Association of Vitamin D Receptor Gene Polymorphism with Susceptibility to Graves’ Disease in Eastern Croatian Population: Case-control Study

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Aim
To evaluate the effect of vitamin D3 receptor (VDR) gene BsmI/ApaI/TaqI restriction fragment length polymorphisms on Graves’ disease susceptibility in a subset of patients from Eastern Croatia.

Methods
Graves’ disease patients (n = 110) and ethnically matched euthyroid controls (n = 99) with no clinical evidence or family history of thyroid or autoimmune diseases were genotyped for VDR gene polymorphisms by BsmI/ApaI/TaqI endonuclease digestion after polymerase chain reaction amplification with sequence-specific primers. Data were analyzed by χ²-test, and crude odds ratios (OR) with 95% confidence interval (95% CI) were calculated.

Results
The Apal “AA” (14.5% vs 30.3%, patients vs controls, respectively, OR = 0.39, 95% CI [0.2-0.77], P = 0.01) and BsmI “BB” (7.3% vs 23.2%, OR = 0.26 [0.11-0.61], P = 0.002) genotypes were significantly underrepresented in patients, whereas Apal “aa” (28.2% vs 9.1%, OR = 3.92 [1.76-8.74], P = 0.001) and TaqI “TT” (51.8% vs 31.3%, OR = 2.36 [1.34-4.16], P = 0.004) genotypes were significantly more frequent in patients than controls. The genotype combination, which conferred the strongest protection against Graves’ disease, was “BBAAtt” (2.7% vs 17.2%, OR = 0.14 [0.04-0.48], P = 0.001).

Conclusion
These findings suggest that VDR gene BsmI/ApaI/TaqI polymorphisms are associated with Graves’ disease susceptibility in a subset of patients from Eastern Croatia. The Apal and BsmI “AA” and “BB” genotypes, respectively, as well as combined “BBAAtt” genotype, appeared to confer protection against Graves’ disease, whereas Apal “aa” and TaqI “TT” genotypes were associated with an increased risk for Graves’ disease. However, the true mechanisms of association remain to be elucidated.

Graves’ disease is an organ-specific autoimmune thyroid disease in which thyrotropin (TSH)-receptor autoantibodies cause hyperthyroidism (1). The susceptibility to Graves’ disease is conferred by multiple genetic and environmental factors. So far, genetic studies have identified over 20 potential candidate loci involved in the pathogenesis of Graves’ disease (2,3). Among those, genes involved in immune system regulation remain important candidate genes (4,5). Vitamin D receptor (VDR)-related endocrine system has been shown to influence immune regulation through multiple pathways (6,7). Briefly, 1α, 25-dihydroxyvitamin D3, which exerts its biological effects through VDR, inhibits differentiation of dendritic cells into antigen-presenting cells (8), suppresses T-cell proliferation (9), inhibits secretion of proinflammatory Th1-related cytokines such as interleukin (IL)-1 (10), IL-2, IL-6, IL-12, tumor necrosis factor α, interferon γ (11) and down-regulates the expression of HLA-DR (12,13) and CD13 antigen in mononuclear phagocytes (13).
The presence of VDR in most cells of the immune system, in particular dendritic cells, as well as CD8+ lymphocytes, CD4+ T-lymphocytes (14), and cells of the monocytic-macrophage lineage (15) further underscores its immunoregulatory properties.

Meta-analyses of genetic studies support a contribution of common variants to susceptibility to common diseases (16). A cluster of readily detectable biallelic polymorphisms exists in the 3'-region of the VDR gene, two of which, located in the intron between exon 8 and exon 9, are defined by restriction endonucleases Apal and BsmI (17). The enzyme Taql identifies another polymorphism found in the 3'-coding sequence in exon 9. Indeed, variants of the VDR gene have been associated with susceptibility to several autoimmune processes, such as insulin-dependent diabetes mellitus (18-20), Addison disease (21), primary biliary cirrhosis, autoimmune hepatitis (22), Crohn disease (23), and multiple sclerosis (24). Recently, a relationship between VDR variants and susceptibility to Graves’ disease was also reported in female Japanese patients (25). In addition, 1α, 25-dihydroxyvitamin D3 administration was able to prevent the development of thyroiditis in animal models (26), whereas in humans, it was reported to ameliorate Graves’ disease (27). However, associations or linkage between polymorphisms of the VDR gene and autoimmune thyroid disease found in some reports have been difficult to replicate in other populations (28). Furthermore, in different populations, the same polymorphisms can confer increased or decreased risk to disease susceptibility. Recently, VDR polymorphisms were shown to affect susceptibility to insulin-dependent diabetes mellitus in Dalmatian region of Croatia (29).

