Associations of Tibial Lead Levels with *Bsm*I Polymorphisms in the Vitamin D Receptor in Former Organolead Manufacturing Workers

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We evaluated associations of tibial lead levels with polymorphisms in the vitamin D receptor (VDR) in 504 former organolead manufacturing workers with past exposure to lead. In this cross-sectional study, we measured tibial lead by ^{109}Cd K-shell X-ray fluorescence. Tibial lead was evaluated in subjects with different *VDR* genotypes defined using the *Bsm*I restriction enzyme, adjusting for confounding variables. Study participants had a mean age ± SD of 57.4 ± 7.6 years. A total of 169 (33.5%) subjects were homozygous for the *Bsm*I restriction site (designated *bb*), 251 (49.8%) were heterozygous (*Bb*), and 84 (16.7%) were homozygous for the absence of the restriction site (*BB*). Among all of the study subjects, tibial lead concentrations were low, with a mean ± SD of 14.4 ± 9.3 µg Pb/g bone mineral. There were only small differences in tibial lead concentrations by *VDR* genotype, with mean ± SD tibial lead concentrations of 13.9 ± 7.9, 14.3 ± 9.5, and 15.5 ± 11.1 in subjects with *bb*, *Bb*, and *BB*, respectively. In a multiple linear regression model of tibial lead concentrations, the *VDR* genotype modified the relation between age and tibial lead concentrations; subjects with the B allele had larger increases in tibial lead concentrations with increasing age (0.37, 0.48, and 0.67 µg/g per year of age in subjects with *bb*, *Bb*, and *BB*, respectively; the adjusted *p*-value for trend in slopes = 0.04). The *VDR* genotype also modified the relation between years since last exposure to lead and tibial lead concentrations. Subjects with *bb* evidenced an average decline in tibial lead concentrations of 0.10 µg/g per year since their last exposure to lead, whereas subjects with *Bb* and *BB* evidenced average increases of 0.03 and 0.11 µg/g per year, respectively (the adjusted *p*-value for trend in slopes = 0.01). Polymorphisms in the vitamin D receptor modified the relations of age and years since the last exposure to lead with tibial lead concentrations. Although controversy remains on the influence of the *VDR* genotype on bone mineral density, the data suggest that variant *VDR* alleles modify lead concentrations in bone, either by influencing lead content or calcium content or both. *Key words*: bone lead, vitamin D receptor, X-ray fluorescence. *Environ Health Perspect* 108:199–203 (2000). [Online 20 January 2000]

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Interactions between lead and calcium have long been recognized. For example, both calcium deficiency and calcitriol (1α,25-dihydroxyvitamin D) result in increased lead absorption from the gastrointestinal tract (*1,2*); blood lead levels are lower in children with higher dietary calcium intakes (*3*); dietary calcium can reduce bone lead accumulation and its mobilization during pregnancy and lactation in animals (*4*); low calcium intake during pregnancy and lactation results in greater mobilization of maternal skeletal lead stores in humans (*5,6*); and lead accumulates in bone, a calcium-rich tissue (*7–9*). Lead also appears to affect parathyroid hormone and calcitriol levels in serum, with moderate lead levels increasing the levels of parathyroid hormone and calcitriol (*10*), and toxic levels decreasing the renal synthesis of calcitriol (*11*).

In target tissues, calcitriol exerts its effects after binding to the vitamin D receptor (VDR). Several restriction fragment length polymorphisms (RFLPs) have been identified in the *VDR* gene (*12*). The RFLPs are in the nontranslated region of the *VDR* gene, and thus would not be expected to influence the binding affinity of the receptor for its ligand, calcitriol (*13*). However, one study did not find a difference in duodenal mucosal receptor density by *VDR* genotype, which was thought to be another way the *VDR* polymorphisms could influence bone mineral density (*13*). These RFLPs have been associated with differences in circulating osteocalcin levels (*12*) and in bone mineral density (*14–18*). However, the association of *VDR* polymorphisms and bone mineral density remains controversial (*19–24*).

Most studies have focused on the *Bsm*I polymorphism; this restriction enzyme results in three genotypes: *bb, Bb*, and *BB*. The absence of the restriction site, termed *BB*, has a prevalence in Caucasian populations ranging from 7 to 32% (*15*). Study subjects, mainly women, with the *BB* genotype have up to 10–15% lower bone mineral densities than subjects with the *bb* genotype (*15*). A meta-analysis concluded that the average effect size across all published studies, comparing bone mineral densities in subjects with *BB* to those with *bb*, was 2.4% lower at the hip, 2.5% lower at the spine, and 1.7% lower at the distal radius (*15*).

