



Association Study of Vitamin D Deficiency and its Receptor Gene Polymorphism in Diabetic Nephropathy progression

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ABSTRACT

Vitamin D deficiency and polymorphisms in the vitamin D receptor (VDR) gene have been linked to type 2 diabetes (T2DM) and diabetic nephropathy. This study aimed to evaluate the prevalence of vitamin D deficiency and VDR gene polymorphisms (Apal rs7975232 and FokI rs2228570) in Egyptian patients with T2DM and to determine their associations with the development of diabetic nephropathy. A total of 75 patients with end-stage renal disease (ESRD), 75 patients with T2DM, 75 patients with diabetic nephropathy, and 30 normal controls were included in the study. Biochemical analysis was performed, including measurements of 25-hydroxy vitamin D [25(OH)D], fasting blood sugar (FBS), postprandial sugar (PPS), homeostatic model assessment of insulin resistance (HOMA-IR), fasting insulin, glycated hemoglobin (HbA1c), blood urea, serum creatinine, estimated glomerular filtration rate (eGFR), albumin-creatinine ratio, total calcium, phosphorus, and lipid profile. VDR gene polymorphisms were detected using the PCR-RFLP technique. The results showed significantly lower levels of vitamin D in patients with T2DM (31.9 ± 8.1 ng/ml) and diabetic nephropathy (10.8 ± 8.1 ng/ml) compared to control subjects (44.9 ± 20.7 ng/ml) ($P < 0.001$). Vitamin D insufficiency was more prevalent in patients with T2DM and diabetic nephropathy. There was a significant increase in the frequencies of the (ff) and (AA) genotypes and the (f) and (A) alleles in the T2DM group. In conclusion, our study found a high prevalence of vitamin D deficiency in Egyptian patients with T2DM and diabetic nephropathy. There were also significant differences in the distribution of FokI genotypes and alleles between patients with T2DM and controls, suggesting a potential risk factor for Egyptian patients with T2DM. However, the (a) allele of the Apal genotype appeared to have a protective effect in Egyptian patients.

1. Introduction

Nowadays, Type 2 diabetes mellitus disease is a chronic disease prevalent all over the world. Due to our Lifestyle in our country increased the number of T2DM patients for many years and due to vitamin D receptor (VDR) expressed in the pancreatic β cells and kidneys that increased our interest in studying the correlation between VDR polymorphism and T2DM, and VDR polymorphism with diabetic nephropathy disease. Vitamin D and its metabolite is a lipid-soluble steroid hormone, mediated through vitamin D receptor (VDR) [1].

The prohormone of vitamin D is activated by two hydroxylation processes occurring in the liver and kidneys to form 1,25-dihydroxyvitamin D, which is dependent on sun exposure [2]. Many bodily tissues that express the vitamin D receptor (VDR) were not previously considered to be target tissues for vitamin D. However, it has been discovered that numerous organs and cells outside of the kidney, such as the skin, glands, bone cells, and immunological and cardiovascular tissues, are also activated by vitamin D. The active form of vitamin D binds to the vitamin D binding protein (VDBP) and enters target cells, where it attaches to cytoplasmic vitamin D receptors (VDRs). The VDR then transports vitamin D to the cell nucleus, where it interacts with transcription factors and changes its conformation. When this binding interacts with the vitamin D response element (VDRE) in the promoter region of genes, it creates a transcriptional regulatory unit. Gene expression is regulated by this relationship. The VDR is part of a superfamily of nuclear hormone receptors that mediates the genomic effects of vitamin D and stimulates the production of target genes. The VDR is present in numerous organs, including those involved in glucose metabolism, such as pancreatic beta cells. So, the deficiency in vitamin D leads to the development of diabetes mellitus disease [3].

Vitamin D receptors are widely expressed in different tissues. Its gene contains 11 exons along with introns and the VDR-encoding gene is located on chromosome 12 (12q13.11). The best-studied VDR gene polymorphisms are Apal (rs7975232), and FokI (rs2228570) whereas, Apal is a silent genetic variant that increases mRNA stability. While FokI gene is located on exon 2 and results in a protein shortened by three amino acids of the initiation codon [4].

2. Subjects and methods

2.1. Subjects

This study included 30 normal control subjects, 75 diabetic patients, 75 diabetic nephropathy patients, and 75 diabetic patients with end-stage renal disease (with a diabetic onset of 5 to 10 years), all diagnosed according to the Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2006. The 75 diabetic nephropathy patients were undergoing hemodialysis. Under ethical permission number (IDE00244), the patients were chosen from the National Institute of Diabetes and Endocrinology (NIDE) outpatient clinic in Cairo, Egypt, between November 2018 and December 2019. Only oral hypoglycemic medications were administered to the individuals in the diabetic groups, and a thorough medical history was obtained, with particular emphasis placed on any coexisting conditions. All subjects were informed about the nature and target of the study, and before their participation, their written consent was obtained. The National Institute of Diabetes and Endocrinology's ethics committee gave the go-ahead for this investigation.

Group I (DM)

This group consisted of 75 male patients with type 2 diabetes who were being managed with conservative drug treatment. Their mean ages were 42 ± 3.1 years and their Albumin/creatinine ratio was less than 30 ± 25 mg albumin/g creatinine.

Group II (DN)

This group included 75 male patients with diabetic nephropathy, with mean ages 38 ± 4.6 years and an Albumin/creatinine ratio ranging from 30 to 300 mg albumin/g creatinine.

Group III (HD)

This group consisted of 75 male patients with type 2 diabetes and end-stage renal disease (ESRD) who were undergoing regular hemodialysis treatment. Their mean ages were 40 ± 5.4 years and they had been on hemodialysis for 4 hours per session, three times weekly, for 3 to 10 years. Their serum creatinine level was 8.0 ± 3.5 (4.5-11.5) mg/dl.

Control group

This group included 30 normal male subjects with mean ages 31 ± 2.3 years and a vitamin D (25 hydroxycholecalciferol) level of 32 ± 10 ng/mL. In this study, the patient data included age, parental consanguinity, family history of renal disease, occupation, education, smoking, and laboratory investigations.

2.1.1. Inclusion Criteria

This study includes only non-smoking male patients with diabetes who are being treated with oral hypoglycemic agents. Patients in the diabetic group may also be undergoing hemodialysis for end-stage renal disease (ESRD).

