

Allelic Variation at the Interleukin-1 Receptor Antagonist Gene Is Associated With Early Postmenopausal Bone Loss at the Spine

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Genetic factors play an important role in determining bone mineral density (BMD) in later life, with the genetic influence mediated through effects on both peak mass and on age- and menopause-related bone loss. At menopause there is an increase in the production and activity of various cytokines and growth factors within the bone microenvironment. The activity of interleukin-1 (IL-1), a powerful stimulant of osteoclastic bone resorption, is increased in estrogen-deficient states with increased production of IL-1 and inhibition of the IL-1 receptor antagonist (IL-1ra). Treatment with IL-1ra blocks the bone loss associated with ovariectomy in animals and the IL-1 receptor antagonist gene (IL-1RN) is therefore a potential candidate gene for the regulation of postmenopausal bone loss. We examined the relationship between annual rates of change in BMD over 5 years and an 86 bp variable number tandem-repeat polymorphism of the IL-1RN gene in 108 early postmenopausal women. All women were within 5 years of a natural menopause at the study's onset, healthy, and not on hormone replacement therapy or other medication known to affect bone metabolism. BMD was measured annually over the 5 year study period at the lumbar spine and femoral neck using dual-energy X-ray absorptiometry. Three alleles were identified (A1 = 4 repeats, A2 = 2 repeats, A3 = 5 repeats), with five genotypes observed: A1A1 (41.7%), A1A2 (45.4%), A2A2 (6.5%), A1A3 (2.8%), and A2A3 (3.7%). For analysis, alleles were collapsed into a biallelic system grouping the A1 and A3 alleles. There was no significant relationship between the IL-1RN genotypes and baseline bone mass at either the spine or hip. IL-1RN genotype was significantly associated with annual rates of change in spinal bone mass ($p < 0.05$), and this finding remained significant after adjustment for age, weight, and baseline BMD. Carriage of at least one copy of the A2 allele was associated with reduced bone loss at the spine (mean change in BMD \pm SD: $-0.81 \pm 1.46\%$ /year) when compared with noncarriage of the A2 allele (mean change $-1.38 \pm 1.48\%$ /year), $p = 0.05$. We therefore conclude that allelic variation at the IL-1RN locus is associated with differential rates of early postmenopausal bone loss at the spine. Further research will be required to clarify the mechanisms underlying these findings and to determine

whether this association translates into a significant long-term effect on BMD and fracture in later life. (Bone 23: 367–371; 1998) © 1998 by Elsevier Science Inc. All rights reserved.

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Introduction

Bone mineral density (BMD) in later life is a strong predictor of subsequent osteoporotic fracture⁶ and is determined by both the peak value achieved during skeletal growth and by age- and menopause-related bone loss. Family and twin studies suggest a strong genetic component in the determination of peak bone mass, with 50%–85% of the population variance in BMD being attributable to genetic factors.^{22,24} Twin studies in postmenopausal and elderly women also support a persistent and significant genetic influence on bone mass in later life.^{1,8} This may represent either a strong residual effect from the genetic contribution to peak bone mass or an independent genetic effect on the regulation of bone loss. Indirect assessment of bone turnover through biochemical markers suggests a genetic regulation of bone metabolism that may translate into differing effects on bone loss,^{9,15,29} although to date only two twin studies have directly attempted to explore the genetic contribution to age- and menopause-related bone loss, with conflicting and uncertain results.^{4,16}

Osteoporosis is a complex disease that is likely to have a polygenic etiology,¹¹ and candidate gene analysis has demonstrated that polymorphisms of the vitamin D receptor (VDR) locus,²⁶ the estrogen receptor (ER) locus,¹⁸ and the collagen type I $\alpha 1$ (COL1-A1) locus¹⁰ are all potential genetic markers for bone mass and perhaps bone loss. Estrogen deficiency is associated with an increase in local production of various cytokines and growth factors within the bone marrow and bone cells, and these inflammatory factors appear to play an important role in the development of postmenopausal osteoporosis.²³ Interleukin-1 (IL-1) is a powerful stimulant of bone resorption and a well-recognized inhibitor of bone formation.⁷ The observed postmenopausal increase in IL-1 activity results from an effect of estrogen on the production of both IL-1 and the IL-1 inhibitor, IL-1 receptor antagonist (IL-1ra). These data, therefore, raise the possibility that the IL-1 receptor antagonist gene (IL-1RN) may be a potential candidate for genetic regulation of early postmenopausal bone loss. In this study we have therefore examined the relationship between bone mass, early postmenopausal bone loss,