Given the association of VDR variants with insulin-dependent diabetes mellitus and thyroid autoimmunity, we investigated the distribution and potential effects of VDR gene variants on Graves’ disease susceptibility in a subset of patients from Eastern Croatia.

Subjects and Methods

Subjects

The study included 110 unrelated patients (97 women, 13 men, median age at diagnosis 41 years, interquartile range 31-48 years) from Eastern Croatia, diagnosed and treated at Department of Nuclear Medicine, Radiation Protection and Pathophysiology, Osijek University Hospital, Osijek, Croatia, from 1997 to 2003. Graves’ disease was defined by the presence of biochemical hyperthyroidism, and at least two of the following criteria: diffuse goiter, elevated 24 h radiiodine thyroid uptake, positive thyroid peroxidase- and/or TSH-receptor autoantibodies, and thyroid-associated orbitopathy. None of the patients had any other immune-related disease previously found to be associated with VDR gene polymorphisms.

The control group consisted of 99 ethnically matched (79 women, 20 men, median age 36 years, interquartile range 25-47 years), unrelated, euthyroid, thyroid peroxidase autoantibodies-negative subjects, without clinical evidence or family history of autoimmune and endocrine disease. All control subjects had normal ultrasonic findings of the thyroid gland. Informed consent in written form was obtained from all participants prior to the testing. The study was approved by the hospital’s ethical committee and performed from March 2003 to December 2004.

Materials

High Pure PCR Template Preparation Kit, LightCycler FastStart DNA Master SYBR Green I Kit and DNA Molecular Weight Marker VIII were purchased from Roche Diagnostics (Mannheim, Germany). Primer 1 (5’-GGGAGACGTAGCAAA GG-3’), primer 2 (5’-AGAGGTCAAGGGTCACT G-3’), primer 3 (5’-CAGAGCATGGACAGGGA GCAAG-3’), and primer 4 (5’-GCAACTCCTCAT GGCTGAGTCTCA-3’) were obtained from Invitrogen (Paisley, UK). Restriction enzymes BsmI, Taql and Apal were Sigma (Taufkirchen, Germany) products.

Genotyping

Genomic DNA was extracted from 200 µl EDTA blood with a DNA isolation kit (Roche Diagnostics) according to manufacturer’s instructions. Each DNA sample was subjected to 40 cycles of capillary polymerase chain reaction (PCR) performed with a LightCycler (Roche Diagnostics). Mastermixes were optimized for the LightCycler and contained the following: 2 mmol/L MgCl2; 0.5 µmol/L of the primers 1 and 2, or 0.8 µmol/L of the primers 3 and 4; and 2 µl of FastStart mixture, that contained FastStart Taq polymerase, deoxyribonucleoside triphosphates,
and SYBR Green dye. The quality of PCR products was confirmed using melting curve analysis. The restriction fragment length polymorphisms (RFLPs) were coded as Bb (BsmI), Aa (Apal), or Tt (TaqI), the uppercase letter signifying the absence of the site and lowercase letters signifying the presence of the restriction site.

Detection of the BsmI restriction site was achieved by amplifying a region spanning the site with one primer originating in exon 7 (primer 1) and the other originating in intron 8 of VDR gene (primer 2). Amplification was started with an initial denaturation at 95°C for 10 min followed by 40 amplification cycles of 10 s at 95°C, 5 s at 51°C, and 15 s at 72°C. PCR products were 359-bp long. After amplification, PCR products were digested with 5 U of BsmI restriction enzyme for two hours at 65°C and then electrophoresed through 3% agarose gels containing ethidium bromide. Bands were visualized on an ultraviolet transilluminator (Pharmacia Biotech, Uppsala, Sweden). The presence of the BsmI restriction site on both alleles (defined as bb) generated 182 and 177 bp fragments, whereas the absence (BB) yielded one undigested 359 bp fragment.

