# DIFFERENTIAL EXPRESSION OF VITAMIN D BINDING PROTEIN IN TYPE 2 DIABETES MELLITUS PATIENTS

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## ABSTRACT

Vitamin D binding protein (VDBP) gene polymorphisms have been found associated with risk of developing Type 2 diabetes mellitus (T2DM) in a Pakistani population. This study aims to evaluate VDBP expression in T2DM patients as plausible alteration in its expression might contribute to vitamin D deficiency.We analyzed VDBP expressionin T2DM patients and healthy controls by 2-DE followed by its validation via western blotting. *In Silico*PPI analysis was performed to evaluate role of VDBP and its interacting proteins with disease pathology.2-DE analysis revealed significant upregulated expression of VDBP in T2DM patients compared with controls (Fold change >1.7, **p*<0.05). Similarly, significant upregulated expression of VDBP was confirmed by western blotting. We foundslightly increased expression of VDBP in females compared to males, however, difference remained insignificant. *In Silico* PPI analysis revealed interaction of VDBP with other proteins mainly related to vitamin D binding, molecular processes in vitamin D pathway and cellular component proteins. Our data suggest that upregulated VDBP expression possibly affects vitamin D related glucose-mediated insulin secretion pathways in T2DM patients due to low functional vitamin D. Clinical monitoring of VDBP, 1,25(OH)2D and 25(OH)D is essential to accurately assess vitamin D status so that therapeutic interventions can be implemented to avoid functional vitamin D deficiency.

**Keywords**: Type 2 diabetes mellitus, VDBP, Protein differential expression, vitamin D deficiency, 2-DE

## INTRODUCTION

Type 2 diabetes mellitus (T2DM) and hypovitaminosis D prevail worldwide. Hypovitaminosis D or sub-optimal vitamin D levels [25(OH)D < 20 ng/mL] are commonly observed in patients suffering with T2DM (Lips *et al.,* 2017). Hypovitaminosis D is also associated with several traits of T2DM including insulin resistance, systemic inflammation, β-cell dysfunction, glucose intolerance (Chagas *et al.,* 2012). Recent data suggest that different pathological and physiological conditions can affect the concentration of one of the major component of vitamin D pathway i.e. VDBP (Vitamin D binding protein) which in turn can affect circulating 25(OH) D levels (Yousefzadeh *et al.,* 2014).

Vitamin D binding protein (VDBP) encoded by *GC* (Group Specific component) gene is a 52-59kDa, multifunctional plasma protein predominantly synthesized in the liver. The prime responsibility of VDBP is to bind to 85-90 % of circulating vitamin D and its metabolites. Remaining 10-15% of vitamin D binds to another member of the VDBP family i.e. albumin, while only <0.1% exist in unbound form (Bikle *et al.,* 1985;Chun *et al.,* 2008). The fraction of vitamin D bound to albumin and exists in unbound/free state is the bioavailable form of vitamin D (Gozdzik *et al.,* 2011). According to free hormone hypothesis, it is the unbound form of vitamin D that executes biological response in target cells (Chun *et al.,* 2014). VDBP has three phenotypic isoforms/variants: Gc-1f (fast), Gc-1s (slow), and Gc-2 due to two common polymorphisms rs7041 and rs4588 in *GC* gene. (Arnaud *et al.,* 1993). These phenotypic variants differ with respect to their binding affinity with vitamin D and its metabolites. Gc-1f has the highest binding affinity , Gc-1s has intermediate affinity , while Gc-2 has the lowest binding affinity with all vitamin D metabolites (Lauridsen *et al.,* 2001). Besides transporting vitamin D and its storage, VDBP performs several other roles such as: (1) scavenge actin molecules; (2) bind both saturated and unsaturated fatty acids; (3) activate macrophages; (4) stimulate osteoclasts function; (5) enhance the chemotactic effects of C5-derived peptides; (6) associate with immune system, such as T and B cells. Even after binding with vitamin D and its metabolites, 98–99% VDBP binding sites remain vacant, which suggests its role beyond vitamin D transport (Gomme *et al.,* 2004).

