

VITAMIN D-RECEPTOR GENE POLYMORPHISMS AND BONE DENSITY
IN PREPUBERTAL AMERICAN GIRLS OF MEXICAN DESCENTJESUS SAINZ, PH.D., JAN M. VAN TORNOUT, M.D., M. LUIZA LORO, M.D., JAMES SAYRE, PH.D., THOMAS F. ROE, M.D.,
AND VICENTE GILSANZ, M.D., PH.D.**ABSTRACT**

Background Bone mass is under strong genetic control, and recent studies in adults have suggested that allelic differences in the gene for the vitamin D receptor may account for inherited variability in bone mass. We studied the relations of the vitamin D-receptor genotype to skeletal development and variation in the size, volume, and density of bone in children.

Methods We identified three allelic variants of the vitamin D-receptor gene using the polymerase chain reaction and three restriction enzymes (*Apal*, *Bsml*, and *TaqI*) in 100 normal prepubertal American girls of Mexican descent. We then determined the relations of the different vitamin D-receptor genotypes (AA, Aa, aa, BB, Bb, bb, TT, Tt, and tt) to the cross-sectional area, cortical area, and cortical bone density of the femoral shaft and the cross-sectional area and density of the lumbar vertebrae.

Results The vitamin D-receptor genotype was associated with femoral and vertebral bone density. Girls with aa and bb genotypes had 2 to 3 percent higher femoral bone density ($P=0.008$ and $P=0.04$, respectively) and 8 to 10 percent higher vertebral bone density ($P=0.01$ and $P=0.03$, respectively) than girls with AA and BB genotypes. There was no association between the cross-sectional area of the vertebrae or the cross-sectional or cortical area of the femur and the vitamin D-receptor genotype. The chronologic age, bone age, height, weight, body-surface area, and body-mass index did not differ significantly among girls with different vitamin D-receptor genotypes.

Conclusions Vitamin D-receptor gene alleles predict the density of femoral and vertebral bone in prepubertal American girls of Mexican descent. (*N Engl J Med* 1997;337:77-82.)

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DEFINING the genetic and environmental factors responsible for variations in bone mass during skeletal growth should aid in the identification of children at risk for osteoporosis and fractures later in life.¹ Although the influence of environmental factors such as nutrition and physical exercise on the amount of bone that is gained during childhood has been the subject of several studies,² knowledge of the genetic components of bone mass is limited to studies of mother-daughter pairs and twins.³⁻⁵ Consequently, great interest was generated by a recent report suggesting that allelic differences in the gene for the vitamin D

receptor account for inherited variability in bone mass.⁶ However, not all subsequent studies confirmed the relation, and the results of positive studies differed with regard to the magnitude of the association.⁷⁻⁹ Further controversy arose from data suggesting that vitamin D-receptor gene polymorphisms were not related to bone mass but were related to variations in diaphyseal cross-sectional growth resulting from periosteal apposition of new bone.¹⁰⁻¹² These discrepancies could, in part, be due to the technique used to study bone. Measurements of bone density by absorptiometry are based on a two-dimensional projection of a three-dimensional structure, cannot accurately account for variations in cross-sectional area, and are influenced not only by bone mass, but also by the size of the bone.¹³

Quantitative computed tomography (CT) allows accurate assessment of both the size of the bones and the various components that influence bone mass.¹⁴ In this study, we used quantitative CT to investigate whether there is an association between vitamin D-receptor genotype and the skeletal development of prepubertal girls and, if so, whether this link is related to variations in the size of the femurs or the lumbar vertebrae, the volume of cortical bone in the femurs, or the density of cortical bone in the femurs or of cancellous bone in the vertebrae.

METHODS**Subjects**

We studied 100 normal, prepubertal, American girls of Mexican descent who were between 6.7 and 11.7 years of age and were recruited from schools in Los Angeles County, California. The protocol was approved by the Children's Hospital of Los Angeles Institutional Review Board, and informed consent was obtained from all subjects and their parents or guardians.

Subjects were excluded if they had any chronic illness, had been ill for more than two weeks during the previous six months, had ever been hospitalized, or had taken any medications, vitamin preparations, or calcium supplements within the previous six months. Girls were also excluded if their parents or grandparents were not of Mexican descent.

All the children underwent a physical examination by a pediatric endocrinologist to determine their stage of sexual development. Only girls who were prepubertal and whose height and weight were between the 5th and 95th percentiles for the mean age-adjusted normal values for white girls were enrolled. Body-

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surface area and body-mass index were calculated as previously described.¹⁵ Skeletal maturation was determined by the method of Greulich and Pyle¹⁶; girls whose chronologic and bone age differed by more than one year were also excluded.

