

## ASSOCIATION OF POLYMORPHISM AT THE TYPE I COLLAGEN (COL1A1) LOCUS WITH REDUCED BONE MINERAL DENSITY, INCREASED FRACTURE RISK, AND INCREASED COLLAGEN TURNOVER

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**Objective.** To examine the relationship between a common polymorphism within intron 1 of the COL1A1 gene and osteoporosis in a nested case-control study.

**Methods.** We studied 185 healthy women (mean  $\pm$  SD age  $54.3 \pm 4.6$  years). Bone mineral density (BMD) was measured using dual x-ray absorptiometry, and fractures were determined radiographically. The COL1A1 genotype was assessed using the polymerase chain reaction and *Bal I* endonuclease digestion.

**Results.** Genotype frequencies were similar to those previously observed and in Hardy-Weinberg equilibrium: SS 61.1%, Ss 36.2%, and ss 2.7%. Carriage of at least one copy of the "s" allele was associated with a significant reduction in lumbar spine BMD ( $P = 0.02$ ) and an increased risk of total fracture ( $P = 0.04$ ). Urinary pyridinoline levels were significantly elevated in those with the risk allele ( $P < 0.05$ ).

**Conclusion.** These data support the findings that the COL1A1 gene polymorphism is associated with low BMD and fracture risk, and suggest a possible physiologic effect on total body turnover of type I collagen.

Osteoporosis is a common condition characterized by low bone mineral density (BMD), deterioration

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of the skeletal microarchitecture, and a consequently increased risk of fragility fracture. Family and twin studies have demonstrated that 50–85% of the population variance in BMD is attributable to genetic factors, findings that are consistent with the hypothesis that BMD is under strong genetic determination (1–5). Twin studies have also suggested that this genetic regulation of BMD may be mediated, at least in part, by an effect on bone turnover (6,7).

Type I collagen is the major protein constituent in bone, and in a few individuals with severe osteoporosis, coding sequence defects similar to those observed in osteogenesis imperfecta have been demonstrated (8). These structural mutations are, however, rare and unlikely to account for more than a small portion of the clinical cases seen (9). Sequence changes in regulatory regions of the type I collagen gene, rather than gross structural mutations within the protein-coding region, may also predispose to osteoporosis, with alterations in type I collagen metabolism. A single basepair polymorphism at an Sp1 binding site within the promoter region of the type I collagen  $\alpha 1$  gene (COL1A $\alpha 1$ ) has recently been associated with low BMD and vertebral fracture risk in 2 UK patient groups (10). Association studies may, however, be confounded by population admixture, heterogeneity, or stratification (11), and experience with another candidate gene, the vitamin D receptor, suggests that such preliminary results require confirmation in other populations (12). The candidacy of the potential locus is also enhanced if a functional significance can be demonstrated. We therefore examined the relationship between the COL1A1 polymorphism and BMD, osteoporotic fracture risk, and biochemical markers of type I collagen resorption in a cross-sectional analysis of women from a UK population cohort.

## SUBJECTS AND METHODS

**Study subjects.** The study design was a nested case-control study of subjects selected from a large general population cohort of 1,003 white women, with a mean ( $\pm$ SD) age for the total cohort of  $54.2 \pm 6.0$  years (13). This represents a good and efficient study design because the outcome was not known when subjects were recruited to the study, thereby minimizing bias, and only relevant individuals would require genotyping.

Women in the age range of 45–64 had been selected from a large single general practice in Chingford, northeast London (total of 11,000 registered patients), to participate in a longitudinal epidemiologic study of rheumatic diseases. A total of 1,353 women were found to be in the age range specified, and of these, 78% (1,003) agreed to participate in the study. The area is predominantly middle class, 98% are white, and the population is similar to UK normal subjects in terms of height, weight, smoking status, hysterectomy rates, and use of hormone replacement therapy (HRT). All women who participated in the present study were healthy and had no history of physical illnesses known to affect bone metabolism. All subjects gave informed consent, and the study protocol was approved by the local ethics committee.

**Questionnaires.** All subjects had completed a nurse-administered questionnaire detailing medical and gynecologic/obstetric histories, current and past medications including HRT, smoking status, alcohol consumption, dietary calcium intake, and exercise levels. Self-reported personal history of appendicular fracture was taken for the 10-year period preceding initiation of the study (1978–1988). Trauma details associated with the fracture event were also recorded by administration of a previously validated questionnaire (14).