Region of VDR gene containing Apal and TaqI restriction site was obtained in PCR reaction using primer 3, originating in intron 8, and primer 4 originating in exon 9 of VDR gene. Amplification was performed with a 95°C initial denaturation for 10 min followed by 40 cycles of 10 s at 95°C, 5 s at 69°C, and 30 s at 72°C. PCR products were 740 bp long. For detection of TaqI and Apal restriction sites, each PCR product was subjected to digestion with 3 U of BsmI for two hours at 65°C or with Apal enzyme using 10 U for two hours at 30°C. After digestion with Apal, according to the presence or absence of restriction site, genotypes were identified as AA (one undigested PCR fragment of 740 bp), aa (two fragments of 515 and 225 bp) and heterozygous Aa. The TaqI digestion revealed one obligatory restriction site, the homozygous TT (absence of the specific TaqI restriction site) yielding fragments of 490bp and 245 bp. The homozygous tt exhibited fragments of 290, 245, and 205 bp and the heterozygous Tt provided 490, 290, 245, and 205 bp fragments.

Statistical analysis

After testing for normality of data distribution (Kolmogorov-Smirnov test) and homogeneity of variances (Levene test), unpaired, continuous variables, expressed as medians (interquartile ranges), were analyzed with the Mann-Whitney U test because the assumption of equality of variances was not met. Genotypes and alleles in patients and controls, given as absolute and relative frequencies, were compared with Pearson’s χ²-test with Yates’ correction for 2×2 and 3×2 tables or by Fisher exact test whenever appropriate. All tests were two-tailed. The strength of association was estimated by crude odds ratio (OR), with 95% confidence interval (95% CI). Significance was accepted at P<0.05 or, in case of k multiple independent comparisons, at P<0.05/k (Bonferroni’s correction). The values given in tables and throughout the text are true P values. All calculations were performed with SPSS software, release 9.0 (SPSS Inc., Chicago, IL, USA).

Results

The allele and genotype frequencies for Apal, TaqI, and BsmI variants in our controls were similar to those previously reported in the Croatian population (29). No difference was observed in age (Z = -1.797, P = 0.072, Mann-Whiney U test) or gender-related composition between patients and control group (χ² = 15.899, P = 0.001). The VDR “aa” genotype occurred more frequently in the patients than in the controls, whereas “AA” genotype was significantly undertransmitted to the patients. Also, the “A” allele was more frequent among the controls.

Similarly, comparing the distribution of BsmI polymorphisms in the patients and the controls (Table 2), significantly fewer patients than expected carried the “BB” genotype. Furthermore, the “B” allele was also significantly underrepresented among the patients.

The distributions of TaqI genotype frequencies (Table 3) differed significantly between the patients and the controls. The “TT” genotype occurred more frequently in the patients, with the “T” allele significantly overrepresented among the patients.
Stratified genotype-wise analysis by gender revealed that the associations were essentially unchanged in women, despite a reduction in sample size (for Apal \( \chi^2 = 15.512, P < 0.001 \); for TaqI \( \chi^2 = 11.848, P = 0.003 \); for BsmI \( \chi^2 = 11.221, P = 0.004 \)). Repeated allele-wise comparisons did not provide any additional information on susceptibility. The number of men in both groups was too small to draw meaningful conclusions.

No difference was observed in genotype distributions of RFLPs when the patients with an age of onset of Graves’ disease at or above the median value for the group were compared to the patients with an age of onset of disease below the median value (for Apal \( \chi^2 = 1.306, P = 0.52 \); for TaqI \( \chi^2 = 2.059, P = 0.357 \); and for BsmI \( \chi^2 = 0.583, P = 0.747 \)).

An extended combined genotype analysis was performed to further test for the associations observed: 17 of 27 possible combined BsmI/Apal/TaqI genotypes with non-zero frequencies in either patients or control group were identified; four most commonly represented genotypes with relative frequencies \( \geq 10\% \) in either group (accounting for \( \geq 67\% \) of cases in both groups) are presented in Table 4. Multiple comparisons by repeated \( \chi^2 \) tests were performed (k = 17, threshold for significance at \( P < 0.0029 \)) disclosing significant underrepresentation of the “BBAAtt” genotype in patients (\( \chi^2 = 10.949; \text{OR} = 0.14; 95\% \text{ CI} [0.056, 0.357] \)) and carriers of the “BBAAtt” genotype in patients (\( \chi^2 = 0.583, P = 0.747 \)).

