

Research Article

Volume 6 Issue 3 – March 2018
DOI: 10.19080/CRDOJ.2018.06.555688

Curre Res Diabetes & Obes J

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VDR Gene Haplotypes Associate with Type 1 Diabetes and Suggest Interaction with HLA, IL2RA and CTLA4 loci in Colombian Families



Natalia Gomez Lopera¹, Alejandra Rodriguez¹, Martin Toro², Maria Victoria Lopera², Alejandra Velez³, Juan Manuel Alfaro^{1,2} and Nicolas Pineda Trujillo^{1*}

¹Grupo Mapeo Genético, Departamento de Pediatría, Facultad de Medicina, Universidad de Antioquia, Colombia

²Seccion de Endocrinología, Departamento de Pediatría, Universidad de Antioquia, Colombia

³Pontificia Universidad Bolivariana, Facultad de Medicina, Colombia

Submission: December 22, 2017; **Published:** March 14, 2018

***Corresponding author:** Nicolas Pineda Trujillo, Grupo Mapeo Genetico, Departamento de Pediatría, Facultad de Medicina, Universidad de Antioquia, Medellín, Carrera 51 D # 62-29, Colombia, Tel: +574 2196065; Email: nicolas.pineda@udea.edu.co

Abstract

Vitamin D and its receptor are possibly related to T-cell-mediated autoimmune diseases and influences susceptibility to Type 1 diabetes (T1D). The aim of this study was to assess the contribution of vitamin D receptor (VDR) polymorphisms to the susceptibility to T1D in a Colombian sample. Associations of VDR gene variants with T1D were investigated in a sample of 200 familial trios from Colombia. VDR polymorphisms FokI (rs2228570), BsmI (rs1544410), ApaI (rs7975232) and TaqI (rs731236), were determined using PCR-RFLP methodology. For interaction tests, we used data obtained in this sample for other T1D candidate loci, such as HLA, PTPN22, INS, IL2RA, SUMO4, CLEC16A, CTLA4 and IFIH1 genes. Allele and genotypic transmission disequilibrium tests were performed; haplotypes and gene-gene interactions were also analyzed. For characterization of the sample, the probands were tested for autoantibodies against tyrosine phosphatase-like 2 (IA-2) and anti-glutamic acid decarboxylase 65 (GAD65). We found that two haplotypes at loci BsmI-ApaI-TaqI have a protective effect in our sample [Haplotypes A-G-T OR=0.219, ci95% (0.12-0.519), p-value=5.326x10⁻⁵, and G-T-C OR=0.376, ci95% (0.18-0.786)]. In addition, locus BsmI shows a trend of interaction with SNP rs3087243 (at CTLA4 gene, p-value=8x10⁻³). Likewise, it was observed a suggestion of interaction of VDR variants with IL2RA, HLA-I and HLA-II. In conclusion, this family-based study presents evidence of two haplotypes and interaction of VDR variants with other loci associated with T1D in Colombian families.

Keywords : VDR gene; Interaction; Haplotype; Colombian families

Introduction

Type 1 diabetes (T1D) is a complex disease characterized by autoimmune and progressive destruction of insulin-secreting pancreatic beta-cells. It is well known that T1D is caused by complex interactions between many genetic and environmental factors [1]. The most firmly established known genetic contribution to T1D susceptibility has been the human leukocyte antigen region (HLA) on the short arm of chromosome 6. However, several other non-HLA regions have been identified as predisposing factors [2]. Among these susceptibility genes, the VDR gene is one of the most widely studied and increasing evidence suggests that vitamin D and its receptor are possibly related to T-cell-mediated autoimmune disease and influence susceptibility to T1D [3].

During the last years, diverse studies have emerged to analyze a possible relationship between T1D and polymorphisms

in the Vitamin D receptor (VDR), yielding conflicting results; some of them showed significant association while others failed to reach statistical significance. A more recent meta-analysis found that BsmI and FokI polymorphisms are associated with a significantly increased risk of T1D [4], whereas the ApaI, and TaqI polymorphisms do not appear to have a significant association with overall T1D risk. However, in a later meta-analysis, Tizaou et al. [5] found that individual VDR polymorphisms seemed not to be associated with T1D risk. However, they found that haplotypes contributed significantly to disease susceptibility.