Genotypic Analysis of Polymorphisms of the Vitamin D–Receptor Gene

The genotype for three restriction-fragment-length polymorphisms of the vitamin D–receptor gene was determined by polymerase-chain-reaction (PCR) amplification and enzymatic digestion of the products with *ApaI*, *BsmI*, and *TaqI*. The primers for the *BsmI* polymorphism have been described previously.⁶ The forward primer for the *ApaI* and *TaqI* polymorphisms, located in exon 7, was the same as that used for amplification of the *BsmI* polymorphism: 5'CAACCAAGACTACAAGTACCGCGTCAGTGA3'. The reverse primer for the *ApaI* and *TaqI* polymorphisms was located in exon 9: 5'CACTTCGAGCACAAAGGGCGTTAGC3'. PCR was performed with a Biometra Trio thermoblock (Floral City, Fla.) under standard conditions, for 35 cycles, and with 65°C as annealing temperature. With the enzymes *ApaI*, *BsmI*, and *TaqI*, the respective genotypes were defined as A, B, T (indicating the absence of the restriction site) or a, b, t (indicating the presence of the restriction site). The PCR product for the *BsmI* polymorphism was 825 base pairs (bp) long, and the restriction fragments were 650 bp and 175 bp long. The PCR products for the *ApaI* and *TaqI* polymorphisms were 2000 bp long; the lengths of the fragments after digestion with *ApaI* were 1700 and 300 bp, and the lengths of the fragments after digestion with *TaqI* were 1800 and 200 bp.

Biochemical Studies

After an overnight fast, blood was taken for measurement of serum biochemical values, calcitropic hormones, and markers of bone turnover. Serum parathyroid hormone, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, alkaline phosphatase, bone-specific alkaline phosphatase, and osteocalcin were measured at Corning Nichols Institute, San Juan Capistrano, California.

Techniques and Definitions of Bone Measurements

All CT bone measurements were performed with the same instrument (CT-T 9800, General Electric, Milwaukee) and mineral reference phantom (CT-T bone densitometry package, General Electric). The density of cancellous bone and the cross-sectional area of the vertebrae were measured at the midportion of vertebral bodies L1 through L3, and the cross-sectional area, the area of cortical bone, and the density of cortical bone were measured at the midshaft of both femurs, as previously described.^{17,18}

The size of the axial skeleton was defined as the cross-sectional area at the midportion of the vertebral body in square centimeters, and the density of cancellous bone was defined as the amount of bone and marrow in milligrams per cubic centimeter per pixel, the CT unit of measurement. Because of the small size of the trabeculae as compared with that of the pixel, the CT values for cancellous bone density reflect not only the amount of mineralized bone and osteoid, but also the amount of marrow per pixel.¹⁴ These measurements are analogous to in vitro determinations of the volumetric density of trabecular bone, which are obtained by washing the marrow from the pores of a specimen of cancellous bone, weighing it, and dividing the weight by the volume of the specimen, including the pores.¹⁹

The size of the appendicular skeleton was defined as the cross-sectional area at the midshaft of the femur in square centimeters, the volume of cortical bone as the cortical bone area at the midshaft of the femur in square centimeters, and the density of cortical bone as the amount of bone in milligrams per cubic centimeter per pixel. Because of the thickness and relative lack of porosity of cortical bone at the midshaft of the femur, the CT values reflect the density of the bone (the amount of collagen and mineral in a given volume of bone).¹⁷ These measurements are analogous to in vitro determinations of the intrinsic mineral den-

sity of bone, which are commonly expressed as the ash weight per unit volume of bone.²⁰

The coefficients of variation for repeated CT measurements of femoral cortical bone density, cortical bone area, and cross-sectional area and of vertebral cancellous bone density and cross-sectional area were 0.6 to 2 percent.^{17,18} The time required for the procedure was approximately 10 minutes, and the radiation exposure was approximately 100 to 200 mrem (1 to 2 mJ per kilogram), localized to the midportions of the first three lumbar vertebrae and the femurs; the effective radiation dose was approximately 8 mrem (0.08 mJ per kilogram).^{21,22}

Statistical Analysis

Anthropometric, biochemical, and bone measurements were assessed by analysis of variance with the Bonferroni correction for multiple comparisons and by linear regression analysis.²³ Linkage disequilibrium was assessed by a chi-square test with the null hypothesis of no linkage disequilibrium between the different polymorphic alleles. To estimate haplotype frequencies, the computer program Estimated Haplotype (Linkage Program version 5.1) was used.²⁴ All statistical tests were two-sided.

