Research article

The vitamin D receptor polymorphism in the translation initiation codon is a risk factor for insulin resistance in glucose tolerant Caucasians

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Abstract

Background: Although vitamin D receptor (VDR) polymorphisms have been shown to be associated with abnormal glucose metabolism, the reported polymorphisms are unlikely to have any biological consequences. The VDR gene has two potential translation initiation sites. A T-to-C polymorphism has been noted in the first ATG (f allele), abolishing the first translation initiation site and resulting in a peptide lacking the first three amino acids (F allele). We examined the role of this polymorphism in insulin sensitivity and beta cell function. This study included 49 healthy Caucasian subjects (28 females, age 28 ± 1 years old, body mass index 24.57 ± 0.57 kg/m^2, waist-hip ratio 0.81 ± 0.01 cm/cm). They were all normotensive (less than 140/90 mmHg) and glucose tolerant, which was determined by a standard 75-gm oral glucose tolerance test. Their beta cell function (%B) and insulin sensitivity (%S) were calculated based on the Homeostasis Model Assessment (HOMA). Their genotypes were determined by a polymerase chain reaction-restriction fragment length polymorphism analysis. Phenotypes were compared between genotypic groups.

Results: There were 18 FF, 21 Ff, and 10 ff subjects. Since only 10 ff subjects were identified, they were pooled with the Ff subjects during analyses. The FF and Ff/ff groups had similar glucose levels at each time point before and after a glucose challenge. The Ff/ff group had higher insulin levels than the FF group at fasting (P=0.006), 30 minutes (P=0.009), 60 minutes (P=0.049), and 90 minutes (P=0.042). Furthermore, the Ff/ff group also had a larger insulin area under the curve than the FF group (P=0.009). While no difference was noted in %B, the Ff/ff group had a lower %S than the FF group (0.53 vs. 0.78, P=0.006). A stepwise regression analysis confirmed that the *Fok I* polymorphism was an independent determinant for %S, accounting for 29.3% of variation in %S when combined with waist-hip ratio.

Conclusions: We report that the *Fok I* polymorphism at the VDR gene locus is associated with insulin sensitivity, but has no influence on beta cell function in healthy Caucasians. Although this polymorphism has been shown to affect the activation of vitamin D-dependent transcription, the molecular basis of the association between this polymorphism and insulin resistance remains to be determined.

Introduction

Although conflicting findings about bone mineral density (BMD) in patients with type 2 diabetes have been reported, there are substantial data which support the notion that type 2 diabetes is associated with increased BMD [1,2,3]. The Rotterdam Study [2], which involved 5,931 subjects, including 243 men and 355 women with type 2 diabetes, provides the most convincing evidence. They found that diabetic men and women had increased BMD independent of age, obesity, the use of estrogen, thiazide, or loop diuretics, impairment in the ability of daily living, and smoking [2]. Furthermore, hyperinsulinemia has been reported to be associated with an increased BMD in diabetic [4] and non-diabetic subjects [5]. From the Rancho Bernardo Study [5], the level of fasting insulin was significantly and positively associated with BMD in non-diabetic female subjects, where each 10 μU/ml increase in fasting insulin level was associated with an increase of BMD by 0.33 g/cm^2 in the radius and 0.57 g/cm^2 in the spine. However, no independent association between fasting insulin level and BMD was noted in males [5]. Since fasting insulin level is an indicator of insulin resistance, it is tempting to hypothesize that insulin resistance (decreased insulin sensitivity) is associated with increased BMD.

BMD is known to have strong genetic determinants. Our understanding of the relationship between BMD and VDR gene polymorphism is based on a twin study of serum osteocalcin level [6]. The synthesis of osteocalcin, the most abundant non-collageous protein in bone, is induced by calcitriol (the active form of vitamin D) through the VDR. Since a variation in serum osteocalcin level was shown to have a strong genetic component mediated through the VDR, the relationship between VDR polymorphisms and serum osteocalcin level was found. Furthermore, this relationship was shown to be independent of age or menopausal effect [7]. Subsequently, the VDR gene polymorphisms were found to account for 75% of the total genetic effect on BMD in healthy people [8]. However, to date there are more than 50 studies on the relationship between VDR gene polymorphisms and BMD [9]. About half of the studies found a significant association, and the other half found no association [9]. The most convincing evidence comes from a study of peak BMD in a group of pre-pubertal girls [10], which confirms the relationship between VDR gene polymorphism and BMD.