To date, no studies have evaluated bone lead concentrations by *VDR* genotype. Bone lead content can be measured by X-ray fluorescence (XRF), thus providing an estimate of lifetime exposure to lead (*7–9*). In the XRF technique, bone lead concentration is normalized to bone mineral content, providing an estimate in micrograms lead per gram bone mineral. We report a study of tibial lead concentrations by *VDR* genotype in former organolead manufacturing workers whose last occupational exposure to lead occurred an average of 18 years before their bone lead measurement.

Methods

Study design and overview. Data for the study were derived from a 4-year prospective evaluation of central and peripheral nervous system function in current and former employees of a chemical manufacturing facility in the eastern United States that produced tetraethyl and tetramethyl lead (*25–27*). All subjects had past exposure to organic and inorganic lead and none were currently exposed to lead. Subjects were enrolled over a 3-year period and were followed from 2 to 4 years. This work is a cross-sectional analysis of tibial lead levels obtained in the third year of the study.

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Selection and recruitment of study subjects. The recruitment and selection of study subjects have been previously described by Schwartz et al. (*25–27*). Of the 703 former lead workers enrolled in the study, 84% of the eligible subjects completed tibial lead measurements. Of the 543 subjects who completed tibial lead measurements, 93% completed *VDR* genotyping.

Data collection. The study was approved by the Committee for Human Research at the Johns Hopkins School of Hygiene and Public Health (Baltimore, MD), and all subjects provided informed written consent. Data collection procedures have been described by Schwartz et al. (*25,26*). In the first year of the study, subjects provided a 10-mL blood specimen by venipuncture. The specimen was stored at -70°C as plasma, buffy coat, red blood cells, and whole blood. *VDR* genotyping and blood lead levels were measured from this sample obtained in the first year of the study. During the third year of the prospective study, tibial lead was measured by ^{109}Cd K XRF at the midtibial shaft (*7–9*).

Tibial lead measurements. Schwartz et al. (*25*) described the details of tibial lead measurement in the former lead workers. Tibial lead was assessed (in micrograms lead per gram bone mineral) via a 30-min measurement at the left midtibial shaft using ^{109}Cd in a back-scatter geometry to fluoresce K-shell X-rays from lead. These X-rays were then quantitated to estimate the concentration of lead in bone, after normalization to the elastic scatter peak (due mainly to bone mineral content), in micrograms lead per gram bone mineral (*7–9*). ^{109}Cd-based K-shell XRF has been validated against atomic absorption spectrometry of lead in bone samples (*7,28*). For quality control and calibration, bone lead phantoms constructed of plaster-of-Paris with known concentrations of lead ranging from 0 to 122 µg Pb/g plaster were regularly measured by the XRF system. Seven subjects had point tibial lead concentration estimates that were < 0. All point estimates were retained in the statistical analyses, including negative values, because this method minimizes bias and does not require censoring of data (*29*).

Vitamin D receptor genotyping. Genomic DNA was extracted from whole blood by using the QIAamp Blood Kit (QIAGEN, Hilden, Germany). The *Bsm*I polymorphic site in intron 8 was amplified by polymerase chain reaction (PCR) using the primers originating in exon 7 (primer 1: 5′-CAACCAAGACTACAAGTAC-CGCGTCAGTGA-3′) and intron 8 (primer 2: 5′-AACCAGCGGGAAGAG-GTCAAGGG-3′). The reaction was completed in a 50-µL reaction volume containing 0.3 ng DNA, 0.2 mM of each primer, 1.25 units Amplitaq DNA polymerase (Perkin-Elmer, Branchburg, NJ), 1.25 mM dNTP, 10 mM Tris-HCL, pH 8.3 (at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% (weight/volume) gelatin. We used the following PCR cycle conditions: holding at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. The amplification produced 825 base pair fragments. After PCR amplification, 10 mL was digested with 5 units *Bsm*I (New England Biolabs, Beverly, MA) at 65°C, as described by the supplier. The digested samples were electrophoresed in 3% MetaPhor agarose gels (FMC Bioproducts, Rockland, ME) in tris base EDTA buffer. Gels were stained with ethidium bromide, visualized on a transilluminator under ultraviolet light, and photographed.