Vitamin D exerts its effects through the vitamin D receptor (VDR). The VDR is found in almost all cells of the immune system, especially the antigen-presenting cells (macrophages and dendritic cells), activated T lymphocytes, as well as in pancreatic islet beta-cells [6]. It is acknowledged that

Vitamin D is an effective immunosuppressant via inhibition of lymphocyte activation and cytokine production and prevents or markedly suppresses the development of several autoimmune diseases in animal models [7]. For example, at the level of the pancreatic islets, vitamin D decreased in-vivo and in-vitro proinflammatory chemokine and cytokine expression (e.g., IL6), which are implicated in the pathogenesis of T1D, making beta-cells less chemo attractive and less prone to inflammation [6]. Furthermore, Vitamin D decreases *HLA* class I expression leading to reduced vulnerability of islet Beta-cells to cytotoxic T lymphocytes [8].

The human *VDR* gene is located on chromosome 12q13.11, spanning 63.495 bp and includes eight protein-coding exons (exons 2-9) and six untranslated exons (exons 1a-1f), which are alternatively spliced [9]. Four common single nucleotide polymorphisms (SNPs) in the *VDR* gene have been investigated extensively: FokI C>T (rs2228570), BsmI G>A (rs1544410), ApaI T>G (rs7975232), and TaqI T>C (rs731236) [10]. Allele T of the FokI SNP creates an alternative ATG initiation codon in exon 2 leading to a three amino acids longer of the *VDR* protein. The shorter form of the protein (424 aa) is more active than the long form (427 aa). The BsmI and ApaI SNPs are both located in intron 8, and the TaqI is a silent SNP in exon 9. These three polymorphisms lead to silent variants associated with increased *VDR* mRNA stability [11].

The above apparent discrepancies among different studies could be a result of the effect of ethnic differences related to the distribution of *VDR* polymorphisms amongst populations, as well as to interactions with other genetic or environmental factors involved in the pathogenesis of type 1 diabetes [12]. These discrepancies demonstrate that the role of *VDR* polymorphisms in T1D pathogenesis is unclear. For this reason, the aim of this study was to assess the contribution of vitamin D receptor polymorphisms to the susceptibility to T1D in a Colombian population. Our results may help clarify whether *VDR* gene variants contribute or not to the susceptibility for being affected by T1D. Also, they could contribute to elucidating whether the role of such variants is allelic or genotypic.

Patients and methods

Subjects

The subjects for the present study are from Antioquia, Colombia. This population corresponds to a genetically special population, described as a mixed population with linkage disequilibrium sources and founder effects [13-15], which makes it ideal for mapping complex traits, such as T1D. The study group consists of 200 trios (two parents and their affected child). The cases are children diagnosed with T1D before 15 years old.

Patients were identified in the Pediatric Endocrinology Program (Universidad de Antioquia and Hospital San Vicente Fundación), IPS universitaria-UdeA, Universidad Pontificia Bolivariana, and Instituto Antioqueño de Diabetes. This study

was approved by the ethics committee of the Medicine school of Universidad de Antioquia, and informed consent was obtained from patients and their parents prior blood sampling.

Inclusion criteria were as follows: 1) At least six great-grandparents of affected children originated in any of the Colombian states of Antioquia, Risaralda, Quindío, Caldas, North of Cauca Valley, North of Tolima, which indicates self-reported "Paisa" ancestry. 2) Both parents willing to participate in the study.

DNA isolation and *VDR* genotyping

Genomic DNA was isolated from EDTA whole peripheral blood samples using phenol-chloroform protocol [16]. Genotypes for FokI (rs2228570), BsmI (rs1544410), ApaI (rs7975232) and TaqI (rs731236) of *VDR* gene were obtained by DNA amplifications with standard PCR and specific sets of primers [17], followed by restriction fragment length polymorphism method (RFLP). All amplifications were carried out in a total volume of 15µL, containing 30ng of purified DNA, 1.5µL 10X reaction buffer containing Tris-HCl, 10mM of each dNTPs, 100nM of each primer and 1.15 U of Taq Polymerase; 1.5mM of MgCl₂ for FokI, TaqI and ApaI; and 2.0mM for BsmI. All PCRs were done in a BioRad Diad thermal cycler.