**Fracture validation.** A self-reported personal history of appendicular fracture was obtained for the 10-year period preceding the beginning of the study. Peripheral fractures were subsequently validated from radiographs and from the records of both the general practitioner and the hospital (14). Lateral view radiographs of the thoracic (T4–T12) and lumbar (L1–L4) spine were obtained on all women according to standardized procedures. Past vertebral fractures were ascertained by morphometric analysis using a semiautomated digitizer and a validated algorithm that utilized standard deviation cut-offs of anterior and posterior height (15). A fracture was defined as at least two 1-SD deformities or one 3-SD deformity. These criteria have been shown to be associated with low BMD in population studies and are similar to more stringent cut-offs used by other groups of investigators (16,17). Women were categorized as cases for the present study if they had radiologic evidence of prevalent vertebral fracture and/or a validated appendicular fracture. Control subjects were those who had no radiologic evidence of vertebral fracture and no self-reported history of peripheral fracture.

**Bone densitometry.** BMD was measured at the lumbar spine (L1–L4) and femoral neck using dual x-ray absorptiometry (model QDR-1000; Hologic, Waltham, MA). Reproducibility, expressed as the coefficient of variation from duplicate measurements in healthy volunteers, was 0.8% at the lumbar spine and 1.6% at the femoral neck.

**Biochemical assessment.** Urinary collagen crosslinks pyridinoline and deoxypyridinoline were measured in urine

**Table 1.** Characteristics of 185 women, according to prevalent fracture status (cases or controls)

Variable*	Cases (n = 55)	Controls (n = 130)	P
Age, mean $\pm$ SD years	56.4 $\pm$ 5.2	53.4 $\pm$ 4.0	<0.0001
BMI, mean $\pm$ SD kg/m <sup>2</sup>	26.7 $\pm$ 4.7	25.6 $\pm$ 3.8	0.10
Menopause duration, median (interquartile range) years	8 (4–12)	4 (2–5)	<0.0001
No. (%) postmenopausal	52 (95)	122 (94)	0.85
No. (%) ever used HRT	15 (27)	6 (0.5)	<0.0001
No. (%) ever smoked	21 (38)	59 (45)	0.41
BMD, mean $\pm$ SD gm/cm <sup>2</sup>			
Lumbar spine	0.881 $\pm$ 0.154	0.975 $\pm$ 0.149	0.0001
Femoral neck	0.699 $\pm$ 0.122	0.763 $\pm$ 0.131	0.003

\* BMI = body mass index; HRT = hormone replacement therapy; BMD = bone mineral density.

samples obtained in the early morning following an overnight fast from a subgroup of women from the total cohort population. Urinary collagen crosslinks were assayed using ion-pair reverse-phase high-performance liquid chromatography in the presence of 1-octanesulfonic acid (18). Inter- and intraassay variations were <10% for both pyridinoline and deoxypyridinoline. This assay had been validated against standard gradient systems, where correlations of 0.95 (pyridinoline) and 0.92 (deoxypyridinoline) were observed between the two techniques in 27 normal women.

**Genotype assignment.** High molecular weight DNA was obtained from peripheral blood leukocytes by use of a phenol and chloroform extraction system. Polymerase chain reaction was performed using published primers, and the product was digested with the restriction enzyme *Bal* I (Promega, Madison, WI) as described by Grant et al (10). Alleles were coded as "S" (absence of restriction site) and "s" (presence of site).

**Statistical analysis.** Differences in demographic variables between the fracture cases and the controls, as well as between the COL1A1 genotypes were initially compared using either analysis of variance for normally distributed variables or chi-square test for categorical variables. The COL1A1 genotype frequencies were compared between the fracture and nonfracture cases using Fisher's exact test, with the odds ratio for fracture associated with COL1A1 genotype modeled using logistic regression. Because previous work had suggested a dominant pattern of risk associated with the "s" allele (10), analysis was subsequently performed on the combined "Ss" and "ss" genotype groups.

The study was designed to have 80% power ( $\alpha = 0.05$ ) to detect a 2-fold increased fracture risk associated with carriage of the "s" allele, and a 0.4 SD decrease in mean lumbar spine BMD between the "SS" and "Ss/ss" genotype groups. Preliminary analysis suggested that urinary collagen crosslink values were not normally distributed, and for subsequent analysis, natural logarithmic transformation was required to normalize the data. All analysis was performed using the PC software statistical program STATA.

