCAACCGGCGTATCC; R: GGGGTTTCTTTCTGGCTCTCA	53	$(T)_{40}$TTTATAGTTCTTTCCTAGAC
G	.35				
SRP9:					
75707[d] (+79106):					
A	.60	.34	F: CCAGCAATGTTGAGGAAT; R: CGCAGGAAGGTGTGGAGA	53	$(T)_{46}$CTGCTCAGCAAGCAGTTCCA
G	.40				

[a] Numbers in parentheses indicate position relative to *SOST* initiation site. *SOST* translational start site corresponds to nt 48 of GenBank accession number AF326739.
[b] Number of cycles = 37.
[c] Position of polymorphism in GenBank accession number AF326736.
[d] Position of polymorphism in GenBank accession number AF397423.
[e] NA = not applicable.

al. 1996). This allowed us to determine the phase of the alleles at each of the RFLP loci. As a result, three frequent haplotype alleles were discerned—encoded 1 (baT; frequency in whites 48%), 2 (BAt; 40%), and 3 (bAT; 10%)—that combined to become six genotypes encoded 11, 12, 13, 22, 23, and 33. We excluded the less frequent haplotypes (4 and 5) from the analysis. Women with genotypes containing these two haplotypes represent 1.5% of this population. Detailed information on haplotype alleles and genotype frequencies in the Rotterdam Study can be found elsewhere (Uitterlinden et al. 1996). The Sp1 polymorphism of *COLIA1* was detected by PCR with a mismatched primer that introduces a diallelic restriction site, as described elsewhere (Uitterlinden et al. 1998). The test discriminates two alleles, G and T, at position exon 2 −348, which is the first base in the last of four Sp1-binding sites located in the first intron of *COLIA1* on 17q21 (Mann et al. 2001).

Statistical Analysis

Linkage disequilibrium (LD) and haplotype analysis.—The LD coefficient (D') between each pair of SNPs was calculated with the help of the software programs PHASE (Stephens et al. 2001) and HaploXT. PHASE was also used to reconstruct haplotypes over the *SOST* gene region (SRP1–6) for each individual. Hardy Weinberg equilibrium (HWE) was calculated, in accordance with standard procedures, by use of the χ^2 analysis.

Association analysis.—Subjects were grouped on the basis of genotype. We grouped subjects by allele copy number (0, 1, and 2, corresponding to noncarriers, heterozygous carriers, and homozygous carriers, respectively) for each allele (or haplotype). We allowed for three possible genetic models to explain differences between groups—that is, an allele-dose effect, a dominant effect, or a recessive effect. "Allele dose" was defined as the number of copies of a certain allele in the genotype. In case of a consistent trend reflected as an allele-dose effect, we performed a linear regression analysis to quantify the association. In case of a dominant or recessive effect of the test allele, analysis of (co)variance (ANCOVA) was performed to test for differences between two genotype groups. For dominant alleles, we compared test-allele carriers versus noncarriers, whereas, for recessive effects, subjects homozygous for the test allele were compared with heterozygous carriers combined with noncarriers.

To study interaction of genetic variants (i.e., *SOST* with *VDR* and *SOST* with *COLIA1*), subjects were analyzed in four groups on the basis of presence or absence of risk alleles for each gene. For reasons of power, we combined heterozygous and homozygous subjects for the risk allele into carriers of at least one copy of the risk allele.

Odds ratios (ORs) with 95% CIs were calculated by logistic-regression analyses to estimate the relative risk of fractures by genotype for the risk allele; the reference group comprised those with no copies of the risk allele. We first calculated crude ORs and then adjusted for potentially confounding factors (e.g., age, BMI, BMD, and age at menopause). To estimate nonvertebral fracture risk by genotype, we used Cox proportional hazard models, thereby taking into account potential differences in follow-up time. To estimate the risk of vertebral fractures, ORs with 95% CIs were calculated using logistic-regression models. We were not able to use Cox proportional hazard models for vertebral fractures, since the exact time of the events were not known. For reasons of power, all vertebral-fracture analyses were done with combined prevalent and incident vertebral fractures.

All statistical analyses were performed using SPSS version 10.1.0. P values are two-sided, and $P \leq .05$ was considered significant.

Results

SOST *Genotype, Allele Frequencies, and HWE*

We identified a number of informative polymorphisms in the *SOST* gene/VBD region by a variety of methods (see the "Subjects and Methods" section). We found only a single coding-region polymorphism that affected the amino acid sequence of sclerostin. This G11988A variant (SRP4) results in a Val→Ile change in the signal sequence. Our analysis of the *SOST* intron and flanking regions identified an additional 30 SRPs (fig. 1). We determined allele frequencies for 15 of the SRPs in a set of 90 normal DNA samples, which represent 10 individuals of African, 30 of Asian, and 50 of white descent. Nine SRPs were chosen for further study (SRP1–9) on the basis of their distribution across the *SOST* gene locus and their representation of a wide range (2%–50%) of minor-allele frequencies. SRP1 (C4102G), SRP2 (C10356T), and SRP3 (10565insGGA) are located in the 5′ flanking region; SRP4 (G11988A) is in the sclerostin signal sequence; and SRP5 (C17965G), SRP6 (A18292G), SRP7 (T42722C), SRP8 (A58874G), and SRP9 (A75707G) are located 3′ of the gene, the latter three falling within the region that is deleted in subjects with VBD (fig. 1) (Staehling-Hampton et al. 2002). There were no differences found in allele frequencies between men and women (results not shown).