2.1.2. Exclusion Criteria

Patients with cirrhosis, gastrectomy, parathyroidectomy, hysterectomy, or any form of cancer evaluation are not eligible for this study. Moreover, patients who have received vitamin D supplementation within the last six months were also excluded.

2.2. Methods

2.2.1. Blood samples

Ten ml of venous blood samples were collected from patients and normal controls in the morning after fasting. Each blood sample was divided into the following portions: a) Two ml of blood was added to sodium fluoride tubes and centrifuged immediately at 5000 rpm for 10 minutes and the plasma samples were separated rapidly for determination of fasting blood sugar levels (FBG). b) Two ml of blood was collected on EDTA coated tube for estimation of HbA1c. c) Two ml of blood sample was kept in a clean glass tube without additives to clot at 37 °C for 20 minutes and then centrifuged at 3000 rpm for 10 minutes. The serum was then separated into aliquots and stored at -70 °C to be thawed only once on demand for the determination of biochemical investigations (as Vitamin D, total calcium, serum phosphorous, Serum creatinine, blood urea, Lipid profile [Total Cholesterol, Triglycerides, HDL-C, LDL-C, VLDL-C, and atherogenic index] also insulin and HOMA-IR using commercially available kits supplied for human samples. d) The Rest blood samples were taken in an EDTA-containing tube solution for the detection of gene polymorphisms. e) After 2 hours of eating blood samples were collected to determine postprandial serum sugar levels (PPG). In addition, a five ml urine sample was collected for each subject in a clean container. Then centrifuged at 3000 rpm, the supernatants are separated in dry clean glass tubes and stored at -20°C for the determination of microalbumin and creatinine level.

2.2.2. Urine Samples

Random urine samples were collected from patients and normal controls to determine the Albumin creatinine ratio (ACR).

2.2.3. Biochemical analyses

Serum levels of vitamin D were determined using the manufacturer's instructions using VIDAS® auto-analyzer (bioMérieux S.A., Marcy l'Étoile, France). This assay employs the Enzyme-linked Fluorescent Assay (ELFA). Fasting blood sugar (FBS), fasting insulin, postprandial glucose (PPG), creatinine, blood urea, lipid profile, total calcium, and phosphorus levels were examined according to the manufacturer's instructions using a commercially available kit obtained from Spinreact (Spain). Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated equation, [HOMA-IR = (insulin × glucose) / 405] For glycemia in mg/dL. Quantitative determination of Albumin creatinine ratio (ACR) in urine was performed using a commercial assay kit purchased from (ABC Diagnostics, Egypt) [5].

Using the equation [Concentration of microalbumin (µg/ml) = ([A1s – A2s]/ [A1st- A2st]) × 50]. Also, the Quantitative Determination of estimated-Glomerular Filtration Rate (e-GFR) is measured by the CKD-EPI creatinine equation [6], where creatinine clearance was expressed in milliliters per minute, age in years, weight in kilograms, and serum creatinine in mg/dL.

$$\text{GFR} = 141 \times \min(\text{Scr}/k, 1)^\alpha \times \max(\text{Scr}/k, 1)^{1.209} \times 0.993^{\text{age}} \times 1.018 (\text{if female}) \times 1.159 (\text{if black}).$$

Where, K= 0.7 if female

K= 0.9 if male

$\alpha = -0.329$ if female $\alpha = -0.411$ if male

min = The minimum of Scr/k or 1

max = The maximum of Scr/k or 1

Scr = serum creatinine (mg/dl).

2.2.4. PCR-RFLP analysis of FokI (rs2228570) and Apal (rs7975232)

Genotypes were determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for the FokI (rs2228570) and Apal (rs7975232) polymorphisms. The primers sequences used for FokI genotyping were forward, 5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3'; and reverse, 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'. PCR reactions were performed in a total volume of 25 µl, including 2.5 µl 5× PCR buffer, 1.5 mM MgCl₂, 200 µM dNTPs, 20 pmol of each primer, 200 ng genomic DNA, and 0.5 units of Taq polymerase (My Taq Red DNA Polymerase, Bio line Germany). The PCR conditions for rs2228570 were 94 °C for 4 min, followed by 35 cycles of 60 sec at 94 °C, 60 sec at 58 °C, and 2 min at 72 °C, with a final elongation of 7 min at 72 °C.

The products were digested overnight with specific restriction endonucleases purchased from (New England Biolabs, UK) according to the manufacturer's guidelines and separated by 2.5% agarose gel electrophoresis For the FokI polymorphism, three distinct genotypes were identified: FF wild-type (265 bp), FF homozygous (196 and 69 bp), and Ff heterozygous (265, 196, and 69 bp). The primers sequences used for Apal were forward, 5'CAACCAAGACTACAAGTACCGCTCAGTGA-3'; reverse, 5'CACTTCGAGCACAAGGGGCGTTAGC-3'. The PCR conditions were 30 seconds at 94 °C, 30 sec at 65 °C, and 2 minutes at 72 °C, with a final elongation of 4 min at 72 °C. After PCR, the products were digested overnight with specific restriction endonucleases purchased from (New England Biolabs, UK) according to the manufacturer's guidelines and separated by 2.5% agarose gel electrophoresis For the Apal polymorphism, three distinct genotypes were identified: AA homozygous (2000 bp), aa homozygous (1700 and 300 bp), and Aa heterozygous (2000, 1700, and 300 bp).

2.2.5. Statistical Analysis

The Analysis of variance (ANOVA) and descriptive statistics were used to assess differences in clinical characteristics between the groups. Pearson correlation coefficient (PCC) was determined between parameters in all study groups. The genotype and allele frequencies were evaluated and showed no deviation from Hardy-Weinberg equilibrium (HWE), and all statistical analyses were conducted using SPSS 26, with a significance level of $P < 0.05$ [7].

3. Results

3.1. Levels of biochemical parameters in the study groups

There was an augmented elevation in fasting blood glucose in the DM group by 92.9% with a dramatic significant elevation in the DN group by 109.2%, and a highly significant increase by 58.7% in the HD group compared to the control group. Regarding its concentration in the normal control group, postprandial blood glucose levels recorded a dramatic increase which amounted to 138.8%, 144.3%, and 102.7% for DM, DN, and HD groups respectively compared to the control group. Also, there was a highly significant increase in HbA1c in DM, and DN groups by 50.9%, and 60.7% respectively with a significant increase of 33.3% in the HD group compared to the control group.

