

## Serum vitamin D levels and gene polymorphisms (Fok1 and Apa1) in children with type I diabetes and healthy controls

Maimoona Nasreen, Khalid Pervaiz Lone, Saba Khaliq, Shagufta Khaliq

### Abstract

**Objective:** To compare the pattern of Vitamin D receptor (VDR) polymorphisms (Apa I and Fok I) in Type I Diabetes mellitus (T1DM) as cases vs healthy population as control and to investigate the association of VDR polymorphism with vitamin D levels in cases and controls.

**Methods:** The hypothesis of the study was "VDR gene polymorphisms (Fok 1 and Apa 1) and vitamin D levels are associated with the T1DM". The case-control study was carried out on 44 cases and 44 controls. Clinically diagnosed unrelated cases were recruited from the Diabetic Clinic of Jinnah Hospital, Lahore during Aug. 2012 to Jan 2013. Unrelated controls with normal glucose levels and no first-degree family history of T1DM were selected by convenient sampling. Vitamin D levels of both cases and controls were measured using Enzyme Linked Immunosorbant Assay (ELISA). Genotyping was performed by Restriction Fragment Length Polymorphism (RFLP)-PCR method and the data were analyzed statistically with IBM-SPSS 21.

**Results:** Our results demonstrated suboptimal vitamin D levels in whole of our sample population, whether control or cases ( $p = 0.529$ ). There was no statistically significant difference in 25-Hydroxyvitamin D3 levels between cases ( $11.351 \pm 5.92$ ) and controls ( $12.335 \pm 6.64$ ). VDR polymorphism was not associated with susceptibility to T1DM in our sample population. Similarly, no association between VDR polymorphism and vitamin D levels was observed i.e. FokI  $p=0.507$  and  $p=0.543$  and ApaI  $p=0.986$  and  $p=0.307$  for cases and controls respectively.

**Conclusion:** There is an overall deficiency of Vitamin D levels in cases and control subjects while SNPs association studies suggested that in our sample population there was no association of VDR gene polymorphisms Fok I and Apa I with T1DM.

**Keywords:** Case-Control study, T1DM, Vitamin D receptor Polymorphism, SNP Fok1 and Apa1, Pakistani Population. (JPMA 66: 1215; 2016)

### Introduction

Diabetes, caused by insulin deficiency following destruction of the insulin-producing pancreatic beta cells, has been recognized as the fastest growing disease. According to the World Health Organization, it is estimated that the total number of people with diabetes will double from 171 million in 2000 to 366 million by 2030.<sup>1</sup> Type 1 diabetes mellitus (T1DM) is the most common form of diabetes in childhood, accounting for approximately two-thirds of new diagnoses of diabetes in patients  $\leq 19$  years of age in the United States.<sup>2</sup> Diabetes is a multifactorial disease influenced by both genetic and environmental factors. There is evidence for worldwide epidemics of both vitamin D deficiency and T1DM, and vitamin D has been identified as a contributing factor to DM especially T1DM.<sup>3</sup> Vitamin D, among its many roles, acts as a modulator of the immune system by promoting monocyte differentiation and inhibiting lymphocyte proliferation and cytokine secretion.<sup>4</sup> Few observational cohort studies in individuals with T1DM have shown

associations between low vitamin D levels as compared to age, gender, ethnicity matched healthy controls.<sup>5-8</sup> Various studies have shown that sufficient intake of vitamin D during the early childhood and pregnancy period reduces the risk of developing type 1 diabetes.<sup>9-11</sup>

Vitamin D acts via the nuclear vitamin D receptor (VDR) that plays a crucial role as a transcription factor that regulates the beta cell secretion of Insulin. Polymorphisms within the VDR gene have been associated with altered gene expression or gene function.<sup>12</sup> Four single-nucleotide polymorphisms (SNP) in the vitamin D receptor (VDR) gene have been studied in reference to T1DM: FokI F>f (rs10735810, NM\_000376.2: c.2T>C), BsmI B>b (rs1544410, NM\_000376.2: c.1024+283G>A), ApaI A>a (rs7975232, NM\_000376.2: c.1025-49G>T), and TaqI T>t (rs731236, NM\_000376.2: c.1056T>C).<sup>13,14</sup> Despite several studies, the role of VDR polymorphisms in T1DM pathogenesis has been unclear. Several studies have suggested association between one or more of these SNPs and T1DM,<sup>15-18</sup> but others have failed to confirm this finding.<sup>19,20</sup> Meta-analysis by Guo, et al. (2006) reported that Fok 1 polymorphism was associated with increased risk of insulin dependent

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Department of Physiology & Cell Biology, University of Health Sciences, Lahore.