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Table 1. Mean characteristics (\pm SD) of 125 subjects according to IL-1RN genotype

Variable	Total (n = 125)	A1A1 (n = 45)	A1A2 (n = 49)	A2A2 (n = 7)	A1A3 (n = 3)	A2A3 (n = 4)
Age (yr)	53.1 (3.5)	52.2 (3.4)	53.2 (4.0)	52.6 (2.8)	53.0 (4.0)	55.8 (1.5)
Age at menopause (yr)	50.4 (3.1)	49.8 (3.1)	50.2 (3.9)	50.6 (3.4)	50.0 (4.0)	51.5 (1.0)
Menopause duration (yr)	2.7 (1.7)	2.4 (1.8)	3.0 (1.6)	2.0 (1.8)	3.0 (0.0)	4.3 (0.5)
BMI (kg/m ²)	26.0 (4.8)	26.4 (4.7)	26.1 (5.3)	25.4 (3.9)	24.7 (3.1)	23.8 (2.1)
No. ever smoking	54 (43)	20 (43)	23 (47)	0 (0)	1 (33)	3 (75)
Lumbar spine BMD (g/cm ²)	0.969 (0.144)	0.960 (0.145)	0.985 (0.139)	0.888 (0.118)	0.982 (0.082)	0.912 (0.123)
Femoral neck BMD (g/cm ²)	0.763 (0.130)	0.748 (0.129)	0.775 (0.131)	0.699 (0.100)	0.741 (0.170)	0.743 (0.110)
Change in lumbar spine BMD (%/yr)	-1.1 (1.5)	-1.4 (1.5)	-0.7 (1.5)	-1.6 (0.8)	-1.8 (0.5)	-0.9 (1.9)
Change in femoral neck BMD (%/yr)	-0.50 (2.2)	-0.9 (2.3)	0.0 (2.2)	-1.2 (0.9)	-0.4 (1.4)	-2.0 ^a (-)

^aOnly one subject in this group with annual rate of hip loss data.

and a variable number tandem repeat (VNTR) in the IL-1RN gene.

Patients and Methods

Subjects were selected from a large general population cohort of 1003 white women, with a mean (\pm SD) age for the total cohort of 54.2 ± 6.0 years.¹² Women in the age range 45–64 years had been selected from a large, single general practice in Chingford in northeast London (total 11,000 registered patients) to participate in a longitudinal epidemiological study of rheumatic diseases. A total of 1353 women were found to be in the age range specified and, of these, 78% (1003) agreed to participate. The area is predominantly middle class, 98% are white, and the population similar to UK normals in terms of height, weight, smoking status, hysterectomy rates, and use of hormone replacement therapy (HRT). From this total cohort, 125 women were selected for the present study if they were within 5 years of a natural menopause and currently not on HRT or other medication known to affect bone metabolism. If hormonal treatment was initiated during the study period, results were included up to that timepoint.

Throughout the study no women received concurrent treatment with medication known to affect bone metabolism such as steroids, bisphosphonates, calcium, or vitamin D. Postmenopausal status was defined as an absence of menstruation for at least 6 months and was confirmed by measurement of serum follicular stimulating hormone and estradiol levels. All women were healthy with no history of physical illnesses known to affect bone. All subjects had completed a nurse-administered questionnaire detailing medical and gynecological/obstetric histories, current and past medications including HRT, smoking status, alcohol consumption, dietary calcium intake, and exercise levels. Informed consent was obtained from all women and the study protocol was approved by the local ethics committee.