PCR products were analyzed on 2% agarose gels containing 5mg/mL ethidium bromide and visualized under a UV transilluminator. The amplified products were digested using the restriction enzymes BsmI, FokI, ApaI and TaqI, according to the manufacturer's instruction. Briefly, 10µL of each related PCR product was mixed with 5 U of the corresponding restriction enzyme, 2µL of 10X buffers and 8µL H₂O and then incubated 16h at 37 °C for BsmI and ApaI, 3h at 55 °C for FokI and 3 h at 65 °C for TaqI. Digested samples were resolved on 2.5% agarose gels as described above. Genotypes were scored blindly. Moreover, as a quality control, 10% of the samples were amplified and digested twice and second scored. Moreover, genotypes previously obtained in this sample at candidate genes consistently associated with the disease in different populations (HLA, INS, PTPN22, CTLA4, IL2RA, SUMO4, IFIH1, and CLEC16A) [18,19] were used, for the interaction tests. Genotyping was performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) or tetra-primer amplification-refractory mutation system (ARMS)-PCR [20].

Autoantibodies evaluation

In this study, autoantibodies (AABs) for glutamic acid decarboxylase 65KDa (GAD65) and tyrosine phosphatase-like 2 (IA-2) were already tested for 100 cases [21,22]. For the present study, the remaining 100 samples were tested for the same two AABs, using a commercially ELISA-based Kit (AESKULISA, Mikroforum-Germany).

Data analysis

The study had over 80% power according to genetic power calculator [23]. Prior to association analysis, we performed QC

of the obtained genotypes. Thus, data were tested for Mendelian inheritance using pedcheck [24]. Hardy-Weinberg equilibrium (HWE) for individual loci were assessed in parents using the Pearson chi-square test. Single-point and haplotype association analyses were carried out using two approaches: the transmission disequilibrium test (TDT) as implemented in Unphased [25] and conditional logistic regression as implemented in the “trio” R package [26]. A Bonferroni correction was applied for multiple testing, so $p=0.05/12=0.004$ (4 tests for individual SNPs plus 8 haplotypes).

Linkage disequilibrium (LD) was calculated and LD blocks were plotted using Haploview4.2 [27]. Haplotype analysis was performed using the 2- or 3- markers sliding windows method implemented in Unphased [23]. The sample was stratified according to the presence or absence of AABs and age at onset, as either early (≤ 5 years) or late (> 5 years). For the gene-gene interaction, two-way gene-gene interaction of unlinked loci was tested using a conditional logistic regression as implemented in the “trio” R package [24].

Results

Sample description

This study included 200 nuclear families from Colombia. 105 out of 200 patients (52.5%) were male and the remaining 95 (47.5%) were females. Gender differences did not reach statistical significance (p -value 0.33). The average age at diagnosis was 7.8 years (Table 1). Sixty one children (30.5%) developed the disease before 5 years age, with an average age for this category of 2.66 years. While the children with age at or above 5 yo had an average onset of 10.28 yo. Regarding the auto-antibodies, it was found that 78% (156) of the patients had

at least one specific autoantibody related with T1D triggering (GAD-65 or IA-2), while 22% (44) were negative for these two AABs.

Table 1: Characteristics of individuals affected with type 1 diabetes mellitus.

Characteristics	Sample (200)
No Males (%)	105 (52.5%)
No Females (%)	95 (47.5%)
Age at diagnosis	7.8±4.6
Age at onset before 5 (%)	30.50%
Age at diagnosis after 5 (%)	69.50%
Presence of AABs(IA-2 or GAD65)	78.00%
Absence of AABs(IA-2 and GAD65)	22.00%

Unless indicated otherwise, data are given as the mean±SD or as the number of subjects in each group with percentages in parentheses.

Association and linkage disequilibrium analyses

Marker descriptions are given in Table 2. Data quality control showed that the genotyping rate was $>98\%$ and no Mendelian errors were found. The four SNPs were found to be in Hardy-Weinberg equilibrium. TDT single-point analysis did not detect any significant transmission distortion (Table 2). On the other hand, genotypic TDT based on 3 Pseudo controls (Table 3) revealed that genotype TT at SNP rs7675232 (*Apal*) has a trend towards a protective effect (OR=0.59, 95% CI=0.34-1.02; $p=0.058$). Not significant deviation of transmissions was observed when considering the presence or absence of AABs, or age at onset.