Statistical analysis. Our first goal of the analysis was to determine whether there were differences in mean tibial lead levels among the three *VDR* genotype groups. This initial analysis revealed that age and the years since the last exposure to lead were important confounding variables. Thus, our second major goal was to determine if the *VDR* genotype modified relations between age and the years since the last exposure to lead and tibial lead concentrations. SAS statistical software programs were used for the data analysis (SAS Institute, Inc., Cary, North Carolina). Mean tibial lead levels were compared in subjects with the *bb*, *Bb*, and *BB* genotypes by one-way analysis of variance. Next, we used multiple linear regression to control for potential confounding variables. In modeling tibial lead levels (in microgram lead per gram bone mineral), we started with our previously published model (*25*) that controlled for age, height, tobacco use (current vs. never and previous vs. never), lead exposure duration (with both linear and quadratic terms to account for nonlinear relation), diabetes (yes vs. no), regular exercise that induced sweating (yes vs. no), and height. The *VDR* genotype was then added to this model in one of two ways: first, as a pair of indicator variables in which *bb* served as the reference group, and second, as a trinary variable (0, 1, 2) corresponding to *bb*, *Bb*, and *BB*, to test for trends. Effect modification of the *VDR* genotype with age and years since the last exposure to lead was determined by examining the significance of cross-product terms of *VDR* genotype indicators with age and years since the last exposure, respectively. Nonlinear relations for age or years since the last exposure were evaluated by inclusion of quadratic terms, but because no significant curvilinear relations were identified, only the linear results are presented. All regression relations were evaluated for the assumptions implicit in

Table 1. Characteristics of 504 former organolead manufacturing workers who completed tibial lead measurements and *VDR* genotyping, 1996–1997.

Characteristic	Mean ± SD[a]	Range
Age (years)	57.4 ± 7.6	41–73
Exposure duration (years)	8.3 ± 9.7	0–40
Duration since last exposure (years)	17.9 ± 11.6	1–49
Blood lead (µg/dL)	4.6 ± 2.6	1–20
Tibial lead (µg Pb/g bone mineral)	14.4 ± 9.3	-2–51[b]
Race, Caucasian (%)	93	
Current tobacco use (%)	20	
Current alcohol use (%)	74	

[a]Values shown are mean ± SD except where indicated. [b]K-shell XRF can provide negative estimates of bone lead concentration in subjects with low levels; all values were used in the analysis (*31*).

Table 2. Results of linear regression modeling of tibial lead levels, evaluating interactions between *VDR* genotype and age (model 1) or years since the last exposure (YSLE) to lead (model 2) in 504 former organolead manufacturing workers, 1996–1997.

Independent variables	Units of β-coefficient	β-Coefficient	β SE	*p*-Value[a]
Model 1[b]				
Age	Bone mineral per year (µg Pb/g)	0.368	0.087	< 0.01
Years since last exposure	Bone mineral per year (µg Pb/g)	0.005	0.042	0.91
VDR-Bb	Bone mineral (µg Pb/g)	-5.542	6.013	0.36
VDR-BB	Bone mineral (µg Pb/g)	-15.910	8.277	0.06
Age × *VDR-Bb*[c]	Bone mineral per year (µg Pb/g)	0.107[d]	0.104	0.30
Age × *VDR-BB*[c]	Bone mineral per year (µg Pb/g)	0.302[d]	0.143	0.04
Model 2[b]				
Age	Bone mineral per year (µg Pb/g)	0.476	0.055	< 0.01
Years since last exposure	Bone mineral per year (µg Pb/g)	-0.096	0.060	0.11
VDR-Bb	Bone mineral (µg Pb/g)	-1.544	1.440	0.28
VDR-BB	Bone mineral (µg Pb/g)	-2.240	1.912	0.24
YSLE × *VDR-Bb*[c]	Bone mineral per year (µg Pb/g)	0.122[d]	0.068	0.07
YSLE × *VDR-BB*[c]	Bone mineral per year (µg Pb/g)	0.205[d]	0.088	0.02

[a]From β/SE β; *p*-values may not correspond exactly because of rounding, to three significant digits, of the β-coefficients and the SEs. [b]These models also controlled for exposure duration (linear and quadratic terms), tobacco use (current vs. never and past vs. never), history of diabetes, height, and exercise that induced sweating, to be consistent with prior research (*25*). [c]Interaction terms evaluating whether influence of age (model 1) or YSLE to lead (model 2) differs by *VDR* genotype. [d]*p*-Values for trends in slopes with age (model 1) across *VDR* groups = 0.04, and for years since the last exposure to lead (model 2) across *VDR* groups = 0.01.

linear regression, including dependent variable normality, linearity, lack of multicolinearity, and equality of variances. One subject with the *bb* genotype who had an extreme tibial lead value was removed from the analysis because of the disproportionate influence this value had on the regression modeling.