In addition, there was a dramatic increase in HOMA-IR in DM, DN, and HD groups by 356.6%, 637.3%, and 452.9% respectively compared to the control group, with a dramatic increase in fasting-insulin in DM, DN, and HD groups by 125.9%, 252.8%, and 232%. Multiple comparisons between DN, and HD with DM groups as a positive control group recorded a non-significant change in FBS levels in the DN group and a significant decrease of 17.7% in the HD group. Postprandial blood glucose and HbA1c showed a non-significant change in the DN group. In addition, Postprandial blood glucose and HbA1c showed a significant decrease in the HD group by 15.1% and 11.6% respectively compared to the DM group. In the meantime, there was a highly significant increase in HOMA-IR in the DN group by 61.4% and a non-significant change in the HD group compared to the DM group. In addition, fasting-insulin recorded a highly significant increase in DN and HD groups by 56.14% and 46.9% compared to the DM group respectively.

Additionally, blood urea levels recorded a dramatic increase of 295.2% in the HD group compared to the control. While the same parameter in DM and DN groups recorded a non-significant change. Serum creatinine was dramatically elevated by 632.6% in the HD group but a non-significant change was seen in DM and DN compared to the control group. In addition, the total calcium recorded a significant increase of 9.6% in the DM group and 6.45% in the DN group but a non-significant change in the HD group was seen compared to the control group. In the meantime, phosphorus was significantly increased by 22.6% in the DM group and highly significantly increased by 44.7% in the HD group. However, there was a non-significant change in phosphorous in the DN group compared to the control group.

At the same time, a dramatic elevation of micro-albumin in urine in DN and HD groups by 583.9% and 4226.4%. However, a non-significant change in micro-albumin in the DM group was seen compared to the control group. In addition, the estimated glomerular filtration rate (eGFR) was non significantly changed in the DM and DN groups with an augmented decrease recorded in the HD group by 84.86 % compared to the control group. Multiple comparisons between DN and HD groups with the DM group showed a dramatic increase in the HD group by 302% for blood urea. Also, no significant change in the DN group for blood urea compared to the DM group.

Serum creatinine level showed a dramatic elevation of 585.1% in the HD group. Moreover, a non-significant change was seen in the DN group compared to the DM group. In the meantime, a non-significantly change in total calcium in the DN group and a significant decrease was seen in the HD group by 12.7% compared to the DM group. In addition, there was a significant decrease of phosphorous in the DN group by 9.8% with a significant increase in the HD group by 18.0% compared to the DM group. Urinary micro-albumin showed a dramatic increase in DN and HD groups this increase amounted to 224.3% and 1951.9% respectively compared to the DM group. At the same time, the estimated glomerular filtration rate (eGFR) showed a non-significant change in the DN group and an augmented decrease in the HD group by 84.4% compared to the DM group.

A significant increase in total cholesterol levels in the DM and the DN groups by 34.8% and 36.3% respectively. However, a non-significant change was seen in the HD group compared to the control group. In the meantime, triglycerides showed a dramatic increase in the DM and the DN groups by 109.9% and 108.3% respectively. Although, a non-significant change was seen in the HD group compared to the control group. HDL-c was significantly decreased in the DM, the DN, and the HD groups by 18.4%, 15.9%, and 13.8% respectively compared to the control group. LDL-c was a highly significant increase in the DM and the DN groups by 49.5% and 51.27% respectively and a non-significantly change was seen in the HD group compared to the control group. VLDL-c was significantly increased in the DN group by 4.43% with a significant decrease seen in the DM and the HD groups by 5.49% and 5.2% respectively compared to the control group.

Risk I was an augmented increase in the DM group by 71.59% and a highly significant increase in the DN group by 65.6%, while showed a significant increase in the HD group by 42.01% compared to the control group. Risk II showed an augmented increase in the DM and the DN groups by 87.8% and 77.6% respectively, while showed a significant increase in the HD group by 47.2% compared to the control group. In the multiple comparisons between DN and HD groups with the DM group, a non-significantly change in total cholesterol level was seen in the DN group. While a significant decrease in total cholesterol was seen in the HD group by 14.6%. There was a non-significant change in triglyceride levels seen in the DN and the HD groups.

In addition, a non-significantly change of the HDL-c was seen in the DN and the HD groups. LDL-c, VLDL-c, Risk I, and Risk II showed a non-significant change in the DN group. Also, LDL-c showed a significant decrease in the HD group by 29.02% compared to the DM group. However, VLDL-c showed a non-significant change in the HD group compared to the DM group. In addition, Risk I was significantly decreased in the HD group compared to the DM group. While Risk II showed a significant decrease in the HD group compared to the DM group.

This current study showed a highly significant decrease in vitamin D levels in the DM, DN, and HD groups by 66.6%, 55%, and 70.8% respectively compared to the control group, in addition, these findings showed a significant increase in vitamin D levels in the DN group by 34.7% and a non-significant decrease by 12.6% in the HD group compared to the DM group as a positive group (Table 1).

3.2. Correlation analyses

The correlation analysis showed a highly significant positive correlation between vitamin D level and HDL-cholesterol, between vitamin D and total calcium, and also a highly significant positive correlation between vitamin D and eGFR. However, there was a highly significant negative correlation between vitamin D level and (FBS, PPS, HbA1C, HOMA-IR, Fasting-insulin, serum creatinine, blood urea, serum phosphorus, total cholesterol, triglycerides, and LDL-cholesterol) of all studied groups (Table 2).

3.3. Genotyping of FokI (rs2228570)

Due to the relatively low frequency of the (Ff) genotype, we combined both (Ff) and (ff) genotypes into one group (Ff+ff) and compared it to the (FF) genotype group (Fig. 1). The results showed that the homozygous genotype (FF) was distributed as follows: 21 (70%), 49 (65%), 44 (59%), and 40 (54%) for the control, DM, DN, and HD groups, respectively. The (Ff+ff) genotypes were distributed as follows: 9 (30%), 26 (35%), 31 (41%), and 35 (46%) for the control, DM, DN, and HD groups, respectively. There was no significant change in the distribution of (Ff+ff) genotypes in the DM, DN, and HD groups compared to the control group. However, there was a significant increase in the (f) allele, with a frequency of 44% in the HD group compared to the control group. There was no significant change in the frequency of the (f) allele in the DM and DN groups compared to the control group (Table 3).