BMD was measured at the lumbar spine (L1–4) and femoral neck using dual-energy X-ray absorptiometry (Hologic QDR-1000, Hologic, Waltham, MA). Short-term reproducibility, assessed by duplicate measurements in healthy volunteers, was 0.8% at the lumbar spine and 1.6% at the femoral neck. Annual measurements of BMD were made at 0, 12, 24, 36, 48, and 60 months. In calculating the rate of change in BMD it was assumed that the expected change in BMD is linear with time for each subject with variation in slopes and intercepts from subject to subject, and that the deviations of the measured BMD for a subject from the expected BMD have a zero mean and constant variance and are uncorrelated. Under these assumptions, a linear regression equation, where BMD is a dependent variable expressed as a linear function with time (in years), was fitted for individual subjects. Using this model, the annual percentage

change in BMD for each subject was then derived by dividing the regression slope by the intercept at time zero.

High-molecular-weight DNA was obtained from peripheral blood leukocytes using a phenol and chloroform extraction system. PCR was performed using two flanking primers to amplify an 86 bp tandem repeat region in intron 2 of the IL-1RN gene (5'-CTC AGC AAC ACT CCT AT-3' and 5'-TCC TGG TCT GCA GGT AA-3'). PCR reactions were performed in a total volume of 20 μ L under standard conditions²⁷ using a Corbett Research FTS-1 thermocycler programmed as follows: 1 min at 96°C; 30 cycles of 1 min at 94°C; 1 min at 60°C; 2 min at 70°C; and a final extension of 3 min at 72°C. Final PCR products were separated by electrophoresis using 1.2% agarose gels at 100 V for 1 h. To visualize DNA, gels were stained with ethidium bromide and transilluminated under ultraviolet light. Alleles were sized relative to a 1 kb DNA marker and subsequently coded using published nomenclature.²⁷

For analysis, the IL-1RN alleles were collapsed into a biallelic system based on the number of repeats. The IL-1RN genotype A1 (four repeats) was grouped with genotype A3 (five repeats), and compared with genotypes A2 (two repeats) and A4 (three repeats). This reduces the problems associated with small numbers of subjects in several genotype groups, and has a plausible biological basis as the larger alleles will have probably arisen because of mutation from a common ancestor allele with fewer repeats. Differences in demographic variables between the IL-1RN genotypes were compared using analysis of variance (ANOVA) for normally distributed variables and the chi-square test for categorical variables. Adjustment for potential confounders was performed using analysis of covariance (ANCOVA). Where ANOVA values were significant ($p < 0.05$), the Bonferroni test for multiple comparisons was used to search for evidence of differences between individual genotype groups. Analysis for allele-specific effects was performed using carriage of the collapsed allele classes under dominant and recessive risk models, with comparisons between the variable means using the two-sided Student *t*-test. All analyses were performed using the PC software statistical program STATA (Stata Corp., TX).

Results

Characteristics of the 125 women entered into the study are shown in **Table 1**. Full clinical and genotype results were available on 108 of these women. No significant differences were observed between subjects with genotype results and those in whom DNA was either unavailable or attempts at PCR unsuccessful.

Analysis of the genotypes in the 108 women showed evidence of three alleles, which corresponded both in size and frequency to those previously identified (**Table 2**). From the three alleles

Table 2. IL-1RN alleles identified through DNA amplification of 108 unrelated individuals (comparison with published data)

Allele	Size (bp)	Number of repeats (n)	Allele frequency (%) ^a	Allele frequency (%) ^b
A1	410	4	65.8	73.6
A2	240	2	31.0	21.4
A3	500	5	3.2	3.6
A4	325	3	—	0.7
A5	595	6	—	0.7

^aCurrent study (N = 108 subjects).

^bSee Tarlow et al.²⁷ (N = 70 subjects).

observed we were able to demonstrate five of six possible genotypes (Table 1). There was no significant difference between the five genotype groups in anthropomorphic or environmental variables known to influence bone mass.