Results

The 504 subjects who completed tibial lead measurements and *VDR* genotyping were primarily Caucasian (93%). Their mean age ± SD was 57.4 ± 7.6 years, ranging from 41 to 73 years (Table 1). Tibial lead concentrations and blood lead levels were low, ranging from -2 to 51 µg/g and from 1 to 20 µg/dL, respectively (Table 1). A total of 169 (33.5%) subjects were homozygous for the *Bsm*I restriction site (designated *bb*), 251 (49.8%) were heterozygous (*Bb*), and 84 (16.7%) were homozygous for the absence of the restriction site (*BB*).

There were only small differences in unadjusted tibial lead concentrations by *VDR* genotype, with mean ± SD tibial lead levels of 13.9 ± 7.9, 14.3 ± 9.5, and 15.5 ± 11.1 in subjects with *bb*, *Bb*, and *BB*, respectively. Relationships between age, lead exposure duration, height, tobacco use, diabetes, exercise, and tibial lead levels have been described by Schwartz et al. (*25*). After adjustment for these factors, the trend of increasing tibial lead levels across the *bb*, *Bb*, and *BB* groups did not achieve statistical significance (adjusted *p*-value for linear trend = 0.16).

Linear regression indicated that the *VDR* genotype modified the relations between age and tibial lead concentrations and years since the last exposure to lead and tibial lead concentrations (Table 2). After adjusting for the previously identified confounding variables (*25*), on average, subjects with the B allele had larger increases in tibial lead concentrations with increasing age [0.37, 0.48, and 0.67 µg/g per year of age in subjects with *bb*, *Bb*, and *BB*, respectively; adjusted *p*-value for trend in slopes = 0.04; model 1 (Table 2), and Figure 1A]. *VDR* also modified the relationship between years since the last exposure to lead and tibial lead concentrations. Subjects with *bb* had, on average, a decline in tibial lead concentration of 0.10 µg/g per year since their last exposure to lead, whereas subjects with *Bb* and *BB* had slight increases [0.03 and 0.11 µg/g per year, respectively; adjusted *p*-value for trend in slopes = 0.01; model 2 (Table 2) and Figure 1B]. The correlation between age and years since the last exposure to lead was only moderate (Pearson's $r = 0.33$, $p < 0.01$).

In former lead workers without ongoing exposure to lead, bone lead stores are the main contributor to current blood lead levels. However, the *VDR* genotype did not modify the relation between blood lead levels (dependent variable) and tibial lead concentrations (data not shown).

Discussion

Because calcitriol binds to the vitamin D receptor, and because there is evidence that the *VDR* genotype influences, for example, bone mineral density and serum osteocalcin levels, it is likely that the *VDR* genotype influences the kinetics of calcium. Lead is a cation that behaves like calcium in biologic systems; therefore, we hypothesized that the *VDR* genotype influences lead uptake and retention in bone storage pools. In previous studies, the *BB* genotype was associated with bone mineral densities that were on average 2–10% lower than those in individuals with *bb* (*15*). Few studies have evaluated the association between the *VDR* genotype and bone mineral density in men, but one recent study reported that men under 50 years of age with *BB* had forearm bone densities that were 7% lower, on average, than densities in men with *bb* or *Bb*, possibly because of larger bone size rather than reduced bone mass (*30*). Our data reveal that the *VDR* genotype modifies the apparent kinetics of lead in bone in men with ages ranging from 40 to 70 years. The associations of both age and years since the last exposure to lead with tibial lead concentrations differed by genotype. The findings