**Correspondence:** Khalid Pervaiz Lone. Email: khalid.lone@gmail.com

diabetes.<sup>16</sup> Recent meta-analysis in Asian population shows that association of Bsm 1 polymorphism in T1DM is more significant in East Asia whereas Fok 1 is associated with the disease in population of West Asia.<sup>17</sup> Recent study from Egypt reported that Fok 1 and Bsm 1 polymorphism of the VDR gene was associated with T1DM.<sup>15</sup> Limited data is available to conclude the association between VDR polymorphism in T1DM for association South Asian population. Additional research is required to find out the effect of VDR gene polymorphism in type 1 diabetes. The Focus of the present study is to evaluate the association of VDR polymorphism (Fok 1 and Apa 1) in T1DM and also to assess their levels of vitamin D.

## Methods

**Study population:** The study was conducted in the Department of Physiology and Cell Biology and Centre for Research in Endocrinology and Reproductive Sciences (CRERS), University of Health Sciences, Lahore. Properly diagnosed cases of T1DM, according to American Diabetes Association criteria<sup>21</sup> were recruited from the Diabetic clinic of Jinnah hospital in Lahore city. Unrelated cases recruited for the present study were < 18 years of age with either gender who were diagnosed at the age of 2-12 years. Similarly, unrelated controls with normal glucose levels and having no first-degree family history of T1DM were selected by convenient sampling. Study subjects, which had other non-diabetic related autoimmune disorders, or had undergone an immunosuppressive therapy or have used glucocorticoids / vitamin D / calcium supplements were excluded from the study. Relevant information (age, sex, demographic characteristics.) was recorded and a complete general physical and systemic examination was conducted. The sample size was statistically calculated with the confidence level of 90% and taking the case-control population proportions of 0.05 and 0.15 respectively based on the earlier study.<sup>18</sup> Thus, overall 88 study subjects were selected with 44 cases and 44 controls.

**Ethics:** Prior to the sampling a written informed consent was taken from the subjects' parents. The Study was undertaken as per Helsinki declaration after approval by the Ethical Review Committee of University of Health Sciences Lahore and Ethical Committee of Jinnah hospital, Lahore.

**Serological Analysis:** From each study subject, 4 ml venous blood was collected in EDTA coated vacutainer tubes while 1 ml was collected in serum vacutainer. From the serum vacutainer, Vitamin-D (25-OH-D3) was measured by enzyme-linked immunosorbent Assay (DIAsource; Belgium, 25-OH Vitamin D Total ELISA kit).

**DNA Isolation and Genotyping:** DNA was extracted from whole blood samples using chloroform/ phenol method. Genotyping was performed by using PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) method. The primers used for Fok I are forward 5' AGCTGGCCCTGGCACTGACTCTGCTCT'3 and reverse 5'ATGGAAACACCTTGCTTCTTCTCCCTC'3 and for Apa I are forward 5' CAGAGCATGGACAGGGAGCAA'3 and reverse 5'GCAACTCCTCATGGCTGAGGTCTC' 3. A final reaction volume mixture of 20 µl contained 100 ng of template DNA, 5xPCR Buffer with 2.5 mM MgCl<sub>2</sub>, 2.5 mM of each deoxynucleotide triphosphate (dNTPs), 5 pmol of each primer and 5U of Taq DNA Polymerase (Fermentas) was used for each PCR. The cycling conditions of PCR reactions were as follows: 94°C for 5 minutes followed by 35 cycles for 94°C for 45 seconds, 45 seconds at 58°C for Fok I and 66°C for Apa I and 72°C for 45 seconds with a final extension of 72°C for 7 minutes. The product achieved for Fok I was of 265bp and for Apa I was about 740bp. For Fok I SNP, restriction digestion was performed by Fok 1 fast digest enzyme (Fermentas, USA) at 37°C for 15 min. While for Apa 1, restriction digestion was performed by conventional Apa I restriction enzyme (Fermentas, USA) for 16 hours, both the enzymes were deactivated after the described time at 65 °C for 5 min. PCR products after digestion were visualized on 2% agarose gel electrophoresis. For Fok I SNP, the undigested product of 265 bp determined the presence of common "F" allele while the digested product of 169 bp and 96 bp analyzed the rare allele "f". Similarly, for Apa I SNP, undigested product of 740 bp determined the presence of common "A" allele while the digested product of 530 bp and 210 bp analyzed the rare allele "a".