The relationship between VDBP variants rs7041 and rs4588 and T2DM has been studied in a Pakistani population and was found associated with increased susceptibility of developing T2DM(Iqbal *et al.,* 2017).Literature also suggests that South Asians are having positive association between VDBP polymorphisms and T2DM (Wang *et al.,* 2014; Shao *et al.,* 2012). However, the expression of vitamin D binding protein (VDBP) in T2DM patients has received limited attention. Therefore, it would be worth noting the expression of VDBP in a T2DM population as

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plausible alteration in its expression might contribute to vitamin D deficiency. The aim of the present study was to evaluate the expression of vitamin D binding protein (VDBP) in T2DM patients and healthy controls.

## METHODOLOGY

### Study Participants

Plasma samples utilized in this study for analysis of VDBP expression were previously collected for a case control study. Hence, the study participant's recruitment criteria along with their clinical characteristics are described elsewhere. Briefly, 165 T2DM patients were recruited from the Endocrinology Clinics of the Aga Khan University Hospital (AKUH) with informed consent after obtaining ethical approval from institutional ethical review committee. Additionally, 165 age and gender matched (within 5 years) healthy controls were recruited from the personnel of the same university and other healthcare institutions in Karachi (Iqbal *et al.,* 2017).

### Plasma Protein Estimation

Ten mL venous blood were collected from patients and healthy controls in a tube containing EDTA as an anticoagulant. Plasma was separated after centrifugation of blood samples and stored immediately at -80°C until analysis. Total plasma proteins were estimated by Bradford protein assay using bovine serum albumin as a standard (Bradford, 1976).

### Two-Dimensional Gel Electrophoresis (2-DE)

Two-Dimensional Gel Electrophoresis (2-DE) was carried out as described previously (Ferrero *et al.,* 2005). Briefly, Plasma samples from DM patients (n=3) as well as healthy controls (n=3) were diluted 1:10 in a solubilisation buffer (9M urea, 2% CHAPS, 65mM DTT, 2% immobilized pH gradient (IPG) buffer pH 3-11 and 0.05% bromophenol blue). The volume of the sample containing 90 µg of the solubilized plasma protein was then dissolved in reswelling buffer (8M urea, 2% CHAPS, 13mM DTT and 2% immobilized IPG buffer pH 3-11) to achieve a final volume of 250µL This mixture was then applied to rehydrate an 11cm pH 3-11 nonlinear IPG gel strip overnight at room temperature. Isoelectric focusing (IEF) was carried out on Multiphor™ II Electrophoresis System (GE Healthcare Bio-Sciences AB) at 20°C using following parameters: 500 V for 1 h 1000 V for 1 h gradual increase to 4000 V and kept constant for 32000 Vh. Prior to second dimension, IPG strips were incubated in 10ml equilibration buffer containing 6 M urea, 2% (w/v) sodium dodecyl sulphate (SDS), 50 mM tris-HC1 pH 6.8, 30% glycerol (v/v), 65 mM DTT, and 0.01% bromophenol blue for 15 minutes. 2-DE was carried out on a linear 12% polyacrylamide gel without a stacking gel, instead the gel strip was fixed with a 3mL solution of 0.5% agarose dissolved in running buffer. The second-dimensional gels were run at constant 100V for 6 hours. The 2D gels were silver stained and scanned using a desktop scanner. The gels were prepared in triplicates or more to ensure reproducibility.

### Silver Staining

Silver staining of the all 2D gels were performed according to Blum (Helmut *et al.,* 1987) Briefly, gels were first immersed in a fixing solution containing 40% methanol with 10% acetic acid for 20 minutes followed by 3 times washing with wash solution containing 30% ethanol for 20 minutes. The gels were then incubated in freshly prepared solution of 1.2mM Sodium thiosulfate for 1 minute and then washed with deionized water. Subsequently, the gels were stained by incubating in staining solution containing silver nitrate and 2µL (37%) formaldehyde for 20 minutes followed by three times washing with deionized water. Finally, gels were developed by immersing in developing solution containing 30g Sodium carbonate, 5mg Sodium thiosulfate and 500µL formaldehyde until protein spots became visible. The reaction was stopped by incubating gels in stop solution of 5% Acetic acid.