### Table 1. Distribution of vitamin D receptor (VDR)-Apol polymorphisms in patients with Graves’ disease and their matched controls

<table>
<thead>
<tr>
<th>VDR-Apol genotypes and alleles</th>
<th>No. (%) of participants</th>
<th>( \chi^2 )</th>
<th>( P )</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype-wise comparison:†</td>
<td>AA 16 (14.5)</td>
<td>30 (30.3)</td>
<td>15.899</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>Aa 63 (57.3)</td>
<td>60 (60.6)</td>
<td>31.282</td>
<td>(0.05-1.51)</td>
</tr>
<tr>
<td>Allele-wise comparison:</td>
<td>A 95 (43.2)</td>
<td>120 (60.6)</td>
<td>11.978</td>
<td>(0.001)</td>
</tr>
<tr>
<td></td>
<td>a 125 (56.8)</td>
<td>78 (39.4)</td>
<td>2.02 (1.37-2.99)</td>
<td></td>
</tr>
</tbody>
</table>

†: Genotype-wise comparison:†

### Table 2. Distribution of vitamin D receptor (VDR)-BsmI polymorphisms in patients with Graves’ disease and their matched controls

<table>
<thead>
<tr>
<th>VDR-BsmI genotypes and alleles</th>
<th>No. (%) of participants</th>
<th>( \chi^2 )</th>
<th>( P )</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype-wise comparison:‡</td>
<td>BB 8 (7.3)</td>
<td>23 (23.2)</td>
<td>11.505</td>
<td>(0.003)</td>
</tr>
<tr>
<td></td>
<td>Bb 54 (49.1)</td>
<td>46 (46.5)</td>
<td>1.78 (1.01-3.15)</td>
<td></td>
</tr>
<tr>
<td>Allele-wise comparison:</td>
<td>B 70 (68.2)</td>
<td>92 (46.4)</td>
<td>8.811</td>
<td>(0.003)</td>
</tr>
<tr>
<td></td>
<td>b 25 (25.6)</td>
<td>106 (53.6)</td>
<td>1.86 (1.25-2.77)</td>
<td></td>
</tr>
</tbody>
</table>

‡: Genotype-wise comparison:‡

### Table 3. Distribution of vitamin D receptor (VDR)-TaqI polymorphisms in patients with Graves’ disease and their matched controls

<table>
<thead>
<tr>
<th>VDR-TaqI genotypes and alleles</th>
<th>No. (%) of participants</th>
<th>( \chi^2 )</th>
<th>( P )</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype-wise comparison:‡</td>
<td>TT 57 (51.8)</td>
<td>31 (31.3)</td>
<td>9.515</td>
<td>(0.001)</td>
</tr>
<tr>
<td></td>
<td>Tt 42 (38.2)</td>
<td>50 (50.5)</td>
<td>0.50 (0.22-1.12)</td>
<td></td>
</tr>
<tr>
<td>Allele-wise comparison:</td>
<td>T 156 (70.9)</td>
<td>112 (56.6)</td>
<td>8.705</td>
<td>(0.003)</td>
</tr>
<tr>
<td></td>
<td>t 64 (29.1)</td>
<td>86 (43.4)</td>
<td>1.87 (1.50-2.274)</td>
<td></td>
</tr>
</tbody>
</table>

‡: Genotype-wise comparison:‡
population. Consistently, the combined increased risk of Graves' disease in the Croatian and
ethnicity to Graves' disease, whereas genotypes “AA” appeared to be associated with increased susceptibil-
ity to confer increased risk in Japanese cohort, it ap-
peared protective in Croatian population. Further-
more, although significant associations were evi-
dent in both studies, differences in the nature of as-
ociations were observed for BsmI RFLP, the “B” allele being overrepresented in Japanese patients sample, whereas reverse association is suggested by our own data.

No relation was detected between any other combined genotype and Graves' disease phenotype.