**Statistical Analysis:** All the statistical analyses were performed by IBM SPSS version 21. Genotype frequencies were tested for Hardy-Weinberg equilibrium by  $\chi^2$  test. Mean  $\pm$  SD was given for quantitative variables (age of participants, age of onset of disease, serum vitamin D levels). Frequencies and percentages were given for qualitative variables such as genotypes etc. Pearson chi-square and Fisher exact test was applied to observe associations between Fok 1 and Apa 1 genotype and vitamin D levels. Odds ratio at 95% confidence interval (CI) was determined to describe the strength of association by Logistic regression analysis. P value less than 0.05 was considered significant.

## Results

### Clinical and Biochemical Characteristics of Study Subjects

Table-1 summarizes the clinical and biochemical

**Table-1:** Biochemical and Clinical characteristics of the study subjects.

Parameters	Normal Subjects (n)	T1D subjects (n)	P value
n (Males/Females)	44 (25/19)	44 (23/21)	NA*
Age (y)	14.81 + 2.7	17.92 + 2.8	0.862
Age at Diagnosis (y)	NA	09.58 + 2.5	NA
Duration of Illness (y)	NA	9.16 ± 25.0	NA
No. of Insulin units/day	NA	24.91 ± 9.4	NA
Serum Vitamin D level (ng/ml)	12.33 ± 6.6	11.35 ± 5.9	0.529

\*NA= Not applicable. T1D= T1DM. y= Years.

characteristics of the study subjects with respective p values obtained after students' t-test.. Majority of T1DM cases (54.50%) at the time of diagnosis presented with symptoms of hyperglycaemia (polyuria, polyphagia, and polydipsia), 27.3% presented with Diabetic ketoacidosis (DKA) and 18.2% presented with complaints of weight loss. Serum Vitamin D levels show statistically significant inverse correlation with the episodes of Diabetic ketoacidosis ( $r = -0.509$ ,  $p < 0.001$ ) and number of Insulin units administered per day ( $r = -0.350$ ,  $p = 0.020$ ). On the Contrary, overall there was no significant difference in serum Vitamin D levels of controls and T1DM the study subjects ( $p = 0.529$ ).

## Association of Fok I and Apa I polymorphisms with Type I Diabetes

The Hardy-Weinberg equation studies show that our study population was not in the Hardy Weinberg equilibrium. Table-2 summarizes the allelic and genotypic frequencies of the Fok I and Apa I polymorphism among our study subjects. The "f" allele of Fok I do not show any association with the disease while the "a" allele of Apa I is slightly higher among the diabetic patients but it is still not statistically significantly associated. Regression analysis indicate that overall the odds ratio for being heterozygous for Fok I is 2.008 (95% CI, 0.203-1.220) with  $p = 0.127$  while in Apa I polymorphism there are equal likely odds present for either a heterozygote or rare homozygote pair to be present i.e. 1.207 (95% CI, 0.263-5.550) with  $p = 0.809$  and 1.059 (95% CI, 0.419-2.679) with  $p = 0.904$ .

## Association of Fok I and Apa I polymorphisms with Vitamin D levels among study subjects

For an association of both the variants with Vitamin D levels, the study subjects were categorized into three subsets based on their detected vitamin D levels as described by Need et al., 2008.<sup>22</sup> Study subjects with vitamin D levels <10 ng/ml were labeled as having

**Table-2:** Frequency of the Fok I and Apa I polymorphism and association of these variants with type 1 diabetes in the study subjects.