Since the VDR is expressed in tissues, namely muscle and pancreatic islets [11,12,13,14], which are involved in the regulation of glucose metabolism, we hypothesized that the VDR gene could play a role in glucose metabolism. In this study, we examined the role of the *Fok I* polymorphism in the translation initiation codon of the VDR gene in 49 healthy, glucose tolerant Caucasians who underwent a standard oral glucose tolerance test. The insulin sensitivity and beta cell function were estimated using a homeostasis model assessment (HOMA) [15]. We found that the *Fok I* polymorphism is associated with insulin resistance in a Caucasian population.

Results

Characteristics of the study population

Since hypertension is associated with insulin resistance [16], only those with normal blood pressure (systolic blood pressure less than 140 mmHg and diastolic blood pressure less than 90 mmHg) were enrolled into the study. Because we were also concerned about the effect of glucose toxicity on beta cell function and insulin sensitivity [17], all subjects underwent a standard 75-gm oral glucose tolerance test. Only those subjects with a fasting plasma glucose level less than 6.1 mM, interval plasma glucose levels (at 30, 60, and 90 minutes) less than 11.1 mM, and two-hour plasma glucose level less than 7.8 mM were enrolled in this study.

This study included 49 healthy Caucasian subjects, of which 28 were female. Clinical features of the subjects were: mean age 28 years old (range: 19-39 years), mean body mass index 24.26 kg/cm^2 (17.58-34.26 kg/cm^2), mean waist-hip ratio 0.80 cm/cm (0.65-1.03 cm/cm), mean systolic blood pressure 113 mmHg (94-135 mmHg), mean diastolic blood pressure 67 mmHg (53-83 mmHg), mean fasting plasma glucose level 4.71 mM (3.88-5.54 mM), mean 30-minute post challenged plasma glucose level 7.40 mM (5.34-9.66 mM), mean 60-minute post challenged plasma glucose level 9.09 mM (3.79-10.20 mM), mean 90-minute post challenged plasma glucose level 6.30 mM (3.12-9.02 mM), and mean two-hour post challenged plasma glucose level 5.71 mM (2.94-7.60 mM).

Genotype, phenotype, and biochemical association

After genotyping, we identified 18 FF, 21 Ff, and 10 ff subjects. In this Caucasian population, the allele frequency was 58% for the F allele and 42% for the f allele. The distribution of genotypes was in compliance with the Hardy-Weinberg equilibrium (p=0.873). Since only 10 ff subjects were identified, they were pooled with the Ff subjects. Two groups (FF vs. Ff/ff) had compatible gender distribution, age, body mass index, systolic and diastolic blood pressure, fasting lipid profiles and fasting plasma glucose level (Table 1). However, the Ff/ff subjects had a higher mean waist-hip ratio and a higher mean fasting plasma insulin level, compared to the FF subjects (p=0.042 and p=0.006, respectively). These results indicate that the Ff/ff subjects are more obese and more insulin resistant than the FF subjects.

Table 1: Clinical features and glycemic parameters by the VDR genotypes

		Ff/ff		FF	
		Mean (n)	(95% CI)	Mean (n)	(95% CI)
N		31		18	
Gender	F/M	16/15		12/6	
Age	year	28	(26, 30)	27	(24, 30)
Body mass index	kg/m2	25.01	(23.45, 26.56)	23.82	(22.06, 25.59)
Waist-hip ratio 1	cm/cm	0.83	(0.79, 0.86)	0.77	(0.73, 0.82)
Systolic blood pressure	mmHg	113	(110, 117)	112	(107, 117)
Diastolic blood pressure	mmHg	68	(65, 70)	66	(62, 70)
Triglycerides	mM	1.357	(1.083, 1.631)	1.045	(0.731, 1.360)
Total cholesterol	mM	4.055	(3.757, 4.353)	4.093	(3.725, 4.461)
HDL cholesterol	mM	1.226	(1.108, 1.343)	1.322	(1.132, 1.512)
LDL cholesterol	mM	2.373	(2.106, 2.641)	2.392	(2.099, 2.685)
Oral glucose tolerance test					
Fasting plasma glucose	mM	4.75	(4.64, 4.85)	4.63	(4.43, 4.84)
Fasting plasma insulin 2	pM	70	(63, 77)	54	(46, 62)
%B		162	(144, 180)	152	(112, 192)
%S 3		0.53	(0.47, 0.59)	0.78	(0.52, 1.06)