3.4. Genotyping of Apal (rs7975232)

Due to the relatively low frequency of the (Aa) genotype, we combined both (Aa) and (aa) genotypes into one group, (Aa+aa), and compared it to the (AA) genotype group. Table 5 shows the distribution of the homozygous genotype (AA) as follows: 8 (28%) for control, 42 (56%) for DM, 40 (53%) for DN, and 31 (41%) for HD. The (Aa+aa) genotypes were distributed as follows: 22 (72%) for control, 33 (44%) for DM, 35 (47%) for DN, and 44 (59%) for HD. There was a significant decrease in the (Aa+aa) genotypes in DM and DN, but not in HD, compared to control.

Additionally, there was a significant decrease in the (a) allele, with 42% and 44.6% in DM and DN, respectively, compared to control. However, there was no significant change in the (a) allele in HD compared to control (Table 4). In addition, these findings showed a highly significant increase in serum vitamin D levels in individuals with (Ff+ff) genotypes in DM and DN by 56.8% and 51.7% respectively, with an augmented increase of 75.9% in the HD group compared to individuals with the (FF) genotype. In contrast, there was a non-significant change in serum vitamin D levels in individuals with (Aa+aa) genotypes in all groups compared to individuals with (AA) genotype (Table 5).

Table 1. Clinical Parameters of all studied groups

Parameters	FBS (mg/dl)	PPS (mg/dl)	HBA1c %	HOMA-IR	Fasting-insulin (mIU/ml)	Vitamin D (ng/mL)	S. creatinine (mg/dl)	Bl.urea (mg/dl)	Total calcium (mg/dl)	Phosphorous (mg/dl)	A/C Ratio (mgAlb/g creat)	eGFR (ml/min/1.73 m ²)
Groups												
G I (DM)												
Range	99-362	145-586	4.8-12.5	4.6-37.4	16-54	8.0-20.5	0.8-1.37	16-53	8.6-11.4	3.4-6.9	24-55	63-164
Mean±S.D	171±20.9	235±32	7.7±1.5	9.91±1.3	22.8±3.2	9.5±2.8	1.1±0.2	29±4.2	10.2±1.3	4.66±0.55	40.7±6.1	106±14
P<	0.001	0.001	0.001	0.001	0.001	0.001	0.01	0.01	0.001	0.001	0.01	0.01
Percent change of (DM) vs Control	92.8	138.8	50.9	356.6	125.9	-66.6	6.9	-1.6	9.6	22.6	110.8	-2.4
G II (DN)												
Range	83-40	110-451	5.3-14	4.6-38	17.7-58	8.1-31.9	0.6-2.06	15-77	8.5-11	3.2-5.8	36-297	43-194
Mean±S.D	185.6±27	240.4±3	8.2±1.2	16±2.4	35.6±4.9	12.8±5.7	1.1±0.2	31.5±4.5	9.9±0.47	4.2±0.63	132±18	106±14
P<	0.001	0.001	0.001	0.001	0.001	0.001	0.01	0.01	0.001	0.01	0.001	0.01
Percent change of (DN) vs control	109.2	144.3	60.7	637.3	252.8	-55.08	6.9	6.7	6.45	10.5	583.9	-3.2
Percent change of (DN) vs (DM)	8.4	2.2	6.4	61.4	56.14	34.7	0	8.6	-2.9	-9.8	224.3	-0.7
G III (HD)												
Range	84-23	122-347	4.5-10.7	5.9-32.3	22-55	8.1-10.8	2-12	45-190	6.4-12	3.3-7.9	494-1509	7.1-69.3
Mean±S.D	140.8±21	200±28	6.8±1.0	12.0±1.8	33.5±5	8.3±0.6	7.4±1.1	116.6±17	8.9±1.22	5.5±0.7	835±120	17±2.3
P<	0.01	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.01	0.001	0.001	0.001
Percent change of (HD) vs control	58.7	102.7	33.3	452.9	232	-70.8	632.6	295.2	-4.3	44.7	4226.4	-84.86
Percent change of (HD) vs (DM)	-17.7	-15.1	-11.6	21.08	46.9	-12.6	585.1	302	-12.7	18	1951.9	-84.4

(P>0.05) non-significant (NS); (P<0.05) significant (S); (P<0.001): highly significant (HS)

Parameters	TOTAL CHOLESTEROL (mg/dl)	TRIGLYCERIDES (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	VLDL-C
Control	136 - 185	62 - 191	40 - 65	77 - 118.6	12.4 - 38.2
Range	160.6 ± 2	94.1 ± 12	47.6 ± 7	94.2 ± 13	18.8 ± 2.6
Group I (DM)	122 - 332	60 - 83	22 - 57	58.4 - 248	12 - 107.2
Range	217 ± 32	198 ± 28	39 ± 4.5	141 ± 21	36.1 ± 5.0
Mean±S.DP<	0.001	0.011	0.005	0.001	0.01
Percent change of GI (DM) to control	34.8 %	109.9 %	-18.4 %	49.5 %	-5.49 %
Group II (DN)	125 - 400	48 - 565	22 - 55	70 - 269.8	9.6 - 88
Range	219 ± 31	196 ± 29	40 ± 5.2	143 ± 20	37.7 ± 4.8
Mean±S.DP<	0.001	0.10	0.012	0.001	0.001
Percent change of GII(DN) to control	36.3 %	108.3 %	-15.9 %	51.27 %	4.43 %
Percent change of GII(DN) to G I(DM)	1.1 %(NS)	-0.7 %(NS)	3.09 %(NS)	1.1 %(NS)	4.4 %(NS)
Group III (HD)	96 - 277	65 - 440	22 - 65	24.8 - 197	13 - 88
Range	185 ± 26	171 ± 25	41 ± 6.0	110 ± 15	34.2 ± 5.0
Mean±S.DP<	0.151	<0.05	0.01	<0.05	0.01
Percent change of GIII(HD) to control	15.1 %	81.7 %	-13.8 %	15.9 %	-5.2 %
Percent change of G III(HD) to G I(DM)	-14.6 %(NS)	-13.4 %(NS)	5.67 %(NS)	-29.02 %(HS)	-5.2 %(NS)