RESULTS

Characteristics of the Study Population

Table 1 shows the anthropometric characteristics of the 100 girls. By design, the average values for bone age were similar to those for chronologic age. Also by design, the heights and weights of the girls were between the 5th and 95th percentiles for age; mean height was at the 50th percentile, and mean weight was at the 70th percentile.

The most common vitamin D–receptor genotypes were Aa (55 percent), Bb (42 percent), and TT (54 percent). Genotypes aa and bb were more frequent (24 percent and 44 percent, respectively) than AA and BB (21 percent and 14 percent, respectively). When the genotypes for all polymorphisms were combined, the most frequent were AaBbTt (29 percent) and aabbTT (23 percent).

Genotype, Phenotype, and Biochemical Association

There were no significant differences in developmental status among girls with different vitamin D–receptor genotypes, and the mean values for age, bone age, height, weight, body-surface area, and body-mass index were similar in all groups (Table 1). There were significant relations between vitamin D–receptor genotype and the range of values for bone density in both the femurs and the vertebrae. Girls with genotypes aa and bb had 2 to 3 percent higher femoral bone density ($P=0.008$ and $P=0.04$, respectively) and 8 to 10 percent higher vertebral bone density ($P=0.01$ and $P=0.03$, respectively) than those with AA and BB genotypes (Fig. 1). The presence of these homozygous genotypes predicted higher femoral ($r=0.35$, $P=0.03$) and vertebral ($r=0.44$, $P=0.02$) bone density. Although the bone densities did not differ according to *TaqI* genotype, there was a trend toward higher values for vertebral and femoral bone density in girls with the

TABLE 1. VITAMIN D-RECEPTOR GENOTYPE, AGE, AND ANTHROPOMETRIC CHARACTERISTICS IN 100 NORMAL PREPUBERTAL AMERICAN GIRLS OF MEXICAN DESCENT.*

RESTRICTION ENZYME AND GENOTYPE†	NO. OF GIRLS	AGE	BONE AGE	HEIGHT	WEIGHT	BODY-SURFACE AREA	BODY-MASS INDEX‡
		yr		cm	kg	m ²	
<i>ApaI</i>							
AA	21	9.2±1.3	9.3±1.4	132.1±6.2	31.1±5.2	1.08±0.12	17.9±2.5
Aa	55	9.3±1.4	9.4±1.3	135.5±10.2	34.7±8.8	1.15±0.20	18.4±2.9
aa	24	9.0±1.4	9.1±1.5	131.7±9.5	31.1±7.4	1.07±0.17	17.9±2.7
<i>BsmI</i>							
BB	14	9.2±1.2	9.2±1.2	131.8±5.2	31.4±7.0	1.09±0.12	18.0±2.4
Bb	42	9.3±1.5	9.4±1.3	135.8±10.5	34.3±9.1	1.14±0.20	18.3±3.2
bb	44	9.1±1.4	9.2±1.5	132.7±9.2	32.4±5.2	1.10±0.16	18.1±2.4
<i>TaqI</i>							
TT	54	9.2±1.4	9.3±1.5	134.2±9.8	32.9±8.1	1.10±1.12	18.0±2.6
Tt	38	9.2±1.4	9.4±1.3	134.3±9.9	33.8±8.4	1.14±1.14	18.5±2.9
tt	8	9.1±1.2	9.1±1.2	129.9±6.0	30.3±6.0	1.05±1.06	17.9±3.4
All	100	9.2±1.4	9.3±1.4	133.9±9.6	33.0±8.0	1.12±0.18	18.1±2.7

*Plus-minus values are means ±SD.

†AA, BB, and TT denote homozygosity for the absence of the *ApaI*, *BsmI*, and *TaqI* sites, respectively; aa, bb, and tt denote homozygosity for the presence of the *ApaI*, *BsmI*, and *TaqI* sites; and Aa, Bb, and Tt denote heterozygosity.

‡The body-mass index was defined as the weight in kilograms divided by the square of the height in meters.