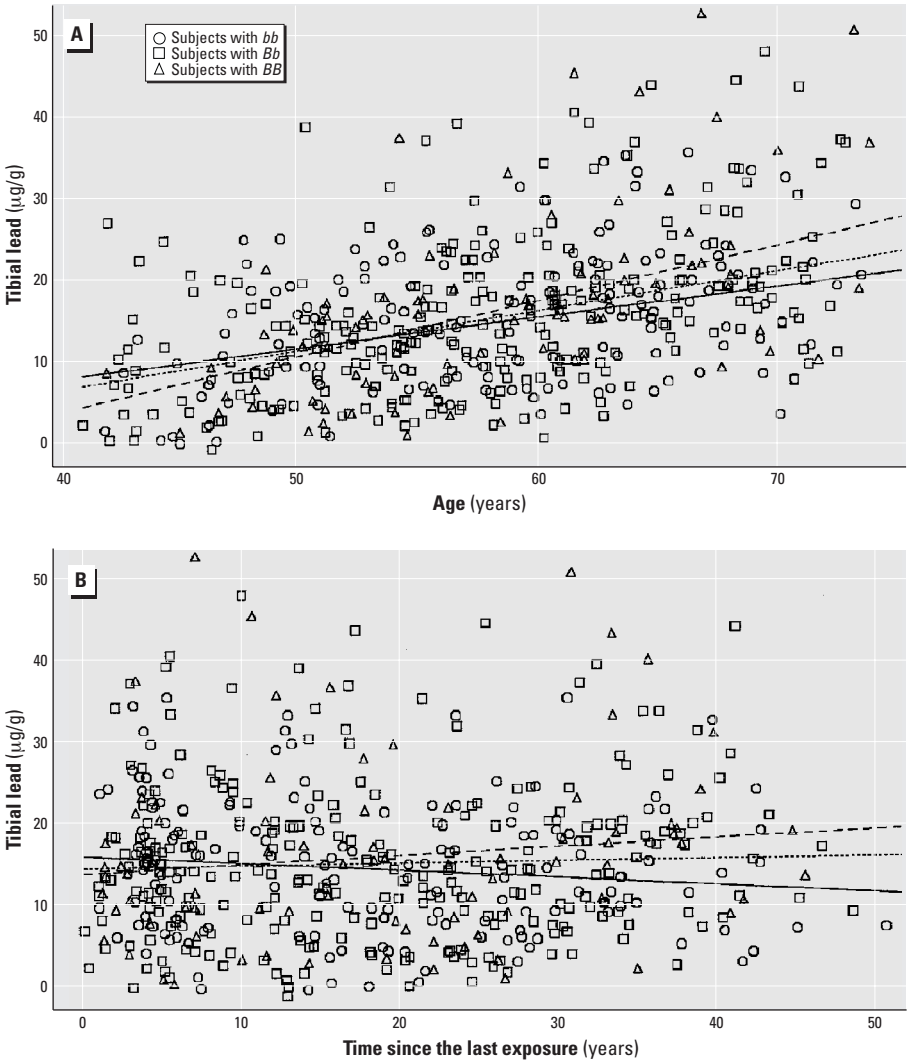

Figure 1. (*A*) Plot of the results of model 1, Table 2, for the relation of age and tibial lead levels by *VDR* genotype, adjusting for confounding variables, in 504 former organolead workers. The solid line is for subjects with *bb*, the dotted line is for subjects with *Bb*, and the dashed line is for subjects with *BB*. The slopes of the lines are 0.368, 0.475, and 0.670 µg lead/g bone mineral per year of age, in subjects with *bb*, *Bb*, and *BB*, respectively (*p*-value for trend in slopes = 0.04). (*B*) Plot of the results of model 2, Table 2, for the relation of years since the last exposure to lead and tibial lead levels by *VDR* genotype, adjusting for confounding variables, in 504 former organolead workers. The solid line is for subjects with *bb*, the dotted line is for subjects with *Bb*, and the dashed line is for subjects with *BB*. The slopes of the lines are -0.096, 0.026, and 0.109 µg lead/g bone mineral per year since the last exposure to lead, in subjects with *bb*, *Bb*, and *BB*, respectively (*p*-value for trend in slopes = 0.01).

also exhibited trends with the number of copies of the B allele. More copies of B were associated with larger increases in bone lead concentrations with increasing age, and no declines in concentrations with increasing years since the last exposure to lead, relative to subjects with the *bb* genotype.

For our study subjects, peak tibial lead concentrations should have been achieved at the end of the occupational exposure to lead, then remained stable or slowly declined with increasing duration since the last exposure to lead, unless a significant environmental exposure pathway was present. Numerous studies have documented that in cross-sectional analysis of bone lead and age, bone lead concentrations increase with increasing age (*31*). However, one recent longitudinal study reported that tibial lead concentrations evidenced no changes when measured twice over a 3-year period in 70 community exposed men with an average age of 66 years (*31*), suggesting that the apparent association with age represents a birth cohort effect rather than a true increase in tibial lead concentration over time. Thus, a decrease in tibial lead concentration with increasing years since the last exposure to lead is the most likely scenario as the lead slowly clears from bone stores.