### Validation of Identified VDBP by Western Blotting

VDBP identification was subsequently confirmed by immunoblotting as previously described (Towbin *et al.,* 1992). Briefly, extracted protein samples (20 µg for each) were loaded on 10% polyacrylamide gel along with molecular weight markers and run at constant 100 V. The proteins were electrotransferred onto polyvinylidine difluoride (PVDF) membrane (Amersham GE, Munich, Germany) at 300 mA for 180 min using Wet Electroblotting System (Bio-Rad, Hercules, CA, USA) followed by blocking with 5% non-fat dry milk (BSA) in Phosphate-Buffered Saline and Tween 20 (TBST) for 1 h at room temperature (24°C). After washing the membrane with PBS containing 0.05% Tween 20, the VDBP immobilized PVDF blot was incubated in primary antibody Anti-Vitamin D Binding protein antibody (ab81307) overnight at 4°C. The membrane was washed three times with TBST and incubated with secondary antibody goat anti rabbit IgG HRP (1:10,000) for 1 h at room temperature. Finally, the

protein bands were detected using TMB reagent and visualized on Gel Documentation System (Bio-Rad, Hercules, CA, USA).

**Image Analysis**

2-DE gel images were densitometrically analyzed using Melanie™ 9.0 software (GeneBio, Geneva Switzerland) and merged to create a single master gel. The expression levels of the spots were demarcated by measuring the volume of each spot divided by the total volume of all spots in the gel. This method is named as Total Spot Volume Normalization. For each spot, the relative volume intensity was averaged and specified as the mean ± SEM. The spots having significantly different intensities were selected by Student's t-tests ($p < 0.05$).

For western blot images, relative intensities of each band were calculated using Quantity One 1-DAnalysis Software (Bio-Rad, Hercules, CA, USA) and data were expressed as mean ± SEM. Reproducibility was ensured by preparing gels in triplicates or more from each patient and control sample.

***In Silico* Protein-Protein Interaction Analysis**

The functional association of the differentially expressed VDBP was generated through protein interaction network STRING 10.5 (https://string-db.org).This database provides the comprehensive description of the proteins in various cellular mechanisms and functions. Acquired accession numbers of the identified protein was entered in search column and possible interaction of the entered proteins were acquired at high confidence score i.e. 0.7. Each node represents a protein with edged interactions with others.

## RESULTS AND DISCUSSION

2-DE expression analysis revealed 27 differentially expressed proteins in T2DM patients compared to healthy controls. Among these, 09 spots were found to be upregulated while 18 were down-regulated in diabetic patients compared with healthy controls. The observed protein spots were initially matched with the human plasma 2D PAGE map accessed from ExPASy. As described previously by Ferrero et al.,(Ferrero *et al.,* 2005) VDBP spots were identified based on MW, PI and their position with respect to other spots belonged to that area. Image analysis of VDBP revealed increased expression of VDBP in T2DM patients compared to healthy controls, and the difference was statistically significant [Fold change >1.7, *$p$<0.05] (Fig. **1**). Proteins were considered upregulated with fold change ≥ 1.5 and down-regulated with foldchange <0.5. Furthermore, Differential expression of VDBP is also represented by expression graphs created through GraphPad Prism 7 by using normalized volume obtained through statistical analysis of 2D images (Fig. **1**).

VDBP expression was validated by western blotting in 4 independent gels prepared in triplicates. A 54 kDa band of VDBP was detected in all samples from every member of group. Comparison of VDBP band intensities showed significant upregulated expression of VDBP in T2DM group compared with healthy controls ($p < 0.05$, Fig. **2**). When these two groups were compared with respect to gender, slightly higher expression was observed in diabetic females compared to healthy females, but the difference was non-significant.

Previous recent reports have shown that vitamin D deficiency is more prevalent in poor glycemic controlled T2DM patients (Iqbal *et al.,* 2016) and VDBP gene polymorphisms rs7041 and rs4588 have strong positive association with risk of developing T2DM in Pakistani population (Iqbal *et al.,* 2017) which suggests that VDBP expression could be altered in T2DM patients. Herein, we report significant upregulated expression of VDBP in T2DM Patients compared to healthy controls in the same population.

Our findings depicting upregulated VDBP expression in T2DM patients are comparable with other previously published studies. Recently, Fawzy *et al*. (2018) showed increased serum and urinary VDBP levels in T2DM patients suffering with Diabetic nephropathy (DN). In addition, Chou *et al.* (2015) showed that VDBP levels are increased in diabetic rat serum, kidney and liver and VDBP is phosphorylated in diabetic rat serum. Similarly, Cho *et al.* (2007) reported upregulated expression of serum VDBP in microalbuminuric type 2 DM patients compared to normoalbuminuric patients. In Contrary, Thrailkill *et al.* (2011) demonstrated increased urinary excretion of VDBP with no significant change in serum VDBP levels in T1DM patients. Whereas, Blanton *et al.* (2011) found decreased serum VDBP levels in T1DM patients. However, these last 2 observations were based on data collected from T1DM patients.