1 p=0.042 for geometric means; 2 p=0.006 for geometric means; 3 p=0.006 for geometric means

Beta cell function (%B) and insulin sensitivity (%S) were estimated from the HOMA based on the average of three fasting plasma glucose and insulin levels before the oral glucose tolerance test. No difference was noted in %B between the two groups (p=0.23). However, there was a drastic difference in %S between the two groups (p=0.006). After an oral glucose load (Figure 1), the Ff/ff subjects had similar plasma glucose levels at each time point compared to the FF subjects. However, the Ff/ff subjects had higher postchallenged insulin levels at 30, 60, and 90 minutes than the FF subjects (p=0.009, p=0.049, and p=0.041, respectively). Furthermore, the Ff/ff subjects had a higher insulin area under the curve than the FF subjects (p=0.009). These results again indicate that the Ff/ff subjects are more insulin resistant than the FF subjects.

Discussion

We found that in healthy, normotensive Caucasian subjects with normal glucose tolerance, the *Fok I* polymorphism in the vitamin D receptor gene accounted for 15.2% of the variance in %S, but had no impact on %B. Although the Ff/ff subjects were more obese than the FF subjects (p=0.042 for waist-hip ratio), the difference in %S between the two groups decreased after adjusting for waist-hip ratio (0.50 vs. 0.69 before and 0.52 vs. 0.65 after adjusting for waist-hip ratio). However, this polymorphism remained an independent determinant for %S after adjusting for waist-hip ratio (p=0.039 Figure 2). Therefore, we concluded that the *Fok I* polymor-

Stepwise regression analysis

Various covariates have been shown to affect either beta cell function or insulin sensitivity or both. To examine the influence of the available covariates in addition to the *Fok I* polymorphism on beta cell function and insulin sensitivity, we employed a stepwise regression analytical approach. Age, gender, body mass index, waist-hip ratio, and systolic and diastolic blood pressure were entered into the model and a backward stepwise regression analysis was performed. It revealed that the *Fok I* polymorphism was an independent determinant for %S, but not for %B (Table 2). The *Fok I* polymorphism, combined with waist-hip ratio, explained 29.3% of the variance in %S. This result confirms that this polymorphism is an independent risk factor for insulin resistance.

phism was a risk factor for insulin resistance, independent of obesity.

The allelic frequencies of the present study (58% for the F allele and 42% for the f allele) are very similar to the reported frequencies in a Japanese (56% for the F allele and 44% for the f allele) and a Caucasian (59% for the F allele and 41% for the f allele) population [18,19]. In the present study, we pooled the ff subjects with Ff subjects for its small sample size (10 ff vs. 18 Ff and 21 FF). To examine whether there is a difference among the three genotypic groups, we examined the influence of genotypes on %S and %B by ANOVA. We found a significant
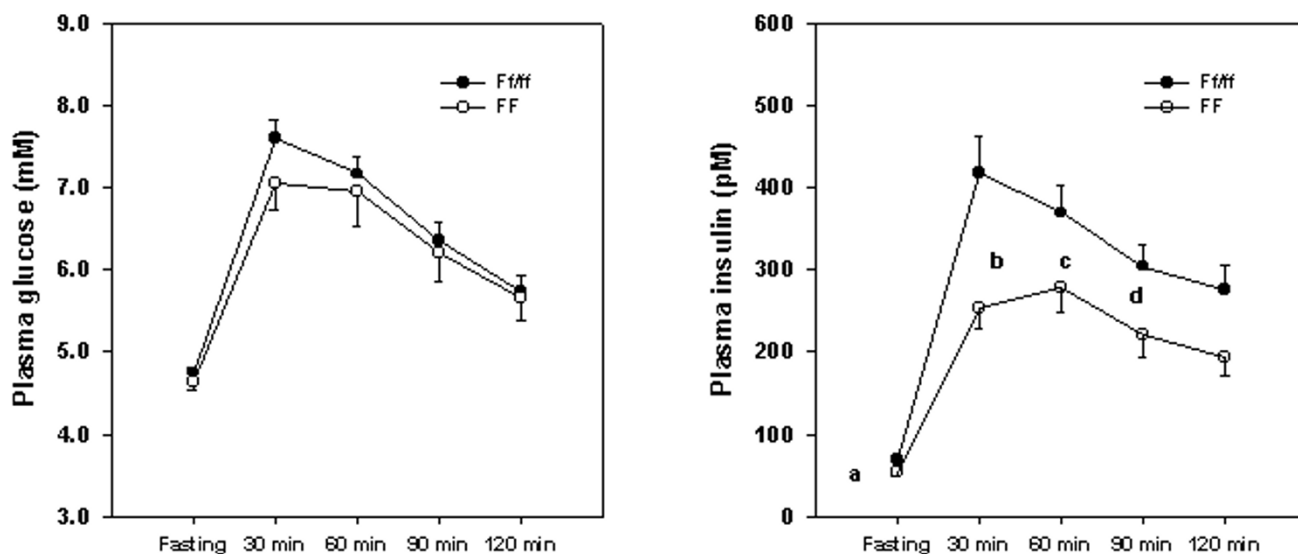
Figure 1