Although present in the NIGMS samples at a frequency of 1.7%, the minor allele of SRP4 (Val/Ile in SOST signal sequence) was not detected in a subset of the Rotterdam elderly white sample and so was excluded from the association study. The genotype distributions for the remaining eight SRPs in the Rotterdam Study were found to be in HWE ($P > .05$), except for SRP2 ($P = .02$) (table 1).

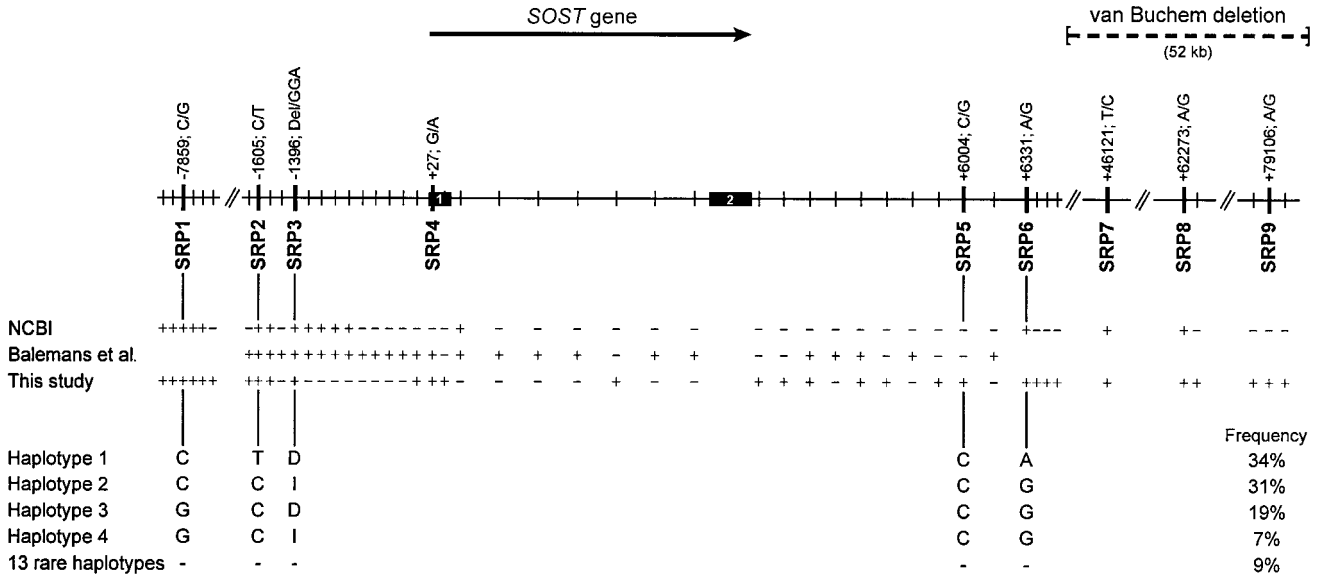

Figure 1 Structure of *SOST* gene region and overview of polymorphisms. The SRPs described in this study span ~87.3 kb of genomic sequence. The two *SOST* exons are indicated by solid boxes, and the 52-kb VBD deletion region is indicated by the heavy horizontal dashed line. Nucleotide positions are relative to the *SOST* initiation codon (+1) and GenBank sequences AF326736 and AF397423. Polymorphisms identified in the course of this study are compared with those reported by Balemans et al. (2002*a*) and those present in the NCBI SNP database and are indicated below the gene map as well as by their presence (+) or absence (−). Lack of a plus sign (+) or minus sign (−) indicates that the corresponding region was not included in the study. The four most common haplotypes across SRP1–3 and SRP5–6 identified in the Rotterdam Study population are also shown, with their respective frequencies (where n = 3,878 alleles).

LD and Haplotype Analysis

To get more insight into the pattern of LD between alleles at polymorphic loci, pairwise disequilibria measures (D') were calculated (fig. 2). Strong (although not complete) LD was observed between the SRPs surrounding the *SOST* gene (SRP1–3 and SRP5–6) and, to a lesser extent, between SRP7, -8 and -9. Using the PHASE program, we reconstructed haplotype alleles for each subject on the basis of the SRPs surrounding the *SOST* gene (SRP1–6). These are shown in figure 1, together with their frequencies in the 1,939 subjects from the Rotterdam Study. Because of the strong LD, we observed 4 frequent (>5% population frequency) haplotype alleles and 13 less frequent haplotype alleles among the 32 possible haplotypes. The four frequent haplotypes accounted for 91% of the chromosomes in our population. No differences in frequencies were observed between men and women.