Table 2: Characteristics of the SNPs tested and allelic disequilibrium test.

SNP	Position	Alleles	MAF	HW p_value	Genotyping Rate (%)	P-value	OR (CI 95%)
rs2228570 (FokI)	10416201	C/T	0.3595	0.743	98.6	0.461	0.897(0.671-1.198)
rs1544410 (BsmI)	10383141	G/A	0.3801	0.1093	98.3	0.586	1.089(0.802-1.477)
rs7975232 (ApaI)	10382143	T/G	0.387	0.01425	98	0.628	1.052(0.815 -1.358)
rs731236 (TaqI)	10382063	T/C	0.2841	0.9018	98.8	0.234	0.828(0.606 -1.131)

MAF: Minor Allele Frequencies; HE: Hardy-Weinberg Equilibrium; OR: Odds Ratio; CI: Confidence Interval

Table 3: Regression analysis for the SNPs tested in the 200 familial trios.

SNP	Coef	RR	Lower	Upper	SE	Statistic	p-Value	Trios
rs2228570	-0.562	0.570	0.318	1.022	0.298	3.560	0.059	71
rs731236	0.081	1.084	0.660	1.781	0.253	0.102	0.749	71
rs7975232	-0.527	0.590	0.342	1.019	0.278	3.581	0.058	82
rs1544410	-0.027	0.974	0.512	1.848	0.327	0.007	0.935	47

Results are for the recessive inheritance mode. Coef: Coeficiente; RR: Relative Risk; SE: Standard Error

Linkage disequilibrium (LD) analysis revealed that 3 SNPs (rs1544410, rs7975232, rs731236) were in LD (Figure 1). Two haplotypes composed by alleles at these three loci were found to be associated with T1D in our sample (Table 4). These haplotypes were characterized by alleles A-G-T and G-T-C at at rs1544410-rs7975232-rs731236, respectively. Such haplotypes were under-transmitted from parents to affected children (OR=0.219; ci95%=0.12-0.519 and OR=0.376; ci95%

0.18-0.786; p value=5.326x10⁻⁵). The most frequent haplotype was G-G-T (0.322), followed by haplotype G-T-T (0.235). A-G-T haplotype only reached 0.041 frequency. Haplotype stratified analysis according to the autoimmunity and age at onset revealed a significant associations (P-value=2.0x10⁻³). However, no significant confidence intervals were obtained (data not shown).

Table 4: Haplotypic analyses for SNPs in LD.

Global analysis							
SNPs	Haplotype	Trans	Untrans	Odds-R	95%Lo	95%Hi	P-value
rs1544410 - rs7975232 rs731236	A-G-T	9.21	28.67	0.219	0.12	0.519	5.326x10 ⁻⁵
	A-T-C	84.85	56.09	1.36	0.883	2.095	
	A-T-T	36.94	50.29	0.578	0.327	1.02	
	G-G-T	119.8	106.3	1	1	1	
	G-T-C	15.15	26.96	0.376	0.18	0.786	
	G-T-T	82.06	79.71	0.842	0.569	1.245	

Trans: Transmissions; Untrans: Untransmissions

Bold values represent significant findings

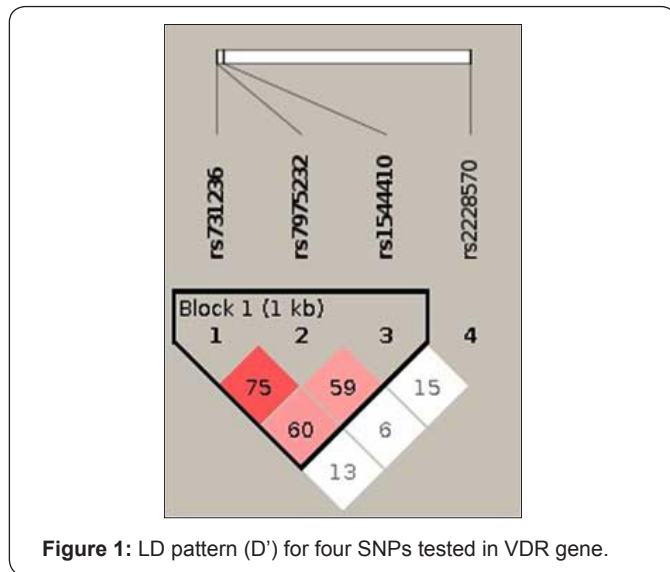


Figure 1: LD pattern (D') for four SNPs tested in VDR gene.