Plasma glucose and insulin levels during the oral glucose tolerance test by the FokI polymorphism at the vitamin D receptor gene locus. The data presents arithmetic means ± standard errors. The Ff/ff group is shown as the line with the solid dots and The FF group is shown as the line with open dots. The plasma insulin levels were different between the two groups at fasting (a, p=0.006), 30 (b, p=0.009), 60 (c, p=0.049) and 90 (d, p=0.041) minutes. The insulin areas under the curve were also different (p=0.009) between the two groups.

Table 2: Stepwise regression analysis

Dependent Variable	Covariate Entered	Covariate Removed	r 2	p
%S			0.293	
	Waist-hip ratio			0.004
	Fok I polymorphism			0.039
		Diastolic blood pressure		0.121
		Body mass index		0.140
		Systolic blood pressure		0.152
		Gender		0.566
		Age		0.715
%B			-	
		Age		0.146
		Fok I polymorphism		0.229
		Waist-hip ratio		0.475
		Body mass index		0.715
		Gender		0.786
		Diastolic blood pressure		0.916
		Systolic blood pressure		0.985

difference in %S (p=0.016) and again no difference in %B (p=0.408). However, no difference in %S was noted between the Ff and ff groups (p=0.159). The main difference in %S is between the FF and Ff groups (p=0.007).

Although we cannot exclude the possibility of inadequate power to detect the difference from the present sample size, this observation is consistent with the functional study of the *Fok I* polymorphism in cultured hu-

man peripheral blood mononuclear cells [20]. In this functional study, they compared the inhibitory effect of 1,25-dihydroxyvitamin D_3 on the growth of cultured human peripheral mononuclear cells isolated from subjects with different *Fok I* genotypes and found significant differences among the three genotypes, but found no difference between the Ff and ff groups [20].

Obesity is well known to be associated with insulin resistance [21]. In the present study, we also found that the *Fok I* polymorphism was associated with obesity assessed by waist-hip ratio but not by body mass index (Table 1). However, the association between the Ff/ff genotypes with both insulin resistance and obesity prompted us to further examine the interaction among this polymorphism, obesity, and insulin resistance. Multivariate analysis revealed that this polymorphism had no independent impact on waist-hip ratio (p=0.1468) after adjustment for age (p=0.0203), gender (p=0.0007) and body mass index (p < 0.0001), while systolic and diastolic blood pressure had also no impact on waist-hip ratio (p=0.5549 and p=0.3806, respectively). However, from the multivariate analysis result of %S (Table 2), we concluded that this polymorphism had an important impact on insulin sensitivity, which was independent of the influence of waist-hip ratio. Although gender was excluded as an independent covariate for %S in the present study after adjustment for waist-hip ratio, which was affected by gender, the interaction between gender and this polymorphism, if any, needs to be addressed in a study with a much larger sample size.