	Cases n (%)	Controls n (%)	ODDS Ratio (95% CI)	Chi-square ( $\chi^2$ )	P-value
<b>Fok I Polymorphism</b>					
Genotype ff	0 (0)	0 (0)	--	--	--
Genotype Ff	12 (27.3)	19 (43.2)	2.008 (0.203-1.220)	2.321	0.127
Genotype FF	32 (72.7)	25 (56.8)	Reference	--	--
Frequency of f allele	14 %	22%	0.537 (0.176-1.634)	1.222	0.408
<b>Apa I Polymorphism</b>					
Genotype aa	05 (11.4)	04 (9.1)	1.207 (0.263-5.550)	0.058	0.809
Genotype Aa	25 (56.8)	25 (56.8)	1.059 (0.419-2.679)	0.153	0.904
Genotype AA	14 (31.8)	15 (34.1)	Reference	--	-
Frequency of a allele	40 %	38%	1.100 (0.468-2.583)	0.047	0.998

**Table-3:** Association of Fok I and Apa I polymorphisms with the Vitamin D levels of study subjects.

	Normal Subjects (Vitamin D level)		p value	Diabetic Subjects (Vitamin D level)		p value
	Deficiency n (%)	Insufficiency n (%)		Deficiency n (%)	Insufficiency n (%)	
<b>Fok I Polymorphism</b>						
Genotype FF	10 (50.0)	15 (62.5)	0.543	15 (78.9)	17 (68.0)	0.507
Genotype Ff	10 (50.0)	09 (37.5)		04 (21.1)	08 (32.0)	
Genotype ff	--	--		--	--	
<b>Apa I Polymorphism</b>						
Genotype AA	05 (25.0)	10 (41.7)	0.307	06 (31.6)	08 (32.0)	0.986
Genotype Aa	12 (60.0)	13 (54.2)		11 (57.9)	14 (56.0)	
Genotype aa	03 (15.0)	01 (04.2)		02 (10.5)	03 (12.0)	

"vitamin D deficiency", subjects with vitamin D levels between 10-30 ng/ml were classified as having "vitamin D insufficiency" and the ones with vitamin D levels 30-150 ng/ml were considered as having "vitamin D sufficiency". Strikingly, none of the study subjects was having the levels above 30 ng/ml so there were no study subjects with Vitamin D sufficiency. The Table-3 describes the association results of both the variants Fok I and Apa I with the two present categories of Vitamin D levels. When studied as whole, in Fok I polymorphism there was an equal proportion of subjects i.e. 64.1% with deficiency and 65.3% with insufficiency had FF genotype ( $p = 0.118$ ). On the contrary, there was a slight change of pattern for Apa I polymorphism where 59.0% with deficiency and 55.1% with insufficiency were heterozygotes ( $p = 0.607$ ) and on both homozygotes the data were dispersed in small percentages. The analysis of subjects as cases and controls also showed similar results as shown in Table-3. There was no striking association of both the polymorphism with the Vitamin D levels in the study subjects on both sides.

## Discussion

The pathogenesis of T1DM is not completely understood. Various observational and experimental studies have shown that vitamin D may be involved. Studies from Saudi Arabia,<sup>20</sup> showed that vitamin D levels were low in T1DM as compared to healthy controls. Greer, et al. (2013) reported that 43% Australian T1DM had low vitamin D levels as compared to their controls.<sup>23</sup>

In contrast, we found no significant difference in serum vitamin D levels in T1DM and controls. However, our study reports low serum vitamin D levels both in T1DM and the healthy young controls. This may be one of the reason for both no difference between diabetics and control children. Two studies are in agreement with our results. A research conducted in Chile by Garcia, et al. (2007) found no difference in 25-OH D levels in T1DM and healthy controls.<sup>24</sup> They reported 26.8 ( $\pm 7.7$ ) ng/ml in T1DM and 28.8( $\pm 4.1$ ) ng/ml in healthy controls. The other study was carried out in Florida by Bierschenk, et al. (2009) which reported similar results like our study.<sup>25</sup> They reported suboptimal vitamin D levels in the whole study population despite the fact that Florida receives ample sunshine. In their study 76.1% cases and 70.1%, controls had vitamin D levels  $\leq 30$ ng/ml. A study from Iran highlighted low serum vitamin D levels in 77% of T1DM.<sup>26</sup> Their finding was in agreement with ours as none of their research participants had adequate vitamin D levels but their study lacked a control group.