(P>0.05) non-significant (NS); (P<0.05) significant (S); (P<0.001): highly significant (HS)

Table 2. Correlations between vitamin D level and biochemical data of all studied groups

parameter	FBS (mg/dl)	PPS (mg/dl)	HBA1c %	HOMA-IR	Fasting-insulin mIU/ml	Serum creatinine (mg/dl)	BLurea (mg/dl)	Total calcium (mg/dl)	Phosphorous (mg/dl)	eGFR (ml/min/1.73m ²)	Total cholesterol l(mg/dl)	TAGs (mg/dl)	HDI-c (mg/dl)	LDL-c (mg/dl)
Vitamin D (ng/ml)														
Group I (DM)	-0.741	-0.67	-0.696	-0.674	-0.677	-0.520	-0.637	0.663	-0.701	0.525	-0.627	-0.686	0.551	-0.662
-Pearson correlation coefficient (r-value)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)
-(P-value)														
G II (DN)	-0.933	-0.952	-0.926	-0.929	-0.968	-0.947	-0.903	0.919	-0.940	0.922	-0.939	-0.979	0.975	-0.948
-Pearson correlation coefficient (r-value)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)
-(P-value)														
G III (HD)	-0.949	-0.930	-0.943	-0.893	-0.919	-0.965	-0.965	0.943	-0.866	0.976	-0.958	-0.916	0.951	-0.972
-Pearson correlation coefficient (r-value)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)	0.001 (HS)
-(P-value)														

(P>0.05) non-significant (NS); (P<0.05) significant (S); (P<0.001): highly significant (HS)

Table 3. Allele and genotype frequencies of FokI (rs2228570) polymorphism for studied groups.

Groups	Genotypes		P-value (1 df)	Alleles		P-value (1 df)
	FF	Ff+ff		Allele F	Allele f	
Control (N=30)	21 (70%)	9 (30%)	0.647	44 (73.3%)	16 (26.6%)	0.604
G I (DM) (N=75)	49 (65%)	26 (35%)		105 (70%)	45 (30%)	
G II (DN) (N=75)	44 (59%)	31 (41%)	0.314	95 (63.3%)	55 (36.6%)	0.159
G III (HD) (N=75)	40 (54%)	35 (46%)	0.103	84 (56%)	66 (44%)	0.019

(P>0.05) non-significant (NS); (P<0.05) significant (S); (P<0.001): highly significant

Table 4. Allele and genotype frequencies of Apal (rs7975232) polymorphism for DM, DN, HD, and control groups.

Groups	Genotypes		P-value (1 df)	Alleles		P-value (1 df)
	AA	Aa+aa		allele A	Allele a	
Control (N=30)	8 (28%)	22 (72%)	0.009	19 (31.6%)	41 (68.3%)	0.001
G I (DM) (N=75)	42 (56%)	33 (44%)		87 (58%)	63 (42%)	
G II (DN) (N=75)	40 (53%)	35 (47%)	0.011	83 (55.3%)	67 (44.6%)	0.001
G III (HD) (N=75)	31 (41%)	44 (59%)	0.14	62 (41.3%)	88 (58.6%)	0.163

(P>0.05) non-significant (NS); (P<0.05) significant (S); (P<0.001): highly significant

Table 5. Vitamin D levels and genotypes in all groups

Vitamin D level (ng/mL)				
Groups	Control	G I (DM)	G II (DN)	G III (HD)
Genotypes				
FF Mean ±S.D	9.7±3.8	8.82±1.8	8.7±0.9	8.3±1.8
Ff+ff Mean ±S.D	29.4±7.3	13.8±4.7	13.2±2.7	14.6±2.7
P-value	0.001 (HS)	0.001(HS)	0.001(HS)	0.001(HS)
Percent change of(Ff+ff) to FF	203.09 %	56.8 %	51.7 %	75.9 %
AA Mean±S.D	13.4±9.9	10.1±3.16	9.9±2.8	11.5±3.97
Aa+aa Mean±S.D	16.4±10.7	11.2±4.6	11.2±2.8	10.9±93.9
P- value	0.497(NS)	0.255(NS)	0.061(NS)	0.557(NS)
Percent change of (Aa+aa) to AA	22.3 %	10.8%	13.1 %	-5.2 %

(P>0.05) non-significant (NS); (P<0.05) significant (S); (P<0.001): highly significant