The role of VDR gene polymorphisms has been examined in four groups of diabetic patients, however their roles in insulin sensitivity have not been examined. In 93 Southern Indian families with type 1 diabetes, transmission disequilibrium testing analysis demonstrated the preferential transmission of the "b" allele of the *Bsm I* polymorphism to affected subjects [22]. However, it was less certain for the *Taq I* and *Apa I* polymorphisms in this sample set. Nonetheless, this study indicates that the *Bsm I* polymorphism at the VDR gene locus is a risk factor of type 1 diabetes. In a study of 152 Caucasian families with type 1 diabetes, there was an excessive transmission of bT and bAT extended haplotypes to affected individuals but no evidence of excessive transmission of *Taq I* and *Apa I* alleles alone [23]. Recently, we confirmed the association of this marker with type 1 diabetes in a Taiwanese population [24]. These studies in two different populations indicate that the *Bsm I* polymorphism at the VDR gene locus confers susceptibility to type 1 diabetes. The study of type 2 diabetes with the VDR gene included 164 Bangladesh Asians who were at risk for type 2 diabetes and 25% of them were either diabetic or impaired glucose tolerant. An association between the *Apa I* polymorphism and insulin secretion index was noted [25]. These studies suggest that the VDR gene locus is a genetic marker for both type 1 and type 2 diabetes.

Since the *Bsm I* polymorphism is located in intron 7, the *Apa I* polymorphism in intron 8, and the *Taq I* polymorphism in exon 9 within the 3'-untranslated region of the VDR gene, they are less likely to have any biological consequences in causing the disease and affecting glucose metabolism. Furthermore, functional studies of these polymorphisms show no clearly demonstrable difference among them [26,27,28,29]. The T-to-C transition polymorphism at the translation initiation codon of the human VDR gene not only destroys a *Fok I* site but also abolishes the first translation initiation codon of this gene. The alternative translation initiation codon, which also adheres the first-AUG rule by Kozak [30], is located downstream by three amino acids. In the present study, we examined the *Fok I* polymorphism, which not only affects the translation of the peptide, but also affects the biological activity tested in Hela Cells [31]. This T-to-C transition resulted in the synthesis of a smaller (F allele, three amino acids less than the f allele) protein with increased vitamin D-dependent transcriptional activation [31]. In addition, the different response to 1,25-dihydroxyvitamin D is also demonstrated in cultured human peripheral blood mononuclear cells from subjects with different *Fok I* genotypes [20]. We found that the subjects with homozygous F allele had increased insulin sensitivity compared to those with the f allele.

Although the molecular basis of this association is unclear, the study of the relationship between the level of vitamin D and glucose tolerance [32] provides some insight to the underlying mechanism. Glucose tolerance and vitamin D level were assessed in 142 elderly Dutchmen. Fasting insulin and postchallenged insulin levels decreased with increased vitamin D levels [32]. As a higher insulin level is a surrogate for decreased insulin sensitivity, their observation suggests that a higher vitamin D level is associated with increased insulin sensitivity. Since vitamin D binds to the vitamin D receptor to initiate the downstream action, a higher vitamin D level would lead to an increase in vitamin D-dependent transcription activation. Our observation that the subjects with homozygous F alleles (associated with an increased activity) are more insulin sensitive than those with the f allele is consistent with the notion above.

Our original hypothesis was that the allele, which is associated with increased BMD, was also associated with higher insulin levels and insulin resistance. However, since the F allele is associated with increased BMD [31,33], our observation of the association between the F
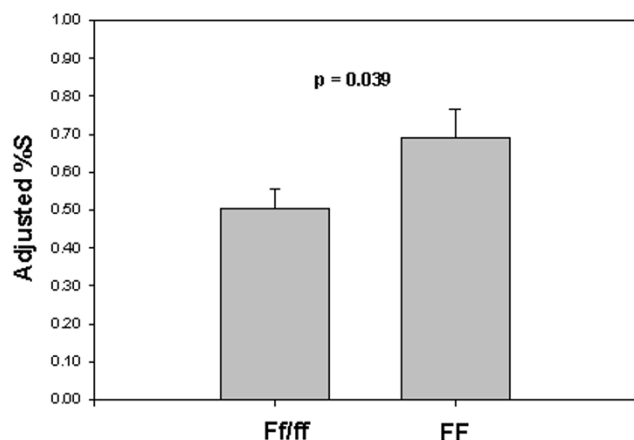
Figure 2
Adjusted insulin sensitivity (%S) by the Fok I polymorphism at the vitamin D receptor gene locus. They were logarithmically transformed before analysis and expressed as geometric mean ± standard error. Insulin sensitivity was adjusted for waist-hip ratio as described in the methods and results sections and in Table 2.

allele and increased insulin sensitivity is not consistent with our original hypothesis. On the other hand, not all reports support the association between the F allele and increased BMD [34,35]. Nonetheless, our observation shows that this polymorphism is an independent determinant for insulin sensitivity and is in agreement with the notion that a higher level of vitamin D is associated with increased insulin sensitivity [32]. Furthermore additional studies in the Caucasian and other ethnic populations are required.