Association between Genotype and BMD

We next analyzed the association of *SOST* gene polymorphisms with bone characteristics for the individual polymorphisms and for the haplotype alleles. We found a significant association of BMD with SRP3 in women and with SRP9 in men but not with the other six polymorphisms. Tables 2 and 3 show baseline characteristics by SRP3 genotypes for women and SRP9 genotypes for men, respectively. For both SRP3 and SRP9, the three genotype groups did not differ significantly in age, anthropometric characteristics, dietary calcium intake, or, for women, age at menopause.

Table 2 shows FN and LS BMD measurements in postmenopausal women, grouped by the three SRP3 genotypes. At both sites, BMD was highest in the homozygous DEL-DEL group and lowest in the homozygous GGA-GGA group; heterozygotes had intermediate values, which indicates an allele-dose effect. BMD in the DEL-GGA group was 2% less than the DEL-DEL group at both sites, whereas, in the GGA-GGA group, BMD was 4% less than that in the DEL-DEL group (table 2) (P = .01 for FN; P = .004 for LS). The effects of the SRP3 genotype on BMD at the Ward's triangle and trochanter sites were similar (data not shown). When the BMD values were adjusted for possible confounders, the differences decreased somewhat. In men, no association of SRP3 with BMD was found. When we compared SRP3-allele frequencies between groups composed of women in the highest and lowest quintiles of BMD, we found the GGA-insertion allele to be overrepresented in the lowest BMD quintile (data not shown).

The three SRP9 genotype groups differed significantly in FN BMD and LS BMD in men, but no association was found in women. At both sites, BMD was highest in the