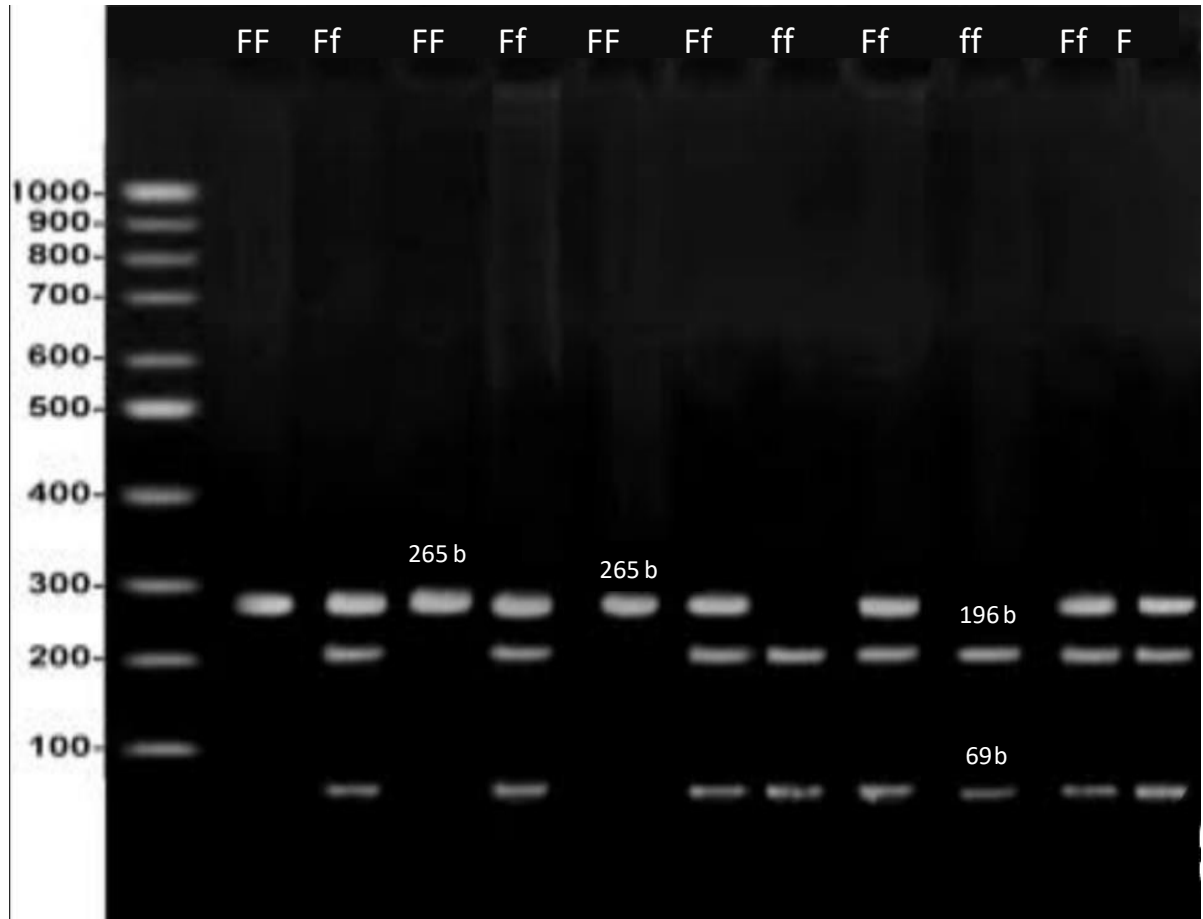


Fig. 1 PCR-RFLP analysis of FokI (rs2228570) polymorphism. The agarose gel (2.5%) was stained with ethidium bromide used for genotyping. The determined genotypes were above the panel.

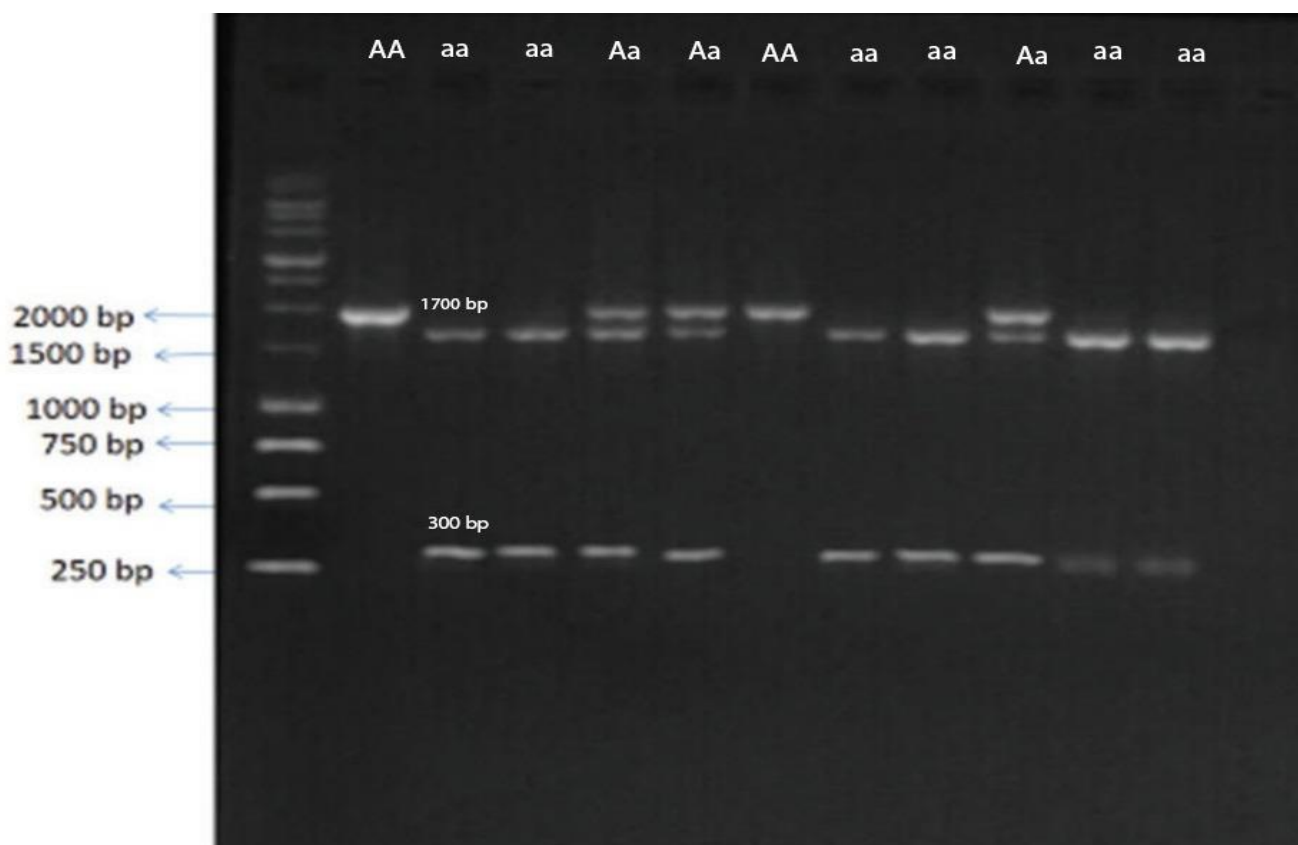


Fig. 2 PCR-RFLP analysis of Apal (rs7975232) polymorphism. The agarose gel (2.5%) was stained with ethidium bromide used for genotyping. The determined genotypes were above the panel.