Gene-gene interactions

Regarding the gene-gene interaction analysis, it showed a trend of interaction of VDR with CTLA4, IL2RA, HLA-I and HLA-II genes (Table 5). We identified GxG interactions between SNPs rs1544410 in VDR gene with rs3087243 in CTLA4 gene (p=8.9x10⁻³), rs7975232 in VDR and rs2040410 in HLA-II (p=0.01). In addition, SNP rs731236 in VDR appears to interact with SNP rs706778atIL2RA (p=0.01), and also interacts with SNPs rs7745906 and rs2524074 at HLA-I (p=0.01 and 0.02 respectively). INS, PTPN22, SUMO4, IFIH1, and CLEC16A genes did not show significant interaction with VDR gene variants.

Table 5: Gene-Gene interactions of VDR gene variants with four other T1D loci.

SNP1 (VDR)	SNP2	LL(with IA)	LL(w/oIA)	Statistic	P-Value
rs7975232	rs2040410 (HLA-II)	-349.2	-355.3	12.19	0.01
rs731236	rs7745906 (HLA-I)	-353.8	-360.4	13.19	0.01
rs731236	rs2524074 (HLA-I)	-369.8	-375.7	11.7	0.02
rs731236	rs706778 (IL2RA)	-364.6	-370.7	12.2	0.01
rs1544410	rs3087243 (CTLA4)	-356.5	-363.2	13.53	8x10 ⁻³

LL: Log Likelihood; IA: Interactions

Discussion

The purpose of this study was to test for association of VDR polymorphisms in a Colombian sample with T1D, all originated from Antioquia, Colombia. To our knowledge, this is the first study on the association between VDR SNPs and T1D among Colombian subjects. We examined the association of VDR gene polymorphisms at four positions (FokI, BsmI, TaqI and ApaI) with T1D. Associations between VDR polymorphisms and T1D have been seen in other countries including Hungary [17], Brazil [28], India [29], Croatia [30], Chile [31], Iran [12], Egypt [32], and other [4,33,34]. Meanwhile, other studies have not confirmed these findings and no associations were found neither in Portuguese [35] nor Danish populations [36], indicating that different genetic backgrounds may be causative of these discrepancies.

In the present study, we found that *VDR*Apal gene polymorphism (rs7975232) has a trend to protect in the recessive state. In agreement with our results, a meta-analysis found a significant association between Apal polymorphism and T1D risk in East Asians [37]. We did not find any allelic association with T1D susceptibility. In fact, another recent meta-analysis showed that FokI, BsmI, Apal and TaqI polymorphisms, when taken separately, seemed not to be associated with T1D risk, but particular haplotypes such as G-T-T and A-G-C (BsmI-Apal-TaqI) showed significant associations [5]. None of these two haplotypes were found to be associated in our study. G-T-T haploypete presented 0.235 frequency in our study (data not shown).

In addition, our results showed that haplotypes A-G-T and G-T-C protect significantly against T1D. Although different, others have also reported protective effects of specific vitamin D receptor (*VDR*) haplotypeS characterized by BsmI-Apal-TaqI loci [38]. Even more, Uitterlinden et al. [11] found that haplotype A-G-T is one of the most frequent ones in Caucasians with a 50% prevalence. They also suggested that the variation of the degree of linkage disequilibrium between restriction fragment length polymorphism loci in the studied population might influence the degree of association between haplotypes and phenotypes. Our finding extends the repertoire of haplotypes associated with T1D, in different populations. This suggests a functional role of the *VDR* receptor in the susceptibility to T1D (perhaps extensive to autoimmunity as a whole), even in different haplotype contexts.

On the other hand, complex relationships may exist between *VDR* polymorphisms and other T1D related genes. Such interactions may explain why several previous findings regarding *VDR* polymorphisms have been inconsistent. In some cases, the effect might be missed if the gene is examined in isolation without allowing for its potential interactions with these other factors [39]. Our data showed a possible interaction effect between one of the *VDR* polymorphisms with *CTLA4*, *IL2RA*, *HLA-I* and *HLA-III*loci.