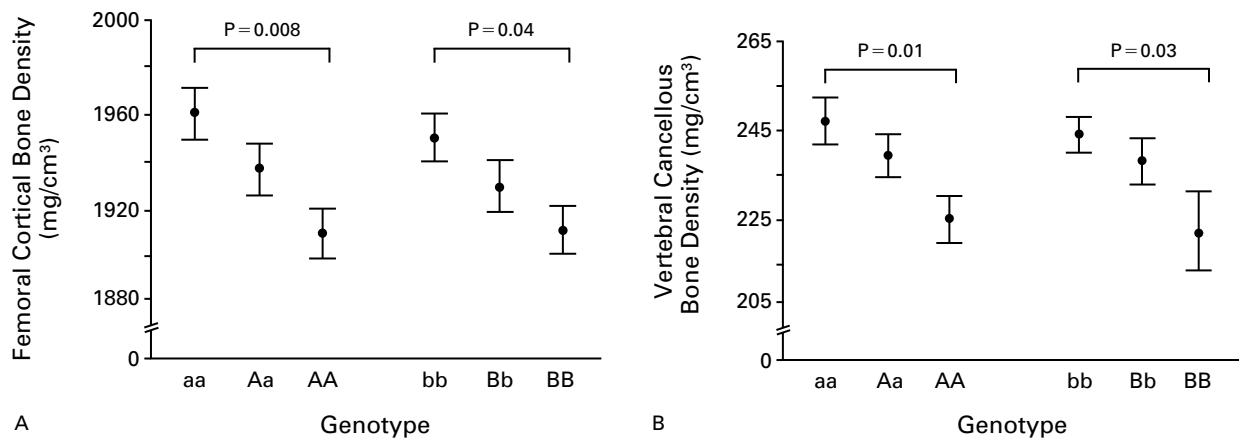


Figure 1. Femoral Cortical (Panel A) and Vertebral Cancellous (Panel B) Bone Density in Relation to Vitamin D-Receptor Genotypes Determined with the Restriction Enzymes *ApaI* and *BsmI* in 100 Normal Prepubertal American Girls of Mexican Descent.

AA and BB denote homozygosity for the absence of the *ApaI* and *BsmI* sites, respectively; aa and bb, homozygosity for the presence of the *ApaI* and *BsmI* sites; and Aa and Bb, heterozygosity. Values shown are means ±SE. P values are for differences between homozygosity genotypes by multiple-comparison tests.

TABLE 2. VITAMIN D-RECEPTOR GENOTYPE AND CT MEASUREMENTS OF THE FEMORAL SHAFTS AND LUMBAR VERTEBRAE IN 100 NORMAL PREPUBERTAL AMERICAN GIRLS OF MEXICAN DESCENT.*

RESTRICTION ENZYME AND GENOTYPE†	NO. OF GIRLS	FEMUR			VERTEBRAE	
		CORTICAL BONE DENSITY	CROSS- SECTIONAL AREA	CORTICAL BONE AREA	CANCELLOUS BONE DENSITY	CROSS- SECTIONAL AREA
		mg/cm ³	cm ²	cm ²	mg/cm ³	cm ²
<i>ApaI</i>						
AA	21	1909±51‡	3.04±0.58	1.77±0.28	225±30§	6.65±0.69
Aa	55	1936±77	3.22±1.20	1.88±0.48	239±34	7.09±1.13
aa	24	1960±62‡	3.02±0.75	1.69±0.35	247±28§	6.48±0.82
<i>BsmI</i>						
BB	14	1912±41¶	3.14±0.54	1.82±0.30	224±32	6.66±0.62
Bb	42	1929±88	3.21±1.04	1.85±0.41	240±36	7.02±1.15
bb	44	1951±65¶	3.06±0.86	1.77±0.39	242±29	6.76±0.95
<i>TaqI</i>						
TT	54	1942±69	3.14±1.08	1.83±0.44	241±30	6.82±0.97
Tt	38	1931±78	3.10±0.97	1.82±0.44	237±36	6.90±1.15
tt	8	1921±45	3.26±0.65	1.66±0.30	229±38	6.70±0.54
All	100	1936±70	3.13±1.00	1.81±0.42	239±32	6.84±1.00

*Plus-minus values are means ±SD. P values were calculated by multiple-comparison procedures.

†AA, BB, and TT denote homozygosity for the absence of the *ApaI*, *BsmI*, and *TaqI* sites, respectively; aa, bb, and tt denote homozygosity for the presence of the *ApaI*, *BsmI*, and *TaqI* sites; and Aa, Bb, and Tt denote heterozygosity.

‡P=0.008 for the difference between homozygous *ApaI* genotypes.

§P=0.01 for the difference between homozygous *ApaI* genotypes.

¶P=0.04 for the difference between homozygous *BsmI* genotypes.

||P=0.03 for the difference between homozygous *BsmI* genotypes.

TT genotype, and toward lower values in girls with the homozygous tt genotype (Table 2).

The 23 girls with the most favorable genotype (aabb) had 2 percent higher femoral bone density (P=0.03) and 12 percent higher vertebral bone density (P=0.01) than the 14 girls with the least favorable genotype (AABB). The estimated frequencies of the AB and ab haplotypes indicate that these alleles were in linkage disequilibrium (P<0.001).²⁴ Neither age nor anthropometric measurements contributed to the variance in bone density (data not shown).