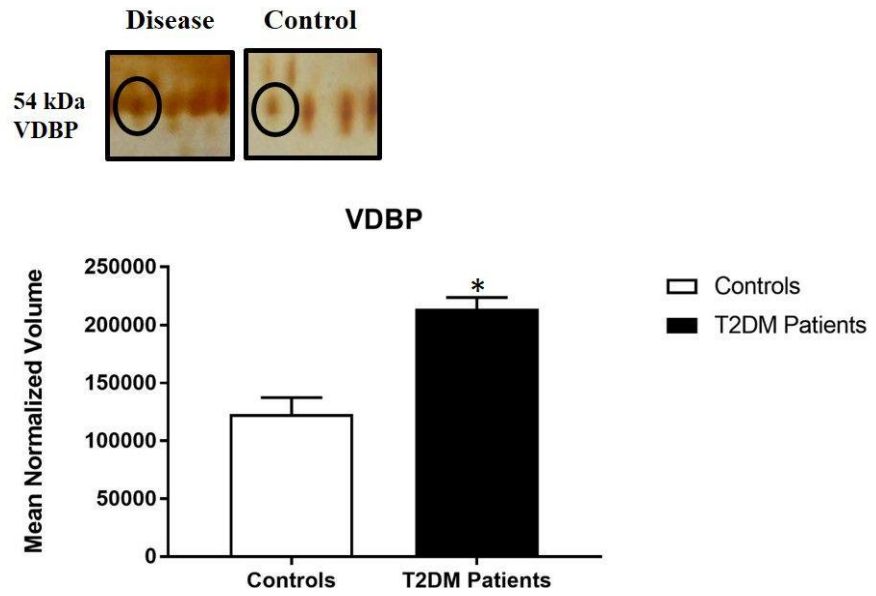


Fig. 1. Representative VDBP by 2-DE demonstrating differential expression in T2DM patients compared with controls. IEFof 90µg of plasma protein was done by using IPG strips of pH 3-11NL, and separated on 12.5% polyacrylamide gel followed by silver staining. Densitometric analysis was done by using Melanie™ 9.0 software and differential expression was statistically analyzed using student's t-test, *$p < 0.05$.