**Table 2.** Characteristics of 185 women, according to COL1A1 genotype group

Variable*	SS (n = 113)	Ss (n = 67)	ss (n = 5)	P
Age, mean $\pm$ SD years	54.0 $\pm$ 4.6	54.8 $\pm$ 4.5	55.0 $\pm$ 6.3	0.53
BMI, mean $\pm$ SD kg/m <sup>2</sup>	26.0 $\pm$ 5.2	25.8 $\pm$ 3.9	26.4 $\pm$ 3.2	0.90
Menopause duration, median (interquartile range) years	4 (2-5)	4 (2-8)	5 (4-5)	0.76
No. (%) postmenopausal	106 (94)	63 (94)	5 (100)	0.85
No. (%) ever used HRT	11 (10)	9 (13)	1 (20)	0.62
No. (%) ever smoked	46 (41)	30 (45)	4 (80)	0.21
No. (%) with fracture	28 (25)	27 (40)	0 (0)	0.03
BMD, mean $\pm$ SD gm/cm <sup>2</sup>				
Lumbar spine	0.965 $\pm$ 0.163	0.912 $\pm$ 0.147	0.924 $\pm$ 0.087	0.14
Femoral neck	0.754 $\pm$ 0.146	0.726 $\pm$ 0.105	0.757 $\pm$ 0.097	0.38

\* BMI = body mass index; HRT = hormone replacement therapy; BMD = bone mineral density.

## RESULTS

Complete clinical and genotype data were available on 185 predominantly postmenopausal women, with a mean ( $\pm$ SD) age of 54.3  $\pm$  4.6 years. Fifty-five women had validated prevalent fractures. Descriptive details of the fracture cases and controls are shown in Table 1. In total, 21 women reported previous or current use of HRT, with a median duration of therapy of 9 months (interquartile range 3-12 months).

The COL1A1 genotype frequencies observed in the total group were similar to those previously reported in white subjects, and were in Hardy-Weinberg equilibrium: SS 61.1%, Ss 36.2%, and ss 2.7%. No significant differences in demographic characteristics were observed between the COL1A1 genotype groups (Table 2).

BMD was significantly reduced in the fracture cases compared with those without a history of prevalent fracture at both the spine and the hip (Table 1). No clear relationship between the 3 COL1A1 genotype groups and BMD at either the spine or the hip (Table 2) was seen, although this probably reflects the small number of subjects with the "ss" homozygous genotype. Pooling of the "Ss" and "ss" genotype groups under a dominant inheritance model, however, showed a relationship between the "s" allele and bone mass. BMD was significantly reduced at the lumbar spine in subjects with at least 1 copy of the "s" allele (mean difference 0.047 gm/cm<sup>2</sup>, 95% confidence interval [95% CI] 0.001, 0.093;  $P = 0.02$ ) (Figure 1). A similar trend was also seen at the femoral neck, although this difference was not significant (mean difference 0.026 gm/cm<sup>2</sup>, 95% CI -0.013, 0.065;  $P = 0.10$ ).

As previously stated 55 fractures were validated: 28 vertebral deformities and 27 low trauma appendicular fractures. We found that the risk genotypes ("Ss/ss") were higher in the prevalent fracture cases compared with the nonfracture cases, with frequencies of 49%

versus 33%, respectively ( $P = 0.04$ , by Fisher's exact test). Carriage of at least 1 copy of the "s" allele was associated with an increased risk of any fracture, with this increase in risk being modestly reduced after adjustment for BMD (Table 3). Site-specific analysis suggested a consistent trend of fractures at both the spine and appendicular sites being more commonly associated with the "s" allele, although these findings were not statistically significant for the individual skeletal sites because of the small numbers of cases.

The mean values for the urinary collagen crosslinks in the COL1A1 genotype groups are presented in Table 4. Pyridinoline concentrations were significantly elevated in subjects with the "Ss/ss" genotypes compared with the homozygous "SS" genotype. Levels of deoxypyridinoline did not, however, differ significantly between the genotype groups. These findings were essentially unaltered after adjustment for potential confounders (age, menopause duration, body mass index, ever use of HRT, smoking).

## DISCUSSION

Type I collagen is the major protein constituent of bone and is therefore a strong and plausible candidate gene for the regulation of BMD. Our data show an association between a single basepair polymorphism (G $\rightarrow$ T) within the regulatory region of the COL1A1 gene and risk of osteoporosis. The "s" allele was associated with reduced BMD at the spine, with a more modest reduction observed at the hip. These findings are similar to those observed in the 2 other populations studied from Aberdeen and London (10). As in our work, this earlier study had shown that the genetic effect associated with the COL1A1 locus appeared stronger at the spine. We found that the risk genotypes ("Ss/ss") were overrepresented in subjects with prevalent fracture