It has been reported a biological interaction between vitamin D and *CTLA4*. *CTLA4* is a critical suppressive protein that is expressed constitutively by regulatory T cells (Treg) and is induced on conventional T cells following activation. It functions to restrain inappropriate activation of autoreactive T cells and to restore T cell homeostasis following activation [40]. Jeffery et al. [41], found that vitamin D increases *CTLA-4* expression through direct effects on the T cell, suggesting that the suppressive effect of vitamin D on T cell activation might be further enhanced through *CTLA4*-mediated removal of CD80/86, by trans-endocytosis. A possible explanation, why only SNP rs1544410 (BsmI) and not the complete haplotype in LD interacts with *CTLA4*, is that the LD presented corresponds to D' and not to R2. R2 values are much smaller (data not shown).

VDR-1,25-(OH)2D3 complex has been shown to play a significant role in interfering with the signaling of transcription

factors involved in the regulation of immunomodulatory genes[42] as well as expression of HLA class-II alleles on monocytes and human bone cells [43]. The interaction between *VDR* and HLA alleles may be mediated by the *VDRE* (*VDR* enhancer) present in the promoter region of *HLA-DRB1*0301* allele [29]. Israni et al. [27] proposed that the insufficient amount of Vitamin D in early life in the predisposed individuals with *HLA-DRB1*0301* can lead to an impaired expression of the allele in the thymus, resulting in escape from thymic deletion of autoreactive T cells. Furthermore, 1,25(OH)2D3 decreases MHC class I expression leading to reduced vulnerability of islet beta-cells to cytotoxic T lymphocyte [8].

On the other hand, it has been seen that *IL2RA* gene is regulated by vitamin D in CD4+ T cells. *IL2RA* is central to immunoregulation as an important modulator of immunity. *IL2RA* expression on regulatory T cells is essential for their function in suppressing T cell immune responses and autoimmune disease [44]. Exogenous addition of 1,25(OH)2D3 to CD4+ T cells in vitro induced the expression of *IL2RA*. This change in gene expression correlated with increased cell-surface expression of the *IL2RA* in activated CD4+ T cells [45]. For instance, in Multiple sclerosis patients, there was a significant correlation between *IL2RA* expression in CD4+ T cells and serum levels of 25 (OH)D3 [45].

This research has an important limitation, which regards to the sample size. It is clear that small sample sizes reduce the power to detect significant associations with less frequent variants or with variants with a small effect size on T1D. Also, all this study relies on the PCR-RFLP, which might not be the most reliable and thorough method, since other methods based on fluorescence could provide more reliable data. However, our study does have some strengths, such as the sample selection and the statistical approach (TDT). All of the individuals in this study belong to the most differentiated population in Colombia, which is a homogenous population living Northwest in the country. This population has reported sources of linkage disequilibrium, founder effects and ancestry characterization from its three-hybrid component, mainly European, followed by Amerindian and finally the African component [13-15,46,47]. Using the TDT, we have expected the associations not to reflect any false positive finding due to population stratification, since this only accounts the transmissions from heterozygous parents [48].

Conclusion

In conclusion, our study points to a protective role of the vitamin-D receptor gene in type 1 diabetes. It was found that two haplotypes at this gene (BsmI-Apal-TaqI) protect significantly against T1D in our population. Furthermore, our results suggest an interaction between three *VDR* polymorphisms with *HLA-II*, *HLA-I*, *IL2RA* and *CTLA4* SNPs, contributing to shed light on the plausible biological and biochemical pathways that underpin the disease. These suggestive interactions might be more clearly defined in a larger sample size than ours.

Acknowledgment

We are very grateful to the families who participated in this study. We are also very grateful to Dr Jorge Garcia-Ramírez for contributing patients. This study received financial support from Colciencias (grant contract # 0331-2013) and a doctoral scholarship (NG-L) from Colciencias call # 727. Also Universidad de Antioquia contributed to finance this study.

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DOI: [10.19080/CRDOJ.2018.06.555688](https://doi.org/10.19080/CRDOJ.2018.06.555688)

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