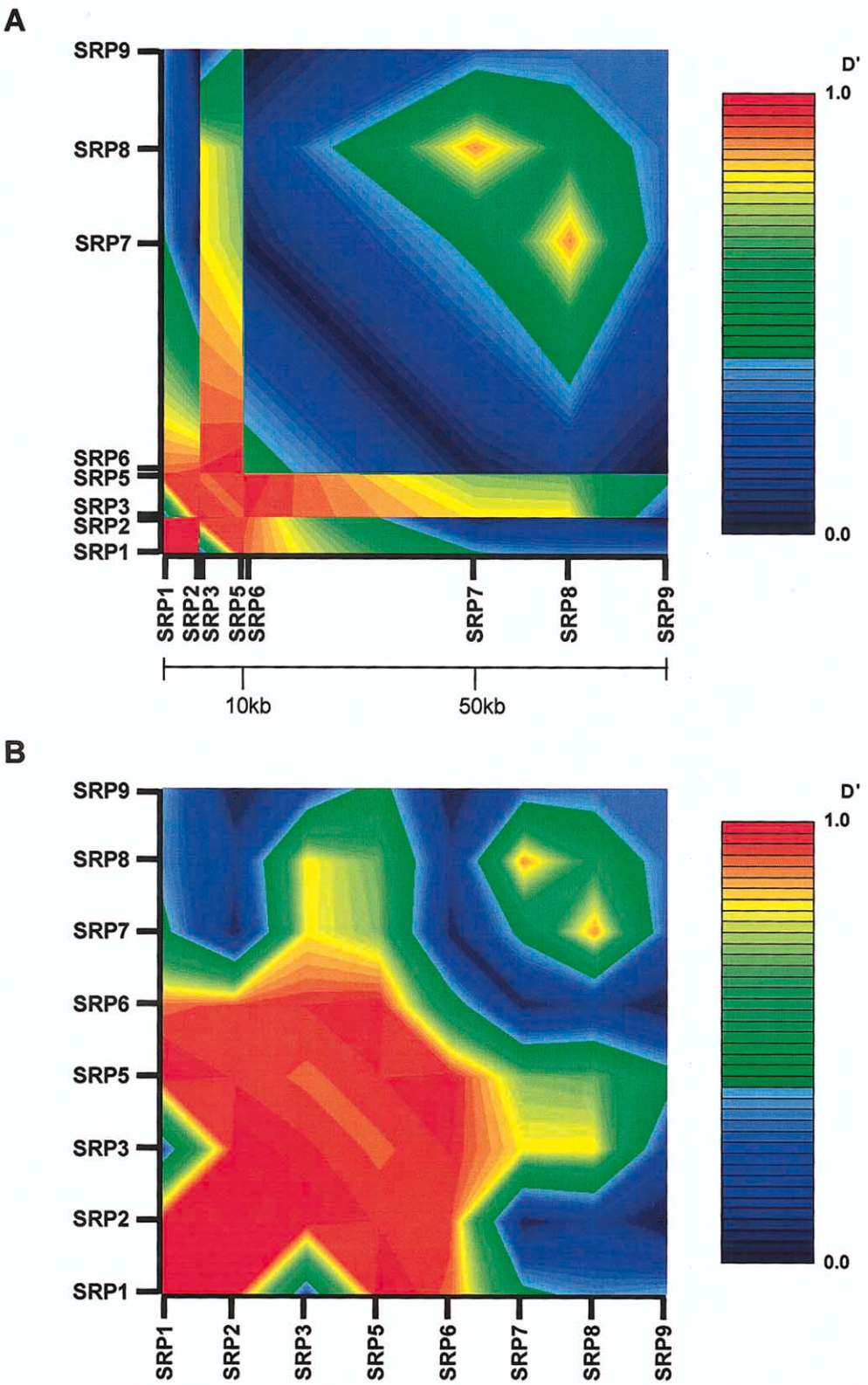

Figure 2 Pairwise LD *D′* values of eight SRPs across the *SOST* gene region. The software program PHASE was used to calculate values by use of true genomic distance (*A*) and by use of the SRPs at equal distance from each other (*B*).

Table 2

Characteristics of 1,016 Postmenopausal Women, by SRP3 Genotype

Characteristic	Findings by SRP3 Genotype			
	DEL-DEL (n = 380)	DEL-GGA (n = 503)	GGA-GGA (n = 133)	P
Frequency (%)	37	50	13	
Age (years)	67 ± 7	67 ± 7	68 ± 7	.27[a]
Height (cm)	162 ± 7	162 ± 6	162 ± 6	.80[a]
Weight (kg)	68.7 ± 10.0	68.6 ± 10.5	67.4 ± 9.7	.40[a]
Age at menopause (years)	48.7 ± 5.1	48.7 ± 4.9	48.3 ± 4.7	.69[a]
Dietary calcium intake (mg/d)	1,110 ± 389	1,104 ± 340	1,081 ± 291	.74[a]
Current smoker (%)	23	22	23	.91[a]
BMD (g/cm^2):				
FN:				
Unadjusted	.81 ± .13	.80 ± .12	.78 ± .12	.01[b]
Adjusted[c]	.81 ± .12	.80 ± .11	.79 ± .11	.05[b]
LS:				
Unadjusted	1.03 ± .17	1.01 ± .17	.99 ± .18	.004[b]
Adjusted[c]	1.03 ± .16	1.01 ± .16	.99 ± .16	.01[b]

[a] P value calculated with an ANCOVA test.
[b] P value calculated with a linear-regression model.
[c] Values are adjusted for age, weight, and height.

GG group and lowest in the AA group (table 3). BMD in the GA group was 1% less than that in the GG group at both sites, whereas, in the AA group, BMD was 3% less than that in the GG group at both sites (P = .02 for FN; P = .03 for LS). The effects of the SRP9 genotype on BMD at the Ward's triangle and trochanter sites were similar (data not shown). When the BMD values were adjusted for possible confounders, BMD measurements did not change essentially for LS, but differences increased somewhat for FN. When we compared SRP9 allele frequencies between groups composed of men in the highest and lowest quintiles of BMD, we found the A allele to be overrepresented in the lowest BMD quintile (data not shown).

We repeated the analyses using reconstructed haplotypes that contained SRP3 for each subject, estimated by the PHASE program. These analyses showed the associations we observed to be driven by the individual polymorphism (SRP3) rather than by a particular haplotype (data not shown). We also tried the same analyses with haplotypes of SRP7–9, but reconstruction of the haplotypes in each individual could not be estimated reliably by PHASE.

Association between LS-BMD and SRP3 in Females Is Age Related

BMD declines with age, and genetic effects on BMD could theoretically be mediated by differences in the age-related rate of bone loss as well as by differences in peak bone density. We therefore assessed the effect of age on the relationship between the SRP3 and SRP9 genotypes and BMD. The study subjects were stratified by age into tertiles (aged 55–63, 64–71, and 72–80 years). Figure 3 illustrates that, for SRP3, the differences in LS BMD became larger with increasing age in females only. There were no differences in LS BMD between the different genotypes in the youngest age group, whereas an intermediate difference in BMD appeared in the middle age group. In the oldest age group, the difference in BMD between the DEL-GGA group and the DEL-DEL group was 4% (P = .05 [ANCOVA]), and the difference in BMD between the GGA-GGA group and DEL-DEL group was 8% (P = .004 [ANCOVA]). We found significant evidence to suggest an allele-dose effect of the SRP3 GGA-insertion allele with decreased LS BMD (P = .006). When we tested for interaction between genotype and age, the interaction term did not reach significance (P = .13).

No age-related association between BMD and SRP3 genotypes was found at the FN site. Similar analyses for SRP9 genotype in men found no effect of age on the BMD differences between the different genotype groups.