In the present study, we report an association between a polymorphism at the translation initiation codon of the human VDR gene and insulin sensitivity in glucose tolerant and normotensive Caucasians. This polymorphism is an independent determinant of insulin sensitivity, explaining 15.2% of the variance in %S and with waist-hip ratio, explaining 29.3% of the variance in %S. Although the speculated mechanism of our observation is in agreement with the published association between vitamin D levels and insulin sensitivity, further studies are required to confirm our observation in other ethnic groups as well as in another Caucasian population. To explore the underlying molecular basis of the *Fok I* polymorphism at the VDR gene locus, as well as the association between vitamin D level and insulin sensitivity, also warrants further study.

Subjects, materials and methods
Study subjects
We confirm that the study has complied with the recommendations of the Declaration of Helsinki. The study was approved by the institutional review board and a

written informed consent was obtained from each participant at the beginning of the study. Subjects were recruited through an advertisement on a campus media of this institution. Only healthy subjects were invited to participate in this study. They were prescreened via telephone and confirmed again on the day of the visit. They were biologically unrelated.

Oral glucose tolerance test and physical examination
Subjects were instructed to fast for at least 14 hours before the study visit. On the morning of the visit, an indwelling angiocatheter was inserted into an antecubital vein. All subjects had fasting blood samples drawn at -15, -10, and -5 minutes. These samples were used for glucose, insulin, lipid measurement and genomic DNA isolation. Fasting plasma glucose and insulin levels were calculated as the average of three fasting samples. After the oral administration of 75-gm glucose, postchallenged blood samples were drawn at 30, 60, 90, and 120 minutes for glucose and insulin measurements. A limited physical examination was performed on all study participants, including measurements of weight, height, waist, hip, and blood pressure. After resting quietly for 30 minutes, three consecutive measurements of systolic and diastolic blood pressures were taken one minute apart, and the average of the three measurements was reported.

DNA extraction and genotyping of the Fok I polymorphism
Genomic DNA was extracted from peripheral lymphocytes as previously described [36]. The genomic DNA fragment flanking the *Fok I* polymorphism was amplified using two primers flanking exon 2 of the VDR gene: AGCTGGCCCTGGCACTGCTCTGCTCT and ATGGAAACACCTTGCTTCTTCTCCCTC. Polymerase chain reaction (PCR) was carried out in a total volume of 11 μl containing 0.5 pM of each primer, 0.2 mM dNTP, 2 mM $MgCl_2$, 5% dimethysulphoxide (DMSO), 0.275 U Taq polymerase, 50 mM KCl, 10 mM Tris-HCl pH 8.3, and 0.001% gelatin. The region of interest was amplified by an initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, and concluded with a final extension at 72°C for 10 minutes. Then, 5 μl of the PCR product (265 base pairs) was digested in 15-μl of reaction volume containing 1 U of *Fok I* (New England Biolabs Inc., Beverly, Massachusetts, USA) with the buffer supplied by the vender. The digested PCR products were resolved on 2.0% agarose gels. *Fok I* digested the first ATG and yielded two products, 69 and 196 base pairs (f allele), while the T to C transition destroyed the *Fok I* site (F allele).

Statistical analysis
SYSTAT version 8.0 for Windows of the SPSS Inc. (Chicago, Illinois, USA) was used for statistical analysis. Var-

iables with skewed distributions were logarithmically transformed before analysis. The variables transformed were body mass index, waist-hip ratio, plasma insulin levels, insulin area under the curve, %B, and %S. Data were presented as mean (or geometric means when appropriate) with 95% confidence intervals, unless otherwise specified. Two-sided t-tests or chi-square tests were used to evaluate the differences between the two groups. To determine the interaction of the genotype of interest with the other covariates, a stepwise regression analysis was performed with a backward option and an alpha-to-remove or alpha-to-enter setting at 0.10. The covariates with the highest p value were removed one at a time, until all the remaining covariates had a p value less than 0.10.

Abbreviations

HOMA, Homeostasis Model Assessment; VDR, vitamin D receptor; BMD, bone mineral density; PCR, polymerase chain reaction.

Acknowledgements

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