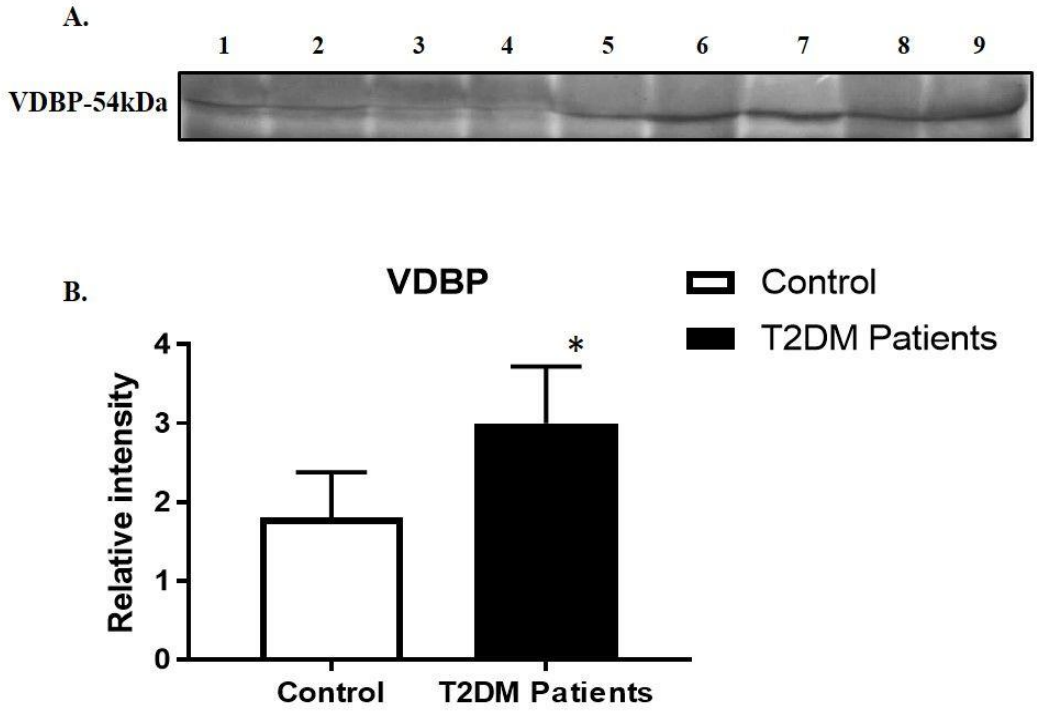


Fig. 2. Representative VDBP validated by westernblotting demonstrating differential expression in T2DM patients compared with controls. 20µg plasma protein were loaded in each well of a 10% SDS-PAGE gel, transferred to PVDF membrane followed by immunodetection using anti-VDBP antibody. (A) Lane 1-4 represents healthy controls whereas Lane 5-9 T2DM patients. (B) VDBP bands were densitometrically analyzed using Quantity One 1-D Analysis Software Differential expression was statistically analyzed using student's t-test, *$p < 0.05$.

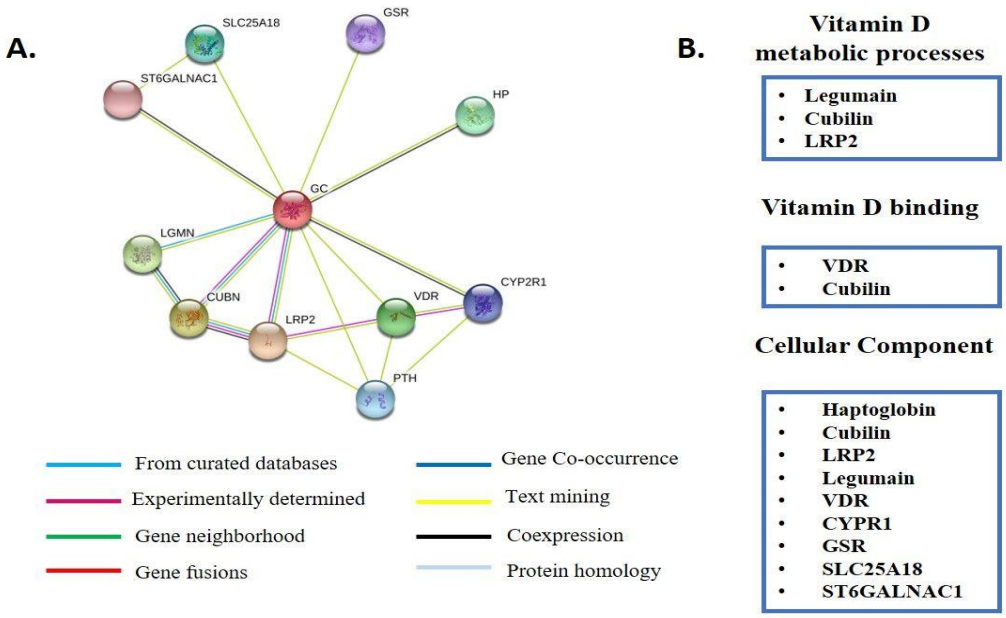


Fig. 3. Vitamin D binding protein (VDBP/GC)-protein interaction by string database. (A) Proteins are represented as nodes whereas the interaction between proteins are indicated by the connecting line. (B). Classification of proteins based on Gene Ontology (GO) biological processes [data source: https://string-db.org, version 10.5]

The functional mechanism behind increased VDBP levels is not known. One possibility is that VDBP is increased as a compensatory mechanism as previously speculated (Kalousova *et al.,* 2015). VDBP performs various other essential metabolic and immunological roles in addition to vitamin D transport and acting as its reservoir (Haddad, 1995). For instance, low grade systemic inflammation is one of the hallmark of T2DM (Lowe *et al.,* 2013) and it could be considered that VDBP is increased in response to prevailing pro-inflammatory state. Moreover, hyperglycemia mediated increased VDBP expression is another plausibility as suggested by Chou *et al.* (2015). Approximately 85-90% of vitamin D and its metabolites circulates bound to VDBP. Thus, with our observed increased expression of VDBP and previously reported increased total 25(OH)D levels (21.67 ± 10.7 in T2DM patients versus 16.70 ± 11.3 in healthy controls; $p < 0.001$) in the same diabetic population (Iqbal *et al.,* 2017), it can be speculated that majority of circulating 25(OH)D was bound to VDBP and a low fraction of free form of vitamin D was available in diabetic patients compared to control .Although, 25(OH)D is the major circulating form of vitamin D and clinically considered as a biomarker to determine vitamin D status, however, it cannot elicit biological response (Chun *et al.,* 2014). Therefore, increase in serum VDBP might cause a functional vitamin D deficiency even in the presence of higher 25(OH) D levels in T2DM patients compared to control (Havens *et al.,* 2013). Hence , it can be speculated that due to low availability of biologically active form, plausible glucose-mediated insulin secretion mechanisms are hampered in this diabetic population (Norman *et al.,* 1980).