4. Discussion

Vitamin D receptor (VDR) expressed in the pancreatic β cells and its polymorphisms have a direct effect on blood glucose levels and increase the progression of T2DM. Apal is a silent genetic variant that increases mRNA stability, while FokI gene is located on exon 2 and results in a protein shortened by three amino acids of the initiation codon [4]. The present study showed that there was a highly significant decrease in Apal VDR polymorphism (Aa+aa) genotypes in the DM group compared to control and there was a highly significant decrease in (a) allele frequency in the DM group compared to control. In agreement with Alkhedaide et al., who suggested that the (A) allele of Apal VDR polymorphism was statistically associated with T2DM while the (a) allele of Apal VDR polymorphism is a protective allele in the Saudi Arabian population (Taif city) [8]. Thus, (aa) is a wild genotype which prevalent with a high percentage in the current study control group and has a high protection effect rather than the (AA) genotype which is prevalent in T2DM patients.

In addition, Maulood et al. recorded that there was a non-significant association between the Apal VDR polymorphism and the risk of T2DM in the Erbil city population [9]. That may be due to the (aa) genotype being more prevalent than the (AA) genotype which increases T2DM risk disease. In contrast, Al-Hazmi et al. demonstrated that there was no correlation between Apal and T2DM risk disease in the Saudi Arabian population [10], this difference between studies may be due to the differences in gene expression, epigenetic inheritance, and may be due to different races, region, lifestyle. In contrast, there was a disagreement between our results with Rasoul et al., who recorded that there was a non-significant difference in the frequencies of Apal genotypes between T1DM patients and the controls in Kuwaiti T1DM patients [11], This may be due to the following reasons: First, the difference in the type of diabetes mellitus disease, type 1 diabetes mellitus is a genetic disease that often shows up early in life but type 2 diabetes mellitus is mainly lifestyle-related and develops over time; Second, the different in the type of medication of the different diseases; Third, the difference in races, region, and lifestyle.

Diabetic nephropathy (DN) is a serious chronic microangiopathic complication of DM, appearing in about 20-40% of diabetic patients, and is considered the first cause of end-stage renal failure in patients with DM. About 380 million patients are expected to be diagnosed with DM by the year 2025 causing an increase in the prevalence of DN. The major clinical manifestations of DN are proteinuria, hematuria, and progressive reduction of renal function: The United States Renal Data System registered that approximately 35–50% of ESRD cases are secondary to DN complications [12].

The present study showed that there was a highly significant decrease in (Aa+aa) genotypes compared to the (AA) genotype in the DN group and there was a highly significant decrease in (a) allele frequency in the DN group compared to the control group with a non-significant change in (Aa+aa) genotype and (a) allele in HD group. In agreement with Song et al., who reported that there was a link between the VDR gene Apal and DN susceptibility in comparison with diabetic patients without DN in the Asian population [13]. In contrast, Yang et al., demonstrated that there was a non-significant association between Apal VDR polymorphism and chronic kidney disease susceptibility in the Han Chinese population in the absence of other chronic diseases such as diabetes mellitus [14]. In addition, Yin et al., reported that there was no association between diabetic nephropathy risk and Apal VDR polymorphism in T2DM in comparison with the control group [15], the reason for differences in results of studies that focus on the association of VDR gene polymorphisms with T2DM may be due to genetic, and environmental differences in population.

In the meantime, VDR polymorphism correlated with the serum level of vitamin D and it may be (increase, decrease, or not affect) the serum vitamin D deficiency in the body. According to Apal VDR polymorphism, the current study showed that there was a non-significant change in serum vitamin D levels in patient's carriers of (Aa+aa) genotypes of Apal VDR polymorphism in the study groups. Whereas, (Aa+aa) genotypes and (a) alleles did not affect serum vitamin D levels in Egyptian diabetic patients. This is in agreement with Divanoglou et al., [16], and Hassan et al., [17], who suggested that no effect was identified for Apal VDR polymorphism on serum vitamin D levels in a Greek rural population. That may be due to Apal genes being a silent gene that didn't affect serum vitamin D levels when translated.

The single nucleotide polymorphisms (SNPs) of FokI (T > C) can change the length of the amino acid sequence, when the initiation codon is mutated from ATG to ACG (F allele), it loses the role of starting the translation, and only wait for the next initiation codon to translate, which will result in lack of three amino acids in activation domain of VDR protein positions, the VDR protein lacked three amino acids is easier to activate its effector genes [18]. At the same time, FokI VDR polymorphism had a different effect on serum vitamin D by increasing its level in different genotypes and alleles. These current findings showed that there was a highly significant increase of the (f) allele in the HD group, while a non-significant change of the (f) allele in the DN group was found compared to control group with a non-significant change of (Ff+ff) genotypes in DN and HD groups compared to control group. These results were in agreement with Al-Shaer et al., who demonstrated that there was a high frequency of the (FF) genotype in comparison to the control group and considering the (FF) genotype and (F) allele as a reference with a significantly lower incidence of (ff) genotypes and (f) allele, in Egyptian hemodialysis (HD) patients without other accompanying disease [19]. That proved the current findings whereas, the (f) allele of FokI VDR polymorphism increased susceptibility to T2DM and confirmed the increase of the (f) allele in the HD group due to the presence of chronic T2DM.

Current data is also in harmony with the theoretical perspectives of Li Li et al., who mentioned that there was no association between FokI VDR gene polymorphism and chronic renal failure in the dialyzed ESRD patients without T2DM of Caucasian populations [20]. That may be due to the risk of chronic renal failure and ESRD increased in the presence of other chronic diseases such as diabetes mellitus (DM) which increased in the case of single nucleotide polymorphism of FokI VDR causing a change in the length of amino acid sequence producing FokI single nucleotide polymorphism and increasing (f) allele. Confirming the hypothesis of Dong et al., who suggested that diabetic patients who carry the (f) allele were at 1.459 times risk higher than those without for kidney disorders, where single nucleotide polymorphisms (SNPs) change the activity of VDR, and (ff) genotype may be a susceptible factor for DKD in Yunnan Han population [18]. In addition, this was in agreement with Bouksila et al., who demonstrated that the FokI (ff) genotype was associated with a lower risk of ESRD without diabetes mellitus [21].

That may be due to the (ff) genotype having a positive correlation with T2DM risk disease. The results of this study were not in the same line with Yin et al., who demonstrated that there was a non-significant change in FokI polymorphism between diabetic nephropathy patients and diabetic patients without nephropathy in a study conducted in Caucasian and Asian populations [15]., this difference between studies may be due to the differences in gene expression and epigenetic inheritance, and may be due to different races. In addition, current results are in the same line with Liu et al., [22], who suggested that there was a non-significant change in FokI VDR polymorphism in hemodialysis patients without diabetes mellitus.