Collapse of the IL-1RN VNTR into a biallelic system gave rise to three genotype groups: A1A1 and A1A3 (n = 48); A1A2 and A2A3 (n = 53); and A2A2 (n = 7). No demonstrable effect of IL-1RN genotype class was observed on baseline BMD at either the lumbar spine or femoral neck. There was, however, a significant association between rates of change in BMD at the lumbar spine and the collapsed IL-1RN genotypes (ANOVA *F* value = 3.17, *p* < 0.05), although not at the femoral neck (ANOVA *F* value = 1.58, *p* > 0.2). The significant finding at the spine remained after adjustment for age, weight, and baseline BMD, and also after adjustment for multiple testing using the Bonferroni test statistic. Subjects who were heterozygous for the A2 allele (genotypes A1A2 and A2A3) tended to have reduced rates of spinal bone loss when compared to subjects with the genotypes A1A1 and A1A3 (Bonferroni-adjusted *p* value = 0.06). There were, however, too few subjects with the genotype A2A2 to observe a clear allele dose effect across the three genotype groups. Pooling of the A2 heterozygous and homozygous genotype groups allowed testing of the association between carriage of at least one copy of the A2 allele and rates of bone loss. These genotype groups were also well matched for baseline characteristics, and analysis demonstrated reduced rates of spinal bone loss in subjects carrying the A2 allele when compared with noncarriage (Table 3, Figure 1). A similar, but nonsignificant, trend was also seen in the femoral neck. These findings remained unaltered after adjustment for potential confounders.

Table 3. Mean (±SD) characteristics of subjects according to carriage of A2 allele

Variable	Carriage of A2 allele	
	- (n = 48)	+ (n = 60)
Age (yr)	52.3 (3.4)	53.4 (3.8)
Age at menopause (yr)	49.8 (3.1)	50.4 (3.7)
Menopause duration (yr)	2.5 (1.8)	3.0 (1.6)
BMI (kg/m ²)	26.5 (4.6)	25.8 (4.9)
No. ever smoking (%)	22 (46)	27 (45)
Lumbar spine BMD (g/cm ²)	0.966 (0.140)	0.969 (0.140)
Femoral neck BMD (g/cm ²)	0.752 (0.130)	0.764 (0.127)
Change in lumbar spine BMD (%/yr)	-1.43 (1.47)	-0.80 (1.47) ^a
Change in femoral neck BMD (%/yr)	-0.90 (2.26)	-0.23 (2.14) ^b

^a*p* < 0.05 (vs. noncarriage).

^b*p* = 0.18 (vs. noncarriage).

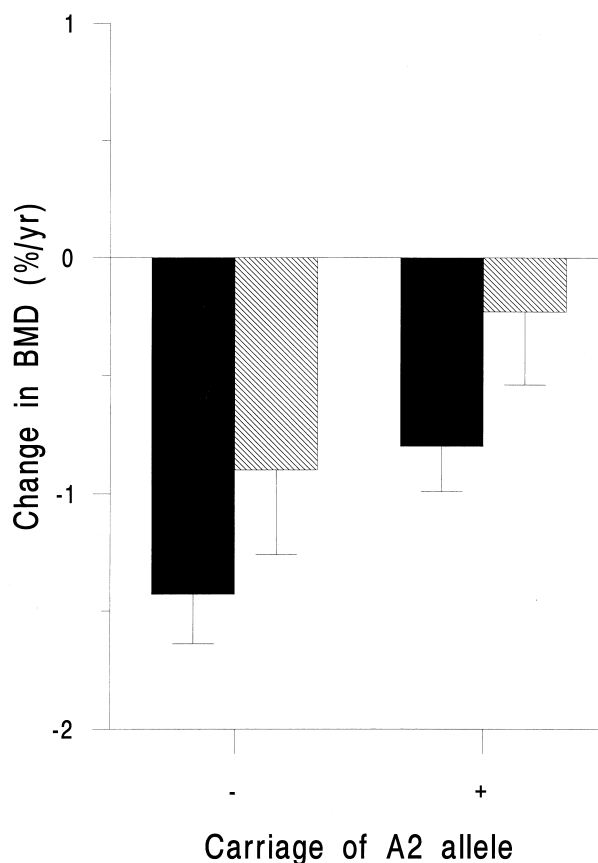


Figure 1. Relationship between carriage of the A2 IL-1RN allele and mean (±SEM) bone loss from the lumbar spine (solid bars) and femoral neck (hatched bars). Carriage of the A2 allele was associated with reduced bone loss at the spine (*p* < 0.05) with a similar nonsignificant trend at the hip (*p* = 0.18).