Tibial lead levels are normalized to bone mineral content, so observed differences in tibial lead concentrations could be due to differences in bone lead content, bone mineral density, or both. Because bone mineral is the denominator of the XRF technique, a decrease in bone mineral density would yield an apparent increase in tibial lead concentration. Peak mineral density of cortical bone is probably attained at 30–35 years of age. After 40–50 years of age, cortical bone mineral density declines by approximately 0.3 to 0.5% per year, but the tibia is not a high mineral loss site (*32*). In our study subjects, on average, tibial lead concentrations increased by approximately 0.37 µg/g per year of age for subjects with *bb*; the increases with age for subjects with *Bb* and *BB* were 29 and 105% higher per year, respectively. These estimates are consistent with those of previous studies, in which the *VDR* genotype was not considered (*33,34*). These studies reported that tibial lead increased, on average, 0.38 µg/g per year of age for subjects from 20 to 55 years of age (*33*) and 0.63 µg/g per year of age in middle-aged and elderly men with a mean age of 67 years (*34*). Among study subjects, the mean tibial lead level was 14 µg/g; therefore, increases in tibial lead concentrations of 0.37, 0.48, and 0.67 µg/g per year of age for subjects with *bb*, *Bb*, and *BB*, respectively, represent increases of 2.6, 3.4, and 4.8% per year of age, respectively. These values seem too high to be solely due to bone mineral loss. It is thus likely that the *VDR* genotype not only influences measured tibial lead concentrations, possibly by affecting bone mineral content, but also the actual accumulation and/or retention of lead in bone.

With increasing years since the last exposure to lead, on average, subjects with *bb* had declines in tibial lead concentrations of approximately 0.10 µg/g per year since their last exposure. In contrast and on average, subjects with *Bb* and *BB* evidenced slight increases in tibial lead concentrations with increasing durations since their last exposure to lead. The correlation between age and years since the last exposure to lead was only moderate; variability in age only accounted for 11% of the variability in years since last exposure (Pearson's $r = 0.33$, $r^2 = 0.109$); therefore, it is unlikely that the association of years since the last exposure and tibial lead concentration is merely due to the correlation with age. The mean tibial lead concentration in subjects with *bb* was 14 µg/g, so an annual decline of 0.10 µg/g is approximately 0.7% per year. The increases in tibial lead concentrations with increasing duration since the last exposure for subjects with *Bb* and *BB* (0.2 and 0.8%, respectively) are close to the average fractional loss of bone mineral content with age. This suggests that the association of tibial lead concentration with years since the last exposure to lead, modified by the *VDR* genotype, could be due to a greater average loss of bone mineral content with age in subjects with the B allele.

Subjects with *BB* had the highest unadjusted (and adjusted) average current tibial lead concentrations. They also exhibited the largest increases in tibial lead concentrations with age and duration since the last exposure. These relatively higher tibial lead concentrations could be due to higher initial accumulation, longer retention, or both. The cross-sectional data do not allow determination of the likely mechanism for these observations. If the current results from this cross-sectional study are representative of the actual clearance in individuals with different *VDR* genotypes, the higher current levels and slower clearance imply that the *BB* genotype promotes the retention of lead in bone. However, because the *VDR* genotype likely influences bone mineral density, with the *BB* genotype promoting the loss of bone mineral (which would produce an artifactual increase in measured tibial concentration), it is possible that at least a portion of the apparent slower clearance of the *BB* genotype is due to the loss of both lead and bone mineral, with disproportionately more rapid loss of bone mineral with time, as compared to that of the *bb* and *Bb* genotypes.

Thus, the data suggest that the *VDR* genotype modifies current tibial lead concentrations, possibly by influencing both the kinetics of lead in bone and bone mineral content. The results have implications for epidemiologic studies that use bone lead measurements to predict health effects: this genetic cause of interindividual differences in tibial lead concentrations may need to be assessed and adjusted for so that health effects modeling would be more accurate. If the *VDR* genotype affects the accumulation and release of lead from bone, it may also be an important source of inter-individual susceptibility to the health effects of lead.

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