An increase of the (f) allele and (Ff+ff) genotypes was seen in the DM group of the current study compared to the control group. In agreement with Eweida et al. [23], and Mohamed et al. [24], who suggested that there was a high prevalence of (f) allele of VDR FokI genotype in T2DM in comparison with the controls in the Egyptian population. In addition, Gendy et al. suggested that there was a significant difference between patients with type 2 diabetes mellitus and controls regarding the distribution of FokI genotypes and alleles with an increase in the frequency of (ff) genotype in patients with T2DM [25]. On the contrary Dong et al., suggested that there was a non-statistical difference in FokI VDR polymorphism between DM and DKD in comparison with the normal control group.

Serum vitamin D levels in the current study showed that there was a highly significant increase in patients' carrier of (Ff+ff) genotypes in the study groups, whereas, the higher frequency of (f) allele increased VDR expression along with increased vitamin D serum level. In agreement with Ferraz et al., who suggested that (ff) genotype and (f) allele, increased serum vitamin D levels in a Brazilian population of diabetic patients [26]. This may be due to the (ff) genotype having a translation codon of vitamin D production which increases the serum level of vitamin D. In addition, Borborema et al. suggested that the (Ff+ff) genotypes, increased VDR expression and elevated serum vitamin D levels in the Northeast Brazilian population [27]. Also, Oskoei et al., suggested that the (ff) genotype of FokI polymorphism was associated with the higher expression levels of VDR in breast cancer [28].

In contrast, Kamyshna et al. showed that there was a significant decrease in serum vitamin D levels in autoimmune thyroiditis patients' carriers of (Ff+ff) genotypes in the West-Ukrainian population [29], this difference between studies may be due to the effect of chronic diabetes mellitus in all VDR of the human body but autoimmune thyroiditis effect only on the VDR of the thyroid glands. In addition, Panda et al. suggested that the higher frequency of the (F) allele of FokI VDR polymorphism increased VDR expression along with increased vitamin D levels in household contacts with people with pulmonary tuberculosis patients in the north Indian population [30], this different between studies may be due to the different races with different lifestyle, and different environment.

On comparing all biochemical and clinical data between control, DM, DN & and HD groups of the current study using ANOVA, there was a highly significant negative correlation between vitamin D level and blood glucose levels (FBS, PPS, HbA1C, HOMA-IR and fasting insulin) with p-value 0.001. These results are in the same line with Liu et al., who demonstrated that there was a negative association between Vitamin D levels and HOMA-IR in the United States population [22]. That may be due to vitamin D increases the sensitivity of human body cells to insulin which then decreases serum glucose level causing a negative effect on serum glucose and HOMA-IR. This was in agreement; Hong et al. observed a high deficiency and insufficiency of vitamin D levels among Korean T2DM patients and suggested that increases the risk of DN [31].

In addition, Huu et al., found that there was an increase in vitamin D deficiency and insufficiency in patients with type 2 diabetes, and there was an increase in HOMA-IR, and insulin levels in diabetic groups which recorded a low vitamin D level [32]. In the same line with Amar et al. [33], and Bhatt et al [34], there was a high incidence of vitamin D deficiency in type 2 diabetic patients and there was a negative relationship between vitamin D level and blood glucose level. This current study showed that vitamin D levels have a highly significant negative correlation with (serum creatinine, blood urea, and phosphorous) and a highly significant positive correlation with (eGFR, and total calcium). This study was in the same line with Wang et al., who reported that there was a positive correlation between vitamin D levels and eGFR in hospitalized chronic kidney disease patients. Whereas, vitamin D levels had a positive effect on kidney health [35].

These findings data were in agreement with Helaly et al., who recorded that there was a significant difference in the level of vitamin D between the control group and the diabetic nephropathy patient group where vitamin D level was lower in the diabetic nephropathy group with a positive correlation between eGFR and vitamin D level [36], thus may be due to vitamin D plays an important role in diabetic kidney disease through several pathways such as reducing oxidative stress and inflammation, maintaining kidney health and there was a negative correlation between serum creatinine and vitamin D level. In harmony with the theory of Memon et al. [37], and Herdea et al. [38], who suggested that there was a positive correlation between vitamin D and serum calcium. Thus, vitamin D increases kidney efficacy which then increases serum total calcium level. In the same line with Rastogi et al. [39], and Hu et al. [40], there was a negative correlation between vitamin D levels and serum phosphorus.

In the meantime, this current study showed that the vitamin D level had a highly significant negative correlation with serum (total cholesterol, serum triglycerides, and LDL-cholesterol). While there was a highly significant positive correlation between vitamin D levels and HDL cholesterol. These findings were in the same line with Surdu et al., who suggested that there was a negative correlation between vitamin D level and serum lipid profile (total cholesterol, triglycerides, and LDL-cholesterol) but there was a positive correlation with HDL-cholesterol [41]. In addition, Jin et al. suggested that there was a negative correlation between vitamin D level and lipid profile during pregnancy and there was an improvement in lipid profile in high serum vitamin D level [42]. Similarly, Elshebiny et al., reported that there was a significant negative correlation between vitamin D and lipid profile [43].

5. Conclusion

Vitamin D has been shown to have a significant impact on decreasing blood glucose by improving insulin sensitivity and decreasing HOMA-IR, and its deficiency has been linked to an increased risk of type 2 diabetes (T2DM). The (f) allele of the FokI VDR polymorphism also plays a role in increasing the risk of T2DM and diabetic nephropathy in Egyptian patients. Furthermore, this polymorphism may also be a risk factor for T2DM and diabetic kidney disease.

Interestingly, the expression of the (f) allele of the FokI VDR polymorphism has been found to increase serum vitamin D levels. and the FokI VDR polymorphism does not appear to have an impact on the development of hemodialysis disease or end-stage renal disease (ESRD). However, the (a) allele of the Apal VDR polymorphism does not seem to affect serum vitamin D levels. The (a) allele of the Apal VDR polymorphism may even have a protective effect on Egyptian patients with T2DM.

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