No relation was detected between any polymorphism and serum free thyroxine, free triiodothyronine, thyroid peroxidase- or TSH receptor-autoantibodies levels measured in the patients at the time of diagnosis (data not shown).

### Discussion

Using a case-control study, we observed the association between allelic variants of the VDR gene and phenotype of Graves' disease in a subset of patients from Eastern Croatian population. The Apal genotype “aa” and TaqI genotype “TT” appeared to be associated with increased susceptibility to Graves' disease, whereas genotypes “AA” and BsmI genotype “BB” appeared to confer a decreased risk of Graves' disease in the Croatian population. Consistently, the combined BsmI/ Apal/TaqI genotype with the strongest protective effect was “BBAAtt”. The results presented here provide an independent replication of association of genetic variation at the VDR locus with Graves' disease. An important discrepancy in our findings, however, is the direction of disease risk conferred by given VDR alleles in Croatian patients sample when compared with those observed in a Japanese cohort (25). Whereas the Apal “A” allele seemed to confer increased risk in Japanese cohort, it appeared protective in Croatian population. Furthermore, although significant associations were evident in both studies, differences in the nature of associations were observed for BsmI RFLP, the “B” allele being overrepresented in Japanese patients sample, whereas reverse association is suggested by our own data.

The VDR locus has been extensively studied for the association with susceptibility to numerous autoimmune diseases. Previously, “bb” genotype has been associated with multiple scleroderma in Japanese (24), primary biliary cirrhosis in Germans (22), and an increased risk for type 1 diabetes mellitus in Indian Asians (18), whereas “BB” genotype conferred increased risk for type 1 diabetes mellitus in German population (19). The “T” allele, which was associated with Graves’ disease in our patients, was also associated with autoimmune hepatitis in Germans (22) and type 1 diabetes mellitus in Eastern Europeans (30) and South Indians (18). Conversely, the “t” allele was associated with insulin-dependent diabetes in Germans (19), Crohn (23), and Addison disease (21). Similar contradictory results have been observed in the studies of VDR and its effects on bone mineral density (31). Likewise, the findings are not consistent for Graves' disease, and in the recent extensive, well-conducted study in the United Kingdom, Caucasian patients failed to reproduce any association with VDR gene single-nucleotide polymorphisms (28).

Despite extensive efforts, the results in the identification and inference of genetic effects for complex traits from independent association studies often fail to reach consensus (16,32). In one meta-analysis, significant allelic effects were observed in 11 of 25 examined gene association studies in the direction opposite to the original report of association (16). These findings suggest genetic heterogeneity within the VDR gene in different diseases and populations, possibly due to divergent evolutionary lineages resulting in separate clusters of distinct geography (22,33). In addition, the Apal, BsmI, and TaqI polymorphisms apparently lack any functional effect on the protein product (34), as BsmI and Apal are both found in non-coding region of the gene, whereas both TaqI alleles result in silent, synonymous change at codon isoleucine 352. Furthermore, although alterations in intronic sequences may influence protein expression, intron 8 polymorphisms are not consistently found to affect protein (34) and

### Table 4. The most common combined BsmI/Apal/TaqI genotypes in patients and their matched controls*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. (%) of participants</th>
<th>$\chi^2$</th>
<th>P</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BbAaTt</td>
<td>35 (31.8)</td>
<td>28 (28.3)</td>
<td>0.164</td>
<td>0.685</td>
</tr>
<tr>
<td>bbAaTT</td>
<td>19 (17.3)</td>
<td>14 (14.1)</td>
<td>0.185</td>
<td>0.667</td>
</tr>
<tr>
<td>bbaaTT</td>
<td>23 (20.9)</td>
<td>8 (8.1)</td>
<td>5.81</td>
<td>0.016</td>
</tr>
<tr>
<td>BbAatt</td>
<td>3 (2.7)</td>
<td>17 (17.2)</td>
<td>10.949</td>
<td>0.001</td>
</tr>
</tbody>
</table>