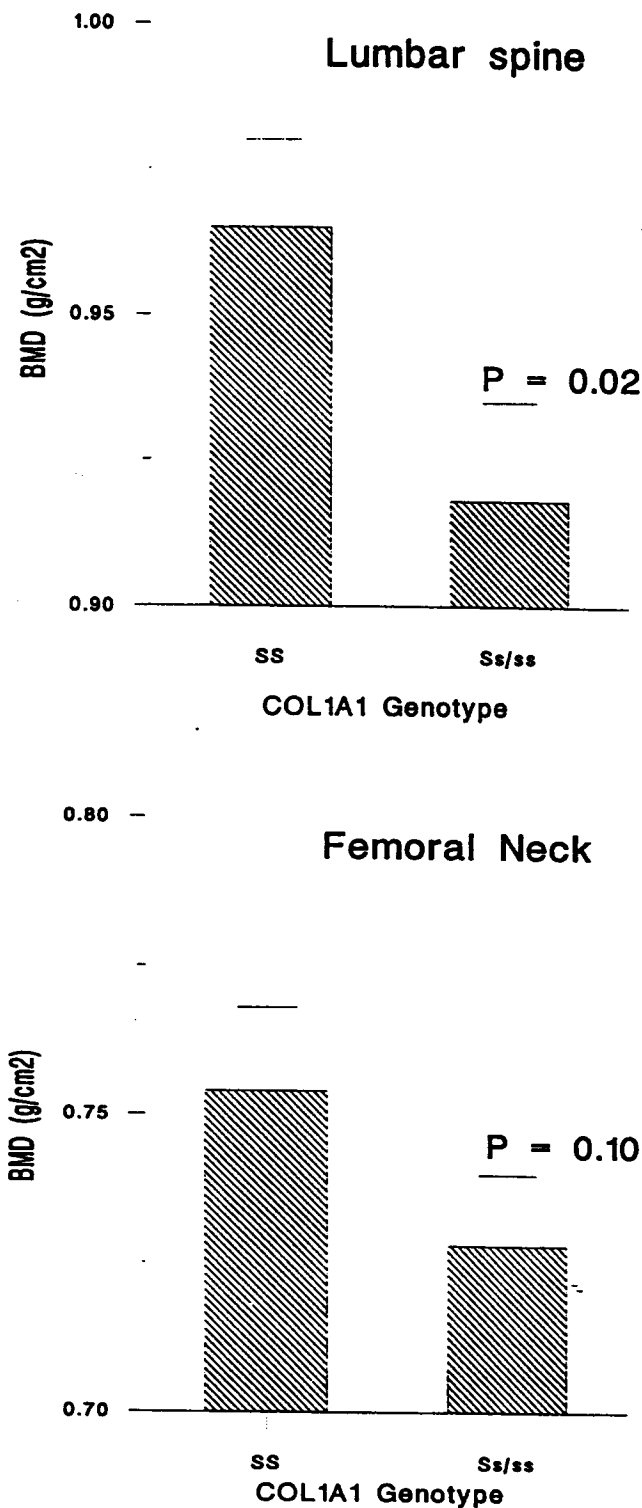


Figure 1. Lumbar spine and femoral neck bone mineral density (BMD) by COL1A1 genotype group (mean  $\pm$  SEM) (n = 113 for the SS group; n = 67 for the Ss group; n = 5 for the ss group).

Table 3. Odds ratio (95% confidence interval) for risk of prevalent fracture (vertebral and appendicular) associated with carriage of the "s" allele

COL1A1 genotype	Crude odds ratio	Adjusted odds ratio*
SS	1.00	1.00
Ss/ss	1.95 (1.01-3.78)†	1.82 (0.91-3.61)‡

\* Adjusted for femoral neck bone mineral density.

†  $P = 0.04$ .

‡  $P = 0.09$ .

cases compared with nonfracture subjects, with the presence of the "s" allele associated with an almost 2-fold increased risk of any fracture compared with the "S" allele. Previous work had shown an association between the COL1A1 genotype and vertebral fracture (55 cases), with an almost 3-fold increased risk of fracture associated with the "Ss" and "ss" genotypes (10). Our data indicate a consistent increased risk for any fracture, although larger studies are required for site-specific analysis. The increased fracture risk associated with the "s" allele appeared largely independent of BMD, and would suggest that the COL1A1 locus may also have effects on bone structure or quality.

Association studies may, however, be confounded by population admixture, heterogeneity, or stratification (11). Specific gene-environment interactions can also modify the genetic effect attributable to a particular locus between population groups. Replication of any initial positive findings is required to confirm the association and reduce the possibility of a Type I error. There has also been concern about the possibility of multiple hypothesis testing in genetic association studies and the use of statistical correction (19). We do not believe that this is an issue in the present study for the following reasons. We have analyzed only 1 biallelic polymorphism within the COL1A1 gene, rather than a highly polymorphic microsatellite with multiple alleles.