Discussion

Family and twin studies demonstrate a strong genetic component to osteoporosis. We have studied the relationship between a VNTR in intron 2 of the IL-1RN gene and both bone mass and annual change in bone mass at the lumbar spine and femoral neck in early postmenopausal women. Our data demonstrate that the IL-1RN gene is a potential candidate for the development of postmenopausal osteoporosis, with allelic variation at the IL-1RN locus associated with differential rates of bone loss from the lumbar spine.

Risk factors for bone loss have yet to be clearly identified in longitudinal studies. We have previously failed to find that combinations of classical epidemiological risk factors²⁵ or biochemical markers¹⁴ were useful predictors of future bone loss. Our data indicate that genetic markers may therefore be useful in predicting bone loss in women at the onset of the menopause. Trabecular bone has a greater metabolic activity than cortical bone, and exhibits an increased response to estrogen withdrawal. This is demonstrated by the higher rates of bone loss observed at the lumbar spine compared with the femoral neck in the total cohort. The association between IL-1RN genotype and spinal bone loss may indicate a specific effect of this genetic locus on trabecular bone, although it may merely reflect a relative lack of power to detect a difference at the hip where the variability in loss rates was higher. Our cohort had a mean age of 53 years and, although women would have already experienced some age-

related bone loss, we feel that the lack of an association between IL-1RN genotypes and baseline BMD suggests that the genetic effect of the locus on osteoporosis risk is not mediated through a major effect on peak BMD. Longitudinal epidemiological studies show continued bone loss in elderly subjects,¹³ and it remains to be determined if the association between the IL-1RN locus and spinal bone loss persists into later life. This may result in significant differences in BMD between the two common alleles, which could translate into differential fracture rates. Association or case-control studies would be required to test this hypothesis.

IL-1ra is a 17 kDa protein that binds to IL-1 receptors and competes with both IL-1 α and IL-1 β without detectable IL-1 agonist effects. IL-1 is a powerful stimulant of bone resorption, activating mature osteoclasts indirectly via a primary effect on osteoblasts and inhibiting osteoclast apoptosis. Monocytes of patients with "high turnover" osteoporosis, the histological hallmark of postmenopausal osteoporosis, secrete increased amounts of IL-1.²¹ Subsequent observations have shown that this postmenopausal increase in IL-1 activity results from an effect of estrogen on the production of both IL-1 and its receptor antagonist IL-1ra. Animal studies have also revealed that treatment with IL-1ra blocks the ex vivo osteoclast formation induced by ovariectomy.¹⁷ The human IL-1RN gene maps to chromosome 2q13-14 and consists of four exons.¹⁹ The VNTR is due to a 86 bp repeat within intron 2 of the gene and this may be of functional significance as the sequence is reported to contain three potential protein binding sites: an α -interferon silencer A; a β -interferon silencer B, and an acute-phase response element.²⁷ Polymorphic VNTR repeats have been shown to influence gene expression at the insulin locus,³⁰ although it is unknown if the IL-1RN VNTR has any direct effect on either osteoclast or osteoblast function. The physical number of repeats may also be important and could affect transcriptional activity and RNA stability. Our data suggest that the A2 allele may be associated with reduced rates of spinal bone loss, and hence reduced osteoclast activity. This contrasts with other studies that have shown the A2 allele to be associated with disease severity in ulcerative colitis,²⁰ systemic lupus erythematosus,² Graves disease,³ and skin diseases.^{5,28} Our study was restricted to postmenopausal women and this may suggest that the A2 allele is specifically associated with reduced inflammatory activity in the estrogen-deficient state. Further studies will need to examine whether there is allele-specific expression of IL-1ra that is modified by hormonal status. In the absence of functional data on IL-1ra activity, however, an alternative hypothesis is that the IL-1RN VNTR is in linkage disequilibrium with a disease-causing gene nearby on chromosome 2, and that the degree of linkage disequilibrium may differ between the patient groups studied.

In summary, our study shows an association between a polymorphic VNTR in intron 2 of the IL1-RN gene and postmenopause-related bone loss from the lumbar spine. This suggests that this genetic locus may play a central role in the regulation of trabecular bone turnover in the estrogen-deficient state. It will be of value in future studies to determine whether this relationship extends beyond the first 5 years of the menopause and whether it translates into a permanent and significant difference in BMD in later life with increased fracture risk.

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