The functional association network of VDBP (GC) was generated through Protein-Protein interaction network database STRING 10.5 (https://string-db.org).STRING enables PPI analysis of network based on both known and predicted interaction between proteins thus providing substantial evidence regarding the complex interactive role of VDBP in T2DM. According to the interaction network (Fig.**3**), a strong interaction is evident between VDBP and interacting proteins with the highest confidence score > 0.9. Strongest and direct interaction of VDBP is mainly evident with proteins involved in vitamin D related metabolic processes such as LRP2 (Megalin), Cubilin and Legumain. Strong and direct interaction of VDBP is also evident with proteins directly binding to vitamin D such as VDR and Cubilin. However, interaction of VDBP is also evident as cellular component with other proteins: Haptoglobin, Cubilin, LRP2, Legumain, VDR, CYPR1, GSR, SLC25A18 and ST6GALNAC.

In this regard, VDBP is a multifunctional plasma protein primarily involved in several physiological functions related to vitamin D metabolism. VDBP interacts with several other proteins directly or indirectly in vitamin D pathway (Fig.**3**). Therefore, altered VDBP expression could affect T2DM related physiological functions of vitamin D. The predominant VDBP interacting partners in vitamin D pathway are: (1) CYP2R1; provides hydroxylase activity for conversion of vitamin D3 or D2 to its biologically active form, (2) VDR; about 38 different tissue types express this protein including Pancreatic β-cell. Bioavailable vitamin D [1, 25(OH)2D3] is capable of interacting

with VDR and can induce transcriptional activation or suppression of several genes related to T2DM features such as insulin resistance, insulin sensitivity and systemic inflammation (Lips *et al.*, 2017), (3) LRP2 (Low density lipoprotein receptor-related protein 2) also known as megalin, along with (4) Cubilin facilitates receptor mediated endocytosis of VDBP-25(OH)D complex in proximal renal tubule cells of kidney for conversion of 25(OH)D to 1, 25(OH)D by renal hydroxylase enzyme (Nykjaer *et al.*, 1999) (5) Legumain is an enzyme encoded by LGMN gene and cleaves VDBP in proximal renal tubule cells(Morita *et al.*, 2007), (6) PTH (parathyroid hormone) maintains calcium homeostasis via regulating circulating 25(OH)D levels. Besides aforementioned possibilities, there might be other alternative unknown mechanisms affected due to upregulation of VDBP in type 2 diabetes.

The present study faced certain limitations such as VDBP spots were not quantitatively analyzed using mass spectrometric analysis. Moreover, present study also comprised of small sample size. Besides aforementioned limitations, to the best of our knowledge this is the first study reporting differential expression of VDBP in a Pakistani population suffering with T2DM. We suggest that in clinical setting, measurement of VDBP and 1, 25(OH)2D levels along with 25(OH)D is potentially beneficial to assess functional vitamin D status, particularly in conditions where VDBP expression is altered.

## CONCLUSION

In conclusion, our data indicates that VDBP expression was upregulated in T2DM patients which might be due to multipleT2DM-related factors still not known. However, increased VDBP expression can ultimately lead towards aberrant vitamin D related glucose-mediated insulin secretion pathways in diabetic patients due to low functional vitamin D levels. Moreover, only 25(OH)D levels determination is not enough to accurately define vitamin D status, particularly in T2DM patients. Therefore, it is suggested that in conditions where VDBP expression is altered, VDBP and 1, 25(OH) 2D3 levels along with 25(OH) D levels should be measured to accurately define vitamin D status so that therapeutic interventions can be implemented to attain sufficient vitamin D levels.

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