No Association between SRP3 and SRP9 Genotypes and Fractures

In our study population, during an average follow-up period of 8.2 years, we captured 234 incident osteoporotic fractures (164 in women; 70 in men) and 146 prevalent vertebral fractures (81 in women; 65 in men). Table 4 shows the distribution of fractures according to the SRP3 genotype in females or the SRP9 genotype in males. No significant differences were found between the genotype groups. In addition, the risk estimates, as calculated by regression analysis, did not show significant

differences from unity, even when adjusted for possible confounding factors such as age, height, and weight (data not shown).

Since the BMD association was observed to be partly dependent on age, we also assessed the effect of age on the fracture-risk estimates. For reasons of power, we stratified the subjects into two age groups (aged 55–67 and 68–80 years). The same analyses as described above were performed, but no significant association was found (data not shown).

Interactions between SOST *and* VDR *or* COLIA1 *Genotypes*

We then studied possible interaction between polymorphisms in the *SOST* gene and two genes previously found to be associated with differences in BMD; that is, the *VDR* gene (12q13) and the *COLIA1* gene (17q21.3). We first analyzed *VDR* genotypes on the basis of haplotypes constructed of three polymorphisms at the 3′ end of the *VDR* gene, as described elsewhere (Uitterlinden et al. 1996), in relation to SRP3 and SRP9 genotypes, but we could not find evidence to suggest interaction or additive effects on BMD or on fracture risk (data not shown).

At the *COLIA1* gene, we analyzed the G→T Sp1 polymorphism for which we previously demonstrated an association with BMD and fracture risk in subjects from the Rotterdam Study (Uitterlinden et al. 1998). In the present study, we defined four groups of women, on the basis of their combined genotype, by presence or absence of risk alleles for *COLIA1* and *SOST*. The groups were those having no risk alleles (reference group), those having at least one copy of the *COLIA1* risk allele but no SRP3 risk allele (the "*COLIA1*" group), those having at least one copy of the SRP3 risk allele (the "SRP3" group), and those having at least one copy of either risk allele (the "*COLIA1*+SRP3" group). We observed a strong additive effect for these two polymorphisms. Whereas SRP3 and *COLIA1* Sp1 by themselves were associated with decreased LS BMD, the combined-risk–genotype group showed an even greater effect. Because both SRP3 and *COLIA1* Sp1 showed modification by age, we next analyzed the additive genotype effect on

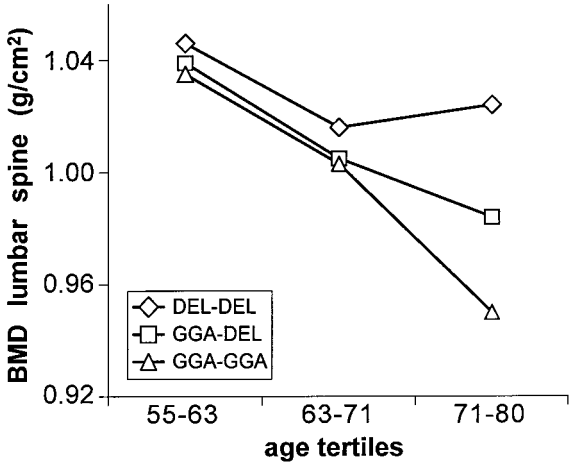

Figure 3 LS BMD in women, stratified by tertiles of age and by SRP3 genotype. The three possible SRP3 genotypes are indicated by diamonds (DEL-DEL), squares (GGA-DEL), and triangles (GGA-GGA). *P* values for linear regression were adjusted for age, height, and weight. The breakdown of subjects aged 71–80 years was DEL-DEL, $n = 118$; GGA-DEL, $n = 176$; and GGA-GGA, $n = 51$.

Table 3

Characteristics of 923 Men, by SRP9 Genotype

Characteristic	Findings by SRP9 Genotype			
	GG ($n = 154$)	GA ($n = 452$)	AA ($n = 317$)	*P*
Frequency (%)	17	49	34	
Age (years)	67 ± 7	67 ± 7	67 ± 7	.84[a]
Height (cm)	175 ± 7	174 ± 7	175 ± 7	.10[a]
Weight (kg)	77.8 ± 9.9	78.0 ± 10.4	78.6 ± 12.4	.65[a]
Dietary calcium intake (mg/d)	1,132 ± 467	1,140 ± 385	1,154 ± 420	.86[a]
Current smoker (%)	30	29	36	.13[a]
BMD (g/cm²):				
FN:				
Unadjusted	.88 ± .13	.87 ± .14	.86 ± .12	.02[b]
Adjusted[c]	.89 ± .12	.88 ± .13	.86 ± .12	.007[b]
LS:				
Unadjusted	1.17 ± .20	1.16 ± .19	1.13 ± .19	.03[b]
Adjusted[c]	1.17 ± .19	1.16 ± .19	1.13 ± .18	.02[b]

[a] *P* value calculated with an ANCOVA test.
[b] *P* value calculated with a linear-regression model.
[c] Values are adjusted for age, weight, and height.