In contrast to the findings for bone density, the vitamin D-receptor alleles in the three genotypic groups were not associated with vertebral cross-sectional area or with femoral cross-sectional or cortical bone area (Table 2). However, these dimensions correlated strongly with age and with all anthropometric measurements; the strongest correlation was with body weight (r=0.76 to 0.80). In the multivariate analysis, once weight was included in the regression model, the predictive power was not improved by the addition of chronologic age, bone age, or height.

The vitamin D-receptor alleles in the three genotypic groups were not associated with the serum concentrations of calcium or other biochemical val-

ues, calciotropic hormones, or markers of bone turnover (data not shown). Similarly, there was no relation between the measurements of bone density and any of the biochemical variables. However, femoral and vertebral cross-sectional areas were weakly correlated with serum concentrations of osteocalcin, alkaline phosphatase, and bone-specific alkaline phosphatase (r=0.24 to 0.28).

DISCUSSION

We found that in normal, prepubertal, American girls of Mexican descent, polymorphisms in the vitamin D-receptor gene accounted for a significant proportion of the variance in the bone density of the femoral shaft and the lumbar vertebrae. The polymorphisms accounted for a difference of more than 1 SD in femoral and vertebral bone density between the groups of homozygotes defined by restriction enzymes *BsmI* and *ApaI*. However, because of the very narrow range of values for femoral bone density, girls with the aa and bb genotypes had femoral bone density that was an average of only 2 percent higher than that in girls with the AA and BB genotypes, whereas vertebral bone density differed by about 9 percent between the same groups.

Femoral bone density was eight times as high as vertebral bone density, a finding consistent with histomorphometric studies indicating an equivalent difference in the porosity of these two forms of bone.^{19,20} Chronologic age, bone age, height, and weight did not influence bone density at either site, a finding that corroborates previous results indicating that skeletal growth in prepubertal children is not associated with significant changes in bone density.^{17,25}

Two characteristics of the population studied should be noted. First, the girls were of Mexican-American heritage, and previous studies have suggested a large degree of genetic homogeneity in this population.²⁶ The genotypic frequencies were very similar to those previously found in France and in people of European descent in Australia and the United States.²⁷⁻²⁹ Second, we chose to study prepubertal children to avoid the confounding effect of the pubertal growth spurt on skeletal development. Differences among girls with different vitamin D-receptor gene alleles might be more difficult to demonstrate if the studies were done during puberty, when large increases in skeletal size and bone mass occur within a short period.^{25,30}

The mechanism or mechanisms by which the vitamin D-receptor genotypes are linked to bone density have yet to be determined, but they are probably related to the established actions of vitamin D. Premenopausal women with the bb genotype have a greater decrease in the serum parathyroid hormone concentration after the administration of calcitriol than women with the BB genotype.³¹ Vitamin D-receptor genotypes have also been linked to the regulation of the intestinal absorption of calcium: when the dietary intake of calcium is low, women with BB alleles may absorb calcium less efficiently than those with bb alleles.³² Thus, differences in allelic status may be related to bone mineralization through the effects of the vitamin D-receptor genotype on the intestinal absorption of calcium.

We found that vitamin D-receptor genotypes were associated with bone density but not with the volume of femoral bone or the cross-sectional area of the femurs or the vertebrae. Our results differ from those of previous studies that used projectional techniques and that were limited by the assumption that the cross-sectional areas of the vertebral bodies and the femoral shaft have a uniform shape.¹⁰⁻¹² Our results also differ from those of previous studies suggesting that the b allele of the vitamin D-receptor gene is associated with lower serum osteocalcin and calcitriol concentrations^{6,28} but agree with the results of other studies that found no such relation.^{27,33,34}

The variations in bone density according to allelic status found in this study have important implications with regard to the structural strength of bone, and they provide guidelines for identifying a subgroup of normal girls who may be at risk for osteoporosis later in life. However, several investigators

have been unable to find an association between bone mass and vitamin D-receptor polymorphisms in elderly women, and others have suggested that the association is present only before menopause.^{9,34,35} Determination of the reasons for possible age-related changes in this association is likely to yield valuable information regarding other factors that determine the strength of bones in adults.

In conclusion, vitamin D-receptor gene alleles predict the density of the lumbar vertebrae and the femoral shaft in prepubertal American girls of Mexican descent. Careful evaluation of the genetic mechanism responsible for the differences in bone density among normal girls may provide a method for identifying girls who are at risk for fractures later in life.

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