Table 4. Relationship of COL1A1 genotype to urinary collagen cross-links\*

Variable	SS (n = 48)	Ss/ss (n = 34)	P
Pyridinoline/creatinine (nmoles/mmoles)	44.6 $\pm$ 24.5	54.1 $\pm$ 24.5	0.05
Deoxypyridinoline/creatinine (nmoles/mmoles)	13.3 $\pm$ 7.0	15.0 $\pm$ 7.2	0.22

\* Values are the mean  $\pm$  SD. P values derived from analysis of variance using log-transformed data.

This reduces the issue of significance attached to individual alleles. We also did not examine all possible genotype and allelic combinations, since our decision to analyze the "Ss" and "ss" genotypes together under a dominant risk model was made a priori and was based on earlier published work. Association was examined only for 3 main phenotypes rather than multiple traits (i.e., BMD at the spine and hip, prevalent fracture status, and biochemical markers of collagen resorption). Since these traits are correlated, they do not strictly represent independent tests, and application of the Bonferroni correction test statistic may therefore not be appropriate (20). Unfortunately, application of too stringent statistical criteria will dramatically reduce power and may preclude the detection of loci with modest effects in complex disease (19).

The findings of our study of women from the UK general population confirm the original findings by Grant et al and support the role of this locus as a candidate in the determination of osteoporosis risk. Although our data would support a dominant inheritance model associated with carriage of the "s" allele, our study cannot exclude the possibility that the genotype risk is actually associated with heterozygous carriage of the "S" allele, suggesting a recessive model of protection for the "SS" genotype. In addition to our work, 2 reports from populations in The Netherlands and Denmark have also demonstrated a significant effect of the COL1A1 locus on BMD and fracture (21,22). In the former study, the genotype effect on fracture was only seen at peripheral sites rather than at the spine, and as in our study, the increase in risk was also independent of BMD. In The Netherlands study, the large sample size also allowed demonstration of a clear additive or codominant effect of the "s" allele on fracture risk and BMD (21). All these studies, therefore, demonstrate a consistent effect of the COL1A1 genotype on BMD, suggesting that the effect of the COL1A1 locus is not so small as to be modified by environmental differences among the 3 countries. To date, however, these studies have all been conducted in white women of Northern European extraction, and it will be important to examine the effect of the COL1A1 locus on osteoporosis risk factors in ethnically diverse populations.

A possible functional significance associated with the COL1A1 locus is suggested by the observation that pyridinoline levels were increased in the "at risk" genotype groups. Deoxypyridinoline, which is reported to be more specific for type I collagen resorbed from bone, was not, however, significantly elevated in subjects with

the "s" allele. This suggests that rather than being bone specific, the COL1A1 locus may have regulatory effects on total body turnover of type I collagen. This could indicate a possible relationship between this locus and type I collagen in skin, which would be of interest in view of the association between skin thickness and osteoporosis (23,24).

The single basepair polymorphism is situated in a transcriptional regulatory region of the COL1A1 gene at a putative Sp1 binding site. It is possible that the polymorphic site may directly affect transcription and, thereby, collagen synthesis. Unfortunately, we did not have serum type I collagen C-terminal extension propeptide (a biochemical marker of collagen synthesis) analyses to further investigate this possibility. Alternatively, the polymorphic site may be in linkage with other sequence changes in the gene which have an effect on type I collagen. As noted, the relationship between COL1A1 genotype and fracture may be due to effects on bone structure and microarchitecture, rather than direct effects on BMD and mineralization. To date, no study has examined the association between the COL1A1 polymorphism and quantitative ultrasound, which is believed to provide information about bone quality.

Significant association may also arise due to linkage disequilibrium between the COL1A1 polymorphism and a nearby novel disease locus on chromosome 17, since families of genes with similar functions often map to similar chromosomal locations. Preliminary data associating COL1A1 genotype with rates of menopausal bone loss would support our findings that this locus has a regulatory effect on collagen resorption (21). This may also explain the preliminary observations from cross-sectional studies that the genetic effect on BMD associated with the COL1A1 locus is stronger in postmenopausal than premenopausal women (21,25).

In conclusion, we have demonstrated that the COL1A1 gene polymorphism is associated with reduced spinal BMD and an increased risk of prevalent fracture. Urinary pyridinoline, a marker of type I collagen resorption, was elevated in subjects with the risk genotypes "Ss" and "ss." These findings aid our insight into the pathogenesis of osteoporosis and support the role of the COL1A1 gene locus as a risk factor for low bone mass and increased risk of fracture.

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