LS BMD by tertiles of age for all women (fig. 4). Our results show that the additive effect is absent in the youngest tertile, is becoming apparent in the middle tertile, but shows greatest effect (0.5 SD) and reaches significance ($P = .02$) in the oldest age tertile. When we repeated the analysis of additive effects on risk of fracture, no genotype-dependent effect was observed, but numbers were small in each of the genotype groups across the age groups.

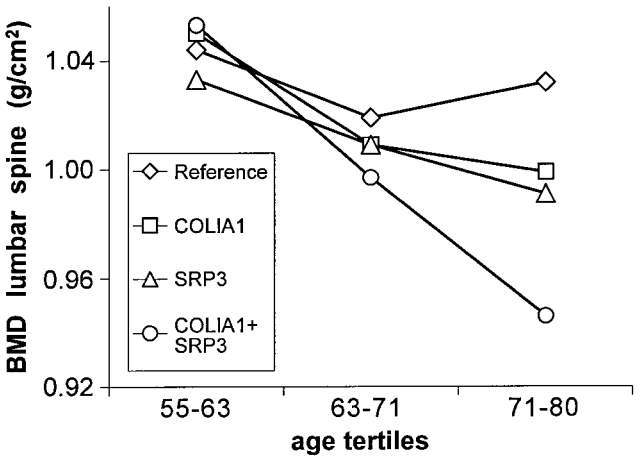

Figure 4 LS BMD in women, stratified by tertiles of age and by combined genotype for SRP3 and *COLIA1* Sp1 polymorphisms. The four possible genotype groups are indicated by diamonds (reference = no risk allele), squares (*COLIA1*), triangles (SRP3), and circles (COLIA1 + SRP3). ANCOVA *P* values were adjusted for age, height, and weight. The breakdown of subjects aged 71–80 years was reference, $n = 76$; *COLIA1*, $n = 41$; SSRP3, $n = 147$; and *COLIA1* + SRP3, $n = 74$.

Discussion

We and others have previously reported that loss-of-function mutations in the *SOST* gene region cause SCL (Balemans et al. 2001; Brunkow et al. 2001) and most likely VBD (Balemans et al. 2002*b*; Staehling-Hampton et al. 2002). These are monogenetic bone disorders with a severe bone phenotype that involve generalized hyperostosis. A number of recent experiments support the claim that the *SOST* gene product, sclerostin, is a key regulator of bone mass (Winkler et al. 2003; Van Bezooijen et al. 2004). On the basis of the observed activities of sclerostin in vitro and in vivo, we hypothesized that polymorphisms that occur in the *SOST* gene region, which have more subtle effects on regulation of expression and function of sclerostin, might influence differences in BMD in the general population. We therefore searched the *SOST* gene for frequently occurring DNA-sequence variations and evaluated whether *SOST* polymorphisms are associated with parameters of osteoporosis—that is, differences in BMD and in fracture risk—in a population of elderly whites.

Of 31 total variants discovered in the area surrounding the *SOST* gene, we chose eight common polymorphisms and one rare polymorphism for further study. The rare variant (SRP4) was the only example of a polymorphism affecting the amino acid sequence of sclerostin (G11988A/Val10Ile in the signal sequence). Although potentially of interest, its frequency was too low (<3% in whites) to allow association studies of sufficient statistical power, even in a population of the size we had available ($n = 1{,}939$). Of the polymorphisms we identified, several were present in the NCBI database or had been published elsewhere (Balemans et al. 2002*a*). The SRPs we identified in the VBD-deletion region are new. The set of nine polymorphisms described in the present study were selected to explore ethnic diversity (SRP1–9) and then to look for association with osteoporosis in whites, as well as to analyze ethnic diversity, LD, and haplotype structure (SRP1–3 and SRP5–9).

For most polymorphisms, the frequencies in the dif-

Table 4

Distribution of Fractures and ORs by Genotype in Women and Men

	Findings for			
	Incident Osteoporotic Fractures		Prevalent Vertebral Fractures	
Deletion Region and Genotype	Percentage with Fracture (No. with Fracture/Total)	OR[a] (95% CI)	Percentage with Fracture (No. with Fracture/Total)	OR[a] (95% CI)
SRP3 (in women):				
DEL-DEL	15.8 (60/380)	1.0	15.6 (36/231)	1.0
DEL-GGA	15.9 (80/503)	1.0 (.7–2.0)	11.5 (33/287)	.7 (.4–1.2)
GGA-GGA	18.0 (24/133)	1.2 (.5–3.0)	14.6 (12/82)	.9 (.5–1.9)
SRP9 (in men):				
GG	7.8 (12/154)	1.0	13.3 (11/83)	1.0
GA	7.5 (34/452)	1.0 (.4–2.2)	11.7 (31/264)	.9 (.4–1.8)
AA	7.6 (24/317)	1.0 (.4–2.3)	12.4 (23/172)	1.0 (.5–2.2)