Vitamin D deficiency is rampant in many populations and

even in those populations that live in sufficient sunshine belt like Lahore where 87.5% of adults had Vitamin D levels less than 15 ng/ml.<sup>27</sup> The mean serum vitamin D level in healthy children of our study population was 12.33 ( $\pm 6.64$ ) ng/ml while the diabetics had 11.35( $\pm 5.92$ ) ng/ml which is in accordance with the findings of studies from Andhra Pradesh (India) which reported that 62-82% and 39% children had vitamin D below 20 ng/ml.<sup>28</sup> A study from Norway reports that 47% infants of Turkish, Pakistani and Somali immigrants had vitamin D below 10 ng/ml.<sup>29</sup>

Our study also provides an insight on the features of T1DM in our sample population. Most of our T1DM presented with symptoms of polyuria, polydipsia and polyphagia which is in agreement with a local study from Karachi by Shera, et al. (2008) and with majority of studies across the globe.<sup>30</sup> There are two studies from Saudi Arabia, which report ketoacidosis as the frequent presentation mode in T1DM. Work by Abdullah, (1989) revealed that 55% of the patients presented with DKA.<sup>31</sup> The frequency and mode of presentation in the lethal condition of DKA reported in the literature is variable from 10 to 80%.<sup>32</sup> About quarter (27.3%) of T1DM of our sample population presented with DKA and 32% of established type 1 patients ended in DKA. Our results are in agreement with a local study conducted in Karachi which shows that 42.7% established and 57.2% newly diagnosed T1DM suffered from lethal DKA.<sup>33</sup> In contrast, Shera, et al. (2008) showed lower frequency. In their study 2% of the T1DM presented with DKA at the time of diagnosis while 21% had positive history of DKA.<sup>30</sup> The reason for low frequency is that the study was carried at an outdoor centre while our study was carried at the tertiary care hospital, which has a lot of patient load pouring in from the whole province because of availability of Diabetic specialists and free insulin.

Studies from Saudi Arabia also yielded variable results. DKA was observed in 49.9% and 77% of the patients in two different studies.<sup>34</sup> Our study highlights the trends of type 1 in Pakistan. Our data are from one diabetic center in Lahore only and more studies nationwide will assist us to determine the incidence of T1DM and DKA in Pakistan.

In our study of single nucleotide polymorphisms (SNP) in exon 2 (Fok 1), intron 8 (Apa I), we did not find any association between VDR gene polymorphism (Fok 1 and Apa 1) and T1D in our sample population. The results of our study are in agreement with research work from Iran<sup>18</sup> and Finland.<sup>19</sup> All these also reported null results for Fok 1 and Apa 1. Research studies from Southern Indian population<sup>35</sup> reported no association for Apa 1

polymorphism in T1DM.

Our findings are contrary to the findings from other populations which yielded positive results, such as study from Romania<sup>36</sup> for Fok 1 polymorphism. Research work from Khorasan province of Iran<sup>37</sup> reported positive result for Fok 1 and Apa 1 polymorphism. Meta-analysis of the available data by Guo, et al. (2006) highlighted Fok 1 to be associated with T1DM while Meta-analysis by Zhang et al. (2012) reported Bsm 1 to be associated with type 1 diabetes.<sup>16,38</sup>

Studies done on VDR polymorphism show conflicting results; some are in favour of a significant association while others like our study report null results. This different outcome may be linked to differences in ethnic variations and diversity in geographical and environmental conditions of the populations investigated. Larger studies probably would produce more definite results. The role of the VDR gene polymorphism should be studied further in other populations, and other polymorphisms, such as the Bsm I, Apa I, Tru 9I and Taq I polymorphisms, should be analyzed for association with T1DM. In this connection, some work is already in progress on the new set of T1DM on Fok I and Bsm I.

## Conclusions

To the best of our knowledge, this is the first study on Pakistani population with T1DM. We report polyuria, polyphagia and polydipsia as the most common mode of presentation in T1DM. There was no significant difference in 25(OH) D (3) between T1DM and healthy controls in our sample population. Interestingly, all study participants had suboptimal levels of vitamin D and similar results have been reported from this lab earlier as well. The patients with recurrent attacks of DKA had very low vitamin D levels. We found a significantly higher requirement of insulin in children with low vitamin D. Overall, our findings specify that frequent sequence variation (Fok 1 and Apa 1) in the VDR gene has no impact in T1DM in our study populations. Further, our study did not reveal any association of 25(OH)D(3) with (Fok 1 and Apa 1) variant of VDR genotype. Due the limited sample size and analysis of only two polymorphisms, a large cohort study is necessary to analyze the effect/importance of all four polymorphisms in VDR gene and its impact on T1DM.

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**Conflict of Interest:** No.

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