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*Relative frequency ≥10 % in either patients or control group. 17 distinct combined genotypes detected.
†CI – confidence interval.
‡$\chi^2$ test with Yates’ correction for 2×2 contingency tables; each genotype versus 16 detected genotypes.
§The threshold for statistical significance at P=0.0029, Bonferroni’s correction for multiple comparisons (k=17)
mRNA levels (35), ligand binding affinity, DNA binding, and transactivation function (36,37). Hence, neither heterogeneity of results nor the functional studies seem to support any direct pathogenetic role for VDR gene variants. Therefore, VDR RFLPs appear to be markers in the linkage disequilibrium with other relevant polymorphisms elsewhere in VDR gene or in its proximity (38), rather than primary susceptibility loci in Graves’ disease. Plausibly, the inverse genetic effects could be generally attributed to different phases of linkage disequilibrium between a marker locus allele and a functional mutation locus (31) that remains unknown. However, the distribution and extent of linkage disequilibrium is affected by differences in genealogical history, ages/frequencies of markers, recombination rates, migrations, expansions and selection (39). Consequently, the structure of linkage disequilibrium differs markedly across genomic regions and populations, and the extent of linkage disequilibrium is highly dependent on the population in which it is measured (40). Thus, the same allele may have different patterns of association with markers and haplotypes in different populations (41).

Even so, there are additional differences/covariates between populations that could affect results. It is well appreciated that population stratification, often difficult to recognize within case-control samples can mask, change, or even reverse the true genetic effects for genes underlying complex traits (42). These adverse effects have been shown to increase markedly with sample size (43). However, for the relatively small number of genotyped individuals, the $\chi^2$-test seems to be conservative (43). Furthermore, although larger control group would be necessary to better estimate true frequencies of VDR genotypes in the Croatian population, genotype distributions and allele frequencies for our controls were similar to that previously reported in the Croatian (29) and some other European populations (21,44). Nevertheless, despite controversies (32), strategies accounting for population stratification may need to be used to improve the estimation of risk in case-control studies.

Gender-specific susceptibility is a prominent feature of Graves’ disease. Gender appears to influence the effects of VDR polymorphisms on the susceptibility to type 1 diabetes mellitus (20) and Graves’ disease (25). This study examined a mixed population. However, after restudying the population composed of women only, the results remained essentially unchanged, despite the reduction in the sample size. We were not able to evaluate the role of VDR gene variants in the cohort of men, large enough to enable meaningful analysis, thereby precluding any further discussion of gender-specific effects in our study.

Phenotypic heterogeneity is another potential explanation for the differences. VDR interacts with environmental exposure to affect disease expression (38). Serum levels of VDR ligand, 1α, 25-dihydroxyvitamin D3, are dependent on different environmental and lifestyle factors. Low vitamin D levels have been implicated in the etiology of several autoimmune diseases (45). In autoimmune hyperthyroidism, 1α, 25-dihydroxyvitamin D3 levels are significantly lower than in toxic nodular goiter (46). Furthermore, serum levels of 1α, 25-dihydroxyvitamin D3 depend on vitamin D binding protein, a serum transport protein recently associated with Graves’ disease (47). Additionally, the level of dietary iodine has been shown to markedly affect the incidence of thyrotoxicosis, Graves’ disease, and hypothyroidism (1,48). In order to compensate for mild-to-moderate iodine deficiency in the 1990s, a new regulation on salt iodination was introduced in Croatia in 1996 (49). In contrast, both United Kingdom and Japan reported iodine-sufficient status (50). This prompted us to restrict the study to the patients diagnosed after 1996, and thereby, reduce, at least partially, stratification of patients with respect to iodine intake at the moment of diagnosis. Nevertheless, the effects of the patient’s country of origin, and a lifetime exposure to vitamin D and dietary iodine cannot be reliably estimated.

In conclusion, with the limitations of the small study sample, our data indicate an association between the VDR gene variants and Graves’ disease in a subset of Eastern Croatian patients. Apal and BsmI genotypes “AA” and “BB”, respectively, as well as combined genotype “BBAAtt” appear to be markers of decreased Graves’ disease susceptibility, whereas Apal “aa” and TaqI genotype “TT” appear to confer an increased risk. However, the mechanisms by which VDR gene variants associate with disease susceptibility remain elusive. Approaches robust to population admixture in testing association, like family based studies and genomic control methods (42), are needed.
to confirm the results and are currently under way in our laboratory.

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