[a] Unadjusted for possible confounders.

ferent ethnic groups were similar (in particular, between whites and Asians) (data not shown). We observed strong LD in the immediate surroundings of the *SOST* gene itself but much less LD with the polymorphisms in the VBD deletion region, which comprises sequences ~35–87 kb downstream of the gene body. The existence of such LD blocks of limited size (10–30 kb) is in line with observations of other areas of the human genome (Kruglyak 1999; Dunning et al. 2000; Abecasis et al. 2001; Daly et al. 2001; Reich et al. 2001). The strong LD is also indicated by the limited number of haplotypes across the *SOST* gene that we could reconstruct for whites in our Rotterdam Study sample by use of the PHASE program. The number of individuals in the other ethnic groups (in the NIGMS panel) is too low to allow for accurate LD measurement and reconstruction of haplotypes. It will, however, be of interest to see if the haplotypes we identified in whites are different from those in Asians and African Americans, a comparison of which could have important consequences for performing and interpreting association studies by use of these polymorphisms and haplotypes in nonwhite groups.

In our association study of elderly whites, we analyzed eight polymorphisms that were, with the exception of SRP2, all found to be in HWE. In view of the number of HWE tests we performed, we consider this finding to be due to chance.

We observed the GGA-insertion allele ($f = 0.38$) of the SRP3 polymorphism to be associated with decreased BMD in women (but not in men) at the FN and at the LS. The difference between extreme genotypes was 0.2 SD, with evidence of an allele-dose effect. For the SRP9 polymorphism, we found the A allele ($f = 0.60$) to be associated with decreased BMD in men (but not in women) at the FN and at the LS. Also, the difference between extreme genotypes was 0.2 SD, with evidence of an allele-dose effect. These associations seem real, given the size of the population, their consistency (effects at several sites of BMD measurement), and the plausible genetic model of gene-dose effects observed. Although the lack of association for a given polymorphism in both sexes would argue against this, such difference between sexes is not unusual. For example, the *COLIA1* Sp1 polymorphism has been found to be associated with BMD only in women and not in men (Uitterlinden et al. 1998; Mann et al. 2001). Although we acknowledge the potential difficulty with multiple comparisons in this study, several of the significance levels we observed are impressive enough to withstand conservative adjustments such as the Bonferroni correction. Together with the arguments presented above, we think the genotype-dependent differences we observe are modest but real. This situation is similar to that seen for effects of common polymorphisms on other complex traits (in addition to BMD and osteoporosis) (Lohmueller et al. 2003). Replication of our findings in other large populations, with a similar age distribution followed by meta-analysis, will help in establishing consistency and true effect size of these polymorphisms (Ioannidis et al. 2001).

When we analyzed the haplotypes across the *SOST* gene and the VBD deletion region, we observed that the associations were driven by the individual polymorphisms SRP3 and SRP9 rather than by a combination of allelic variants present on a haplotype allele. This could imply that there are no other important regulatory polymorphisms either in the sclerostin-promoter region or in the VBD deletion region, apart from the SRP3 and SRP9 polymorphisms, respectively.

So far, no functionality has been established for the SRP3 or the SRP9 polymorphisms. SRP3 is located ~1.4 kb upstream of the *SOST* transcriptional start site and thus may be important for interaction of promoter sequences with the transcriptional machinery. The insertion/deletion polymorphism falls within a short region that is highly conserved between humans and mice (80% identity over 315 bp), which suggests functional significance. In the absence of experimental data defining critical promoter sequences, we searched the TRANSFAC database (Wingender et al. 2000, 2001) for consensus binding sites for relevant transcription factors. SRP3 itself does not appear to fall within a known consensus site; however, it is closely flanked by one HOXA9- and two CBFA2-binding sites. It is also interesting to note that the conserved element falls within a region that appears to have a repressive effect on the activity of the proximal SOST promoter, although it is not clear from the published report which SRP3 allele was studied (Sevetson et al. 2004). The inclusion or exclusion of three base pairs in the chromosome could be envisaged to affect the interaction of DNA-protein complexes upstream and downstream of the region. For example, given the loss-of-function phenotype associated with SCL, one could propose that the SRP3 GGA-insertion allele enhances expression of *SOST* mRNA (through an effect on either positive or negative regulatory factors) and consequently the sclerostin protein, thus leading to decreased BMD. Molecular analysis of the *SOST* promoter region and complete characterization of important regulatory elements is necessary to establish the influence of these subtle polymorphic variants.

Interestingly, Balemans et al. (2002*a*) also analyzed the SRP3 polymorphism (termed "SNP5," which carries a TCC-insertion allele) but did not find any association with BMD in a population of 619 white Scottish perimenopausal women. We believe this can be explained by the age dependency that we observed for the association of SRP3 with BMD in women. In our group of women aged 55–63 years, which is close in age range

to those in the Balemans et al. study, we failed to see an association with BMD. Only when we examined women aged ⩾63 years did the association become apparent, and we observed the genotype effect to further increase with age. The SRP3 genotype effect, therefore, seems to be restricted to older age categories, probably due to modification by age-related factors. Such factors could encompass gene-gene interactions and gene-environment interactions, such as changes in diet, changes in hormonal factors, changes in mechanical loading, etc. In this respect, it is of interest to note that sclerostin is specifically and highly expressed in the osteocyte (Winkler et al. 2003; Van Bezooijen et al 2004), a bone cell that has been implicated in the mechanosensitivity of bone tissue. The evident decrease of mobility with age could therefore play an important role in the age-related increase in the genotype effect we observed. However, this obviously needs further study.

The G→A SRP9 polymorphism is located in the 52-kb VBD deletion region, an area that, when deleted, leads to a phenotype very similar to, albeit less severe than, that seen in SCL (Balemans et al. 2002*b*; Staehling-Hampton et al. 2002). Since there is no evidence of any functional genes within the region, it has been postulated that the VBD-deletion region contains important regulatory sites for the expression of sclerostin (Staehling-Hampton et al. 2002). This notion is supported by recent studies in transgenic mice (G. Loots, personal communication), although the exact mechanism by which *SOST* gene expression is regulated remains to be elucidated. Within the deletion area, several regions conserved between humans and mice have been identified, but SRP9 is not in any of these (B.W.P., S.P., and M.E.B., unpublished data). Therefore, the functional relevance of the SRP9 polymorphism for expression of sclerostin and how it might influence differences in BMD later in life needs further study.

In view of the importance of sclerostin in regulation of BMD and the previous observations of other gene variants that affect BMD, we studied combined effects of such gene variants. In particular, we analyzed sclerostin polymorphisms in relation to the *COLIA1* Sp1 polymorphism and the 3′ *Bsm*I, *Apa*I, and *Taq*I *VDR* polymorphisms. Although we could not see a combined effect of *SOST* with *VDR* polymorphisms, we did see an additive effect of the *SOST* SRP3 and the *COLIA1* Sp1 polymorphisms. In addition, we observed that the combined genotype effect increases with age. As discussed above, SRP3 is likely to affect expression of sclerostin, whereas the *COLIA1* Sp1 polymorphism has been demonstrated to result in increased expression of the COLIA1 protein, which results in a slight excess of COLIA1 homotrimers (Mann et al. 2001). Such homotrimers have been associated with decreased bone quality, including decreased BMD (Mann et al. 2001). Although the molecular mechanism of the additive effect of SOST and COLIA1 has not been established, variations in sclerostin expression are likely to interact with variations in COLIA1 expression. As a BMP antagonist, sclerostin is a key regulator of bone formation, a process that involves production of collagen and subsequent mineralization. Therefore, the production of collagen (including homotrimers) is likely to be under the influence of sclerostin regulation.

It has been well established that a decreased BMD results in increased fracture risk, and several studies have indicated that a 1-SD decrease in FN BMD is associated with 1.5–2.5-fold increase in fracture risk (Schuit et al. 2004). Although we observed genotype effects on BMD, we did not observe any effect of SRP3 or SRP9 on fracture risk. We hypothesize that this lack of association of the *SOST* polymorphisms with fracture risk is explained by the modest effect size of these polymorphisms (0.2-SD difference between extreme genotypes) and the fact that the recognizable effect of the *SOST* polymorphisms is probably restricted to BMD differences. Fracture risk is only partly explained by differences in BMD, which means that other factors, such as bone quality and bone geometry, are at least as important in determination of fracture risk. We analyzed a relatively large population-based study in which 234 incident osteoporotic fractures occurred and 146 prevalent vertebral fractures were diagnosed. This means that the methods set forth in our study have substantial power to detect an increase in fracture risk, given the SRP3 allele frequency of 38%. Yet, in view of the small effect size of SRP3 on BMD, we will need an even larger sample size than the one we had available ($n = 1{,}939$) to detect such effects on fracture risk.

In conclusion, two variants in the *SOST* gene region were associated with BMD differences and were found to be modified by sex (SRP3 and SRP9) and by age (SRP3). The effect size we observed on BMD was too small to be detected as genotype-dependent differences in fracture risk in our population study of 1,939 subjects. The mechanism that underlies the BMD differences remain to be established but is likely to involve modulation of expression of sclerostin. Further studies in additional populations of elderly men and postmenopausal women will be helpful in establishing consistency of our findings and in estimating the true effect size of these regulatory sclerostin polymorphisms.

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Electronic-Database Information

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

GenBank, http://www.ncbi.nih.gov/Genbank/ (for *SOST* [accession numbers AF326736, AF397423, and AF326739])
HaploXT, http://archimedes.well.ox.ac.uk/pise/ (for LD analysis)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for SCL and VBD)

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